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The use of mustard gas in the Iran-Iraq War has stressed the need of reliable methods for retrospective detection of poisoning with chemical warfare agents. We are developing methods to detect adducts of mustard gas to DNA and proteins, which may have a life span of many days after exposure. In order to achieve high sensitivity and specificity, immunochemical detection techniques are developed. As markers to detect adducts, we synthesized N7-(2'-hydroxyethylthioethyl)-guanine, the di-adduct di-(2-guanin-7'-yl-ethyl) sulfide, and N3-(2'-hydroxyethylthioethyl)- adenine, O6-(2'hydroxyethylthioethyl)-guanine and the corresponding 2'deoxyguanosine derivative. N7-(2'-hydroxyethylthioethyl)-guanine was shown to be the major adduct in experiments with double-stranded calf-thymus DNA and human white blood cells exposed to [^{35}S]mustard gas. The N7-guanine di-adduct and the N3-adenine adduct were formed to a lesser extent, whereas the O6-guanine adduct was not detected. (Continued over)

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(continued from reverse)

19. Antiserum was raised in rabbits against DNA treated with mustard gas, which was used for the development of an immunochemical assay for the screening of supernatants of hybridomas for specific antibody activity. Mono- and di-adducts at the N7-position of guanosine-5-phosphate were sythesized for use as haptens to generate monoclonal antibodies against such adducts in DNA. After immunization of mice with the monoadduct hapten bound to a carrier protein, several hybridomas were obtained producing antibodies which recognize the monoadduct of mustard gas containing an intact imidazolium ring and show a low cross-reactivity towards other guanine-adducts. Several milligrams of one of the antibodies are now available. N7-guanine monoadduct with mustard gas could be detected with a specificity of one adduct amongst 5.2×10^6 unmodified nucleotides in single-stranded DNA. A procedure was developed to make double-stranded DNA single-stranded, allowing detection of N7-adducts in single- and double-stranded DNA at a similar sensitivity. Adducts in white blood cells can be detected after exposure of human blood to mustard gas concentrations as low as 2 μM. With the same antibodies an immunofluorescence test was developed which detects local damage in human skin due to exposure to Ct values of mustard gas vapor that do not cause blister formation. Adducts of mustard gas to proteins may also be used to establish exposure. It was shown that much more mustard gas in blood binds to hemoglobin than to DNA. Hemoglobin was alkylated at the amino group of the N-terminal valine of the α-chain after treatment with mustard gas. The N-(2'-hydroxyethylthioethyl)-substituted N-terminal heptapeptide was synthesized. Antibodies raised against this peptide bound to a carrier protein discriminated between hemoglobin and mustard gas-treated hemoglobin, but were of the IgM type. Similar results were obtained when using chicken gammaglobulin alkylated with mustard gas as an antigen. Simple model compounds for nucleophilic amino acids in proteins, $CH_3C(O)NH\text{-}CH(Y)\text{-}C(O)NHCH_3$, have been exposed to mustard gas in order to identify the major reaction products and to study the relative reaction rates of amino acids with mustard gas. Cysteine is by far the most susceptible model peptide with regard to alkylation by mustard gas. Attempts were made to synthesize a tetrapeptide hapten containing the cysteine adduct. Also for use as a hapten, gly-gly-gly-glu-5-(2'-hydroxyethylthioethyl) ester-1-amide was synthesized.

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SUMMARY

The use of mustard gas in the Iran-Iraq War and the rapid proliferation of chemical weapons in several Middle East and Third World countries has stressed the need of reliable methods to establish poisoning with chemical warfare agents. Therefore, we are developing methods for retrospective detection of adducts of mustard gas to DNA and proteins, which may have a life span of several days or even weeks after poisoning. In order to achieve high sensitivity and specificity, immunochemical detection techniques are developed. Such techniques allow a selectivity of one modified nucleotide among about 10^8 unmodified bases in DNA and a minimum detectable amount in the femtomolar range.

For use as markers in the identification of mustard gas-adducts in calf-thymus DNA or in human white blood cells, we have (re)synthesized and characterized several adducts of mustard gas with guanine and adenine. Based on the work of Brookes and Lawley we have obtained N7-(2'-hydroxyethylthioethyl)-guanine, the corresponding di-adduct, i.e., di-(2-guanin-7'-yl-ethyl) sulfide, and N3-(2'-hydroxyethylthioethyl)-adenine. A route of synthesis was developed for O6-(2'-hydroxyethylthioethyl)-guanine and the corresponding 2'-deoxyguanosine derivative. Alkylation experiments of 2'-deoxyguanosine with mustard gas in neutral aqueous solution have shown that the hitherto unreported N1-(2'-hydroxyethylthioethyl)-2'-deoxyguanosine adduct is also formed as a minor reaction product.

The N7-substituted monoadduct of guanine, N7-(2'-hydroxyethyl-thioethyl)-guanine, was shown to be the major adduct in experiments with double-stranded calf-thymus DNA and human white blood cells exposed to [^{35}S]mustard gas. The N7-guanine di-adduct and the N3-adenine adduct were formed to a lesser extent. The O6-guanine adduct was not detected with the available techniques, suggesting that this adduct might be present only in trace amounts (less than 0.5% of total detected radioactivity), if present at all. For double-stranded calf-thymus DNA, the three detectable adduct peaks represented ca. 90% of all radioactivity.

Mustard gas appeared to be a very effective alkylating agent for bases in DNA. Even in blood, with a variety of reactive sites, 1 out of 124 guanine bases was alkylated to form the monoadduct at N7 of guanine upon exposure to 1 mM mustard gas. Crosslinks due to exposure to mustard gas were determined with our recently developed "alkaline elution" method in cultured Chinese hamster cells, as well as in the nucleated cells of exposed human blood. The detection limit of cross-links in the former cells is at 0.5 μM mustard gas, which is in the biologically relevant dose range since 37% of the cells are still able to form colonies after exposure to 1.4 μM mustard gas. At 4 h after exposure, most of the crosslinks had disappeared, either by proper repair or resulting in "other DNA-damages."

After immunization of rabbits with calf-thymus DNA treated with mustard gas, we obtained the antiserum W7/10 with a high specificity

for DNA adducts of mustard gas. With this serum a method for the screening of supernatants of hybridomas for specific antibody activity could be developed and optimized, in which single-stranded calf-thymus DNA exposed to 10 μM mustard gas was used as coating material at 50 ng per well. A competitive ELISA was developed in which mustard gas adducts to DNA could be detected with a minimum detectable amount of a few femtomoles per well, and a selectivity which allows detection of one monoadduct at N7 of guanine amongst 1.3×10^6 unmodified guanines.

The immunochemical methods for this study are aimed to detect damage due to exposure to mustard gas in easily accessible biological samples, i.e., human blood or skin biopsies. In these samples the DNA is present as double-stranded material, in tight conjunction with nuclear proteins. An optimal detection of adducts requires adequate methods to disrupt the cell wall and to release the DNA without changing or destroying the adducts. After exposure of double-stranded and of single-stranded DNA to the same concentration of mustard gas, an approximately 13-fold larger amount of double-stranded than of the single-stranded material is required for effective competition in the ELISA test, although it contained at least as many adducts as the single-stranded DNA. This difference is probably the result of interstrand crosslinks formed by mustard gas and of shielding by the complementary strand which prevents optimal presentation of antigen. A simple alkaline denaturation of double-stranded DNA gave single-stranded material in which the adducts were no longer recognized by the antiserum, probably due to ring opening of the imidazolium ring of the N7-adduct of guanine. Several alternative ways to make DNA single-stranded were tested. After treatment with a combination of low concentrations of formamide and formaldehyde at low ionic strength, the double-stranded DNA gave a stronger inhibition than the single-stranded material, in agreement with a higher content of N7-adducts. When this procedure was applied on DNA from human white blood cells exposed to 1 mM mustard gas, the minimum detectable amount of adduct in the competitive ELISA was about 20 times as high as the detection limit for mustard gas-treated isolated DNA, probably as a result of the above-mentioned complications such as shielding by proteins. It is expected that further improvements can be obtained by developing more efficient methods for release of DNA from cells without destruction of the mustard gas adducts. Presently, adducts in white blood cells can be detected after exposure of human whole blood to mustard gas concentrations as low as 2 μM.

We developed methods for the synthesis of mono- and di-adducts of mustard gas at the N7-position of guanosine-5'-phosphate for use as haptens to generate monoclonal antibodies against such adducts. After immunization of mice with the monoadduct coupled to a carrier-protein via the phosphate moiety, we obtained several hybridomas producing monoclonal antibodies which recognize the N7-guanine monoadducts containing an intact imidazolium ring. The sensitivity of the competitive ELISA with the monoclonal antibodies was comparable to that of the assays performed with polyclonal W7/10.

Two monoclonal antibodies appeared to be quite specific for the N7-monoadduct of mustard gas to guanosine-5'-phosphate with the intact ring. This ring-closed adduct can be detected at a two orders of magnitude lower level than the free guanosine derivative. Furthermore, the antibodies have a low cross-reactivity towards N7-methyl guanine, to the N7-mustard gas adduct to guanine, and, rather strikingly, to the O6-mustard gas guanine adduct. No cross-reactivity was observed with guanosine monophosphate. One subclone has been cultured further, and the antibodies in the supernatant were collected and purified. Several milligrams of these antibodies are now available, of which ca. 2 ng/well are needed in the competitive ELISA. Supernatant and purified antibodies can be stored for months at -20 °C.

In preliminary experiments performed with the same antibodies we demonstrated, by means of immunofluorescence microscopy, the presence of the N7-adduct in human skin biopsies exposed to dosages of mustard gas vapor that do not yet cause blister formation. The adducts were still detectable at 48 h after exposure.

Since mustard gas binds only 1.9 times more efficiently to DNA than to proteins, immunochemical dosimetry of exposure to mustard gas based on protein adducts might be advantageous in view of the almost 300-fold larger amount of protein than of DNA in blood, provided that the protein adducts can be concentrated by way of purification. Therefore, we started the development of an immunochemical assay for the detection of adducts to hemoglobin. We found that 70% of the mustard gas adducts to globin are acid- and/or alkali-labile, which indicates that enzymatic degradation to peptides and amino acids might be a more viable approach to identification of the adducts than the standard approach involving complete degradation into amino acids by means of acid hydrolysis. In this context we have concentrated our efforts on the N-terminal heptapeptide val-leu-ser-pro-ala-asp-lys from the α-chain of hemoglobin, which can be conveniently isolated by means of HPLC after tryptic digestion of the protein. The heptapeptide was obtained by means of the solid phase synthesis method, and mono-substituted with mustard gas specifically at the free α-amino group of valine.

It was demonstrated that the same alkylated heptapeptide is formed when hemoglobin is treated with mustard gas and subsequently digested with trypsin. Mice were immunized with the N-(2'-hydroxyethylthio-ethyl)-substituted heptapeptide coupled to a carrier protein and hybridomas were generated via cell fusion experiments. Three clones were selected which produced antibodies that discriminated between hemoglobin and mustard gas-alkylated hemoglobin in the direct ELISA, but only when hemoglobin has been exposed to high mustard gas concentrations. Moreover, the antibodies were of the IgM type, which cannot be used in a competitive ELISA. New fusions aiming at IgG antibody-producing hybridomas did not yet succeed. As an alternative, chicken gammaglobulin was alkylated with mustard gas. Immunization of a mouse and fusion of spleen cells yielded several hybridomas

hybridomas producing antibodies that discriminated between native and alkylated (5 mM) hemoglobin, but all were of the IgM type.

Since little is known about the reactivity of mustard gas towards individual amino acids in proteins or the structure and stability of the adducts, we have studied the alkylation of simple model peptides, i.e., N-acetyl-amino acid-methylamides [$CH_3C(O)NH\text{-}CH(Y)\text{-}C(O)NHCH_3$], in which Y represents an amino acid side chain that can be alkylated by mustard gas. So far, we have identified the major reaction products with mustard gas in aqueous solution of such model peptides derived from aspartic and glutamic acid, histidine, cysteine, and methionine as well as from the model peptide $NH_2CH(i\text{-}Pr)C(O)NHCH_3$, which served to investigate the reactivity of the α-amino group in an N-terminal valine. The primary reaction products were synthesized by means of independent routes and were used to study the relative reaction rates of the various model peptides with mustard gas in a competition experiment. Mustard gas showed a high preference for reaction with the thiol moiety in the model peptide derived from cysteine.

Since the cysteine model peptide turned out to be the most reactive, we have attempted to synthesize S-(2'-hydroxyethylthioethyl)-cys-gly-gly-gly as a hapten to generate monoclonal antibodies which recognize protein adducts in skin biopsies. For the same purpose, glv-gly-glv-glutamic acid-5-(2'-hydroxyethylthioethyl) ester-1-amide was synthesized.

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

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___ For the protection of human subjects, the investigator(s) have adhered to policies of applicable Federal Law 45 CFR 46.

_____ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

Dec. 11, 1991

PI - Signature Date

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I. INTRODUCTION

Several incidents in the recent past have illustrated the need for unequivocal methods to verify exposure of humans to chemical warfare agents. The most straightforward case was the large scale use of mustard gas, and possibly also of tabun and sarin, in the Iran-Iraq War (1). With severe casualties in hospitals all over the world, analyses of agents and metabolites had to be improvised and gave inconclusive results (2). The controversies with regard to the use of trichothecenes as an agent ("yellow rain") in Southeast Asia which arose from the analyses of environmental and biological samples were widely publicized, and have been reviewed (3). Rather recently, rumors were spread that agents had been used against Unita troups in Angola (4-6). Samples from the casualties were analyzed, with disputable results. In the more distant past, the alleged use of agents in Yemen could not be confirmed, due to lack of adequate methods of analysis (7).

The rapid proliferation of chemical weapons in several Middle East and Third World countries (8) raises the fear that the above-mentioned incidents will not be the last ones. Evidently, the availability of reliable methods for retrospective in vivo detection of exposure to chemical agents is crucial for political and military evaluation when such agents are allegedly used in future conflicts. This need is enhanced by the probability that in many cases biological samples will be the only or at least the most abundant samples available for analysis because the use of agents is first noticed by their effects on casualties. If a total ban on the use, possession and production of chemical agents will eventually materialize, the availability to individual nations and to an International Inspectorate of reliable and sensitive methods for retrospective detection of exposure will further discourage the open or covert use of agents.

In case that the above-mentioned detection methods can be quantified to determine internal dose, their usefulness is enhanced for several applications. For example, they can be used for biomonitoring of workers in facilities set up to destroy stockpiles of agents. In the case of chemical warfare, establishment of the internal dose of agent in casualties can be helpful for triage and treatment. Finally, such methods can be of immediate use in many types of experimental work, ranging from exposure to agents on a cellular level to the development of protection gear.

We have learned from the earlier-mentioned incidents that urine, blood and other biopsies for analysis can often only be obtained several days or even weeks after exposure. Therefore, in vivo verification methods should be very sensitive and relate to long lasting, specific effects of the agents under investigation. Such methods are not yet available for the common chemical warfare agents. For example, intact nerve agents such as soman can be analyzed in blood, brain, and muscle tissues at minimum detectable levels in the low picomolar range, but these levels last only for a few hours after

intoxication at high doses in primates (9). Alternatively, the observation of low levels of cholinesterase activity is not specific for nerve agents. Regeneration of nerve agent from phoshonylated aliesterase with fluoride ions (10, 11) or perhaps analysis of hydrolysis products in urine may provide a more promising approach to retrospective detection of nerve agent exposure.

In view of the large scale use of mustard gas in the Iran-Iraq war and the rapid proliferation of this agent in Third World countries (8), we have selected this agent to develop methods for retrospective detection of exposure. Presently available methods seem unsatisfactory. Recent reports on GC-MS detection of intact mustard gas in an abdominal fat sample obtained from autopsy of an Iranian soldier who died seven days after exposure to mustard gas (12), and in the urine of another soldier exposed seven days earlier (13, 14), need further confirmation. Neither has the report by Stade (15) been confirmed on the presence of intact agent in skin blisters caused by mustard gas. Attempts to verify exposure to mustard gas via analysis in blood or urine of its hydrolysis product thiodiglycol (16), and of thiodiglycol derivatives which are (re)converted into mustard gas with hydrochloric acid (17,18), are complicated by the presence of these products in samples from nonexposed volunteers. Reports on the identity of further metabolites of mustard gas are contradictory. According to Davison et al. (19) the major urinary metabolites in rodents are glutathione conjugates of thiodiglycol, whereas Roberts et al. (20) report bis(cysteinyl) conjugates of mustard gas sulfone as major metabolites. Evidently, the metabolism of mustard gas needs to be reinvestigated. Moreover, 80-90% of the metabolites are excreted within 48 h (19,20).

We have chosen to develop immunochemical detection methods of adducts which are generated by alkylation of DNA and proteins by mustard gas. This choice is based on extensive experimental evidence obtained in the TNO Medical Biological Laboratory and elsewhere which shows that these methods of analysis for DNA-adducts of cytostatic agents and environmental alkylating agents can be highly selective, detecting one alkylated base among $\leq 10^8$ nonalkylated bases (21). The minimum detectable concentration of modified bases is in the low femtomolar range. If cells producing monoclonal antibodies to the adducts can be isolated, detection methods based on these antibody-adduct interactions can be performed on a large scale, with quantitative results. Although alkylated bases in DNA can undergo secondary reactions, e.g., ring-opening in the case of N7 alkylated guanine, and the damage due to adduct formation tends to be repaired, the adducts are detectable for days or even weeks after exposure (21).

In general, biomonitoring methods of alkylating agents based on analysis of protein adducts (for reviews see refs 22-24) are complementary to methods based on analysis of DNA-adducts. In contrast with the immunochemical detection methods for the latter adducts, protein-adducts are usually quantified by GC-MS analysis after total hydrolysis of the protein and derivatization of the alkylated amino acid. Therefore, much less experience has been

obtained with the immunochemical detection of protein-adducts (25,26). A priori, it should be assumed for stoichiometric reasons that in vivo exposure to alkylating agents yields many more adducts of proteins than of DNA. Moreover, it has been shown that the life span of proteins is not shortened by alkylation. Human hemoglobin, with a biological half life of 16-18 weeks, has been proposed as an easily available protein for biomonitoring exposure to various alkylating agents (22-24). In recent experiments the degree of alkylation by ethylene oxide of N-terminal valine in human hemoglobin was determined by means of radioimmunoassay as well as by GC-MS analysis. A good correspondence of the results was found. With ethylene oxide and other directly alkylating agents, a reasonably linear relationship between levels of alkylation of DNA and proteins has also been observed (27).

When mustard gas is used in chemical warfare, the agent affects the skin in liquid or vapor form, whereas inhalation of vapor or aerosol causes extensive damage of the respiratory tract and lungs. Extensive, long-lasting systemic intoxication is also observed due to rapid penetration of agent into the general circulation both via inhalation and the skin (28). Therefore, DNA and proteins from various biopsies may serve as samples to monitor exposure to the agent. Primarily, skin biopsies and nucleated blood cells are convenient to assess damage to DNA. Hemoglobin, albumin, and skin biopsies are logical targets for immunochemical detection of mustard-gas adducts to protein.*

To the best of our knowledge, the products arising from alkylation of DNA due to in vivo exposure to mustard gas have not been investigated. In vitro alkylation of DNA and RNA by mustard gas has been studied by Lawley et al. in the early sixties (30-35). They suggested that foremostly the N7 nitrogen in guanine moieties of DNA and RNA is alkylated by mustard gas, leading to N7-(2'-hydroxyethyl-thioethyl)-guanine (Figure 1a), as well as to the corresponding intrastrand and interstrand (36) di-adduct di-(2-guanin-7'-yl-ethyl) sulfide (Figure 1b). The authors also report that the N-3 nitrogen of adenine in DNA is alkylated to give N3-(2'-hydroxyethylthioethyl)-adenine (Figure 1c). More recently, Ludlum et al. (37) have claimed that traces of the O6 adduct of guanine, i.e., O6-(2'-hydroxyethyl-thioethyl)-guanine (Figure 1d) are also formed. So far, these adducts were characterized on the basis of similarity of their uv spectra and chromatographic behavior with those of analogous alkyl-substituted purines. Further spectroscopic evidence for the structure of the adducts is not available.

With regard to DNA-adducts we studied:
- The synthesis and structural characterization of the above-mentioned adducts.

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* Serological evidence for immunological specificity of protein-mustard gas adducts has been obtained in the past (29).

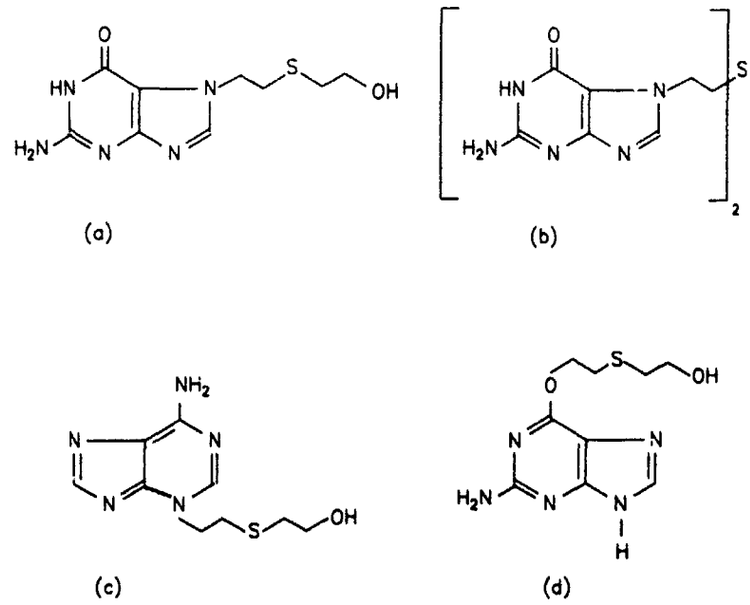

Figure 1. Suggested chemical structures of mustard gas adducts to DNA bases; (a) N7-(2'-hydroxyethylthioethyl)-guanine; (b) di-[(2-guanin-7'-yl-ethyl] sulfide; (c) N3-(2'-hydroxyethyl-thioethyl)-adenine; (d) O6-(2'-hydroxyethylthioethyl)-guanine

- Chromatographic and spectroscopic experiments to detect formation of the adducts upon exposure of calf-thymus DNA or human blood to mustard gas.
- The development of an ELISA based on polyclonal antibodies raised against mustard gas damage to calf-thymus DNA.
- The synthesis of a ribonucleotide derivative of the most abundantly formed adduct.
- The generation and isolation of cell lines which produce monoclonal antibodies against this ribonucleotide hapten.
- The development of an ELISA based on two monoclonal antibodies which have been produced.
- The detection of mustard gas damage in skin biopsies by means of immunofluorescence microscopy based on interaction of mustard gas adducts present in the skin with the monoclonal antibodies.

Evidence has been obtained that the earlier mentioned in vivo stability of alkylated proteins is also observed in case of alkylation by mustard gas. Renshaw (38) applied liquid [^{35}S]mustard gas for 10 min to the skin of human volunteers. At two weeks after the exposure 80% of the activity was still present, most probably bound to protein, whereas 25% activity was left even more than 5 weeks after application. Smith et al. (39) observed binding of [^{35}S]mustard gas to erythrocytes, presumably to hemoglobin, after intravenous administration of mustard gas to human volunteers at a dose of ca. 5 mg/man.

Many investigations have dealt with the analysis of protein-mustard adducts after in vitro alkylation, but the results are much less conclusive than in the case of DNA adducts with mustard gas. This is because virtually all protein studies were performed during the second world war, with the limited techniques available at that time (for reviews see refs 40-43), and further work on this topic was almost (44) not reported. Nevertheless, most investigators agree that mustard gas alkylates primarily the free carboxyl groups of proteins. This conclusion was deduced from the change in titration curves of the proteins in the acidic region due to adduct formation. In the case of pig skin treated with liquid mustard gas it has been observed that 40-50% of the protein-mustard adducts are hydrolyzed at pH > 9, with release of thiodiglycol (38,45). After in vitro reactions of horse oxyhemoglobin with a large excess of mustard gas, Davis and Ross (46) observed that approximately thirty carboxyl groups of the hemoglobin molecule were alkylated. Similarly alkali-labile protein-mustard gas adducts were also reported for ox cornea collagen (47) and several other proteins (48).

Evidence for the alkylation of reactive groups other than carboxyl in proteins by mustard gas is even more indirect. Based on changes in the titration curves of proteins due to reaction with mustard, the alkylation of thiol groups of cysteine and of ring nitrogen in histidine has been invoked. Reactions with model peptides by Moore et al. (49) have shown that the α-amino groups of amino acids and the sulfide moiety of methionine are also alkylated by mustard gas.

In view of the obvious lack of insight into the preferred alkylation sites by mustard gas in proteins, and of the time-consuming effort that would be involved in elucidating these sites, our approach to immunochemical detection of protein-mustard gas adducts is rather pragmatic. With regard to protein-mustard gas adducts we studied:

- The synthesis of the N-terminal heptapeptide of the α-chain of human hemoglobin, alkylated by mustard gas at the N-terminal amino group.
- The identification of this alkylated heptapeptide in a tryptic digest of hemoglobin that had been exposed to mustard gas.
- The generation and production of monoclonal antibodies raised against the alkylated heptapeptide.
- The synthesis of a tetrapeptide hapten in which the 5-carboxylic acid group of C-terminal glutamic acid is alkylated by mustard gas and attempts to synthesize a tetrapeptide hapten in which the side chain of the terminal cysteine is alkylated by mustard gas.
- Reactions of various peptide-like derivatives of single amino acids with mustard gas, in order to identify the reaction products and to determine subsequently the relative reactivities of the model compounds with mustard gas.

II. EXPERIMENTAL PROCEDURES

WARNING: Mustard gas is a primary carcinogenic, vesicant, and cytotoxic agent. This compound should be handled only in fume cupboards by experienced personnel.

II.1. Materials

Technical grade mustard gas was purified by fractional distillation in a cracking tube column (Fischer; Meckenheim, FRG) to a gas chromatographic purity exceeding 99.5%. Hydrogen [^{35}S]sulfide with a specific activity of 8.4 GBq/mmol (227 mCi/mmol) and a total activity of 1,017 MBq (27.5 mCi) was purchased from Amersham International (Houten, The Netherlands) as 2.69 ml (at STP) of gas packed in a break-seal ampoule. The material was diluted with 22 ml (at 25 °C) of chemically pure cold hydrogen sulfide (Baker; Deventer, The Netherlands), giving a total of ca. 1 mmol of labelled hydrogen sulfide. Aqueous solutions of [^{14}C]thymidine (75 mCi/ml) and [^{3}H]thymidine (1.0 Ci/ml) were also procured from Amersham International. Ethylene oxide (lecture bottle, Baker) was used without further purification.

2'-Deoxyguanosine and 3',5'-cyclic-phosphate-2'-deoxyguanosine were purchased from Sigma Chemical Company (St. Louis, Mo, USA). The former product was dried by evaporation with dry pyridine. 5'-Monophosphate-guanosine, guanosine, 5'-monophosphate-2'-deoxyguanosine and adenosine were purchased from Aldrich (Brussels, Belgium) and were used without further purification. O6-ethyl-guanine was purchased form Chemsyn Science Laboratories (Lenexa, Kan, U.S.A.). 2,4,6-Triisopropylbenzenesulfonyl chloride (Janssen, Beerse, Belgium) was crystallized from boiling petroleum-ether 40-60 before use. Triethylorthoformate and levulinic acid (Janssen) were distilled before use. 4-Benzyl-L-aspartate and 5-benzyl-L-glutamate were obtained from Sigma. Benzyloxycarbonyltriglycine, glycylglycine, S-benzyl-N-benzyloxycarbonyl-cysteine, S-benzyl-cysteine-methyl ester hydrochloride, 5-t-butyl-glutamate-1-amide hydrochloride (83.6%) and N-benzoxycarbonyl-leucyl-serine were obtained from Bachem Feinchemicalien (Bubendorf, Switzerland). N-α-Acetyl-histidine monohydrate, N-acetyl-methionine, valine-methyl ester hydrochloride, 2-bromoethylamine hydrobromide, α-bromoisovaleric acid, ethyl chloroformate (97%), aqueous methylamine (40%), dicyclohexylcarbodiimide, chloroacetonitrile, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide, 1-methylpyrrolidine, 4-dimethylaminopyridine, 1,8-diazabicyclo[5.4.0]undec-7-ene, N-methylimidazole, and 10% palladium on charcoal were obtained from Aldrich and were used without further purification. Racemic, (+)-, and (-)-α-phenylethylamine were obtained (Aldrich) and were distilled before use. Trifluoroacetic acid (Aldrich) was distilled before use.

2-Mercaptoethanol, 1,2-dichloroethane (Aldrich) and triethylamine (Merck, Darmstadt, FRG), were commercial products which were purchased as chemically pure materials and dried according to standard methods. Sodium hydride suspension in mineral oil,

bromotrimethylsilane, t-butyldimethylchlorosilane and acetyl chloride were purchased from Aldrich and used as received. Thiodiglycol (Janssen) was vacuum distilled and stored over molecular sieve 4A. Thionyl chloride (BDH; Poole, UK) was distilled at atmospheric pressure from quinoline (Fluka; Buchs, Switzerland) and linseed oil (Brocades; Delft, The Netherlands) successively and finally in a Fischer micro cracking tube column. Dimethylformamide and dimethylsulfoxide were purchased from EGA. (Steinheim/Albuch, FRG). Acetic acid (Merck) was dried and purified by refluxing with acetic anhydride (Merck), followed by fractional distillation. Lithium aluminum hydride, calcium hydride and sodium periodate were purchased from Janssen. Dioxane (distilled from $LiAlH_4$, p.a. quality), poly(ethylene glycol), and ethylene glycol were purchased from Merck.

Toluene (p.a.), diethyl ether and dichloromethane were obtained from Lamers and Pleugers (Den Bosch, The Netherlands). Sodium carbonate (anhydrous, Merck) was dried at 200 °C/40 mbar during 8 h in a vacuum oven. Sodium bicarbonate was purchased from Lamers & Pleugers. Hydrochloric acid gas was purchased from Union Carbide (UCAR; Oevel, Belgium), in a lecture bottle (p.a.). Methanol (Merck, p.a.) for HPLC and Lobar chromatography was used as purchased.

Penicillin and streptomycin were purchased from Gist Brocades (Delft, NL), whereas glutamine was obtained from BDH (Poole, UK). (Sub)class-specific rabbit-anti-mouse antibodies (IgM, IgG1, IgG2a, IgG2b, IgG3, κ, and λ), goat-anti-rabbit-IgG-alkaline phosphatase, goat-anti-mouse-IgG-alkaline phosphatase, calf-thymus DNA, deoxyribonuclease I (EC 3.1.21.1), alkaline phosphatase, type III (EC 3.1.3.1), RNAse A, trypsin, human hemoglobin, bovine serum albumin, chicken gammaglobulin, and hypoxanthine were obtained from Sigma (St Louis, U.S.A.). Proteinase K was procured from Merck (Darmstadt, FRG). Goat-anti-mouse-IgG-FITC was obtained from the Central Laboratory of the Dutch Red Cross Blood Transfusion Service (Amsterdam). Phosphodiesterase, 3',5'-cyclic nucleotide (from beef heart, E.C.3.1.4.37; lot no.:10781830-15), RNAse T1, nuclease P1 (E.C. 3.1.4), 4-methylumbelliferyl phosphate, and 4-nitrophenyl phosphate were obtained from Boehringer (Mannheim, FRG) and keyhole limpet hemocyanin (KLH) from Calbiochem (U.S.A.).

All other chemicals used were dried and/or purified according to standard laboratory practice.

II.2. Methods

Melting points were determined using a Buchi Type S melting point apparatus. Elemental analyses (C, H and N) were performed in duplicate or triplicate using a Heraeus CHN-O-Rapid element analyzer. High efficiency distillations were carried out in a micro cracking tube column (Fischer, type MMS 155, column length 200 mm, 40 theoretical plates, nominal charge 1- 10 ml).
The pH-stat reactions were performed using a Radiometer set, which consisted of a TTA80 titration assembly, a TTT80 titrator with an ABU autoburette containing 0.1 N NaOH, a PHM82 standard pH meter and a

REA270 pH-stat unit. Lyophilization was done with a Virtis Bench Top freeze dryer model 10-030. Optical rotations were measured with a Perkin Elmer 241 polarimeter. UV spectra were recorded on a Beckman UV-7 spectrophotometer.

HPLC analyses were routinely carried out with two Waters 6000 A pumps controlled by a model 660 solvent programmer to give a flow rate of 1 ml/min. Detection was done with an Applied Biosystems 757 variable wavelength absorbance detector, usually set at 285 nm. UV spectra of effluent peaks were obtained using a Waters model 990 diode array detector and wavelength settings of 190-600 nm. Three sets of chromatographic conditions were used for HPLC; system A: reversed phase chromatography on an RP 18 column, with various ratios of aqueous buffer and methanol; system B: ion pair chromatography on an RP 18 column with an aqueous buffer system containing 4 mM $(nBu)_4NHSO_4$ and 0.3 M KH_2PO_4 (pH 6.0) and methanol (2/1, v/v); system C: cation exchange chromatography on a SAX column, with an aqueous gradient running from 1 mM to 300 mM KH_2PO_4 in 30 min. See II.2.6 for the HPLC conditions used to analyze (alkylated) nucleosides resulting from hydrolyzed DNA; see II.15.5 for HPLC conditions to analyze peptides resulting from trypsinized globin and II.15.6 for HPLC analysis of phenyl isocyanate derivatives of amino acids. Semi-preparative HPLC was performed with Lichrosorb RP 18 columns (5 or 7 μm particles, 250 x 10 or 200 x 20 mm), using the same pumps, detector and solvents as for the analytical HPLC apparatus. Flow rates varied from 3-10 ml/min, at pressures up to 5000 psi. Micro-LC analyses of the reaction mixtures of N-acetyl-amino acid-methylamides with mustard gas were performed on a homemade Lichrosorb RP 18 column (7 μm particles, 45 cm x 0.3 mm i.d.) with a Waters 590 pump to give a flow rate of 4 μl/min. Detection was done at 214 nm with the absorbance detector described above. The eluent used was a mixture of 0.01 M ammonium acetate buffer, pH 5.0, containing triethylamine (0.1 ml/l buffer), and methanol (9/1, v/v).
Medium pressure liquid chromatography was carried out using Merck Lobar prepacked glass columns, size C (440 x 37 mm), filled with Lichroprep RP 18 (40- 63 μm), or with Lichroprep Si-60 silicagel. A gear pump (type VZE, Verder, The Netherlands), equipped with Teflon gears, was used to elute the columns at flow rates of 2-20 ml/min, at a maximum pressure of 5 bar. Detection was done with the absorbance detector described above, equipped with a simple stream splitter and set at higher wavelengths to reduce its sensitivity. Slight alterations of the methanol percentage in the eluent were usually necessary, as compared to the analytical HPLC runs, in order to achieve sufficient separation of the components. Sample loads of up to 5 mg proved practicable, although a second separation run followed by recrystallization was usually necessary to obtain analytically pure products.

TLC was performed on Silica Gel (DC-fertigfolien F-1500LS25, Schleicher & Schull). Elution was performed with 8% (system A) or with 14% (system B) methanol/dichloromethane (v/v). Spots on TLC plates were made visible by ultraviolet light (254 nm), using a Raytech UV lamp, model LS-88, or by spraying with 20% H_2SO_4/methanol

and subsequent heating with a hot air blower (Kress,HLG 2000 E). TLC analyses for radioactive products were carried out on Merck silica gel HPTLC plates (60 F 254; 5 x 10 cm), with trichloromethane/acetone (50/40) as the mobile phase. The same solvent system was used for reversed phase TLC analyses on Merck RP 18 plates (5 x 20 cm).

Low pressure ion exchange chromatography was performed using Q Sepharose fast flow material (strong anion exchanger, Pharmacia), using a gradient system of water and a 1 M sodium chloride solution, or with Pharmacia A 25 material (strong cation exchanger), using an aqueous tetraethylammonium bicarbonate gradient system. Gel filtration was performed with Sephadex G-10 material (Pharmacia), using water as eluent. A P-1 pump, a GP-250 gradient programmer, a Frac-100 fraction collector, a single path monitor UV-1 and UV-1/214, two PSV-100 switch valves (all purchased from Pharmacia) and a Kipp & Zonen BD 41 recorder were used for ion exchange and gel filtration. Column chromatography was performed with silicagel 60 (Merck, 230-400 mesh), with dichloromethane/methanol or petroleum-ether/dichloromethane as eluent.

Gas-liquid chromatography (GLC) was performed on a Chrompack model 438A instrument, equipped with an FID detector and a wide bore glass capillary column (50 m, i.d. 0.7 mm) coated with SE 30 (1 μm film thickness). Nitrogen was used as the carrier gas, while the oven temperature was programmed from 70 to 180 °C at 15 °C/min. The temperature of the detector and injector were 250 and 140 °C, respectively.

LC-MS spectra were recorded on a Nermag R10-10C quadrupole instrument, equipped with a TSP ion source (Nermag), which was coupled with the liquid chromatography system via a Vestec TSP interface. The mass spectrometer was operated in the positive ion mode. The temperature of the TSP vaporizer ranged from 250 to 260 °C during gradient elution, while the ion block was maintained at 230 °C. The scan time was 1 s for m/z 100-450. The liquid chromatography system comprised an RP-18 reversed phase column with 0.1 M aqueous ammonium acetate and methanol in varying ratios as eluent.

Electron impact mass spectra were recorded on a VG70-250S mass spectrometer in low resolution mode (RP1000, 10% dal); m/z 25-500, source temp. 300 °C, electron energy 70 eV, direct inlet. The mass spectrometer was coupled to a HP 5890A gas chromatograph, equipped with a CPSIL 5CB fused silica capillary column (length 50 m, i.d. 0.32 mm, film thickness 0.5 μm).

Fast Atom Bombardment mass spectra were recorded at TNO-CIVO, Zeist, The Netherlands, on a Finnigan Mat90 mass spectrometer. The analytes were ionized from a glycerol/thioglycerol matrix with Xenon atoms (7-8 kV acceleration voltage).

^{1}H- and ^{13}C-NMR spectra were recorded at 30 °C using a Varian VXR 400S spectrometer operating at 400.0 MHz and 100.6 MHz, respectively.

All spectra were obtained in the Fourier Transform mode, typically with parameters similar to the following:
for 1H: sweep width 6000 Hz, digital resolution 0.25 Hz, pulse flip angle 50 to 60 degrees, pulse interval 73,
for 13C: sweep width 20000 Hz, digital resolution 1 Hz, pulse flip angle 50 to 60 degrees, pulse interval 25.
Chemical shifts are given in ppm relative to TMS. When DMSO-d_6 was used as a solvent, the solvent signal at 2.525 ppm (residual DMSO-d_5) served as reference for ^{1}H, whereas the signal at 39.6 ppm served as a reference for ^{13}C. When $CDCl_3$ was used as a solvent, the solvent signal at 77.1 ppm served as reference for ^{13}C. When D_2O was used as a solvent, the signals of 3-(trimethylsilyl)-1-propane-sulfonic acid (DSS) served as a reference for ^{1}H and ^{13}C. The inaccuracy of the ^{1}H-^{1}H couplings is estimated at 0.3 Hz.

Gamma irradiations with a ^{60}cobalt source were performed with a Gamma cell 100, Atomic Energy of Canada Ltd, Ottawa, Canada. Radioactive products on TLC plates were scanned with a Berthold model LB 2723 DC scanner, equipped with a windowless proportional counting tube. Radio-GC was carried out on a Packard model 438 gas chromatograph, equipped with a wide bore capillary glass column (30 m, i.d. 0.7 mm) coated with SE 30, and a modified flame ionization detector. The detector outlet was connected to a simple fraction collecting device by means of a piece of teflon tubing which was heated electrically. Packard Carbo-Sorb was used as the SO_2 trapping solution (20 ml per GC fraction collected). Radioactivity measurements were performed on a Packard Tri-Carb Series 4000 Minaxi liquid scintillation spectrometer with Picofluor (Packard) as the scintillation cocktail; counting efficiencies ranged from 90 to 94%. Solutions of the labelled compounds in dichloromethane were measured (100 µl), or 1 ml aliquots of the Carbo-Sorb trapping solution.

II.3. [^{35}S]Mustard gas

II.3.1. Synthesis of [^{35}S]mustard gas (first batch) (50)

Hydrogen [^{35}S]sulfide, cold hydrogen sulfide and ethylene oxide were handled and transferred in a vacuum manifold system evacuated to a pressure of 10^{-3} mmHg. The manifold consisted of a vacuum line to which were connected a reaction flask, the break-seal ampoule containing labelled hydrogen sulfide, a graduated flask with cold hydrogen sulfide and an ampoule containing the cooled ethylene oxide stock. A mercury manometer was used to measure the pressure of the various gases in the system as a means of determining their relative quantities. The reaction of hydrogen sulfide with ethylene oxide was initiated by irradiation with a 500 W halogen lamp, controlled by a thyristor power regulator. The lamp was a cheap general purpose type, purchased on a home-worker market. No further specifications or indication of type and brand name were stated on its casing. Hydrogen sulfide (22 ml at 25 °C) was admitted to the evacuated manifold from the calibrated flask and condensed into the 30 ml reaction flask by cooling the flask in liquid nitrogen. After disconnecting the graduated flask from the manifold, hydrogen sulfide was allowed to

evaporate into the manifold in order to saturate all possible traces of grease, etc. In this way any subsequent loss of labelled material by absorbtion was minimized. Next the break-seal ampoule containing the hydrogen [^{35}S]sulfide was cooled in liquid nitrogen and opened, thus condensing the unlabelled compound with the labelled one in the ampoule. The diluted radioactive hydrogen sulfide was condensed into the reaction flask, the break-seal ampoule was shut off from the system, and the pressure of the gas was measured after the system had adopted room temperature. Hydrogen sulfide was recondensed into the break-seal ampoule and the ampoule was closed temporarily. Ethylene oxide was evaporated slowly into the manifold from the liquefied stock until slightly more than twice the pressure of hydrogen sulfide. Finally, both ethylene oxide and diluted labelled hydrogen sulfide were condensed into the reaction flask and the entire system was heated with a hot air blower for ten minutes to facilitate complete transfer of the reactants.

The reaction flask was then sealed off with a flame and removed from the manifold, after which it was allowed to slowly reach room temperature. The small amount of mobile liquid which remained on the bottom of the flask at room temperature evaporated completely when the flask was heated with a photo lamp to 80 °C, as measured with a thermocouple probe attached to the irradiated outside surface of the flask. In the course of the heating period a condensate reappeared, this time as a viscous liquid. After 48 h the reaction was considered to be complete and the flask was cooled and opened.

Dichloromethane (1 ml) was added and a 1 µl sample of the solution was removed for analyses. The flask was equipped with a magnetic stirring bar and a reflux condensor connected to a drying tube. The solution was refluxed for a few minutes to drive off any remaining hydrogen sulfide and/or excess ethylene oxide. No escaping radioactive gases were detected, which indicated the completeness of the reaction. Next the reaction flask was cooled by immersion into an ice bath and 0.1 ml of thionyl chloride was added at once with stirring. After 2 minutes the evolution of gas ceased and another 0.1 ml of thionyl chloride was added, followed after 2 min by a final portion (0.4 ml). This last addition of thionyl chloride did not cause any evolution of gas. The reaction mixture was allowed to reach room temperature in the course of ca. 1 h. Finally it was refluxed with stirring for 1 h at a bath temperature of 50 °C.

After GC analysis, which confirmed the completion of the reaction, the major part of the dichloromethane was removed by distillation at atmospheric pressure. The last traces of solvent and excess thionyl chloride were removed by vacuum distillation at room temperature and the crude mustard gas was separated from high boiling byproducts by distillation at 80 °C/10^{-2} mmHg in a short path micro distillation apparatus. The yield of crude product was 81 mg, or 51%. A relatively large amount of black tarry residue, probably consisting of polymerization and/or decomposition products, remained in the distillation flask.

Since GC analysis showed the presence of ca. 15% of a higher boiling compound, a second distillation was carried out. This time the distillation apparatus was equipped with a small plug of quartz wool, acting as a fractionating column. Care was taken to avoid the co-distillation of the byproduct by keeping the distillation speed as low as practicable at a bath temperature of 60-70 °C and a pressure of 0.1 mmHg. Two fractions of 24 and 26 mg, respectively, were collected, giving a total chemical yield of pure product of 50 mg (31.5%).

II.3.2. Analysis of [^{35}S]thiodiglycol

The 1 μl sample of [^{35}S]thiodiglycol, removed from the dichloromethane solution, was diluted to 10.0 ml with dichloromethane, and 100 μl aliquots of this solution were counted in Picofluor. A total activity of 1,035 MBq (27.9 mCi) was found, indicating a quantitative recovery (102%) of the radioactivity purchased as hydrogen [^{35}S]sulfide.

Radio TLC analyses revealed the presence of a series of radioactive contaminants. It was suspected that the reaction of hydrogen sulfide with ethylene oxide had introduced ethoxy groups into the thiodiglycol, thus providing a possible explanation for the TLC pattern found. The formation of these byproducts had not been encountered in previous work under identical reaction conditions (51). By integration of the peak surfaces a maximum content of 75% of the desired product was found. To minimize the loss of radioactive material as much as possible it was decided to purify the final product mustard gas, rather than the intermediate thiodiglycol.

II.3.3. Analysis of [^{35}S]mustard gas

GC analysis of the crude reaction mixture surprisingly showed a rather clean chromatogram. Approximately 2% of a single higher boiling compound was present, and contaminants similar to those present in thiodiglycol were not observed. After the first distillation, however, the amount of this byproduct had increased to approximately 15%, as determined by integration of the peak surfaces, while small amounts of two lower boiling compounds were also present. Radio-GC (52) gave poorly reproducible results, as the flow of [^{35}S]SO_2 tended to be retarded by the heated teflon tubing leading from the flame ionization detector to the fraction collector. The resulting memory effect prohibited exact fraction cutting and determination of the radioactivity in the various effluent peaks. An approximation of the activity present in the peaks of the impurities resulted in a quantity of about 15% of the total activity and, therefore, both the chemical and the radiochemical purity were estimated to be 85%.

A sample of 20 ng of the product was further investigated with GC-MS analysis. The mass spectrum (EI) of the major peak was identical with the spectrum of authentic mustard gas. The spectrum of the higher boiling contaminant was identified as 1-(2'-chloroethoxy)-2-(2'-

chloroethylthio)ethane, $Cl\text{-}C_2H_4\text{-}O\text{-}C_2H_4\text{-}S\text{-}C_2H_4\text{-}Cl$, by comparison with the spectrum of material which had been isolated from technical grade mustard gas. The identification confirmed the previously suspected formation of byproducts containing ethoxy groups. Radio-TLC analysis (53) could not separate the byproduct from mustard gas, neither on silica gel plates nor on reversed phase plates (R_F 0.72 and 0.76, respectively). In both systems only one single spot was detected, in addition to some tailing on the plates which is not uncommon for highly radioactive compounds.

After the second distillation the two fractions obtained were examined with GC analysis again. Only the second fraction contained a detectable amount of the high boiling impurity, while both fractions contained small amounts of two lower boiling compounds. Calculation of the peak surfaces resulted in a chemical purity of 95%, while the radiochemical purity was estimated as 98% because only the first of the two low boiling impurities contained any radioactivity (< 2%). The distillation residue contained far more of the high boiling impurity than mustard gas, which was a further indication for the effectiveness of the separation by distillation. Due to the above-mentioned difficulties with radio-GC and radio-TLC no exact figures could be given. As expected radio-TLC analyses of the end product showed again the presence of a single peak in the chromatogram.

The specific activity of the purified mustard gas was determined by measuring the activity of 10 µl aliquots of a solution of 1 µl of fractions 1 and 2 in 1.0 ml of dichloromethane. Assuming a density of mustard gas of 1.27, specific activities of 855 MBq/mmol (23.1 mCi/mmol) and 877 MBq/mmol (23.7 mCi/mmol), respectively, were found, giving a radiochemical yield of approximately 27% (corrected for radioactive decay) and a chemical yield of 31.5%.

II.3.4. Synthesis of [^{35}S]mustard gas (second batch)

The synthesis was repeated as described in II.3.1, starting with 740 MBq hydrogen [^{35}S]sulfide (5.74 GBq/mmol). The material was diluted with cold hydrogen sulfide to ca. 1 mmol, as described before. Reaction with a fresh batch of ethylene oxide yielded thiodiglycol in quantitative yield, but again containing the same impurities. Chlorination with thionyl chloride afforded a reaction mixture in which higher boiling impurities were absent. After a first distillation, however, ca. 15% of the higher boiling impurity was detected with GC analysis. The second distillation removed this impurity, but the yield was as low as in the first run. A quantity of 45 mg of mustard gas was obtained, containing only a small amount of volatile contaminants (purity ca. 98%, GC). The radiochemical yield of the intermediate thiodiglycol was essentially quantitative (758 MBq, purity ca. 75% radio-TLC), whereas 154 MBq of mustard gas was obtained (574 MBq/mmol, 21%). The chemical yield was 28%.

II.4. Synthesis of mustard gas derivatives

II.4.1. 2-Acetoxyethyl 2'-hydroxyethyl sulfide

Thiodiglycol (122 g, 1 mol) was dissolved in dichloromethane (1 l), together with dry pyridine (7.9 g, 0.1 mol). A solution of acetyl chloride (7.85 g, 0.1 mol) in dichloromethane (100 ml) was added dropwise with stirring at room temperature in the course of 1.5 h. After stirring for another 2 h at room temperature the reaction mixture was poured into water (250 ml). The organic layer was washed three times with water to remove excess of thiodiglycol and pyridinium salts. HPLC analysis (system A) showed almost complete removal of thiodiglycol. Distillation of the crude product over a Fischer microcracking tube column gave 8.7 g (49%) of the desired product as a thin, colorless oil, b.p. 131-135 °C/2 mmHg. Isothermal GC analysis at 100 °C of the product on a short (27 m; i.d. = 0.7 mm) column coated with SE-30, as well as HPLC analysis (system A) showed that the purity of the product was ca. 97%, with thiodiglycol and its diacetate as major contaminants in approximately equal amounts.

^{1}H-NMR in $CDCl_3$:
4.24[t,2H,OCH_2], 3.78[t,2H,CH_2OH], 2.78[t,2H,CH_2S], 2.77[t,2H,SCH_2], 2.10[s,1H,OH], 2.08[s,3H,CH_3]

^{13}C-NMR in $CDCl_3$:
170.9[C=O], 63.4[OCH_2], 60.8[CH_2OH], 35.2[SCH_2], 30.3[CH_2S], 20.7[CH_3]

Thermospray-LC-MS:
m/z=165[MH^+], 182[MNH_4^+], 224[MNH_4^+ of di-(2-acetoxyethyl) sulfide]

II.4.2. 2-Acetoxyethyl 2'-chloroethyl sulfide (54)

2-Acetoxyethyl 2'-hydroxyethyl sulfide (20 g, 0.12 mol) was dissolved in dichloromethane (75 ml) and thionyl chloride (15 g, 0.13 mol) in dichloromethane (20 ml) was added with stirring and cooling in ice at 0-3 °C in 45 min. The evolution of gas increased strongly when after 30 min of stirring at 2 °C the temperature was allowed to rise slowly. Occasional cooling was therefore re-applied during 2 h until finally room temperature was reached. The mixture was then gently refluxed, until no more evolution of gas was evident (45 min). Evaporation at atmospheric pressure, followed by a simple vacuum distillation yielded 21.2 g (96.8%) of a slightly yellow oil, b.p. 80-82 °C/0.3 mmHg. GC-MS analysis revealed the presence of ca. 1% mustard gas and ca. 11% di-(2-acetoxyethyl) sulfide. The product was purified to a gas chromatographic purity of 99.7% by a second vacuum distillation over the Fischer microcracking tube column. Yield 17.3 g (79%) of colorless oil, b.p. 72-74 °C/0.2 mmHg.

^{1}H-NMR in $CDCl_3$:
4.23 [t,J=6.7 Hz,2H,$CH_2OC(O)$], 3.65[m,2H,CH_2Cl], 2.92 [m,2H,CH_2S], 2.80 [t, J=6.7 Hz,2H,CH_2S], 2.08[s,3H,$COCH_3$]

^{13}C-NMR in $CDCl_3$:
170.8[CO], 63.6[$\underline{C}H_2OC$], 43.0[CH_2Cl], 34.6[CH_2S], 30.9[CH_2S], 20.9[$\underline{C}H_3CO$]

EI-MS (rel.int.):
m/z= 122(32)[Cl-CH_2-CH_2-S-CH=$CH_2^{+\cdot}$], 109(5)[Cl-CH_2-CH_2-S-CH_2^+], 73(26)[H_2C=CH-S-CH_2^+], 63(11)[Cl-CH_2-CH_2^+], 60(69)[CH_3-$COOH^{+\cdot}$], 43(100)[CH_3-CO^+], 27(27)[$C_2H_3^+$]

II.4.3. 2-t-Butyldimethylsilyloxyethyl 2'-hydroxyethyl sulfide

To a solution of thiodiglycol (10 g, 0.082 mol) and triethylamine (4.7 g, 0.041 mol) in ether/acetone (50 ml/20 ml) was added with stirring t-butyl-dimethylchlorosilane (6.2 g, 0.04 mol) in ether (30 ml) at 20 °C. A slightly exothermic reaction raised the temperature to 23 °C and cooling was applied to maintain a reaction temperature of 20 °C. At the end of the addition thiodiglycol separated from the mixture and acetone (10 ml) was added to dissolve it again. After standing for one night GC-MS, ^{1}H- and ^{13}C-NMR analysis revealed the presence of mono-ether, di-ether and thiodiglycol in a ratio of 10:1:3, respectively. The reaction mixture was filtered and evaporated. The remaining oil was dissolved in ether (150 ml) and the yield of a preliminary synthesis, containing ca. 9 g of 2-t-butyldimethylsilyloxyethyl 2'-hydroxyethyl sulfide, was added. A portion of the latter product had been used for extraction experiments with water, sodium bicarbonate and sodium carbonate, from which it was concluded that thiodiglycol could be removed by washing with water whereas the di-ether content was not changed.

The solution was therefore washed with 3 successive portions of water (50 ml) and evaporated again. The residue was dried by co-evaporation of water with dry acetone and the residue was distilled at the lowest possible temperature and pressure in a molecular still. Despite the poor fractionating power of a molecular still, fractions containing 92% (13.3 g) and 80% (3.0 g) of the desired product were obtained at a distillation temperature of 100 °C and a pressure of 0.001 mmHg. The main fractions of the molecular distillation were pooled and distilled again in the Fischer microcracking tube column, yielding 13.7 g of the desired product (b.p. 91-3 °C/0.1 mmHg) with a gas chromatographic purity of 98%, containing 1.7% of the di-ether.

^{1}H-NMR in DMSO-d_6:
4.74[t,=5.6 Hz,1H,OH], 3.73[t,J=6.9 Hz,2H,CH_2OSi],3.54[dt,J=5.6 Hz and J=6.9 Hz,2H,$C\underline{H}_2OH$],2.64[t,J=6.9 Hz,2H,CH_2S], 2.61[t,J=6.9 Hz,2H,CH_2S],2.61[t,J=6.9 Hz,2H,CH_2S], 0.89[s,9H,$C(CH_3)_3$], 0.07[s,6H,$Si(CH_3)_2$].

^{13}C-NMR in DMSO-d_6:
62.9[Si-O-C], 61.1[C-OH], 34.4[S-C-C-OH], 34.1[Si-O-C-C-S], 25.8[C3-C], 17.9[C3-C], -5.3[Si-C2].

EI-MS:
m/z= 221[M-CH_3], 205[M-(CH_3+CH_4)], 179[M-C_4H_9], 163[M-(C_4H_9+CH_4).

II.4.4. 2-Chloroethyl 2'-hydroxyethyl sulfide (semi-mustard gas) (54)

2-Mercaptoethanol (20 g, 0.25 mol; 95.5% purity) was dissolved in methanol (150 ml, 0.005% water). Sodium hydride (11 g, 0.25 mol, 60% suspension in mineral oil) was added in portions with stirring and cooling in ice within 10 min. After the addition of 1,2-dichloroethane (150 ml, 0.005% water) the solution was left for 5 days in a refrigerator at 5 °C. Next, precipitated sodium chloride was removed by decanting the solution, which was then evaporated at 20 °C/15 mmHg. The residue was dissolved in ether (200 ml) and the solution was washed with ice water (50 ml), dried over $MgSO_4$, filtered and evaporated again. The residue was quickly washed with petroleum ether (4 x 50 ml, 0.004% water) to remove the mineral oil. After degassing the residue for 10 min at 20 °C/15 mmHg, ca. 10 g of crude product remained (28.5%) which was immediately redissolved in ether (200 ml). The gas chromatographic purity was 95%. Precipitated decomposition products were removed by filtration before using the product for further syntheses. The product was deemed too unstable for complete analysis. It was identified by means of GC-MS analysis.

EI-MS:
m/z= 140 ($M^{+\cdot}$), 109 ($ClCH_2CH_2SCH_2^{+\cdot}$), 104 ($M^{+\cdot}$-HCl)

II.4.5. 2- Trimethylsilyloxyethyl 2'-chloroethylsulfide

A solution of semi-mustard gas (ca. 10 g) in ether (200 ml, 0.005% water) was cooled to -60 °C and triethylamine (7.2 g, 72 mmol, 0.01% water) was added with stirring in the course of 10 min. Next a solution of bromotrimethylsilane (10 g, 72 mmol) in ether (50 ml) was added dropwise. The reaction mixture was stirred for 2 h at -50 °C and was left at -20 °C for 2 days. After bringing the mixture to room temperature, the precipitate was removed by filtration. The filtrate was evaporated at reduced pressure and the residue was vacuum distilled. The yield of colorless liquid was 10.0 g (19% overall), boiling point 61-2 °C/0.6 mmHg. The purity according to GC- and ^{13}C-NMR analysis was 93 and 88%, respectively, the main impurity being semi-mustard. The lower purity as determined by NMR was probably due to traces of water in the NMR solvent, which caused partial hydrolysis of the product. Redistillation of the product in the Fischer column did not improve the quality of the product.

Elemental analysis ($C_7H_{17}ClOSSi$; M.W. 212.5):

Calc.:	C	39.23%	Found: 39.36 ± 0.14%
	H	7.95%	8.00 ± 0.05%
	Cl	17.13%	16.89 ± 0.23%
	S	15.85%	15.45 ± 0.37%

^1H-NMR in $CDCl_3$:
3.75[t,J=6.6 Hz,2H,CH_2-OSi], 3.66[m,2H,CH_2-Cl], 2.90[m,2H,SCH_2-CH_2Cl], 2.69[t,2H,J=6.6 Hz,SCH_2CH_2O], 0.13 [s,9H,$Si(CH_3)_3$]

^{13}C-NMR in $CDCl_3$:
62.3 [t,CH_2OSi], 42.6[t,CH_2Cl], 34.2[t,SCH_2], 34.1[t,SCH_2],
-1.0[q,$Si(CH_3)_3$]

EI-MS (rel.int.):
m/z= 176(15)[M-HCl]$^+$, 116(4)[H_2C=CH-O-$Si(CH_3)_3$]$^{+\cdot}$,
103(56)[$H_2COSi(CH_3)_3$]$^+$, 93(35)[$(CH_3)_2SiCl$]$^+$, 73(100)[$(CH_3)_3Si$]$^+$

II.5. Synthesis of guanine and adenine adducts

II.5.1. N7-(2'-Hydroxyethylthioethyl)-guanine (30)

Guanosine (4.8 g, 16.8 mmol) and mustard gas (3.3 ml, 27.6 mmol) in acetic acid (60 ml) were reacted at 100 °C. A clear solution was obtained after 30 min and heating was continued for a total period of 2 h. After cooling, a small amount of solid material precipitated, which was collected by filtration. The filtrate was subjected to evaporation at reduced pressure, which caused the precipitation of large amounts of a very fine off-white solid after a short time. This material was also collected by filtration and the filtrate was evaporated to dryness. After addition of 1 N aqueous HCl (50 ml) to the residue, the solution obtained was extracted with dichloromethane (4 x 10 ml) in order to remove excess mustard gas. Both batches of solids were added to the washed solution, using another 50 ml of 1 N HCl to rinse the filters. The combined solutions were heated at 100 °C for 1 h and aqueous hydrochloric acid was removed by evaporation at reduced pressure. Next the residue was dissolved in water (100 ml) and the solution was neutralized with conc. ammonia (ca. 5 ml). The resulting light brown precipitate was collected by filtration and subsequently extracted with 400 ml of boiling water. The remaining insoluble brown material was filtered off and the filtrate was cooled in ice. The precipitated product was collected. After drying in a vacuum desiccator, a total of 2.1 g of product resulted. According to thermospray-LC-MS analysis the product consisted mainly of the title compound (54%; m/z=256,MH^+), together with guanine (34%; m/z=152,MH^+), traces of the di-adduct (m/z=389,MH^+;411,MNa^+) and some further impurities (12%). The insoluble material contained guanine, together with a small amount of the title compound, a considerable quantity of a product that eluted somewhat later, possibly the corresponding di-adduct, and finally, large amounts of a great number of compounds, eluting only when 100% methanol was used. An amount of 100 mg of raw material was purified on the reversed phase Lobar column in batches of 20 mg each, which were dissolved in 1 N HCl prior to injection onto the column. Elution took place at a flow rate of 4-6 ml/ min with 25 mM NH_4HCO_3 in 25% methanol/water. A total of 45 mg of purified product was obtained by evaporation of the pooled product eluates. This material was further purified by recrystallization from boiling water, which removed an unidentified brown material, together with impurities probably originating from the column. A white crystalline material (35 mg) resulted after drying in a vacuum desiccator, which had a purity of 97% according to HPLC analysis (system A) at 254-300 nm. No impurities were detectable

with ^{1}H- or ^{13}C-NMR. M.p.: decomposition >280 °C. Decomposition above 280 °C has also been reported by Brookes and Lawley (30).

^{1}H-NMR in DMSO-d_6:
10.83[bs,1H,NH], 7.94[s,1H,C8H], 6.17[s,2H,NH_2], 4.78[t,J=5.3 Hz,OH], 4.35 [t,J=6.7 Hz,CH_2N], 3.54[dt,J=±5.6 Hz and 6.5 Hz,2H,$C\underline{H}_2OH$], 2.97[t,J=6.7Hz,2H,N-C-CH_2S], 2.59[t,J=6.7 Hz,2H,SCH_2-C-OH]

^{13}C-NMR in DMSO-d_6:
160.2[C4], 154.6[C6], 152.8[C2], 143.5[C8], 107.9[C5], 60.8[CH_2OH], 46.0 [NCH_2], 33.8[$S\underline{C}H_2$-C-OH], 32.2[N-C-$\underline{C}H_2S$]

Thermospray MS:
m/z=256(MH^+), 270 (MH^+ of product methylated at hydroxyl group; occurs when MeOH is present in eluent), 298(MH^+ of product acetylated at hydroxyl group during thermospray).

UV spectra:
pH=1 : 249 nm (max)
pH=7 : 284 nm (max)
pH=12: 280 nm (max)

II.5.2. Di-(2-guanin-7'-yl-ethyl) sulfide (34)

Mustard gas (0.5 ml, 4.2 mmol) was suspended in a solution of guanosine-5'-phospate di-sodium salt trihydrate (2 g, 4.3 mmol) in water (20 ml). The mixture was stirred magnetically at room temperature for 24 h, during which time the mustard gas slowly dissolved. After the addition of water (10 ml) and conc. HCl (2 ml) the solution was heated at 100 °C for 1 h. The hydrolyzed reaction mixture was left at room temperature overnight and the precipitated solid material was collected by filtration. LC-MS analysis revealed the presence of guanosine (m/z = 152,MH^+), N7-(2'-hydroxyethylthioethyl)-guanine (m/z =256,MH^+), and of the corresponding di-adduct (m/z =389,MH^+). Three successive extractions with diluted HCl (pH 2) during 15 min left a product containing 86% di-adduct. The product was combined with crude product from a similar experiment and was recrystallized from boiling aqueous HCl (pH 2). After drying 115 mg of product was obtained (HPLC 97%, 285 nm; system A), m.p. 230 °C (decomposition). The IR spectrum clearly showed that the di-adduct was obtained as its hydrochloride salt and the product was stored in that form at -20 °C, to minimize the risk of possible instability of the free base.

^{1}H-NMR in CF_3COOD:
8.95[s,2H,2C8H], 4.86[bt,J= ± 5 Hz,4H,2NCH_2], 3.27[bt,J= ± 5 Hz,2CH_2S]

^{13}C-NMR in CF_3COOD (signals of CF_3COOD at 115.7 and 162.9 ppm were used as reference):
154.6[C6], 154.0[C2], 145.9[C4], 140.7[C8], 109.5[C5], 50.0[NCH_2], 32.1 [CH_2S]

Thermospray MS:
m/z= 389(MH^+), 411(MNa^+)

UV spectra:
pH=1 : 249 nm (max)
pH=7 : 284 nm (max)
pH=12: 280 nm (max)

II.5.3. Synthesis of O6-(2"-hydroxyethylthioethyl)-2'-deoxyguanosine

II.5.3.1. 3',5',N2-Triacetyl-2'-deoxyguanosine (55)

To a suspension of 2'-deoxyguanosine (1.33 g, 5 mmol) in dry pyridine (200 ml) was added acetic acid anhydride (4.6 ml, 50 mmol), 4-dimethylaminopyridine (60 mg, 0.5 mmol) and triethylamine (7.6 ml, 50 mmol). The mixture was stirred for 20 h at 50 °C. Next, 50 ml of water was added to the cooled solution. Pyridine was evaporated at reduced pressure and another 100 ml of water was added. Water was evaporated under reduced pressure until crystallization occurred. Yield: 70% of yellow needles (1.4 g, 3.5 mmol). The purity of the product was confirmed by ^{1}H-NMR spectroscopy. M.p.: 203-206 °C [litt. (55): softening at 190 °C; decomposition at 225 °C].

^{1}H-NMR in DMSO-d_6:
11.7[s,1H,NH], 8.2[s,1H,H8], 6.2[t,1H,H1']; 5.3[d,1H,H3'],4.2-4.3[m,3H,H4',H5',H5"], 3.0,2.6[m,2H,H2',H2"], 2.2 [s,3H,NC(O)CH_3], 2.1[s,3H,OC(O)CH_3], 2.05[s,3H,OC(O)CH_3].

^{13}C-NMR in DMSO-d_6:
173.5, 170.1 and 169.9[3C(O)CH_3], 154.8[C6], 148.4[C4], 148.0[C2],137.4[C8], 120.4[C4], 83.0[C1'], 81.8[C4'], 74.4[C3'], 63.6[C5'],35.8 [C2'], 23.8,20.8,20.6(3CH_3).

Thermospray-LC-MS:
m/z= 394(MH^+), 416(MNa^+), 432(MK^+).

UV spectrum (methanol):
Maximum at 256.5 (255) nm; minimum at 224.5 (224) nm; shoulder at 277.5 (278) nm. [Values from litt. (55) between brackets)

II.5.3.2. 3',5',N2-Triacetyl-O6-(2"-acetoxyethylthioethyl)-2'-deoxy-guanosine (56,57)

3',5',N2-Triacetyl-2'-deoxyguanosine (0.8 g, 2 mmol) was dissolved in 8 ml of dry dichloromethane. To this solution, 2,4,6-triisopropylbenzenesulfonyl chloride (1.2 g, 4 mmol), triethylamine (1.2 ml, 8 mmol) and dimethylaminopyridine (15 mg; catalyst) were added. After 30 minutes TLC analysis (eluent 8% methanol in dichloromethane) showed that the reaction was complete. This is deduced from the complete disappearance of the 3',5',N2-triacetyl-2'-deoxyguanosine spot (R_f=0.1), and the appearance of a new, higher running spot (R_f=0.8), after visualization of the spots under UV

light or by spraying with 20% sulfuric acid/methanol and subsequent heating (which produces black spots). The product was purified by flash chromatography on a silica gel column (2x8 cm),eluted with dichloromethane. The intermediate thus obtained was co-evaporated with dry dioxane to dryness and dissolved in 1 ml of dichloromethane. 2-Acetoxyethyl 2'-hydroxyethyl sulfide (3.3 g, 20 mmol; confer II.4.1) was added to this solution and the mixture was cooled tc 0 °C. 1-Methylpyrrolidine (0.93 g, 11 mmol) was added and after stirring for 10 min, 1,8-diazabicyclo(5.4.0)undec-7-ene (DBU, 0.23 g, 1.5 mmol) was added. The mixture was stirred for another 2 h. The solution was diluted with 50 ml of dichloromethane and washed three times with a saturated aqueous solution of ammonium chloride. Dichloromethane was distilled off under diminished pressure and the residue was purified on a column (3x30 cm), which was filled with silica gel and saturated with a mixture of petroleum-ether 40-60 and dichloromethane (1/1,v/v). This mixture was also used to elute apolar impurities from the column, as could be checked with TLC. After this purification step the column was eluted with dichloromethane and methanol. The product was eluted with a gradient starting with 100% dichloromethane and ending up with 10% methanol in dichloromethane. The isolated product was still contaminated with starting material, as was shown with ^{1}H-NMR. Further purification was not deemed necessary.

II.5.3.3. <u>O6-(2"-Hydroxyethylthioethyl)-2'-deoxyguanosine</u>

After preparing a saturated solution of 3',5',N2-triacet ·O6-(2"-acetoxyethylthioethyl)-2'-deoxyguanosine in dioxane in a 100 ml round bottom flask, just enough aqueous ammonia (25%) was added to obtain a homogeneous solution. The flask was closed and set aside at 50 °C for 16 h. Next, the mixture was evaporated to a small volume. A final purification of the product was obtained by means of gel filtration on a G-10 column. The column was eluted at a rate of 1 ml/min and 10 ml fractions were collected. The title compound was collected in fractions 33-47 (checked with HPLC). The combined fractions were concentrated to a small volume and lyophilized. Some fractions which still contained N2 acetylated product were heated again with concentrated ammonia and once more purified on the G-10 column. The lyophilized product is a white fluffy material. The total yield, starting with 2 mmol of 3',5',N2-triacetyl-2'-deoxyguanosine was 90 milligrams (0.24 mmol, 12% overall yield). The product is very hygroscopic and liquefies within minutes when exposed to air. It was stored in a sealed flask in a refrigerator. According to ^{1}H-NMR the purity of the product is > 95%.

^{1}H-NMR in D_2O:
8.04[s,1H,H8], 6.31[dd,J=ca.7 Hz,H1'], 4.64 [m,H3'], 4.62[t,J=6.3 Hz,OCH_2], 4.16[m,H4'], 3.82[m,H5',H5"], 3.79[t,J=6.4 Hz,CH_2OH], 3.04[t,J=6.4Hz,CH_2S], 2.85 (t,J=6.3 Hz,CH_2S], 2.33 and 2.80[dd,H2',H2"].

^{13}C-NMR in D_2O:
163.1[C6], 162.1[C2], 155.2[C4], 141.4[C8], 116.9[C5], 89.8[C4'], 86.9[C4'], 73.9[C3'], 68.7[OCH_2], 64.4[C5'], 63.0[OH2], 41.4[C2'], 36.4[SCH_2], 32.5 [SCH_2].

Thermospray MS:
m/z= 372(MH^+), 256[MH^+ of O6-(2'-hydroxyethylthioethyl)-guanine], 152[MH^+ of guanine].

UV spectra :
pH=1 : 288.5 nm(max), 261 nm(min.), 243 nm(max), 232 nm(min.).
pH=7 : 281 nm(max), 262 nm(min.), 247.5 nm(max), 228 nm(min.).
pH=13: 281 nm(max), 262 nm(min.), 247.5 nm(max), 227 nm(min.).

II.5.3.4. <u>O6-(2'-Hydroxyethylthioethyl)-guanine</u>

O6-(2"-Hydroxyethylthioethyl)-2'-deoxyguanosine (30 mg, 0.08 mmol) was stirred with 25 ml of 0.1 M HCl for 10 min in a 100 ml round bottom flask. HPLC-analysis system A) showed that the starting material had almost completely disappeared. Two new peaks were visible, probably O6-(2'-hydroxyethylthioethyl)-guanine and guanine. The reaction mixture was neutralized with ammonia and evaporated under reduced pressure at 35 °C to a small volume. The residue was applied to a G-10 column. The product was collected in fractions 70-88 (12 ml fractions). The combined fractions were concentrated and lyophilized. The total yield was 8 mg (0.03 mmol, 39% yield). According to ^{1}H-NMR the purity of the product was >95%.

^{1}H-NMR in DMSO-d_6:
7.84[s,1H,H8], 6.2[s,2H,NH_2], 4.56[t,2H,CH_2O], 3.61[t,2H,CH_2OH], 2.96[t,2H,CH_2S], 2.72[t,2H,SCH_2].

^{13}C-NMR in DMSO-d_6:
159.7, 139.0, 65.1[OCH_2], 61.0[CH_2OH], 34.3[SCH_2], 30.1[SCH_2].

Thermospray MS:
m/z= 256(MH^+), 278(MNa^+), 152(MH^+ of guanine).

UV spectra:
pH=1 : 287.5 nm(max), 253 nm(min.).
pH=7 : 281.5 nm(max), 258 nm(min.), 239.5 nm(max), 228 nm(min.).
pH=13: 284 nm(max), 258 nm(min.), 245 nm(sh).

II.5.3.5. <u>Kinetics of dealkylation of O6-alkylated guanines</u>

Reactions were carried out in quartz cuvettes (1x1 cm) thermostatted at 25 °C. After mixing 2.94 ml of 0.05 M KCl solution of which the pH was adjusted to 0.5 with concentrated HCl and 0.06 ml stock solution (1 mg/ml) of O6-alkylated-guanine, UV scans were made every 30 min (2'-hydroxyethylthioethyl compound) or 10,000 min (ethyl compound). Kinetic runs were performed in duplicate. Rate constants were calculated from a plot of the log of the difference between the absorbance at a given time and the absorbance of the dealkylated

product at 248 nm, assuming (pseudo) first-order kinetics. At the selected wavelength, the differences between the absorbance of the dealkylated products and of the corresponding starting compounds are maximal. The absorbance value which was increased less than 0.0005 absorbance units at the next measurement was taken as the absorbance of the dealkylated product.

II.5.4. N3-(2'-Hydroxyethylthioethyl)-adenine (30)

Adenosine (5.0 g, 18.7 mmol) was suspended in acetic acid (65 ml) and mustard gas (3.5 ml, 29.3 mmol) was added. The mixture was stirred and heated to 100 °C. When the temperature had reached 75 °C a clear solution was obtained which was heated for an additional 1.5 h at 100 °C. After cooling and standing overnight at room temperature a small amount of solid material precipitated. The mixture was evaporated at reduced pressure, yielding a semi-solid brown residue. After addition of 1 N HCl (65 ml) the relatively large excess of unchanged mustard gas was extracted with dichloromethane (4 x 10 ml) and the washed solution was heated at 100 °C for 1 h. After 30 min thermospray-LC-MS analysis showed the presence of adenine (m/z= 136,MH^+), together with two new compounds which eluted later. Both products showed MH^+ at m/z= 240, corresponding with (2'-hydroxyethylthioethyl) adducts of adenine.

After cooling and evaporation to dryness the residue was taken up in water and the solution was neutralized with concentrated ammonia, which caused the colour of the solution to darken. After partial evaporation, the solution was left at room temperature during the night. The reaction mixture was evaporated to dryness after filtration and the residue was taken up in warm ethanol/water (6:1, v/v). Upon cooling, a second batch of solid was obtained, which consisted largely of inorganic material (NH_4Cl). The mother liquor, which was freed from more inorganic material by evaporation, dissolving the residue in methanol and precipitating with twice the volume of dichloromethane, was finally evaporated. The crude dark brown residual reaction mixture, containing adenine and the two adducts, was dissolved in water (5 ml). Small amounts of both adducts were isolated by means of reversed phase HPLC (system A) and were tentatively identified as the N3-and the N9-adducts by means of ^1H-NMR. A larger amount of the N3-adduct was isolated by means of liquid chromatography of 10 portions of 0.5 ml each on a reversed phase Lobar column (eluent 25% MeOH, 25 mM NH_4HCO_3, flow 6-10 ml/min, detection at 254 nm). The combined fractions were evaporated at reduced pressure and the residual N3-adduct was freed from column material by extraction with water. The pooled extracts (5 ml) were chromatographed again on the Lobar column. Subsequent evaporation, extraction and freeze drying yielded 51 mg of pale brown solid material. HPLC analysis confirmed the absence of adenosine and N9-adduct, purity ca. 98%. M.p.: slow discoloration to 187 °C, melting and rapid decomposition >188 °C. The product partially turned brown during freeze drying. This same discoloration was observed earlier in different stages of synthesis runs. However, small amounts of N3-

adduct remaining in glass ware and on filters did not turn brown on exposure to light and air for several weeks.

^{1}H-NMR in DMSO-d_6:
8.37[s,1H,C2H], 7.9[b,2H,NH_2], 7.78[s,1H,C8H], 4.49(t,J=6.7 Hz,2H,NCH_2], 3.56 [t,J=6.7 Hz,2H,CH_2OH], 3.12[t,J=6.7 Hz,2H,N-C-CH_2S], 2.64[t,J=6.6 Hz,2H,SCH_2-C-OH)

^{13}C-NMR in DMSO-d_6:
155.1[C6], 152.4[C8], 149.5[C4], 143.8[C2], 120.3[C5], 60.8[CH_2OH], 49.0[NCH_2], 33.9[SCH_2-C-OH], 30.3[SCH_2-C-N)

Thermospray MS:
m/z= 240

UV spectra (58):
pH 1 : 274 nm (max)
pH 7 : 273 nm (max)
pH 12: 272 nm (max)

II.6. Synthesis of 2'-deoxyguanosine 5'-phosphate adducts with mustard gas

II.6.1. Synthesis of N7-(2"-hydroxyethylthioethyl)-guanosine-5'-phosphate and of di-[2-((guanosine-5'-phosphate)-7-yl)-ethyl] sulfide (59)

In a titration vessel of 150 ml, guanosine-5'-phosphate (400 mg, 1 mmol) and mustard gas (0.32 g, 2 mmol) in 100 ml of water were stirred for 16 h. The pH (4.5) was kept constant during the reaction time with a pH-Stat using 1.0 N aqueous NaOH as titrating solvent. The reaction mixture became homogenous after a few hours. At the end of the reaction the release of hydrochloric acid had stopped since no further base was used for titration. According to ion pair HPLC (?14/260 nm; system B) two main products were formed, presumably the mono- and the di-adduct at the N7-position. The presence of starting material and thiodiglycol in the reaction mixture was verified by means of co-injection. The reaction mixture was extracted three times with dichloromethane in order to remove any residual mustard gas, and was subsequently evaporated until 10 ml of solution was left. The reaction mixture was first purified on a column (Pharmacia XK16, 30x2 cm) filled with Sepharose Q fast flow anion exchange material. The column was filled with an emulsion of Sepharose Q in a mixture of 20% ethanol/water (off factory). In order to remove ethanol, the column was flushed with water during 1 h at a speed of 3 ml/min. About 100 mg of crude reaction product dissolved in water (2 ml) was applied to the column, using the P-1 pump. Next, the column was washed for 30 min with water, at a flow of 3 ml/min. In this period the N7-adducts as well as thiodiglycol were flushed off from the column (HPLC). Subsequently, the sodium chloride concentration was increased to 1 M in water in the course of 10 minutes and was kept at this concentration for the next 20 min in order to elute guanosine-5'-phosphate from the column. Finally, the concentration was decreased

to 0% NaCl and the column was washed for 1 h with water in order to prepare the column for the next portion of 100 mg of crude product. After five runs the fractions containing mono- and di-adduct were combined and lyophilized. Reversed phase HPLC showed that almost all of the starting material had been removed.

In order to separate mono- and di-adduct, as well as to remove thiodiglycol and unidentified impurities, the combined fractions were rechromatographed on a Lobar C (440x37 mm) column filled with Q Sepharose fast flow material. This column contains much more anion exchange material than the earlier mentioned column, which was considered to be necessary for separating the mono- and di-adducts. For the gradient system we used two solvents, i.e., solvent A: distilled water and solvent B: 1 M aqueous NaCl. During the first 90 minutes the column was flushed with solvent A. Then, the gradient was increased to 1% B buffer in the course of 10 min and eluted for 230 min. Next, the concentration of B buffer was increased to 10% in 30 min and was held at this concentration for another 30 min. Finally, the amount of B buffer was increased to 30% in 60 min and then to 100% in the course of 60 min. After the eluent had reached a concentration of 100% B buffer the column was run for another 60 min. Next, the salt concentration was decreased to 0% in the course of 60 min and the system was eluted for another 60 min with solvent A in order to clean the column. The eluent was run at a constant speed of 3 ml/min. This purification step was also done batchwise with the slowly increasing gradient as described because the capacity of the column was low for the separation of the mono- and di-adducts, and for the removal of thiodiglycol. The monoadduct was collected between 450 and 580 min after the start of the run, whereas the di-adduct eluted between 580 and 600 min.

In order to separate the mono- and di-adducts from residual thiodiglycol and inorganic salts, gel filtration was used as a final purification step. A column (1 m x 2 cm) filled with Sephadex G-10 matrix was used. Water was used as an eluens at a speed of 1 ml/min and 5-ml fractions were collected. The monoadduct eluted after ca. 80 minutes (UV detection at 214 nm; chloride ions were detected with silver nitrate). The purification of this adduct was rather delicate, because the difference in retention times between product, thiodiglycol and salts was very small. Therefore, only the first 20 ml (four fractions) contained monoadduct which was free from salts and thiodiglycol. Impure fractions were recombined and repurified. The solutions containing pure monoadduct were evaporated to a small volume and were subsequently lyophilized. The product has a white fluffy appearance and is very hygroscopic. Altogether, 120 mg of the monoadduct di-sodium salt was obtained, with a purity of 94% (^{1}H-NMR).

^{1}H-NMR in DMSO-d_6:
9.60[s,1H,H8], 7.43[bs,2H,NH_2], 4.55+3.01+2.66+3.52(all t,J=6 Hz, 8H,$NCH_2CH_2SCH_2CH_2O$], 5.88[d,J=4.6 Hz,1H,H1'], 4.45[t,1H,H2'], 4.24[t,1H,H3'], 4.16[m,1H,H4'], 4.0[m,2H,H2-5']

^{1}H-NMR in D_2O:
9.28[s,1H,H8], 4.7+3.11(m,4H,NCH_2CH_2S], 2.77+3.74[both t,J=6Hz,SCH_2CH_2O], 6.09[d,J=3.6Hz,1H,H1'], 4.7[m,1H,H2'], 4.47[t,J=5Hz,1H,H3'], 4.41[m,1H,H4'], 4.12+4.24[m,2H,H2-5'].

^{13}C-NMR in DMSO-d_6:
154.0[C2 or C6], 150.1[C4], 107.3[C5], 156.5[C6 or C2], 137.2[C8].
49.0+31.5+34.0+61.3[$NCH_2CH_2SCH_2CH_2O$], 89.8[C1'], 75.3[C2'], 70.1[C3'], 85.0[d,J_{POCC}=7Hz,C4'], 63.5[d,J_{POC}=4Hz,C5'].

^{13}C-NMR in D_2O:
157.4(C2], 152.5[C4], 110.2[C5], 158.5[C6], 138.9[C8],
51.2+33.6+36.0+63.0[$NCH_2H_2SCH_2CH_2O$], 92.4[C1'], 77.4[C2'], 72.1[C3'], 86.9[d,J_{POCC}=8Hz,C4'], 66.0[d,J_{POC}=4Hz,C5'].

^{31}P-NMR in D_2O:
1.60

FAB-MS:
m/z= 468(MH^+), 490(MNa^+)

UV spectra:
pH 1 : 258 nm (max)
pH 7 : 258 nm (max)
pH 13 : 266 nm (max)

The di-adduct eluted after ca. 30 min from the G-10 column, which separated the product easily from inorganic material and thiodiglycol. A total of 20 mg of the white, fluffy, and very hygroscopic di-adduct was obtained after lyophilization. According to ^{1}H-NMR, the purity was ca. 90%.

^{1}H-NMR in D_2O:
9.24[s,1H,H8], 4.7+3.12[m,4H,NCH_2CH_2S], 6.09[d,J=4Hz,1H,H1'], 4.7[1H,H2'], 4.46[t,1H,H3'], 4.41[m,1H,H4'], 4.12+4.23[m,2H,H2-5']

^{13}C-NMR in D_2O:
157.2[C2], 152.5[C4], 110.2[C5], 158.5[C6], 139[C8],
51.4+33.3[NCH_2CH_2S], 92.4[C1'], 77.3[C2'], 72.1[C3'], 87.0[d,J_{POCCH}=7 Hz,C4'], 66.0[d,J_{POCH}=4 Hz,C5'].

UV spectra:
pH 1 : 266 nm (max)
pH 7 : 260 nm (max)
pH 13 : 266 nm (max)

II.6.2. 5'-[O-(2"-hydroxyethylthioethyl) phosphate]-2'-deoxyguanosine (59)

A solution of 2'-deoxyguanosine-5'-phosphate (sodium salt; 175 mg, 0.45 mmol) was dissolved in 0.1 M aqueous triethylammonium

bicarbonate* (15 ml) and acetonitrile (2/1, v/v; pH=7.5). Mustard gas (100 mg, 0.63 mmol) was added and the reaction mixture was stirred for 16 h at room temperature. The homogeneous reaction mixture was extracted 3 times with dichloromethane (5 ml) in order to remove unreacted mustard gas. HPLC (system C) indicated that ca. 40% (UV detection at 254 nm) of a reaction product had been formed which eluted more rapidly than the starting material. The product was isolated by means of chromatography on a column (20 x 2 cm) filled with Sepharose Q fast flow anion exchange material. After flushing the column with starting buffer (1 mM triethylammonium bicarbonate) for 1 h at 1 ml/min, the reaction mixture was chromatographed at an elution speed of 1 ml/min, with a linear gradient up to 1 M triethylammonium bicarbonate in the course of 180 min, collecting 5-ml fractions. The reaction product was detected in fractions 22-28 (HPLC). These fractions were concentrated and lyophilized to give 35 mg of reaction product.

^{1}H-NMR in D_2O:
8.0[s,1H,H8], 3.75+2.62+2.62+3.64[m,8H,$POCH_2CH_2SCH_2CH_2O$], 6.3[t,1H,H1'], 2.58+2.86[m,1H,H2'], 4.73[m,1H,H3'], 4.23[m,1H,H4'], 4.03[m,2H,H2-5']

^{13}C-NMR in D_2O:
156.6[C2], 154.4[C4], 119.5[C5], 161.8[C6], 140.7[C8], 86.5[C1'], 41.4[C2'], 74.0[C3'], 88.5[d, J_{POCC}=8Hz,C4'], 68.0[d,J_{POC}=5 Hz,C5'], 68.2(d,J_{POC}=4 Hz) + 34.4(d,J_{POCC}=7 Hz) + 36.7 + 63.4($POCH_2CH_2SCH_2CH_2O$).

^{31}P-NMR in D_2O:
1.1

UV spectra:
pH 1 : 255 nm (max)
pH 7 : 252 nm (max)
pH 13: 267 nm (max)

II.6.3. Attempted alkylation of 2'-deoxyguanosine 3',5'-cyclic phosphate with mustard gas

A suspension of 2'-deoxyguanosine 3'5'-cyclic phosphate (50 mg, sodium salt, 0.16 mmol) in a mixture of 0.1 M aqueous triethylammonium bicarbonate (10 ml) and acetonitrile (5 ml) was stirred at room temperature with mustard gas (100 mg, 0.63 mmol). After a total reaction time of 16 h, the reaction system had become homogeneous, whereas the pH had decreased to 3.0. The reaction mixture was extracted with dichloromethane (3 x 5 ml) in order to remove residual mustard gas. HPLC (system A) and ^{1}H-NMR analysis indicated that a

* A stock solution of 2 M aqueous triethylammonium bicarbonate (pH 7.5) is obtained by bubbling carbon dioxide gas for 8 h through a mixture of triethylamine (550 ml) and water (1450 ml), cooled at 0 °C. The stock solution can be stored at 0-5 °C for several months.

mixture of approximately six reaction products had been formed. Attempts to purify the products were not made.

II.6.4. N1-(2"-Hydroxyethylthioethyl)-2'-deoxyguanosine

2'-Deoxyguanosine (140 mg, 0.5 mmol) in 0.1 M aqueous triethylammonium bicarbonate (10 ml, pH 7.5) and acetonitrile (2/1, v/v) was stirred at room temperature with mustard gas (108 mg, 0.7 mmol). After a total reaction time of 16 h the reaction mixture had become homogeneous. The reaction mixture was extracted with dichloromethane (3 x 5 ml) in order to remove residual mustard gas. HPLC (system A; detection at 254 nm) showed ca. 40% conversion into two reaction products, eluting at 5.6 and 10.3 min (2'-deoxyguanosine: retention time 4.5 min). A few mg of each product was isolated with preparative reversed phase HPLC. The fractions containing the separated product peaks were evaporated several times with water in order to remove the volatile bicarbonate buffer. Finally the residues were co-evaporated with D_2O for ^1H- and ^{13}C-NMR analysis. The early eluting product peak appeared to be a mixture of at least four reaction products, which could not be positively identified. The late eluting minor product peak was identified with ^1H- and ^{13}C-NMR as N1-(2"-hydroxyethylthioethyl)-2'-deoxyguanosine.

^1H-NMR in DMSO-d_6:
7.94[s,1H,H8], 4.15+2.7[both t,4H,NCH_2CH_2S], 2.7+3.60[both t,4H,SCH_2CH_2O], 6.14[,1H,H1'], 2.22+2.58[2H,H2'], 4.36[1H,H3'], 3.83[1H,H4'], 3.55[2H,H5'].

^{13}C-NMR in DMSO-d_6:
153.5[C2], 149.0[C4], 115.9[C5], 156.3[C6], 135.6[C8], 40.8+28.8+34.2+61.0[$NCH_2CH_2SCH_2CH_2O$], 82.3[C1'], 40[C2'], 70.8[C4'], 61.8[C5'].

Thermospray-MS:
m/z= 372(MH^+), 256[MH^+ of N1-(2'-hydroxyethylthioethyl)-guanine]

UV spectra:
pH 1 : 261 nm (max)
pH 7 : 257.5 nm (max)
pH 13: 257.5 nm (max)

II.6.5. Synthesis of ethyl levulinate

A mixture of levulinic acid (58 g, 0.5 mol), anhydrous ethanol (500 ml) and conc. sulfuric acid (1 ml) was refluxed for 5 h. Next, the reaction mixture was poured into a separating funnel, toluene (200 ml) was added, and the solution was washed with aqueous sodium bicarbonate (7.5%, w/v) and water, respectively. The combined organic layers were evaporated to a small volume and the residue was distilled in vacuo to give 57 g (80%) of ethyl levulinate (b.p. 58 ^oC/0.2 mmHg).

^{1}H-NMR in $CDCl_3$:
1.25+4.13[CH_3CH_2O], 2.57+2.75[CH_2CH_2], 2.19[$C(O)CH_3$]

II.6.6. Synthesis of ethyl levulinate-(O-2',3'-guanosine-acetal) (60)

A solution of guanosine (2.8 g, 1 mmol), ethyl levulinate (2.9 g, 20 mmol), triethyl orthoformate (2.5 ml, 15 mmol), dry N,N-dimethylformamide (40 ml), and of 7 M hydrochloric acid in 1,4-dioxane (5 ml) was stirred at room temperature for 24 h. Next, the solution was poured into 400 ml of ether. The ether layer was decanted and the oily residue was washed twice with 100 ml portions of ether. The residue was dissolved in dichloromethane, neutralized with 2% aqueous $NaHCO_3$, and washed with water. The organic layer was dried with $MgSO_4$ and evaporated to a small volume. The oily residue was recrystallized from acetonitrile to give 2.6 g (63%) of product, m.p. 261.5- 264 °C [ref. (60): 262-264 °C].

^{1}H-NMR in DMSO-d_6:
10.8[bs,1H,NH], 7.92[s,1H,H8], 6.6[bs,2H,NH_2], 5.96[d,1H,H1'], 5.31[dd,J=6.5 and 2.3 Hz,1H,H2'], 5.03[dd,J=6.5 and 3.0 Hz,1H,H3'], 4.13[td, J=5.3 and 3.0 Hz,1H,H4'], 3.52[m,2H,H5'], 5.0[OH], 1.32[3H,CCH_3], 2.07+2.46[both t,J=7.5 Hz,4H,CH_2CH_2], 4.10+1.22[5H,CH_2CH_3].

^{13}C-NMR in DMSO-d_6:
153.7[C2], 150.7[C4], 116.8[C5], 156.8[C6], 135.9[C8], 88.4[C1'], 83.7[C2'], 81.2[C3'], 86.9[C4'], 61.6[C5'], 113.7[OCO], 23.6(CH_3], 33.5+28.3[CH_2CH_2], 172.5[C(O)O]

UV spectrum (methanol):
254 nm max; ref. (60): max. at 259 nm (methanol).

II.6.7. Attempted alkylation with mustard gas of ethyl levulinate-(O-2',3'-guanosine-acetal)

Mustard gas (6.3 µl, 0.05 mmol) was added to four NMR tubes, each containing 10 mg (0.025 mmol) of the acetal in 0.8 ml of, respectively, DMSO-d_6, DMF-d_6, DMSO-d_6 with a trace of water, and glacial acetic acid. ^{1}H-NMR indicated that no reaction had taken place after keeping the solutions for 1 week at a temperature of 80 °C. The experiment was discontinued.

II.6.8. Kinetics of imidazole ring opening of N7-alkyl-guanosine-5'-phosphates [alkyl = (2'-hydroxyethylthioethyl) or methyl] (61-64)

Alkaline solutions were adjusted to a pH of 9.8, 11.2 or 11.4 using 50 ml 0.05 M $NaHCO_3$ and sufficient 0.1 M NaOH (about 25 ml) and water until a total volume of 100 ml and the desired pH had been achieved. All reactions were carried out in quartz cuvettes (1x1 cm) thermostatted at 25 °C. After mixing 2.97 ml of buffer solution and 0.03 ml stock solution (4 mg/ml) of N7-alkyl-guanosine-5'-phosphate,

UV scans were made every 15 or 30 minutes. All kinetic runs were performed in duplicate. Rate constants were calculated from a plot of the log of the difference between the absorbance at a given time and the absorbance of the imidazolium ring-opened (i.r.o.) compound at 266 nm, assuming (pseudo) first-order kinetics. At the selected wavelength, the differences between the absorbances of the i.r.o. N7-alkylated compounds and the corresponding starting compounds are maximal. The absorbance value that had increased less than 0.0005 absorbance units at the next measurement was taken as the absorbance of the ring-opened product. A survey of maximum wavelengths and pertaining molar extinction coefficients at pH 11.2 of aqueous ring-closed, of i.r.o. N7-(2"-hydroxyethylthioethyl)-guanosine-5'-monophosphate (N7-HD-GMP), and of the corresponding N7-methyl derivative (N7-Me-GMP) is given in Table 1.

Table 1. Maximum wavelengths and pertaining molar extinction coefficients at pH 11.2 of aqueous ring-closed and imidazolium ring-opened (i.r.o.) N7-(2"-hydroxyethylthioethyl)-guanosine-5'-phosphate (N7-HD-GMP) and of the corresponding N7-methyl derivative (N7-Me-GMP)

Compound	Maximum at (nm)	Molar extinction coefficient (10^3 $l.mol^{-1}.cm^{-1}$)
N7-HD-GMP	284	19
	252	12.5
N7-Me-GMP	282	19
	258	12.5
N7-HD-GMP (i.r.o.)	266	17
N7-Me-GMP (i.r.o.)	266	15

II.7. Synthesis of N-acetyl-amino acid-methylamides*

II.7.1. Synthesis of valine-methylamide (65)

II.7.1.1. N-benzyloxycarbonyl-valine-methyl-ester

Triethylamine (12.2 g, 0.12 mol) was added to a solution of valine-methyl ester hydrochloride (20.0 g, 0.12 mol) in chloroform (170 ml), cooled at 0 °C. Next, benzyl chloroformate (21.3 g, 0.12 mol) was added dropwise in the course of 30 min at the same temperature, with gradual addition of sodium bicarbonate (11.8 g, 0.14 mol). After stirring for an additional hour at 0 °C, the reaction mixture was left at room temperature for 5 h. Next, pyridine (8.5 ml) was added, followed by water (100 ml) 1 h later. The organic layer was washed with 1 N aqueous hydrochloric acid (3x60 ml) and subsequently with water until neutral. After drying on magnesium sulfate, the organic

* In this report it is assumed that all amino acids have the natural L-configuration, unless mentioned otherwise.

layer was concentrated. The oily residue crystallized after trituration with ethanol and n-hexane. This yields 23.0 g (72.5%) of the desired product, m.p. 54-55.5 °C.

Elemental analysis ($C_{14}H_{19}NO_4$,MW 265.3):

Calc.:	C	63.38%	Found: 63.43-63.50%
	H	7.22%	7.26- 7.30%
	N	5.28%	5.23- 5.30%

^{1}H-NMR in $CDCl_3$:
7.27-7.34[m,5H,aromatic H's], 5.30[bd,J=8.9 Hz,1H,NH], 5.11[s,2H,$COOCH_2$], 4.30[dd,J= 4.8 and 8.9 Hz,NHCH], 3.72[s,3H,$COOCH_3$], 2.15[m,1H,$C\underline{H}(CH_3)_2$], 0.96+0.89[both d,J=6.9 Hz,$CH(CH_3)_2$].

^{13}C-NMR in $CDCl_3$:
172.5[COO], 156.3[CONH], 136.4+128.6+128.2+128.1[aromatic C's], 67.1[$COO\underline{C}H_2$], 59.1[CHNH], 52.1[$COO\underline{C}H_3$], 31.4[$\underline{C}H(CH_3)_2$], 19.0+17.6[$CH(\underline{C}H_3)_2$].

II.7.1.2. N-Benzyloxycarbonyl-valine-methylamide

A 40% aqueous solution of methylamine (15 ml) was added to a solution of N-benzyloxycarbonyl-valine-methyl ester (3.0 g, 11.3 mmol) in acetone (5 ml), which resulted in an exothermic reaction. After one day at room temperature, the precipitated reaction product was filtered, washed with water and dried. Yield 2.28 g (76%), m.p. 76-77 °C.

^{1}H-NMR in DMSO-d_6:
7.85[d,1H,$NHCH_3$], 7.42/7.30[m,5H,aromatic], 7.23[d,1H,NHC(O)], 5.06[s,2H,CH_2], 3.8[t,1H,CH], 2.61[d,3H,$NHC\underline{H}_3$], 1.96[m,1H,$C\underline{H}CH_3$], 0.86[d,d,6H,$C\underline{H}_3CH$].

II.7.1.3. Valine-methylamide hydrochloride

Nitrogen gas was bubbled for 5 min through a solution of N-benzyloxycarbonyl-valine-methylamide (1.6 g, 6.1 mmol) in methanol (30 ml). Next, palladium on charcoal (10%; 160 mg) and two drops of concentrated aqueous hydrochloric acid were added to the solution. Hydrogen gas was led over the vigorously stirred solution for 4 h. After filtration and evaporation of solvent, the sticky residue was dissolved in chloroform (3 ml). The solution was extracted with 1 N aqueous hydrochloric acid (6 ml), 0.5 N aqueous hydrochloric acid (3 ml), and with water (2 ml). The aqueous layers were combined and neutralized (pH 7). Next, the aqueous solution was extracted 3 times with chloroform, with addition of concentrated aqueous sodium hydroxide (1 ml) after each extraction. Finally, the combined organic layers were extracted with concentrated aqueous hydrochloric acid. The two layers were separated after addition of water (1 ml). The aqueous layer was concentrated, which left 350 mg (44%) of white, very hygroscopic product, m.p. 159 °C.

^{1}H-NMR in DMSO-d_6:
8.62[bq,1H,N<u>H</u>CH$_3$], 8.30[bs,3H,NH$_3$+], 3.57[m,1H,CHC(O)],
2.67[d,3H,NHC<u>H</u>$_3$], 2.09[m,1H,C<u>H</u>CH$_3$], 0.95[d,6H,C<u>H</u>$_3$CH].

^{13}C-NMR in DMSO-d_6:
168.2[C(O)], 57.5[<u>C</u>HC(O)], 29.6[<u>C</u>HCH$_3$], 25.4[CH$_3$NH],
18.3/18.2[CHCH$_3$].

Thermospray MS:
m/z= 131(MH^+).

II.7.2. <u>Synthesis of N-acetyl-aspartic acid-1-methylamide</u>

II.7.2.1. <u>N-Acetyl-4-aspartic acid-4-benzyl ester</u> (66)

Aspartic acid-4-benzyl ester (6.0 g, 26.9 mmol) was suspended in 30 ml of glacial acetic acid containing 4.2 ml of acetic anhydride. On heating, the suspension dissolved. The solution was refluxed for 5 min (oil bath at 140 °C). Part of the product crystallized on cooling to room temperature. Further crystallization occurred after addition of 30 ml of dry ether and 30 ml of n-hexane. After standing overnight at 5 °C in a refrigerator the crystals were collected on a glass filter with suction, washed with 10 ml of ether/n-hexane (1/1, v/v) and dried in a vacuum desiccator over soda lime and charcoal. Yield 6.30 g (88%), m.p. 258 °C.

Elemental analysis ($C_{13}H_{15}NO_5$, MW 265.3):

Calc.:		Found:	
C	58.86%	C	58.5 ± 0.3 %
H	5.70%	H	5.69 ± 0.02%
N	5.28%	N	5.28 ± 0.06%

^{1}H-NMR in DMSO-d_6:
12.8[b,1H,COOH], 8.26[d,J=8.1 Hz,1H,CONH], 7.32-7.43[m,5H,aromatic],
5.13[s,2H,COOCH$_2$], 4.64[dt,J=6.0 Hz and 7.6 Hz,1H,NHC<u>H</u>], 2.86 and
2.74[both dd,2H,CH$_2$], 1.86[s,COCH$_3$]

^{13}C-NMR in DMSO-d_6:
172.3 [COOH], 170.1[<u>C</u>OOCH$_2$], 169.3[CONH], 136.1, 128.5, 128.1 and
127.9 [aromatic C's], 65.8[COO<u>C</u>H2], 48.6[NHCH], 36.1[<u>C</u>H$_2$COO],
22.4[COCH$_3$]

II.7.2.2. <u>N-Acetyl-aspartic acid-4-benzyl ester-1-methylamide</u> (67)

To a stirred solution of 5.0 g (18.8 mmol) of N-acetyl-aspartic acid-4-benzyl ester and 2.64 ml (18.9 mmol) of triethylamine in 150 ml of 1,4-dioxane was added 1.87 ml (19.0 mmol) of ethyl chloroformate in the course of 0.5 min. Stirring was continued for 15 min at 11 °C. Methylamine (1.8 ml of 40% aqueous solution, 19.6 mmol) was added and stirring was continued for 30 min at 11-15 °C and for 60 min at room temperature. The precipitate of triethylammonium chloride was removed by filtration. The solvent was removed in vacuo and the crystalline residue stirred with 80 ml of water during 2 h. The crystals were

collected on a glass filter, washed with 10 ml of water and dried in a vacuum desiccator over phosphorus pentoxide. Yield 3.36 g, m.p. 140-141 °C. Another 1.51 g (m.p. 140-141 °C) was obtained after concentration of the filtrate in a rotation evaporator and treatment of the residue with 20 ml of water in the same way as above. Total yield: 4.87 g (93%), m.p. 140-141 °C.

Elemental analysis ($C_{14}H_{18}N_2O_4$, MW 278.3):
Calc.: C 60.42% Found: C 60.24 ± 0.05%
H 6.52% H 6.51 ± 0.03%
N 10.07% N 10.01 ± 0.02%

^{1}H-NMR in DMSO-d_6:
8.17[d,J=8.3 Hz,1H,CONHCH], 7.83[bq,1H,J=4.6 Hz,CONHCH$_3$], 7.31-7.44 (m,5H,aromatic], 5.11[s,2H,COOCH$_2$],4.65[dt,J=6.2 and 8.0 Hz,1H,NHCH], 2.82 and 2.61[both dd,4H,CH$_2$], 2.59[d,J=4.8 Hz,3H,NHCH$_3$], 1.85[s,3H,COCH$_3$]

^{13}C-NMR in DMSO-d_6:
170.7[CHCONH], 170.3[C(O)O], 169.3[CH$_3$CONH], 136.2, 128.4, 128.0 and 127.8[aromatic C's], 65.6[COOCH$_2$], 49.4[NHCH], 36.5[CH$_2$COO], 25.8[NHCH$_3$], 22.6[COCH$_3$]

II.7.2.3. N-Acetyl-aspartic acid-1-methylamide (68)

To a solution of 3.5 g (12.6 mmol) of N-acetyl-aspartic acid-4-benzyl ester-1-methylamide in methanol (150 ml) 1 g of palladium on charcoal (10%) was added. During 20 min, nitrogen was passed over the vigorously stirred mixture, followed by a stream of hydrogen during 6 h and again nitrogen during 20 min. The mixture was filtered through a glass filter and the filtrate concentrated in vacuo leaving a crystalline residue. Thermospray-LC-MS analysis showed the presence of an impurity identified as the 4-methyl ester of the product (m/z = 203, MH$^+$). The crystals were recrystallized from methanol/ether (1/5, v/v). According to LC-MS analysis the final product was free from methyl ester. Yield 2.28 g (96%), m.p. 172-173 °C.

Elemental analysis ($C_7H_{12}N_2O_4$, MW 188.2):
Calc.: C 44.68% Found: C 44.72 ± 0.06%
H 6.43% H 6.4 ± 0.1 %
N 14.89% N 14.86 ± 0.08%

^{1}H-NMR in DMSO-d_6:
12.2[bs,1H,COOH], 8.08[d,J=8.1 Hz,1H,CONHCH], 7.74[bq,J=4.6 Hz,1H,CONHCH$_3$], 4.53 (dt,J=6.1 and 7.9 Hz,1H,NHCH], 2.66 and 2.46[both dd,4H,CH$_2$], 2.59[d,J=4.6 Hz, NHCH$_3$], 1.86[s,COCH$_3$]

Thermospray LC-MS:
m/z= 189[MH$^+$], 171[MH$^+$ - H$_2$O].

II.7.3. Synthesis of N-acetyl-glutamic acid-1-methylamide

II.7.3.1. N-Acetyl-glutamic acid-5-benzyl ester (66)

Glutamic acid-5-benzyl ester (5.0 g, 21.1 mmol) was suspended in glacial acetic acid (25 ml) containing acetic anhydride (3.5 ml). On heating, the suspension dissolved. The solution was refluxed for 5 min (oil bath 140 °C). The product crystallized after addition of ether (30 ml) and cyclohexane (30 ml). After standing overnight at 5 °C in a refrigerator, the crystals were collected by filtration, washed with 10 ml of ether/n-hexane (1/1, v/v) and dried in a vacuum desiccator over soda lime and charcoal. Yield 4.75 g (81%), m.p. 121-122 °C.

Elemental analysis ($C_{14}H_{17}NO_5$, MW 279.3):

Calc.:		Found:	
C	60.21%	C	60.0 ± 0.3 %
H	6.14%	H	6.10 ± 0.06%
N	5.02%	N	5.07 ± 0.08%

^{1}H-NMR in DMSO-d_6:
12.6[bs,1H,COOH], 8.13[d,J=7.9 Hz,1H,CONH], 7.31-7.45[m,5H,aromatic], 5.11 [s,2H,$COOCH_2$], 4.25[dt,J=5.1 and 8.4 Hz,1H,NHC<u>H</u>], 2.45[m,2H,CH_2COO], 2.04 and 1.86[both m,4H,$CHCH_2$], 1.87[s,3H,CH_3].

^{13}C-NMR in DMSO-d_6:
173.3[COOH], 172.1[<u>C</u>$OOCH_2$], 169.5[CONH], 136.2,128.5,128.1 and 128.0 [aromatic C's], 65.6[$COO\underline{C}H_2$], 51.1[NHCH], 30.1[<u>C</u>H_2COO], 26.4[$CH\underline{C}H_2$], 22.4[CH_3].

II.7.3.2. N-Acetyl-glutamic acid-5-benzyl ester-1-methylamide (67)

Ethyl chloroformate (1.60 ml, 16.2 mmol) was added in the course of ca. 0.5 min to a stirred solution of N-acetyl-glutamic acid-5-benzyl ester (4.5 g, 16.1 mmol) and triethylamine (2.26 ml, 16.2 mmol) in 1,4-dioxane (150 ml), cooled at 10 °C with dry ice. Stirring was continued for 15 min during which the temperature was kept beneath 11 °C. After addition of 40% aqueous methylamine (1.56 ml, 16.8 mmol), stirring was continued for 30 min at 10-15 °C and 60 min at room temperature. The precipitate of triethylammonium chloride was removed by filtration. The solvent was removed in vacuo and the crystalline residue stirred with water (50 ml) during 1 h. The crystals were collected on a glass filter, washed with water (10 ml) and dried in a vacuum desiccator over phosphorus pentoxide. Yield 3.89 g (83%), m.p.

^{1}H-NMR in DMSO-d_6:
7.99[d,J=8.1 Hz,1H,CONHCH], 7.83[bq,J=4.6 Hz,1H,CONHCH$_3$], 7.31-7.43[m,5H,aromatic], 5.10[s,2H,COOCH$_2$], 4.24[dt,J=5.6 and 8.3 Hz,1H,NHCH], 2.60[d,J=4.6 Hz,3H,NHCH$_3$],2.38[t,J=7.9 Hz,2H,CH$_2$COO], 1.96 and 1.79[both m,2H,CHCH$_2$], 1.87[s,3H,COCH$_3$].

^{13}C-NMR in DMSO-d_6:
172.2[C(O)O], 171.5[CHCONH], 169.4[CH$_3$CONH], 136.3, 128.5, 128.1 and 128.0[aromatic C's], 65.5[COOCH$_2$], 51.8[NHCH], 30.2[CH$_2$COO], 27.3[CH$_2$CH], 25.5[NHCH$_3$], 22.5[COCH$_3$].

II.7.3.3. N-Acetyl-glutamic acid-1-methylamide (68)

Palladium on charcoal (10%, 500 mg) was added to a solution of N-acetyl-glutamic acid-5-benzyl ester-1-methylamide (2.34 g, 8.00 mmol) in 1,4-dioxane (100 ml). Nitrogen was passed for 20 min over the vigorously stirred mixture, followed by a stream of hydrogen during 6 h and again nitrogen during 20 min. The mixture was filtered through a glass filter and the filtrate concentrated in vacuo. The crystalline residue was recrystallized from ethanol (10 ml) and ether (60 ml). Yield 1.47 g (91%) of hygroscopic crystals, m.p. 117-119 °C (with decomposition).

Elemental analysis ($C_8H_{14}N_2O_4$, MW 202.2):
Calc.: C 47.52% Found: C 47.08 ± 0.03%
H 6.98% H 7.12 ± 0.08%
N 13.85% N 13.16 ± 0.13%

^{1}H-NMR in DMSO-d_6:
12.1[s,1H,COOH], 7.96[d,J=8.1 Hz,1H,CONHCH], 7.80[q,J=4.4 Hz,1H,CONHCH3], 4.20[dt,J=5.4 and 8.3 Hz,1H,NHCH], 2.59[d,J=4.4 Hz,3H,NHCH$_3$], 2.22[m,2H,CH2COO], 1.90 and 1.72[both m,2H,CHCH$_2$], 1.87[s,3H,COCH$_3$]

^{13}C-NMR in DMSO-d_6:
173.9[COOH], 171.7[CHCONH], 169.4[CH$_3$CONH], 51.9[NHCH], 30.3[CH$_2$COO], 27.4[CHCH$_2$], 25.6[NHCH$_3$], 22.6[COCH$_3$]

II.7.3.4. Dicyclohexylamine salt of N-acetyl-glutamic acid-1-methylamide

Dicyclohexylamine (0.5 ml, 2.6 mmol) was added to a solution of N-acetyl-glutamic acid-1-methylamide (0.5 g, 2.5 mmol) in ethanol (10 ml). Crystallization occurred after adding ether (40 ml) to the stirred solution. The crystals were collected on a glass filter, washed with ethanol/ether (1/4, v/v) and dried in a vacuum desiccator over charcoal. Yield 910 mg (96%), m.p. 172-173 °C (with decomposition).

Elemental analysis ($C_{20}H_{37}N_3O_4$, MW 383.5):
Calc.: C 62.63% Found: C 61.56 ± 0.05%
H 9.73% H 9.7 ± 0.2 %
N 10.96% N 10.9 ± 0.2 %

II.7.4. Synthesis of N-α-acetyl-histidine-methylamide

II.7.4.1. N-α-Acetyl-histidine-ethyl ester hydrochloride (69)

Acetyl chloride (4.4 g, 56 mmol) was added dropwise to a solution of N-α-acetyl-histidine monohydrate (3.57 g, 17.4 mmol) in dry ethanol, cooled with an ice bath. After setting the solution aside overnight, it was heated at 60 °C for 2 h. Next, the solvent was evaporated in vacuo and the solid residue was redissolved in ethanol at 60 °C. After cooling to 40 °C, ether (30 ml) was added dropwise. The solution was decanted after standing overnight in the refrigerator. After repeating this treatment twice, the solid residue was dried in vacuo, to give 4.1 g (94%) of the desired product, which was considered to be sufficiently pure for use in the next reaction step.

Elemental analysis ($C_{10}H_{16}ClN_3O_3$, MW 261.7):

Calc.: C	45.89%	Found: 43.00	± 0.08%
H	6.16%	6.24	± 0.02%
N	16.06%	15.51	± 0.05%
Cl	13.55%	13.83	± 0.06%

^{1}H-NMR in DMSO-d_6:
1.85[s,3H,CH_3CO], 1.15[t,J=7.1Hz,3H,CH_3C], 3.07[dd,J=9.3 and 15.1 Hz,1H,CHH], 3.15[dd,J=5.6 and 15.1 Hz,1H,CHH], 4.09[s,2H,OCH_2C], 4.55[ddd,J=5.6,7.5 and 9.1 Hz,1H,C<u>H</u>CH_2], 7.45[d,J=1.2 Hz,1H,C-CH], 9.06[d,J=1.4 Hz,1H,N=CH], 8.60[d,J=7.5 Hz,1H,NHC=O], 14.7[broad,2x NH].

II.7.4.2. N-α-Acetyl-histidine-methylamide (70)

N-α-acetyl-histidine-ethyl ester hydrochloride (3.04 g, 11.6 mmol) was dissolved in 40% aqueous methylamine (15 ml). After 2 h a thick precipitate had formed. Anhydrous ethanol (5 ml) was added and solvents were removed after 24 h in a rotafilm evaporator. The residue was dissolved in ethanol (15 ml), which was evaporated after 24 h. After repeating the latter treatment twice, methanol (10 ml) was added to the residue, and precipitated salts were filtered. Acetone was added slowly to the filtrate until the solution became slightly turbid. Upon cooling to 5 °C, a precipitate had formed which was filtered, washed with methanol/acetone (1/1, v/v) and with acetone. The air-dried precipitate was recrystallized twice from methanol to give 1.5 g (64%) of product, m.p. 255-256 °C [with decomposition; ref. 70: m.p. 260 °C].

Elemental analysis ($C_9H_{14}N_4O_2$, MW 210.2):

Calc.: C	51.41%	Found: 51.22	± 0.04%
H	6.96%	6.94	± 0.02%
N	26.66%	26.36	± 0.01%
Cl	0.00%	<0.01%	

^{1}H-NMR in DMSO-d_6:
1.83[s,3H,CH_3CO], 2.57[s,3H,$C\underline{H}_3NH$], 2.74[dd,J=8.6 and 14.7 Hz,1H,CHH), 2.91 (dd,J=5.4 and 14.9 Hz,1H,CHH), 4.41[dt,J=5.4 and 8.4 Hz,$C\underline{H}CH_2$], 6.75[s,1H,C=CH), 7.51[d,J=1.2 Hz,1H,N=CH], 7.77[bq,J=4.6 Hz,1H,$N\underline{H}CH_3$], 8.00[d,J=8.1 Hz,1H,NHC=O], 11.7[broad,>NH].

^{13}C-NMR in DMSO-d_6:
173.0[C-$\underline{C}$H-N], 171.5[NH-$\underline{C}$-CH3], 135.7[N=$\underline{C}$-N], 54.1[$\underline{C}HCH_2$], 30.0[$C\underline{C}H_2CH$], 26.5[CH_3NH], 23.1[$\underline{C}H_3CO$].

Thermospray LC-MS:
m/z=211 (MH^+), 193 (MH^+-H_2O).

UV spectrum (methanol):
Maximum at 213 nm.

II.7.5. Synthesis of N-acetyl-methionine-methylamide

II.7.5.1. N-Acetyl-methionine-ethyl ester

N-acetyl-methionine (2.0 g, 10 mmol) was added to a solution of acetyl chloride (4 ml) in dry ethanol (40 ml), cooled at 4 °C. Next, the solution was stirred for 24 h at room temperature and concentrated in vacuo after an additional period of stirring at 60 °C. According to ^{1}H-NMR, the sticky residue (2.2 g, 96% yield) contained ethanol as the only significant impurity. Therefore, it was used without further purification for conversion into the methylamide.

^{1}H-NMR in DMSO-d_6:
4.70[bs,1H,CH], 4.22[q,J=7.1 Hz,2H,$C\underline{H}_2CH_3$], 2.60[m,2H,CH_2S], 2.40[s,3H,SCH_3], 2.19[m,2H,CH_2CS], 2.11[s,3H,$CH_3C(O)$], 1.30[t,J=7.1 Hz,3H,$OCCH_3$].

II.7.5.2. N-acetyl-methionine-methylamide

Aqueous methylamine (40%) was dropped on sodium hydroxide pellets. The evolving gaseous methylamine was dried over similar pellets in a drying tower and was led through a solution of N-acetyl-methionine-ethyl ester (2.2 g, 10 mmol) in dry ethanol (15 ml) for 30 min at room temperature. After an additional 60 h reaction time at room temperature, the solvent was removed in vacuo in a rotavapor. The solid residue was treated with dichloromethane, and the remaining solids were removed by filtration. Evaporation of solvent from the clear filtrate left 1.94 g of crude product, which was recrystallized twice by dissolution in ethanol, addition of n-pentane until slight turbidity, and cooling overnight at -18 °C. Yield 0.8 g (39%), m.p. 179-180 °C.

Elemental analysis ($C_8H_{16}N_2O_2S$, MW 204.2):

Calc.:	C 47.0%	Found: 46.04 ± 0.12%
	H 7.9%	7.84 ± 0.07%
	N 13.7%	13.57 ± 0.04%

^{1}H-NMR in DMSO-d_6:
8.01[d,J=7.9 Hz,1H,NHCH], 7.82[bq,1H,NHCH$_3$], 4.27[dt,J= 5.1 and 8.4 Hz,1H,HNCHC(O)], 2.59[d,J=4.6 Hz,3H,HCH$_3$], 2.44[m,2H,CH$_2$S], 2.05[s,3H,CH$_3$], 1.87[s,3H,CH$_3$C(O)], 1.76 and 1.90[m,2H,CH$_2$C].

^{13}C-NMR in DMSO-d_6:
171.7[HCC(O)NH], 169.4[CH$_3$C(O)NH], 51.9[NHCHC(O)], 31.8[CH$_2$CH$_2$S], 29.8[CH$_2$S], 25.6[NHCH$_3$], 22.6[CH$_3$C(O)], 14.6[SCH$_3$].

Thermospray MS:
m/z = 205(MH^+), 409 ($2MH^+$).

UV spectrum (H_2O):
Maximum at 206 nm.

II.7.6. Synthesis of N-acetyl-cysteine-methylamide

II.7.6.1. S-Benzyl-cysteine-methylamide hydrochloride

Aqueous methylamine (40%) was dropped on sodium hydroxide pellets (25 g). The evolving gaseous methylamine was dried over similar pellets in a drying tower and was led through a solution of S-benzyl-cysteine-methyl ester (15 g, 57.3 mmol) in methanol (60 ml). After an additional 24 h reaction time at room temperature, the solvent was removed in vacuo in a rotavapor. The residue was treated with acetone (30 ml), and the remaining solids were removed by filtration. Evaporation of solvent of the clear filtrate left 20.6 g of crude sticky product, which could not be solidified. The product was dissolved in ethanol (50 ml) and added to a saturated solution of hydrochloric acid in ethanol (50 ml). After evaporation of the solvent a glassy residue was obtained. Crystallization from acetone (150 ml) gave 8.75 g (59%) of the desired product, m.p. 144-145.5 °C.

^{1}H-NMR in DMSO-d_6:
8.87[bs,1H,NHCH$_3$], 8.53[bs,3H,NH$_3$], 7.24-7.41[m,5H,aromatic], 4.03[t,J=6.7 Hz,1H,CHCH$_2$S], 3.84[s,2H,C$_6$H$_5$CH$_2$S], 2.87[d,J=6.1 Hz,2H,CHCH$_2$S], 2.70[d,J=4.6 Hz, 3H,NHCH$_3$].

^{13}C NMR in DMSO-d_6:
167.7[C(O)NHCH$_3$], 138.0, 129.1, 128.5 and 127.0[aromatic], 51.7[CH$_2$CH], 35.2[C$_6$H$_5$CH$_2$S], 31.7[CHCH$_2$S], 25.6[NHCH$_3$].

Thermospray MS:
m/z = 225(MH^+), 449($2MH^+$).

II.7.6.2. N-Acetyl-S-benzyl-cysteine-methylamide

S-benzyl-cysteine-methylamide hydrochloride (7.5 g, 28.8 mmol) was dissolved in water (50 ml) and a concentrated solution of sodium bicarbonate (2.9 g, 28.8 mmol). The solution was extracted with dichloromethane (3x30 ml). After evaporation of the organic solvent in vacuo, acetic anhydride (50 ml) was added. The solid formed was suspended in the acetic anhydride and, after 3 days at room

temperature, stirred for 3 h at 75 °C. After cooling, the crystals were collected by filtration, washed with water (2x30 ml) and dried in a vacuum desiccator over P_2O_5. Recrystallization from ethanol/water (1/9, v/v) gave 4.6 g (60%) of product, m.p. 155-156 °C (ref. 71: 155-156 °C).

^{1}H-NMR in DMSO-d_6:
8.11[d,J=8.3 Hz,1H,CHN<u>H</u>], 8.03[bq,J=4.6 Hz,1H,N<u>H</u>CH$_3$], 7.20-7.30[m, 5H,aromatic], 4.47[dt,J=6.3 Hz and J=8.0 Hz, 1H,C<u>H</u>NH], 3.76[AB pattern,2H,C$_6$H$_5$C<u>H</u>$_2$S], 2.72[dd,J=13.5 Hz and J=6.3 Hz,1H,<u>H</u>CHCH], 2.62 [d,J=4.6 Hz,3H,NHC<u>H</u>$_3$], 2.54[dd,J=13.5 Hz and J=8.0 Hz,1H,HC<u>H</u>CH], 1.88[s,3H,C(O)CH$_3$].

^{13}H-NMR in DMSO-d_6:
170.7[CH<u>C</u>(O)NH], 169.2[NH<u>C</u>(O)CH$_3$], 138.4, 128.9, 128.4, and 126.8 [aromatic], 52.1[NH<u>C</u>H], 35.2[C$_6$H$_5$<u>C</u>H$_2$S], 33.1[S<u>C</u>H$_2$CH], 25.6[NH<u>C</u>H$_3$], 22.5[C(O)CH$_3$].

Thermospray MS:
m/z = 267(MH^+).

II.7.6.3. <u>N-Acetyl-cysteine-methylamide</u>

Dry ammonia (50-100 ml) was condensed in a flask cooled with solid carbon dioxide-acetone. N-Acetyl-S-benzyl-cysteine-methylamide (200 mg, 0.75 mmol) was added and after stirring for a short period of time the cooling bath was removed. Small pieces of sodium were added until the blue color of the reaction mixture did not disappear within 10 min. The solution was decolorized by adding ammonium chloride. Ammonia was evaporated with a stream of nitrogen. The residue was dissolved in 30% acetic acid/water (1/1, v/v) and evaporated to dryness in vacuo. The crude product contained sodium acetate and approximately 2% of the disulfide, i.e., N,N'-diacetyl-cystine-dimethylamide. The product was isolated by dissolving in degassed dichloromethane, filtration in a nitrogen atmosphere and evaporation to dryness. This treatment did not increase the percentage of disulfide in the product. M.p. 258-259 °C [ref. 71: 185 °C, but probably confounded with the melting point of the disulfide, which was reported as 262 °C; we found a melting point of 190 °C for the disulfide (unpublished results)].

^{1}H-NMR in CD_3OD:
4.42[dd,J=5.8 Hz and J=7.1.Hz,1H,CH$_2$C<u>H</u>], 2.86[dd,J=5.8 Hz and J=14.7 Hz,1H,<u>H</u>CHCH], 2.77[dd,J=7.1 Hz and J=14.7 Hz,1H,HC<u>H</u>CH], 2.74[s,3H, NHC<u>H</u>$_3$], 2.01[s,3H,C(O)C<u>H</u>$_3$].

^{13}C-NMR in CD_3OD:
173.5[CH$_3$<u>C</u>(O)NH], 173.1[CH<u>C</u>(O)NH], 57.4[<u>C</u>HNH], 26.9[S<u>C</u>H$_2$], 26.4 [NH<u>C</u>H$_3$], 22.7[C(O)CH$_3$].

Thermospray MS:
m/z = 177(MH^+), 351(MH^+ of disulfide).

II.8. Reaction of mustard gas with N-acetyl-amino acid-methylamides in aqueous solution at pH 7.5

II.8.1. Reaction of mustard gas with a single N-acetyl-amino acid-methylamide

A calculated amount of neat mustard gas was added from a syringe to a well-stirred, aqueous solution of N-acetyl-amino acid-methylamide (10-50 ml, 0.2-7.7 mM) at room temperature in the titrating vessel of a pH-stat apparatus, yielding a desired molar ratio of the two reaction components. The pH was kept at ca. 7.5 by means of automated addition of 0.1 N aqueous sodium hydroxide. In case of N-acetyl-cysteine-methylamide, the amino acid derivative (20 mg crude product containing 10 µmol of the relevant compound, see II.7.6.3) was dissolved in water (20 ml) through which a stream of nitrogen had been passed for 60 min, whereas mustard gas was added as an 1 mM solution in acetone (1 ml). The reaction had subsided after 30 min (cysteine) or 4-24 h. In one series of experiments the reaction mixture was then analyzed with micro-LC. In a second series of experiments the aqueous solution was concentrated in vacuo in a rotafilm evaporator. Addition of dry ethanol dissolved the organic reaction products while sodium chloride precipitated. After filtration the filtrate was analyzed with thermospray LC-MS.

II.8.2. Competition reactions of N-acetyl-amino acid-methylamides with mustard gas

Stock solutions of valine-methylamide hydrochloride and the N-acetylamino acid-methylamides, except the cysteine derivative, were added to water (5-7 ml). The final methanol concentration was 5-15%. After passing nitrogen gas through the solution, N-acetyl-cysteine-methylamide (see II.7.6.3) was added. Immediately thereafter, neat mustard gas (2-2.5 µl) or a solution of 0.1 M mustard gas in acetonitrile (60 µl) was introduced into the well-stirred solution at room temperature in a titration vessel of a pH-stat apparatus. The pH was kept at 7.5 by means of automated addition of 0.1 N aqueous sodium hydroxide. Samples of the reaction mixture was analyzed with micro-LC after concentration in vacuo.

II.9. Synthesis of adducts of mustard gas and N-acetyl-amino acid-methylamides

II.9.1. N-(2'-Hydroxyethylthioethyl)-valine-methylamide

II.9.1.1. N-(2'-Hydroxyethylthioethyl)-valine-methyl ester

N-(2'-Hydroxyethylthioethyl)-valine (30 mg; 0.14 mmol; purity ca. 80%; see II.10.2.5 and ref. 72) was treated with diazomethane in ether (3 ml; 0.3 mM) at room temperature. After 1 h the reaction was stopped with formic acid/ether (50/1), and solvents were evaporated. ^{1}H-NMR analysis of the residue indicated a ca. 60% conversion to the desired methyl ester. The product was used for the subsequent amidation reaction without further purification.

^{1}H-NMR in CD_3OD:
3.80[s,3H,OCH_3], 3.70[2H,CH_2OH], 3.56[1H,CHNH], 2.69[2H,SCH_2CH_2OH], 2.7-3.0[4H,NCH_2CH_2S], 2.16[1H,$CHCH_3)_2$], 0.98+1.06[dd,6H,$CH(CH_3)_2$].

II.9.1.2. N-(2'-Hydroxyethylthioethyl)-valine-methylamide

N-(2'-Hydroxyethylthioethyl)-valine-methyl ester (150 mg; purity ca. 60%) was reacted with 40% aqueous methylamine (15 ml) for 4 days at room temperature. After evaporation of solvent, the residue was dissolved in methylene chloride (10 ml) and filtered in order to remove hydrolyzed starting material. After evaporation of the solvent, the residue was chromatographed on a reversed phase Lobar column filled with Lichrosorb RP-18, with methanol/water (1/1,v/v) as eluent. This gave 10 mg (11%) of the desired product.

^{1}H-NMR in CD_3OD:
3.67[t,2H,CH_2OH], 2.83[d,1H,CHC(O)], 2.76[s,3H,$NHCH_3$], 2.67[m,4H, NCH_2CH_2S], 2.65[m,2H,CH_2CH_2OH], 1.93[m,1H,$CHCH_3$], 0.95/0.94[d,d,$CHCH_3$].

^{13}C-NMR in CD_3OD:
177.2[C(O)], 69.5[CHC(O)], 62.6[CH_2OH], 48.7[$NHCH_2$], 35.1[SCH_2CH_2OH], 33.1[$NCHCH_2S$], 32.7[$CHCH_3$], 25.9[$NHCH_3$], 18.9/19.6[$CHCH_3$].

Thermospray MS:
m/z= 235(MH^+), 251(MH^+ of sulfoxide; trace).

II.9.2. N-Acetyl-aspartic acid-4-(2'-hydroxyethylthioethyl) ester-1-methylamide and ring closure to 1-methyl-3-acetam .o-succinimide ([73] [74])

II.9.2.1. Alkylation of N-acetyl-aspartic acid-1-methylamide and r .g closure

Mustard gas (80 µl, 640 mmol) was added to a stirred solution of N-acetyl-aspartic acid-1-methylamide (105 mg, 0.56 mmol) in water (20 ml). The pH of the well-stirred mixture was kept at 7.5 with 0.1 N NaOH by means of a pH-Stat. After 4 h the intake of NaOH had stopped. The solution was concentrated in vacuo by means of a rotation evaporator, and the residual oil was further dried in a vacuum desiccator over phosphorus pentoxide. After treatment with dry ethanol the insoluble crystals of NaCl were removed by filtration. The filtrate was analyzed by means of LC-MS.

Thermospray-LC-MS:
m/z= 189(MH^+ of starting material), 171 and 188(MH^+ and MNH_4^+ of 1-methyl-3-acetamido-succinimide), 123 and 140(MH^+ and MNH_4^+ of thiodiglycol), 293[MH^+ of N-acetyl-aspartic acid-4-(2'-hydroxyethylthioethyl) ester-1-methylamide; minor peak].

In another experiment N-acetyl-aspartic acid-1-methylamide (38 mg, 0.20 mmol) was reacted with a fourfold excess of mustard gas (120 µl, 960 mmol) under the same conditions. However, when the reaction was

completed the reaction mixture was not treated to remove NaCl but was analyzed directly. According to HPLC, a larger fraction of the starting material had been converted, but only a small amount of the 4-(2'-hydroxyethylthioethyl) ester was present in the reaction mixture relative to a large amount of the cyclization product.

II.9.2.2. Synthesis of 1-methyl-3-acetamido-succinimide

Acetyl chloride (0.60 ml, 8.4 mmol) was added to a solution of N-acetyl-aspartic acid-1-methylamide (400 mg, 2.1 mmol) in methanol (10 ml), cooled in an ice bath. Reversed phase HPLC analysis showed that the starting material had been completely converted into the corresponding methyl ester after 24 h at room temperature. The reaction mixture was concentrated in vacuo and this procedure was repeated twice after addition of methanol (5 ml) and 1,4-dioxane (10 ml), respectively, in order to remove hydrochloric acid. The residue was redissolved in methanol (10 ml). After addition of triethylamine (0.2 ml, 1.4 mmol), the reaction mixture was set aside at room temperature for 24 h. Concentration of the reaction mixture in vacuo and recrystallization of the residue from ethanol/ether (1/2.5, v/v) gave 230 mg (64%) of the desired product, m.p. 174-176 °C.

Elemental analysis ($C_7H_{10}N_2O_3$, MW 170.2):

Calc.:	C 49.41%	Found:	49.3 ± 0.0%
	H 5.92%		5.9 ± 0.1%
	N 16.46%		16.35 ± 0.05%

^{1}H-NMR in DMSO-d_6:
8.51[bd,J=7.3 Hz,1H,NH], 4.42[ddd,J=9.1,7.8,and 5.2 Hz,1H,CH], 2.94[dd,J=17.6 and 9.2 Hz,1H,ring H-C-H], 2.86[s,3H,NCH_3], 2.52[dd,J=17.5 and 5.2 Hz,ring H-C-H], 1.86[s,3H,$C(O)CH_3$].

^{13}C-NMR in DMSO-d_6:
176.5 and 175.3[both ring C=O], 169.7[$CH_3\underline{C}(O)$], 48.5[CH], 35.1[CH_2], 24.4[NCH_3], 22.2[$\underline{C}H_3CO$].

Thermospray-LC-MS:
m/z= 171(MH^+), 188(MNH_4^+).

II.9.3. N-Acetyl-glutamic acid-5-(2'-hydroxyethylthioethyl)-1-methylamide ester (75)

Dicyclohexylcarbodiimide (0.48 g, 2.3 mmol) was added to a stirred solution of N-acetyl-glutamic acid-1-methylamide (0.47 g, 2.3 mmol), thiodiglycol (0.43 g, 3.5 mmol) and of 4-dimethylaminopyridine (30 mg, 0.25 mmol) in N,N-dimethylformamide (45 ml), cooled to 0 °C in an ice bath. Stirring was continued for 1 h at 0 °C and for 3 h at room temperature. After standing overnight at room temperature the reaction mixture was concentrated in vacuo using a rotation evaporator. The residual semi-crystalline mass was treated with ethyl acetate (40 ml). The crystalline precipitate (dicyclohexylurea, 0.57 g) was removed by filtration and the solution concentrated in vacuo. The residual oil was stirred with ether (40 ml) for 4 h. This

procedure was repeated three times in order to remove thiodiglycol. Finally, the solid residue was re-extracted with ethyl acetate (5 ml). Evaporation of the extract left a sticky residue. Analysis showed that it was the desired product, slightly contaminated with the corresponding di-ester of thiodiglycol with N-acetyl-glutamic acid-1-methyl-amide.

^{1}H-NMR in DMSO-d_6: 8.00[d,J=8.2 Hz,1H,N$\underline{H}$CH], 7.83[bq,J=4.6 Hz,1H,N$\underline{H}$CH$_3$], 4.79[bt,J=4.8 Hz,1H,CH$_2$O$\underline{H}$], 4.22[dt,J=5.6 and 8.2 Hz,1H,NHC$\underline{H}$], 4.16[t,J=6.7 Hz,2H,COOC$\underline{H}_2$], 3.56[dt,J=4.8 and 6.6 Hz,2H,C$\underline{H}_2$OH], 2.76[t,J=6.7 Hz,2H,C$\underline{H}_2$S], 2.62[t,J=6.6 Hz,2,C$\underline{H}_2$S], 2.59[d,J=4.6 Hz,3H,C$\underline{H}_3$NH], 2.31[t,J=7.8 Hz,2H,C$\underline{H}_2$COO], 1.75 and 1.93[both m,2H,C$\underline{H}_2$CH$_2$COO].

^{13}C-NMR in DMSO-d_6:
172.2[COO], 171.5[$\underline{C}$ONHCH$_3$], 169.4[CH$_3\underline{C}$ONH], 63.3[COO$\underline{C}$H$_2$], 61.0[CH$_2$OH], 51.7[CHNH], 34.2[CH$_2$S], 30.1[$\underline{C}$H$_2$COO], 30.0[CH$_2$S], 27.3[$\underline{C}$H$_2$CH$_2$COO], 25.6[CH$_3$NH], 22.5[$\underline{C}$H$_3$CO].

Thermospray MS:
m/z= 307(MH^+), 185(MH^+-thiodiglycol), 491(thiodiglycol di-ester of N-acetyl-glutamic acid-1-methylamide; trace).

II.9.4. <u>N-α-Acetyl-N1-(2'-hydroxyethylthioethyl)-histidine-methylamide</u> (76)

N-α-Acetyl-histidine-methylamide (4.3 g, 20 mmol; see II.4.5) was suspended in dry methanol (15 ml), together with 2-trimethylsilyloxyethyl 2'-chloroethyl sulfide (0.9 ml, ca. 4 mmol) and anhydrous sodium carbonate (1.0 g, 9 mmol). The mixture was refluxed for ca. 7 days with stirring, after which it was cooled and stored in a refrigerator overnight. The precipitate was removed by filtration and washed with methanol. The precipitate was discarded. The combined filtrates were evaporated to dryness at reduced pressure. The residue was extracted with dichloromethane. This left a second batch of mostly unreacted starting material as a solid on the filter, while the filtrate contained (thermospray-LC-MS) starting material, two monoadducts of mustard gas and several late eluting products. One of the late eluting products was identified as a di-adduct containing two mustard gas moieties per histidine moiety. Apparently, the adducts had lost their protective trimethylsilyloxy moieties during the reaction and work-up procedures. The dichloromethane extract was concentrated in vacuo, the residue was separated into two monoadduct fractions and three late eluting products by means of reversed phase chromatography on a Lobar RP 18 column using system A as an eluent at a flow of 4 ml/min (detection at 235 nm). The first eluting adduct was further purified by straight phase chromatography on a Lobar Lichroprep Si-60 silicagel column, using methanol as an eluent. The yield of 95% pure (HPLC, 220 nm; system A) solid material was 34 mg. A clearly defined melting point could not be determined. The product showed sintering and discoloration on heating, starting at 128.5 °C, until finally at ca. 250 °C complete decomposition and charring took place.

^{1}H-NMR in DMSO-d_6:
8.53[d,J= 8.3 Hz,1H,NHCH], 8.27(bq,J=4.6 Hz,1H,NHCH$_3$],
7.57[s,1H,C2H], 6.63[s,1H,C4H], 4.45[dt,J=5.6 Hz and 8.3 Hz,1H,NHCH],
4.09[m,2H,NCH$_2$], 3.55 [t,J=6.5 Hz,2H,CH$_2$OH], 2.96[dd,J=5.7 and 15.2
Hz,1H,HCH-CH], 2.84[t,J=7.2 Hz,2H,CH$_2$S], 2.79[dd,J=8.9 and 15.5
Hz,1H,HCH-CH], ± 2.58[m,2H,CH$_2$S], 2.58[d,J=4.6 Hz,3H,NHCH$_3$],
1.83[s,3H,COCH$_3$].

^{13}C-NMR in DMSO-d_6:
171.4[CONHCH], 169.3[COCH$_3$], 137.4[C2], 127.4[(C5], 126.5[C4],
61.0[CH$_2$OH], 52.2[CHNH], 44.0[CH$_2$N], 34.0[CH$_2$S], 32.2[CH$_2$S],
26.5[CH$_2$CH], 25.6[NHCH$_3$], 22.5[COCH$_3$].

Thermospray MS:
m/z = 315(MH$^+$).

EI-MS:
m/z = 314(M$^+$).

II.9.5. N-α-Acetyl-N3-(2'-hydroxyethylthioethyl)-histidine-methylamide (76)

II.9.5.1. Alkylation with 2-trimethylsilyloxyethyl 2'-chloroethyl sulfide

The pooled and concentrated second eluting monoadduct fractions, obtained by reversed phase Lobar chromatography as described in II.9.4, were further purified by repeated semi-preparative separations with reversed phase HPLC (system A). After lyophilization of the pooled eluates, an oily residue resulted, which solidified after trituration with dry dichloromethane. The yield of solid material was 30 mg, m.p. 134.5- 136 °C. According to reversed phase HPLC (system A) the product contained 2-3% late eluting impurity.

^{1}H-NMR in DMSO-d_6:
8.04[d,J=4.0 Hz,1H,NH-CH], 7.78[bq,J= ± 4.5 Hz,1H,NHCH$_3$],
7.54[s,1H,C2H], 6.89[s,1H,C4H], 4.39[dt,J= ± 5 and ± 8Hz,1H,CHNH],
4.08[t,J=6.8 Hz,2H,NCH$_2$], 3.54[t,J=6.6 Hz,2H,CH$_2$OH], 2.85[t,J=6.8
Hz,2H,CH$_2$S], 2.69 and 2.83[both m,2H,CH$_2$CH], 2.56[d,J=4.4
Hz,3H,CH$_3$NH], 2.56[t,J=6.6 Hz,2H,CH$_2$S], 1.84[s,3H,CH$_3$CO].

^{13}C-NMR in DMSO-d_6:
171.8[CONHCH$_3$], 169.1[COCH$_3$], 137.8[C5], 136.7[C2], 116.3[C4H],
61.0[CH$_2$OH], 52.9[CH], 46.2[NCH$_2$], 33.9[SCH$_2$], 32.5[SCH$_2$],
30.9[CH$_2$CH], 25.6[NHCH$_3$], 22.7[COCH$_3$].

Thermospray MS:
m/z = 315(MH$^+$).

II.9.5.2. Alkylation with 2-acetoxyethyl 2'-chloroethyl sulfide (76)

A mixture of N-α-acetyl-histidine-methylamide (0.46 g, 2.1 mmol), 2-acetoxy-ethyl 2'-chloroethyl sulfide (0.5 ml, ca. 2.4 mmol; see II.4.2), and anhydrous Na_2CO_3 (1 g, 9.4 mmol) in anhydrous methanol (10 ml) was refluxed for 12 h. Ca. 50% of the starting material had reacted, while ca. 25% of (2'-hydroxy-ethylthioethyl)-adducts were present according to LC-MS analysis. The adducts apparently lost the protective acetate group of the alkylating agent during the reaction. No further conversion was achieved by continued refluxing. The bulk of the starting material was removed by repeated extraction of the evaporated residue with dichloromethane. By chromatography on a Lobar Lichroprep Si-60 silicagel column with methanol as eluent, the crude product was separated into three fractions, which were examined with HPLC. The second fraction appeared to be 90% pure N3-adduct: it co-eluted with material of the synthesis as described in II.9.5. Recrystallization from methanol/ether (1/100, v/v; 50 ml) gave 50 mg of 95% pure product (HPLC, 220 nm), m.p. 135-136 °C. The impurities were starting material and an unknown earlier eluting compound. No other adduct was detectable. Analyses of the product were as described in II.9.5. The third fraction eluting from the Lobar column contained the N1-adduct, together with large quantities of earlier eluting starting material.

II.9.6. Isolation of N-acetyl-S-(2'-hydroxyethylthioethyl)-cysteine-methylamide

The product was isolated from the reaction mixture of mustard gas with N-acetyl-cysteine-methylamide obtained as described in II.8.1 by chromatography on a Lobar Lichroprep RP-18 column using methanol/water (1/1, v/v) as an eluent.

^{1}H-NMR in CD_3OD:
4.46[dd,J=6.1 Hz and J=7.8 Hz,1H,C$\underline{H}$NH], 3.69[t,J=6.6 Hz,2H,C$\underline{H}_2$OH], 2.97[dd,J=6.1 Hz and J=13.8 Hz,1H,$\underline{H}$CHS], 2.78 [dd,J=7.8 Hz and J=13.8 Hz,1H,HC$\underline{H}$S], 2.76[s,4H,SC$\underline{H}_2$C$\underline{H}_2$S], 2.74[s,3H,NHC$\underline{H}_3$], 2.69[t,J=6.6 Hz, 2H,SC$\underline{H}_2$CH$_2$OH], 2.00[s,3H,C(O)CH$_3$].

^{13}C-NMR in CD_3OD:
173.4[$CH_3\underline{C}$(O)NH], 173.3[CHC(O)NH], 62.6[CH_2OH], 54.6[NHCH], 35.2 [S$\underline{C}H_2CH_2$OH], 34.6[CH$\underline{C}H_2$S], 33.3 and 33.1[S$\underline{C}H_2\underline{C}H_2$S], 26.4[NHCH$_3$], 22.5 [$\underline{C}H_3$C(O)].

Thermospray MS:
m/z = 281(MH^+).

II.10. Synthesis of haptens: mustard gas adducts with peptides

II.10.1. N-(2'-Hydroxyethylthioethyl)-val-leu-ser-pro-ala-asp-lys

II.10.1.1. Val-leu-ser-pro-ala-asp-lys

The N-terminal heptapeptide of the α-chain of hemoglobin was synthesized as described by Van Denderen et al. (77) using a Biosearch Sam II automatic peptide synthesizer according to the solid phase synthesis method essentially as described by Merrifield (78) with t-butyloxycarbonyl-protected amino acids. Deprotection was performed with trifluoromethanesulfonic acid/thioanisole/m-cresol in trifluoroacetic acid. The peptide was purified using liquid chromatography on G-15 Sephadex (Pharmacia) in 5% acetic acid. Fractions were analyzed on a Beckman Ultrasphere 5 mm Reverse-Phase C18 column using a gradient of acetonitrile with 0.1% trifluoroacetic acid. Fractions containing the peptide of a high purity were pooled and lyophilized twice. In order to confirm the peptide composition, amino acid analyses were performed on the hydrolyzed peptide using precolumn derivatization of the amino acids according to Janssen et al. (79,80).

II.10.1.2. N-(2'-Hydroxyethylthioethyl)-val-leu-ser-pro-ala-asp-lys

See III.11.2 for a description of the N-alkylation of the heptapeptide with mustard gas, as well as for isolation and full characterization of the product.

II.10.2. N-(2'-Hydroxyethylthioethyl)-val-leu-ser (attempted)

II.10.2.1. 2-Aminoethyl 2'-hydroxyethyl sulfide (81)

2-Bromoethylamine hydrochloride (30.7 g, 0.15 mol) in ethanol (75 ml) was added to a mixture of mercaptoethanol (23.5 ml, 0.3 mol), 1 M sodium hydroxide in methanol (150 ml), and ethanol (150 ml). The reaction mixture was heated with stirring and ca. 200 ml of solvent was distilled off. Next, ether (100 ml) was added to the reaction mixture after cooling. After filtration, concentrated aqueous hydrochloric acid (10 ml) and ether (50 ml) were added. After another filtration, the filtrate was concentrated and the residue was distilled at reduced pressure to give 11.9 g (66%) of product, b.p. 125-126 °C/3 mmHg.

Elemental analysis ($C_4H_{11}NOS$, MW 221):

Calc.:	C 39.64%	Found: 39.39 ± 0.13%
	H 9.15%	9.22 ± 0.20%
	N 11.56%	11.40 ± 0.34%

^{1}H-NMR in DMSO-d_6:
3.55[t,2H,C$\underline{H}_2$OH], 2.70[t,2H,C$\underline{H}_2$NH$_2$], 2.58[t,2H,C$\underline{H}_2$CH$_2$OH], 2.55[t,2H,C$\underline{H}_2$CH$_2$NH$_2$].

^{13}C-NMR in DMSO-d_6:
61.0[CH$_2$OH], 41.8[CH$_2$NH$_2$], 35.7[S$\underline{C}$H$_2$CH$_2$NH$_2$], 34.0[S$\underline{C}$H$_2$CH$_2$OH].

Thermospray MS:
m/z = 122(MH$^+$)

II.10.2.2. N-(2'-Hydroxyethylthioethyl)-D,L-valine hydrochloride (82)

A solution of D,L-α-bromoisovaleric acid (1.5 g, 8.3 mmol) and of 2-aminoethyl 2'-hydroxyethyl sulfide (3.3 g, 27.2 mmol) in chloroform (5 ml) was set aside at room temperature for 10 days. Next, a 2.17 M solution of sodium methoxide in methanol (7.6 ml) was added and the solvents were evaporated at reduced pressure. The residue was extracted with acetone. After evaporation of this solvent, the residue was dissolved in 1 M aqueous hydrochloric acid (ca. 5 ml). The solution was used for separation of the products on a Sepharose S Fast Flow cation exchange column (14x1.5 cm). The eluent was composed from solvent A (aqueous hydrochloric acid, pH = 3) and solvent B (0.1 M aqueous sodium chloride). The column was eluted at a rate of 1 ml/min during 60 min with solvent A, for the next 30 min with a linear gradient increasing to 100% solvent B, and finally for 60 min with 100% solvent B. The product eluted after 70-80 min. After lyophilization of the fractions containing the product, most of the sodium chloride in the residue was removed by trituration with ethanol. Evaporation of the solvent left the product as a slightly brownish-colored powder (200 mg, 11%), m.p. 125-128 °C, containing ca. 5.8% sodium chloride according to atomic absorption spectrometry. Addition of NaOD to a solution of the product in D_2O caused a shift to higher field of N-neighbouring hydrogen signals in the ^{1}H-NMR spectrum. It follows that the product was isolated as the hydrochloride salt.

^{1}H-NMR in D_2O:
3.81[d,1H,CHC(O)], 3.76[t,2H,C$\underline{H}_2$OH], 3.33[m,2H,NHC$\underline{H}_2$], 2.93[m,2H,CH$_2$S], 2.77[t,2H,SCH$_2$], 2.33[m,1H,CH$_3$C$\underline{H}$], 1.09 and 2.04[dd,J=4.2, 6.0, and 6.9 Hz,6H,2CH$_3$]

Thermospray MS:
m/z = 222(MH$^+$)

EI-MS:
m/z = 203(M-H$_2$O), 178(M-C$_3$H$_7$), 176(M-COOH), 160(203-C$_3$H$_7$), 130(M-CH$_2$SCH$_2$CH$_2$OH), 105, 102, 84, 36.

II.10.2.3. Optical resolution of D,L-α-bromoisovaleric acid (83)

A solution of (-)-α-phenylethylamine (6.6 g, 0.056 mol) in ether (15 ml) was added to a solution of (±)-α-bromoisovaleric acid (19.5 g, 0.11 mol) in ether (35 ml). The reaction mixture was set aside for 3 days at 5 °C. Next, the precipitated salt was isolated by filtration, washed with ether and dried. Yield 13.3 g (81.6%), $[\alpha]_D$ = -21.9° (c = 0.010, acetone). According to ^{1}H-NMR in $CDCl_3$ [CH-Br, d, 3.72 ppm, (+,-)-isomer; 3.74 ppm, (-,-)-isomer] the product contains 10-15% of the (-,-)-isomer. Four recrystallizations from acetone gave 8.0 g (48%) of the (+,-)-salt, $[\alpha]_D$ = -22.7° (c = 0.012, acetone; ref. 82: $[\alpha]_D$ = -22° , c = 1, acetone), m.p. 130-131 °C, optical purity 99% (^{1}H-NMR).The product was dissolved in 50% aqueous hydrobromic acid (25 ml) and extracted with toluene. The combined organic phases were dried on anhydrous sodium sulfate. Evaporation in vacuo of the solvent yielded (+)-α-bromoisovaleric acid (3.1 g, 65%), $[\alpha]_D$ = +18.9° (c = 0.09, $CHCl_3$), m.p. 41-42 °C.

II.10.2.4. N-(2-Hydroxyethylthioethyl)-D-valine

A solution of (+)-α-bromoisovaleric acid (1.81 g, 10 mmol) and of 2-aminoethyl 2'-hydroxyethyl sulfide (4.08 g, 34 mmol) in chloroform (5 ml) was set aside for 12 days at room temperature. Next, aqueous 2 M hydrochloric acid (12 ml) was added, and subsequently all solvents were evaporated in vacuo. The solid residue was washed with acetone and dissolved in water (5 ml). The product was purified on a Sepharose S cation exchange column as described in II.10.2.2, with replacement of solvent B by aqueous ammonia (pH = 8). The product eluted after 30-40 min, i.e., before application of the gradient. Yield 250 mg (11.3%; Zwitter ion), m.p. 234-235 °C, $[\alpha]_D$ = +18.7° (c = 0.0055, water).

^{1}H-NMR in DMSO-d_6:
3.55[t,2H,CH_2OH], 3.13[d,1H,CHC(O)], 2.63-2.94[m,4H,$2CH_2$], 2.60[m,2H,SCH_2], 2.03[m,1H,CH_3CH], 0.94 and 0.96[dd,J= 6.7 and 4.6 Hz,6H,$2CH_3$].

Thermospray MS:
m/z = 222(MH^+).

II.10.2.5. N-(2'-Hydroxyethylthioethyl)-valine (72)

After addition of aqueous 10 N sodium hydroxide (1.2 ml) to a solution of valine (3 g, 25.6 mmol) in water (20 ml), the solution was heated to 50-70 °C. Next, a solution of 2-acetoxyethyl 2'-chloroethyl sulfide (7.7 ml; see II.4.2) in ether (90 ml) was added to the stirred solution in the course of 1 h. After standing overnight at room temperature, the reaction mixture was washed with chloroform (3x20 ml). Next, the pH of the aqueous solution (pH 12) was lowered to 2.7 by means of addition of concentrated aqueous hydrochloric acid, and the solution was washed with ether (2x30 ml). After evaporation of the aqueous solution to dryness in vacuo, the

white solid residue was rapidly washed with isopropanol (20 ml) and was subsequently extracted with the same solvent in a Soxhlet apparatus for 50 h. Cooling and filtration of the organic solvent gave 2.15 g (38%) of product, m.p. 225-227 °C, purity 92% (^{1}H-NMR).

^{1}H-NMR in D_2O:
3.75[d,2H,CH_2OH], 3.13[d,1H,CHC(O)], 2.63-2.94[m,4H,$2CH_2$], 2.60[m,2H,SCH_2], 2.03[m,1H,CH_3CH], 0.94 and 0.96[dd,J= 6.7 and 4.6 Hz,6H,$2CH_3$]

Thermospray MS:
m/z = 222(MH^+).

II.10.2.6. Chiral ^{1}H-NMR of N-(2'-hydroxyethylthioethyl)-valine (84)

Ytterbium-(S)-carboxymethyloxysuccinate (5.6 mg, 15.5 mmol; gift from Dr. Peters, Technological University, Delft, The Netherlands) dissolved in D_2O/DCl (0.2 ml, pH 3.2) was added to a solution of N-(2'-hydroxyethylthioethyl)-valine (1 mg, 4.5 μmol) in the same solvent (0.8 ml).

II.10.2.7. N-Benzyloxycarbonyl-leu-ser-ethyl ester

Concentrated aqueous sulfuric acid (0.1 ml) was added to a solution of N-benzyloxycarbonyl-leu-ser (3.5 g, 10 mmol) in dry ethanol (50 ml). After a reflux period of 30 min, the solvent was evaporated in vacuo. The residue was dissolved in ether (15 ml) and washed successively with aqueous 0.05 N sodium hydroxide (2 x 5 ml) and water (1 x 5 ml). After drying of the solution on anhydrous magnesium sulfate, evaporation of the solvent left 2.6 g (68%) of product, m.p. 112-113 °C.

^{1}H-NMR in $CDCl_3$:
6.85[d,1H,OC(O)NH], 5.24[d,1H,CHC(O)H], 5.09[m,2H,CH_2O], 4.60[m,1H,$CHCH_2OH$], 4.24[m,2H,OCH_2CH_3], 4.20[m,1H,$CHCH_2CH$], 3.93[d,2H,CH_2OH], 1.7[m,1H,$CHCH_3)_2$], 1.54/1.68[m,2H,$CHCH_2CH$], 1.29[t,3H,CH_2CH_3], 0.94/0.95[dd,6H,$2CH_3$].

Thermospray MS:
m/z = 381(MH^+), 398(MNH_4^+), 363($MH^+ - H_2O$).

II.10.2.8. Leu-ser-ethyl ester hydrochloride (68)

After bubbling nitrogen gas for 5 min through a solution of N-benzyl-oxycarbonyl-leu-ser-ethyl ester (1.0 g, 2.6 mmol) and of concentrated aqueous hydrochloric acid (0.25 ml) in dry ethanol (25 ml), 10% palladium on charcoal (100 mg) was added. Next, the solution was heated to 50 °C and hydrogen gas was led over the solution for 3 h. Filtration of the reaction mixture and evaporation in vacuo of solvent gave a solid product, which was recrystallized from ethanol (10 ml)/ether (65 ml). Yield 350 mg (55%), m.p. 153-154 °C.

Elemental analysis ($C_{11}H_{23}N_2O_4Cl$, MW 282.8):

Calc.:	C	46.71%	Found: 45.23 ± 0.03%
	H	8.20%	8.22 ± 0.14%
	N	9.90%	9.62 ± 0.07%
	Cl	12.55%	11.91 ± 0.18%

^{1}H-NMR in DMSO-d_6:
8.90[d,1H,NHCH], 8.00[bq,3H,NH^+], 5.18[t,1H,CH_2OH], 4.42[m,1H,NHCH], 4.13[m,2H,CH_2CH_3], 3.88[t,1H,$CHCH_2CH$], 3.69/3.80[m,2H,CH_2OH], 1.74[m,1H,$CH(CH_3)_2$], 1.64/1.54[m,1H,$CHCH_2CH$], 1.21[t,3H,CH_2CH_3], 0.93/0.96[dd,6H,$2CH_3$].

Thermospray MS:
m/z = 247(MH^+), 229(MH^+ - H_2O), 201(MH^+ - ethanol).

II.10.2.9. Attempted synthesis of N-(2'-hydroxyethylthioethyl)-D-val-leu-ser (85)

A solution of leu-ser-ethyl ester hydrochloride (10.8 mg, 38 µmol) and triethylamine (5.37 µl, 38 µmol) in N,N-dimethyl-formamide (1 ml) was added to a solution of N-(2'-hydroxy-ethylthioethyl)-D-valine hydrochloride (9.8 mg, 38 µmol) in N,N-dimethylformamide (1 ml). After cooling on ice, dicyclohexyl-carbodiimide (7.88 mg, 38 µmol) was added. The solution was kept for a further 2 h at 0 °C and was subsequently stirred for 24 h at room temperature. Thermospray MS analysis of the reaction mixture indicates <5% conversion to the desired product (m/z = 450,MH^+), whereas the major peak at m/z = 428 pertained to MH^+ of the addition product of N-(2'-hydroxyethylthioethyl)-D-valine and the diimide. A similar reaction between N-(2'-hydroxyethyl-thioethyl)-D-valine (5.7 mg, 26 µmol), leu-ser-ethyl ester hydrochloride (7.3 mg, 26 µmol), and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (4.9 mg, 26 µmol) in N,N-dimethyl-formamide (2 ml) led to the observation in the thermospray mass spectrum of the reaction mixture of MH^+ - H_2O (m/z = 359) of the addition product of N-(2'-hydroxyethylthioethyl)-D-valine and the diimide, without observation of the desired tripeptide product.

II.10.3. Gly-gly-gly-glutamic acid-5-(2'-hydroxyethylthioethyl) ester 1-amide-hydrochloride

II.10.3.1. N-Benzyloxycarbonyl-gly-gly-gly-glutamic acid-5-t-butyl ester-1-amide (67)

Ethyl chloroformate (0.32 ml, 3.35 mmol) was added to a stirred and cooled (-15 °C) solution of cbz-gly-gly-gly (1.06 g, 3.28 mmol) and triethylamine (0.46 ml, 3.3 mmol) in N,N-dimethylformamide (20 ml). After 10 min of stirring, during which triethylammonium choride crystallized, a solution of glutamic acid-5-t-butyl ester-1-amide

(3.36 mmol) in N,N-dimethylformamide (15 ml)* was added. The reaction mixture was stirred for another 20 min at -15 °C and for 1 h at room temperature. After filtration through a glass filter the filtrate was concentrated in a rotation evaporator. The product crystallized by dissolving the residual oil in 30 ml of ethanol and slowly adding 60 ml of water to the stirred solution. The crystals were collected on a glass filter, washed with ethanol/water (1/2, v/v) and dried in a vacuum desiccator containing phosphorus pentoxide. Yield 1.21 g (73%), m.p. 142-150 °C.

^{1}H-NMR in DMSO-d_6:
8.18 and 8.12[both t,J=5.7 Hz,2H,2xNHC=O], 7.9[d,J=8.1 Hz,1H,C(O)NHCH], 7.45[t,J= 5.9 Hz,1H,NHC=O],7.30-7.42[m,5H, aromatic], 7.27 and 7.07[both bs,2H,C(O)NH_2], 5.06[s,2H,CH_2O], 4.21[dt,J=5.0 and 8.5 Hz,1H,CHNH], 3.78[m,2H,CH_2NH], 3.77[d,J=5.6 Hz,2H,CH_2NH], 3.70[d,J=6.C Hz,2H,CH_2NH], 2.22[m,2H,CH_2COO], 1.95 and 1.75[both m,2H,CH_2CH], 1.41[s,9H,3CH_3].

^{13}C-NMR in DMSO-d_6:
173.1[$CONH_2$], 171.7[COO], 169.7, 169.3 and 168.7[3CONH], 156.6[(NHCOO], 137.1, 128.4 and 2x127.8[aromatic C's],79.7[C(CH_3)$_3$], 65.6[$CH_2C_6H_5$], 51.7[CHNH], 43.7, 42.2 and 42.1[3CH_2NH], 31.4[CH_2COO], 27.8[3CH_3], 27.2[CH_2CH].

Thermospray MS:
m/z = 508(MH^+), 400(MH^+ - $C_6H_5CH_2OH$), 344(Cbz-gly-gly-gly-glu-NH_2.H^+ minus $C_6H_5CH_2OH$), 452[Cbz-gly-gly-gly-glu-NH_2.H^+ (possible impurity)].

II.10.3.2. Gly-gly-gly-glutamic acid-1-amide trifluoroacetic acid salt (86)

Over a well stirred solution of cbz-gly-gly-gly-glu-5-t-butyl ester-amide (320 mg, 0.63 mmol) in N,N-dimethylformamide (10 ml), containing 400 mg of 10% palladium on charcoal, a stream of nitrogen was passed for 20 min, followed by a stream of hydrogen for 4 h and again nitrogen for 20 min. The reaction mixture was passed through a glass filter (D4) with suction which afforded only a partial removal of the charcoal. The filtrate was concentrated in vacuo and the residual oil dissolved in trifluoroacetic acid (30 ml). After 16 h at room temperature the mixture was filtered through a glass filter to remove the remaining charcoal and the filtrate was concentrated in vacuo. The semi-crystalline oil was crystallized from absolute ethanol/dry ether (1/5, v/v, 30 ml). The hygroscopic crystals were dried in a vacuum desiccator over charcoal and phosphorus pentoxide. Yield 0.27 g (99%).

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* This "solution" was obtained by adding triethylamine (0.56 ml, 3.87 mmol) to a suspension of glutamic acid-5-t-butyl ester-1-amide hydrochloride (0.99 g, 3.36 mmol) in N,N-dimethylformamide (15 ml). The mixture was shaken until the large crystals of the glutamate had been replaced by the smaller crystals of triethylammonium chloride.

^{1}H-NMR in DMSO-d_6:
8 67[t,J=5.7 Hz,1H,NHC=O], 8.24[t,J=5.7 Hz,1H,NHC=O], 8.07[bs,3H, NH_3^+], 7.95[(d,J=8.1 Hz,1H,C(O)NHCH], 7.34 and 7.08[both bs,2H, $C(O)NH_2$], 4.23[dt,J=5.2 and 8.3 Hz,1H,NHCH], 3.87[d,J=5.8 Hz,2H, $NHCH_2CO$], 3.79[m,1H,$NHCH_2CO$], 3.64[s,2H,$COCH_2NH_3^+$], 2.24[m,2H, CH_2COO], 1.96 and 1.76[both m,$CHCH_2$].

^{13}C-NMR in DMSO-d_6:
174.0[COO], 173.2[$CONH_2$], 168.8, 168.7 and 166.5[3CONH], 51.8[NHCH], 42.0[$2NHCH_2$], 40.2[$CH_2NH_3^+$], 30.2[CH_2COO], 27.3[$CHCH_2$].

Thermospray MS:
m/z = 318(MH^+), 300(MH^+-H2O), 147(H-glu-NH_2.H^+), 346(H-gly-gly-gly-glu-NH_2-5-ethyl ester.H^+; minor impurity due to recrystallization), 391(dioctyl phthalate.H^+)

In order to remove dioctyl phthalate, an aqueous solution of the product was extracted with ethyl acetate and lyophilized.

II.10.3.3. Gly-gly-gly-glutamic acid-5-(2'-hydroxyethylthioethyl) ester-1-amide hydrochloride (69)

By means of heating and occasional shaking 200 mg (0.46 mmol) of gly-gly-gly-glutamic acid-1-amide trifluoroacetic acid salt was dissolved in thiodiglycol (4.0 ml, 39 mmol). After cooling to room temperature acetyl chloride (0.18 ml, 2.5 mmol) was added with shaking. The reaction mixture was kept at room temperature and the disappearance of the starting peptide as well as the formation of the peptide ester was monitored by means of HPLC (system A). After five days the reaction mixture was shaken with ethyl acetate (40 ml). The insoluble oily substance was allowed to settle on the bottom of the flask, after which the supernatant liquid was decanted. This treatment with eth l acetate was repeated twice with solutions of the precipitates in dry ethanol (5 ml). Crystallization occurred when the precipitate was finally treated with acetonitrile (10 ml). However, when collecting the crystals on a glass filter, the apparently very hygroscopic compound turned into an oily substance (ca. 150 mg, 71%), which was analyzed.

^{1}H-NMR in DMSO-d_6:
8.74[t,J= ca.5.8 Hz,1H,NHC=O], 8.3[t,J=5.8 Hz,1H,NHC=O], 7.98[d,J=8.1 Hz,1H, NHCH], 7.38 and 7.10[both s,2H,$CONH_2$], 4.24[dt,J=5.0 and 8.5 Hz,1H,NHCH], 4.17[t,J=6.8 Hz,2H,$COOCH_2$], 3.85[d,J=5.5 Hz,2H,$NHCH_2$], 3.78[m,2H,$NHCH_2$], 3.62[s,2H,$CH_2NH_3^+$], 3.56[t,J=6.7 Hz,2H,CH_2OH], 2.77[t,J=6.8 Hz,2H,CH_2S], 2.63[t,J=6.6 Hz,2H,CH_2S], 2.34[m,2H, CH_2COO], 2.00 and 1.81[both m,2H,$CHCH_2$].

^{13}C-NMR in DMSO-d_6:
173.0[$CONH_2$], 172.3[COO], 168.7[2xCONH], 166.5[$CO(NH)CH_2NH_3^+$], 63.3[$C(O)OCH_2$], 61.1[CH_2OH], 51.6[CH], 42.1[$NHCH_2$], 42.0 [$NHCH_2C(O)NHCH$], 40.3[$CH_2NH_3^+$], 34.2[CH_2CH_2OH], 30.1[CH_2COO], 30.0 [CH_2S], 27.2[CH_2CH].

Thermospray MS:
m/z = 422 (MH^+), 300(MH^+-thiodiglycol), 189(H-gly-gly-gly-$NH_2.H^+$).

II.10.4. Attempted synthesis of cys-gly-gly-gly

II.10.4.1. S-benzyl-N-benzyloxycarbonyl-cysteine-cyano-methyl ester (87)

S-benzyl-N-benzyloxycarbonyl-cysteine (9 g, 26 mmol) was added to a stirred and cooled (0 °C) mixture of triethylamine (5.48 ml, 39 mmol) and chloroacetonitrile (3.29 g, 52 mmol). The reaction mixture was kept at room temperature overnight. Ethyl acetate (50 ml) was added and after stirring for a short period of time the precipitate formed was filtered and washed with ethyl acetate (15 ml). The filtrate was evaporated to dryness. The residue was dissolved in refluxing ether (30 ml), and hexane (45 ml) was slowly added to precipitate the product. The precipitate was filtered, washed with ether/hexane (1/1, v/v) and air-dried to give 6.9 g (69%) of product. m.p. 67-68 °C (ref. 87: 67-68 °C)

^{1}H-NMR in $CDCl_3$:
7.21-7.39[m,10H,aromatic], 5.42[bd,J=6-7 Hz,1H,NHC(O)], 5.12[AB pattern,J=12.2 Hz,2H,$C_6H_5C\underline{H}_2O$], 4.71[s,2H,OCH_2CN], 4.61[bq,J=6-7 Hz,1H,CHC(O)], 3.71[s,2H,$C_6H_5C\underline{H}_2S$], 2.87[m,2H,$CHC\underline{H}_2S$].

^{13}C-NMR in $CDCl_3$:
169.6[$CH\underline{C}(O)O$], 156.1[$O\underline{C}(O)NH$], 137.1, 136.0, 129.0, 128.9[2 signals], 128.8, 128.7, 128.5, 128.3, and 127.6[aromatic], 113.7[CN], 67.5[$C_6H_5\underline{C}H_2O$], 53.5[$NH\underline{C}HC(O)$], 49.2[$O\underline{C}H_2CN$], 36.8[$C_6H_5\underline{C}H_2S$], 33.2 [$CH\underline{C}H_2S$].

II.10.4.2. Glycylglycine-ethyl ester hydrochloride

Acetyl chloride (25 ml) was added dropwise to dry ethanol (250 ml). The temperature of the solution increased to ca. 50 °C. Glycylglycine (13.21 g, 100 mmol) was added, which rapidly dissolved. The reaction mixture was kept overnight at room temperature. After stirring for an additional 2 h at 60 °C, the reaction mixture was cooled and the precipitate formed was filtered and air-dried. Recrystallization from ethanol (100 ml) yielded 14.8 g (75%). M.p. 181-182 °C. HPLC and ^{1}H-NMR analyses showed 10% contamination, probably by the starting compound.

^{1}H-NMR in D_2O:
4.23[q,J=7.2 Hz,2H,$CH_3C\underline{H}_2$], 4.08[s,2H,$NHC\underline{H}_2C(O)$], 3.90[s,2H,$NH_3,C\underline{H}_2C(O)$], 1.27[t,J=7.2 Hz,3H,$C\underline{H}_3CH_2$].

^{13}C-NMR in D_2O:
174.1[$CH_2\underline{C}(O)O$], 170.2[$CH_2\underline{C}(O)NH$], 65.3[$CH_3\underline{C}H_2O$], 43.9[$NH\underline{C}H_2C(O)$], 43.0[$NH_3\underline{C}H_2C(O)$], 15.9[$\underline{C}H_3CH_2O$].

II.10.4.3. N-t-butyloxycarbonyl-glycylglycylglycine-ethyl ester

Triethylamine (2.4 ml) was added to a suspension of glycylglycine-ethyl ester hydrochloride (3.26 g, 16.7 mmol) in dichloromethane (40 ml) which partially dissolved the ester. N-t-butyloxycarbonyl-glycine (2.93 g, 16.7 mmol) was dissolved and next dicyclohexylcarbodiimide (3.5 g) was added. After stirring overnight the precipitate was removed and the filtrate was washed with 1 N HCl, 1 N $KHCO_3$, and water. After drying on magnesium sulfate, the organic phase was evaporated to dryness. The residue was dissolved in acetone (25 ml) and filtered. The filtrate was concentrated to ca. 10 ml to which ether (50 ml) was added. After cooling at -18 °C, the precipitate was collected, washed with ether and dried, yielding 3.2 g (60%). M.p. 98-100 °C.

^{1}H-NMR in DMSO-d_6:
8.24[bt,J=6.0 Hz,1H,N<u>H</u>CH_2C(O)O], 8.06[bt,J=5.8 Hz,1H,N<u>H</u>CH_2C(O)], 6.98[bt,J=5.8 Hz,1H,CH_2N<u>H</u>C(O)O], 4.11[q,J=7.0 Hz,2H,$CH_3C\underline{H}_2O$], 3.85[d,J=6.0 Hz,2H,NHC<u>H</u>$_2$C(O)O], 3.77[d,J=5.8 Hz,2H,NHC<u>H</u>$_2$C(O)], 3.60[d,J=5.8 Hz,2H,OC(O)NHC<u>H</u>$_2$C(O)], 1.41[s,9H,$(CH_3)_3$C], 1.22[t,J=7.0 Hz,3H,$C\underline{H}_3CH_2$].

^{13}C-NMR in DMSO-d_6:
169.8[C(O)NH], 169.7[C(O)O], 169.4[C(O)NH], 155.9[NH<u>C</u>(O)OC], 78.2[<u>C</u>$(CH_3)_3$], 60.5[COO<u>C</u>H_2], 43.4[<u>C</u>H_2C(O)NH], 41.8[<u>C</u>H_2C(O)NH], 40.7[<u>C</u>H_2C(O)O], 28.2[$(CH_3)_3$], 14.1[$CH_2\underline{C}H_3$].

II.10.4.4. Glycylglycylglycine-ethyl ester trifluoroacetic acid salt

N-t-butyloxycarbonyl-glycylglycylglycine-ethyl ester (3.5 g) was added to trifluoroacetic acid (25 ml) inducing a vigorous development of carbon dioxide. After 1 h the trifluoroacetic acid was evaporated in vacuo. The residue was triturated twice with ether (25 ml). Residual ether was removed in vacuo in a rotavapor. Then the oily residue slowly crystallized. The conversion was quantitative. M.p. 99-105 °C. No impurities could be detected by HPLC analysis.

^{1}H-NMR in DMSO-d_6:
8.64[bt,J=6.0 Hz,1H,CH_2N<u>H</u>C(O)], 8.42[bt,J=6.0 Hz,1H,CH_2N<u>H</u>C(O)], 8.02[bs,3H,NH_3], 4.12[q,J=7.0 Hz,2H,$CH_3C\underline{H}_2O$], 3.88[d,4H,J=6.0 Hz, 2x NHC<u>H</u>$_2$C(O)], 3.63[bq,J=5.4 Hz,2H, C<u>H</u>$_2$NH_3], 1.22[t,J=7.0 Hz,3H,CH_3CH_2].

II.10.4.5. S-benzyl-N-benzyloxycarbonyl-cysteinylglycyl-glycylglycine-ethyl ester

Triethylamine (0.66 ml, 4.7 mmol) and two drops of acetic acid were added to a solution of S-benzyl-N-benzyloxycarbonyl-cysteine-cyanomethyl ester (1.72 g, 4.4 mmol) and glycylglycylglycine-ethyl ester trifluoroacetic acid salt (1.5 g, 4.5 mmol). After standing for a week at room temperature the reaction mixture had become solid. After addition of ether (50 ml), the mixture was stirred. The gel

formed was filtered, washed with ether and air-dried. The resulting wax was dissolved in warm acetone (25 ml) and filtered. After cooling at - 18 °C the gel was collected, washed with acetone/ether (1/1, v/v) and with ether, and air-dried. Yield 2.03 g (84%). M.p. 124-125.5 °C.

^{1}H-NMR in DMSO-d_6:
8.35[bt,J=5.4 Hz,1H,CH_2N<u>H</u>C(O)CH], 8.24[bt,J=5.8 Hz,1H,N<u>H</u>CH_2C(O)O], 8.13[bt,J=5.8 Hz,1H,N<u>H</u>CH_2C(O)NH], 7.60[d, J=8.3 Hz,1H,CHN<u>H</u>C(O)], 7.22-7.47[m,10H,aromatic], 5.09[AB pattern,J=12.8 Hz,2H,$C_6H_5C\underline{H}_2O$], 4.33[m,1H,$SCH_2C\underline{H}$], 4.11[q,J=7.1 Hz,2H,$CH_3C\underline{H}_2O$], 3.85[d,J=5.8 Hz,2H,NHC$\underline{H}_2$C(O)O], 3.80[d,J=5.4 Hz,2H,CHC(O)NHC$\underline{H}_2$], 3.79[s,2H,$C_6H_5C\underline{H}_2S$], 3.78[d,J=5.8 Hz,2H,NHC$\underline{H}_2$C(O)NH], 2.84[dd,J=5.0 Hz and J=13.7 Hz,1H,<u>H</u>CHS], 2.61[dd,J=9.1 Hz and J=13.7 Hz,1H,HC<u>H</u>S], 1.21[t,J=7.1 Hz,3H,$CH_2C\underline{H}_3$].

^{13}C-NMR in DMSO-d_6:
170.8[CH<u>C</u>(O)NH], 169.7[$CH_2\underline{C}$(O)O], 169.3[$CH_2\underline{C}$(O)NH], 168.9 [$CH_2\underline{C}$(O)NH], 156.1[O<u>C</u>(O)NH], 138.4, 137.0, 128.9, 128.4(2 signals), 127.8(2 signals) and 126.8[aromatic], 65.7 [O<u>C</u>$H_2C_6H_5$], 60.4[C(O)O<u>C</u>H_2], 54.3[$SCH_2\underline{C}$H], 42.3 [CHC(O)NH<u>C</u>H_2], 41.7[<u>C</u>H_2C(O)NH]. 4.07[<u>C</u>H_2C(O)O], 35.3 [S<u>C</u>$H_2C_6H_5$], 33.3[S<u>C</u>H_2CH], 14.1[$CH_2\underline{C}H_3$].

Thermospray MS:
Two major products were observed. Product 1 (72% of the total product), m/z = 545(MH^+ of the desired compound); product 2 (22% of the total product), m/z = 561(MH^+ + 0, probably the S -> O analog of the desired compound).

II.11. Identification of mustard gas-adducts to calf-thymus DNA and DNA of human white blood cells

II.11.1. Preparation of single-stranded DNA

Calf-thymus DNA was soaked overnight in distilled water (>20 mg/ml). The DNA was then dissolved in phosphate-buffered saline (PBS; 0.14 M NaCl, 2.6 mM KCl, 8.1 mM Na_2HPO_4 and 15 mM KH_2PO_4, pH 7.4; 1 mg/ml). To generate single-stranded DNA, the double-stranded calf-thymus DNA, or DNA isolated from white blood cells (WBC; see section II.11.4), were heated for 10 min at 100 °C. The amount of single-stranded DNA was measured spectrophotometrically (ϵ_{260nm} = 8,580 $l.mol^{-1}.cm^{-1}$; expressed per mol nucleotide).
Single-stranded salmon-sperm DNA was prepared in the same way as described for calf-thymus DNA.

II.11.2. Treatment of DNA with mustard gas

A solution of double- or single-stranded DNA in PBS (1 mg/ml) was treated with mustard gas or [^{35}S]mustard gas in acetone (0.1-1000 μM mustard gas; 37 °C; 30-60 min; final acetone concentration: 1%). The specific radioactivity of the batch at the day of preparation was determined (850 and 316 MBq/mmol) and the specific activity at the day of use was calculated, taking in account a half life of 87 days.

After treatment with mustard gas the DNA was purified by alcohol precipitation (in the same way as described for DNA of human WBC in section II.11.4) and dissolved in a buffer. In certain experiments this solution was sonicated before further use.

II.11.3. Treatment of human blood with mustard gas and the isolation of blood cells

Blood of human volunteers (10 ml) was collected in evacuated glass tubes, containing 15 mg Na_2EDTA. The blood was treated with mustard gas or [^{35}S]mustard gas in acetone (10-1000 μM mustard gas; 37 °C; 30-60 min; final acetone concentration 1%).

Separation of serum and blood cells
The blood was centrifuged (15 min; 4 °C; 480 g) and the serum collected. The cells were washed twice with PBS (4 °C) and the supernatant was removed. The cells were resuspended in PBS.

Separation of erythrocytes and white blood cells (WBC)
Lysis of the erythrocytes was brought about by incubation of the cell suspension at 0 °C with three volumes of freshly prepared lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.4). After centrifugation for 15 min at 400 g (4 °C), the supernatant containing the hemoglobin was removed. The pelleted WBC were washed twice with PBS and finally resuspended in the buffer used in the experiment.

II.11.4. DNA isolation from white blood cells (WBC)

WBC from 10 ml blood, isolated as described in II.11.3., were resuspended in 2.5 ml 10 mM Tris-HCl, 1 mM Na_2EDTA, pH 7.8. Sodium dodecyl sulfate (SDS; final concentration 1% w/v) was added to lyse the cells. Proteinase K (250 μg/ml) was added, to digest protein. The lysates were incubated overnight at 37 °C. DNA was purified by phenol extraction (15 min gently shaking with an equal volume of phenol, saturated with 10 mM Tris-HCl, 1 mM Na_2EDTA, 0.1 M NaCl, pH 7.8, followed by separation and removal of the phenol layer). After addition of 0.1 volume of 3 M sodium acetate, 1 mM Na_2EDTA, pH 5.5, the DNA was precipitated with two volumes of absolute ethanol, pre-cooled at -20 °C. With a glass pipet the DNA was collected, washed in 80% ethanol and dissolved in 2.5 ml of the Tris/Na_2EDTA buffer. RNAse A (final concentration: 75 μg/ml, heated at 80 °C for 5 min to destroy any DNAse activity) and RNAse T1 (Boehringer; final concentration: 75 units/ml) were added to digest the RNA (2 h; 37 °C). The DNA was purified by extraction with an equal volume of chloroform/isoamylalcohol (24:1) and alcohol precipitation as described above. The DNA was dissolved in the buffer used in the experiment. The amount of double-stranded DNA was measured spectrophotometrically (ϵ_{260nm} = 6,600 $l.mol^{-1}.cm^{-1}$, expressed per mol nucleotide).

II.11.5 Preparation of DNA for the competitive ELISA

Various procedures to make mustard gas-treated double-stranded DNA single-stranded were followed in order to obtain an optimal accessibility of the DNA damage for the antibodies without disrupting or destroying the N7-adduct. In all cases the calf-thymus DNA used had been exposed to various concentrations of mustard gas as indicated in section II.11.2. The samples resulting from the various treatments were tested in the competitive ELISA.

Treatment with alkali
Single-stranded calf-thymus DNA (0.15 ml; 100 µg/ml) in PBS was added to 0.8 ml "alkali" solution (1.3 M NaCl, adjusted to pH 12.1 with 1 M NaOH) and the mixture was incubated for 30 min at room temperature. The solution was sonicated for 20 s to prevent re-annealing of the DNA-strands and, subsequently, neutralized with 0.15 ml 250 mM KH_2PO_4. Finally, 0.1 ml 0.2% SDS in PBS was added to prevent adsorption of the DNA to the walls of the polystyrene tubes.

Treatment with 2x SSC/70% formamide
Double- and single-stranded calf-thymus DNA (0.1 ml; 100 µg/ml) in PBS were treated with 0.3 ml SSC/formamide solution, resulting in a final concentration of 2x SSC (0.3 M NaCl, 0.03 M sodium citrate) and 70% formamide. The solution was incubated for 30 min at 56 °C and then directly sonicated for 10 s. A 0.05% SDS solution (0.1 ml) was added. The samples were used as such in the competitive ELISA on the same day.

Heating at low ionic strength
Single- and double-stranded calf-thymus DNA (0.1 ml; 100 µg/ml) in 0.01 M Tris and 1 mM EDTA were heated at 70, 75, or 85 °C for 10 min.

Treatment with formamide and formaldehyde at low ionic strength
Single- and double-stranded calf-thymus DNA or DNA from mustard gas-exposed human white blood cells (700 µl; 100 µg/ml) in 0.01 M Tris, 1 mM EDTA, 4.1% formamide and 0.2% formaldehyde were heated at 52 °C for 25 min and then directly sonicated for 10 s.

Determination of DNA content
A method that is suitable for the determination of small quantities of DNA (10-50 ng/sample) was developed. The method is based upon the binding of the dye Hoechst 33258 to DNA and subsequent fluorometric detection of the DNA-dye complex (excitation: 370 nm; emission: 430 nm). Calibration curves were made with serially diluted DNA (double- or single-stranded calf thymus DNA) in a solution containing 0.14 M NaOH, 9 mM Na_2EDTA, 0.14 M NaH_2PO_4, 0.57 M NaCl and 0.07 µg Hoechst 33258/ml, pH 8. The fluorescence was measured with a Pye Unicam LC-FL detector. At the same DNA concentration, the fluorescence of double-stranded DNA samples was twice as high as that of single-stranded DNA. With this method it is possible to measure DNA concentration ranging from 10-5000 ng/ml. It can also be used to check DNA samples for the presence of double-stranded DNA. For the former purpose, 100 µl of the DNA sample were added to 4.2 ml of alkali buffer (0.06 M

NaOH and 0.01 M Na_2EDTA) and next incubated for 20 min at room temperature to induce complete single-strandedness. After addition of 0.7 ml neutralizing buffer (0.06 M NaOH, 1 M NaH_2PO_4, 4 M NaCl and 0.5 µg Hoechst 33258/ml) fluorescence was measured. The DNA concentration of a sample was derived, by using a calibration curve of single-stranded DNA. For the determination of the relative amounts of double- and single-stranded DNA, an additional measurement was performed. The DNA sample (100 µl) was added to the neutralized buffer and the fluorescence was measured. By comparing the two results, the fraction double-stranded DNA was calculated.

II.11.6. The degradation of mustard gas-treated DNA into nucleosides

DNA isolated from 10 ml WBC or 100-400 µg calf-thymus DNA treated with mustard gas was dissolved in 0.5 ml 10 mM Tris-HCl, 0.1 mM Na_2EDTA, 4 mM $MgCl_2$, pH 7.2, to which was added $ZnSO_4$ (to a final concentration of 0.2 mM), 50 µg nuclease P1 (dissolved in 30 mM sodium acetate, pH 5.3, at a concentration of 1 mg/ml) and 30 units deoxyribonuclease I (dissolved in the Tris-HCl buffer at a concentration of 3000 units/ml). The solution was incubated overnight at 37 °C. Next, the digest was heated for 5 min at 100 °C to inactivate deoxyribonuclease I and to release the N7-alkylated guanines and N3-alkylated adenines. In case the degraded DNA had to be used in ELISA's, before the heat-inactivation, the solution was incubated with 0.35 mg proteinase K (2 h; 37 °C), to destroy also nuclease P1 which is heat-resistant (intact nuclease P1 causes a high background in the ELISA). Proteinase K is inactivated by the subsequent heating. To digest the nucleotides to nucleosides, 1 µl 1 M Tris-HCl, pH 9.0, was added and the solution was incubated for 24 h at 37 °C with 4.5 units of alkaline phosphatase, type III (3.1 units/10 µl in 2.5 M $(NH_4)_2SO_4$). After heating (5 min; 100 °C) and centrifugation, the supernatants were analyzed by means of HPLC; they were injected as such. The amount of nucleosides was measured spectrophotometrically (ϵ_{260nm} = 11,000 $l.mol^{-1}.cm^{-1}$).

II.11.7. HPLC conditions for nucleosides and alkylated nucleosides

The nucleosides and alkylated nucleosides were injected onto HPLC. The HPLC equipment consisted of two pumps (Beckman 114), a gradient mixer (Beckman 340), a gradient controller (Beckman 421), a Rheodyne injector with a 500 µl sample loop, an ODS Reverse-phase column (Beckman; 250 x 16 mm; 5 µm particles RP 18), a UV-Vis detector (Beckman 165 Variable wavelength detector or Pye Unicam LC-UV detector), and an integrator (Spectra Physics 4100). The various compounds were separated by gradient elution of the column. Buffer A contained 25 mM NH_4HCO_3, pH 8, whereas buffer B contained 25 mM NH_4HCO_3 in 80% methanol. The gradient was linear, as follows: 0-20% B from 0 to 20 min, 20% B from 20 to 25 min, 20-60% B from 25 to 45 min. The flow rate was 1 ml/min and the wavelength at which the eluate was monitored was 285 nm. In experiments with DNA treated with [^{35}S]mustard gas, the eluate was collected in 0.5 ml fractions in

6-ml polyethylene vials (Packard) with a fraction collector (Pharmacia, Frac-100). Picofluor 30 (Packard; 4 ml) was added and the radioactivity was determined in a scintillation counter (Mark III, Packard, Searle, USA), connected with a tape deck. The results (cpm) were recorded on a tape, and the disintegrations per minute (dpm) were calculated by external channel-ratio correction on the computer (VAX). The elution pattern of the ^{35}S was combined with the UV-profile to locate the adduct peaks in relation to the unmodified degradation products of DNA.

II.12. Detection of crosslinks in DNA of mustard gas-treated mammalian cells

II.12.1. Cell cultures

Chinese Hamster Ovary (CHO) cells were cultured in monolayer in Ham's F-10 medium (Flow Laboratories, Irvine, UK), supplemented with 15% newborn calf serum (NCS; Flow), $NaHCO_3$ (final concentration: 14.3 mM), glutamine (final concentra-tion: 1 mM), hypoxanthine (final concentration 30 μM), penicillin (final concen-tration: 100 U/ml) and streptomycin (final concentration 0.1 mg/ml), in a humidified atmosphere of 5% CO_2 in air at 37 °C.

II.12.2. Survival of mustard gas-treated cultured cells

CHO cells were cultured in monolayer as described in II.12.1 in cell culture flasks (75 cm^2; Costar). Cells were trypsinized and resuspended in medium (1000 cells/ml). In Petri dishes (Costar: diameter: 6 cm), 100 cells were incubated in 3 ml of medium for 4 h in an incubator (37 °C; 5% CO_2) to become attached to the dishes. Subsequently, the medium was removed and the cells were treated with 3 ml of a mustard gas solution (0.5-2.5 μM mustard gas in F10-medium containing 20 mM HEPES and 1% acetone) for 20 min at 37 °C. For each mustard gas concentration, six dishes were used. CHO-cells, incubated with only F-10 medium with 20 mM HEPES with or without 1% acetone served as control. After exposure to mustard gas the medium was removed and the cells were incubated in complete F10-medium in a 37 °C incubator. After six days of incubation, the cell colonies were stained with 1% methylene blue in F10-medium for 2 h. After the removal of the medium the dishes were dried for 30 min in the open air and then washed with tap water. For each dish, the number of colonies was determined (only colonies consisting of more than 50 cells were counted). Percentage survival was calculated on the basis of the relative colony count.

II.12.3. Detection of crosslinks induced by mustard gas in DNA of cultured cells

Interstrand DNA-DNA crosslinks were detected by application of the alkaline elution technique, which was performed according to Shiloh et al. (88), Van der Schans et al. (89) and Plooy et al. (90). With this technique, single-strand DNA-breaks can be detected on the basis of the elution of alkaline denaturated DNA through membrane filters.

The presence of interstrand crosslinks results in a lower elution rate (see II.12.4). CHO cells were seeded in Petri dishes (Costar; diameter: 3 cm) and labelled for 16 h with [^{14}C]thymidine (0.075 μCi/ml; each Petri dish received 0.06 ml). In parallel cultures meant to serve as controls (to check the method), CHO cells were seeded in 75-cm^2 tissue culture flasks and labelled for 16 h with [^{3}H]thymidine (1.0 μCi/ml; 0.25 ml was added per flask). After a chase period of 1 h with fresh medium, the medium was removed. For 20 min the [^{14}C]-labelled CHO cells were incubated with F10-medium without NCS containing 20 mM HEPES, 1% acetone and mustard gas at concentrations varying from 0-2.5 μM. After treatment with mustard gas, the dishes were placed on ice in cold complete F10-medium (with 15% NCS). The cells were irradiated with 0 or 4 Gy ^{60}Co-γ-rays and scraped off in ice-cold medium. Untreated CHO cells prelabelled with [^{3}H]thymidine were scraped off and to each dish with mustard gas-treated CHO cells the same amount of untreated cells was added. The mixed cell suspension in each dish was transferred onto a polyvinyl chloride membrane filter (Millipore; 25 mm, 2 μm pore size), which already had been washed with ice-cold PBS. The solvent was allowed to pass through the filter by gravity; than the cells were lysed by incubation for 1 h at 20 °C in 3 ml lysis buffer (0.2% sarkosyl, 2 M NaCl, 0.02 M Na_2EDTA, pH 10, and, freshly added, 0.5 mg/ml of proteinase K). Following lysis, the solution was removed by passage through the filter by gravity and the filter was washed once with 3 ml of 0.02 M Na_2EDTA, pH 10. The DNA was eluted through the filter by slow pumping (0.03 ml/min) of a solution containing 0.04 M EDTA, 0.1% SDS and tetrapropylammonium hydroxide to yield a pH of 12.1. Then 2.7-ml fractions (= 90 min) were collected in scintillation vials. The solution remaining on the filter was pumped at high speed in fraction 10, and the filter itself was transferred to a scintillation vial and incubated for 1 h at 70 °C in 0.5 ml of 1.0 M HCl. After cooling to room temperature, 2 ml of 0.4 M NaOH was added. The filter holders and pump lines were each washed four times with 2 ml of 0.4 M NaOH, which was collected to form two additional fractions. To all scintillation vials 14 ml of Picofluor 30 (Packard, USA) was added and the radioactivity was counted in a liquid scintillation counter (MARK III, Packard, Searle, USA), using a double-label programme. Elution patterns for [^{14}C] and [^{3}H] were constructed by plotting the logarithm of the radioactivity remaining on the filter as a function of the fraction number. The logarithm of the ratio between the fractions of [^{3}H]-radioactivity and [^{14}C]-radioactivity retained on the filter at fraction 9 served as a measure for the number of single-strand breaks (SSB; in arbitrary units) in each sample. The amounts of breaks (in arbitrary units) induced by the irradiation was calculated from the averaged slope over the first five fractions of the elution pattern of [^{14}C]thymidine-labelled irradiated but untreated cells, from which the slopes of the unirradiated untreated [^{3}H]-labelled controls were subtracted (Figure 2). From the [^{14}C]-elution pattern the amount of crosslinks present in the DNA of the mustard gas-treated [^{14}C]-labelled CHO-cells was calculated according to the method described below. In this method, a comparison is made between the elution profiles of the treated and untreated irradiated

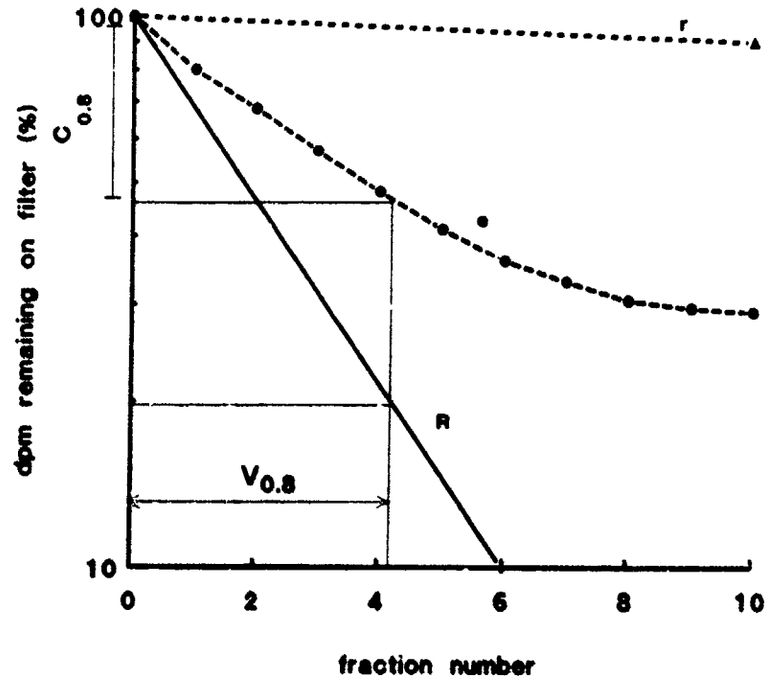

Figure 2. Model for the calculation of DNA crosslinks from the elution pattern obtained by the alkaline elution method. The relative amount of radioactivity remaining on the filter (logarithmic scale) is plotted as a function of fraction number. r, elution pattern of DNA from the unirradiated, untreated control cells; R, elution pattern of DNA from the irradiated, untreated cells; e, elution pattern of the crosslinked DNA from irradiated cells treated with mustard gas. On curve R, the elution volume is determined at which 80.1% of the radioactivity (i.e., 80.1% of the DNA) from the untreated, irradiated cells has passed through the filter ($V_{0.8}$; the slope of line R is a measure of the number of single-strand breaks induced by the radiation). Next, $C_{0.8}$ is read from curve e, by estimation of the proportion of DNA eluted at this volume. $C_{0.8}$ is a measure for the average number of crosslinks, which is half the number of links (x) relative to the average number of radiation-induced breaks (p), both per molecule of single-stranded DNA with molecular weight M, as is described in Appendix A. The value for x/p is obtained with the help of curve c in Appendix A, Figure A1, where $C_{0.8}$ has been plotted as a function of x/p. The $C_{0.8}$ value of the crosslinked samples should not be too small, because in that case the determination of x/p becomes exceedingly inaccurate.

cells, respectively. The reduction in the amount DNA modified by DNA relative to that of the untreated DNA, at the elution volume where 80% of the latter had passed the filter, is a measure of the extent of crosslinking due to mustard gas.

II.12.4. Calculation of the number of interstrand crosslinks in DNA of CHO-cells

Alkaline elution is a method to measure the amount of single-strand breaks or alkali-labile sites induced by alkylating agents or irradiation. However, this method is also suitable for the detection of interstrand crosslinks. When interstrand crosslinks are present in DNA that contains a known number of single-strand breaks (induced by gamma rays), interconnected single-stranded DNA fragments will not separate upon the alkaline denaturation, which results in a slower elution and in a seemingly smaller number of single-strand breaks as calculated from the elution rate compared to untreated DNA with the same number of single-strand breaks. A method to calculate the amount of crosslinks has been described by Van der Schans et al. (91) and a modification of this method is presented in Appendix A of this report. This method resulted in a curve (Figure A1, curve c) which was used for the calculation of the amount of crosslinks. From this curve the ratio x/p, corresponding to the experimentally determined value of $c_{0.8}$ (see Figure 2) was read; x/p is the ratio between the number of interstrand links (x) and the number of γ-ray-induced single-strand breaks (p), both per unit length of single-stranded DNA. Because p had been derived from the elution pattern found for the untreated irradiated cells (see above), x could then be computed. The number of crosslinks for the same length of double-stranded DNA amounts to x/2.

II.12.5. The repair of mustard gas induced crosslinks in CHO cells

To study the repair of crosslinks induced by mustard gas, the cells which had been treated with mustard gas (as described in II.12.3), were incubated in fresh F-10 medium with 15% NCS at 37 °C for 0 to 4 h before irradiation to allow repair processes to proceed. After the selected incubation time, the cells were placed on ice and γ-irradiated. The further procedure was as described in II.12.3 and II.12.4.

II.12.6. Detection of crosslinks induced by mustard gas in DNA of human white blood cells

The alkaline elution method for the detection of mustard gas-induced interstrand crosslinks in DNA of human WBC was a modification of the one described by Schutte et al. (92). Venous blood was collected from volunteers in 10 ml evacuated glass tubes containing 15 mg Na_2EDTA. Portions of 4 ml of total blood were incubated for 30 min at 37 °C with mustard gas at various concentrations (0.5-10 μM mustard gas; final acetone concentration: 1%). Untreated blood and blood incubated with 1% acetone only served as controls. Also blood of four Iranian

patients, who were supposed to have been exposed to mustard gas in the Iran-Iraq War three weeks earlier, was investigated, evidently without further treatment. In the first experiments, the various blood cells (erythrocytes, granulocytes, lymphocytes) were separated by centrifugation through a Percoll-gradient (Pharmacia, Uppsala, Sweden). The blood was diluted (1:1) with a 0.9% NaCl solution. Four ml of diluted blood was added slowly on top of a linear Percoll-gradient (d = 1.055-1.110). The gradient was centrifuged (20 min at 1.8 Kg; 4 °C). The plasma layer containing thrombocytes and lipids was removed, and the lymphocytes forming a broad band half-way down and the granulocytes banding just above the erythrocytes pellet were withdrawn separately by pipetting. The cells were freed from Percoll by dilution and washing twice with PBS and resuspending in PBS. In later experiments, total WBC were isolated by lysis of the erythrocytes. To this purpose, the blood was cooled to 4 °C and diluted with three volumes of freshly prepared ice-cold lysis buffer, containing 155 mM NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM Na_2EDTA, pH 7.4. The blood solutions were mixed gently and after 10 min of lysis of the erythrocytes the blood was centrifuged (15 min; 700 g; 4 °C). The WBC were washed twice with RPMI-medium (RPMI1640, GIBCO) with 10% Foetal Calf Serum (FCS; Flow) and finally suspended in RPMI-medium with 10% FCS to a final concentration of 1.8×10^6 cells per ml and placed on ice. The amount of WBC was counted in a counting-chamber by light microscopy. The cells were irradiated with 0 or 4 Gy ^{60}Co gamma rays. Polycarbonate membrane filters (Nuclepore 25 mm, 5 µm pore size) held in funnels were washed once with PBS and thon 0.5 ml of a solution of 0.2% sarkosyl, 2 M NaCl, and 20 mM Na_2EDTA, pH 10, was applied. The WBC suspension (0.5 ml; 0.9×10^6 cells) was transferred to the filters and incubated for 10 min to induce cell lysis. After 10 min the solution was removed by passage through the filter by gravity. DNA was further released by treatment for 1 h at 20 °C in 3 ml buffer (0.5% SDS, 10 mM NaCl, 10 mM Tris-HCl, 10 mM Na_2EDTA and, freshly added, 0.5 mg/ml of proteinase K to remove proteins possibly crosslinked to DNA). Following this treatment, the solution was allowed to drip through the filter and the filter was washed twice with 0.02 M Na_2EDTA, pH 10. The DNA was eluted through the filter by slow pumping (0.03 ml/min) of a solution containing 0.06 M NaOH and 0.01 M Na_2EDTA, pH 12.6. Six 4.5-ml fractions (= 150 min) were collected in glass vials. The solution then remaining on the filter was pumped at high speed in fraction 6. The filters were transferred to vials and after the addition of 4.5 ml of the elution buffer they were irradiated with 100 Gy of ^{60}Co gamma rays. All fractions were neutralized with a buffer containing 0.6 M NaOH, 1 M NaH_2PO_4, 4 M NaCl, pH 7.4, and Hoechst 33258 (0.5 mg/l). The amount of DNA in the eluted fractions and those of the filters was measured fluorometrically (excitation: 370 nm; emission: 430 nm) in a Pye Unicam LC-FL detector. The background buffer contained 9 volumes of the elution buffer and 1.6 volumes of the buffer used for neutralization. Elution patterns were constructed by plotting the logarithm of the fluorescence remaining on the filter as a function of the fraction number. The untreated blood samples and the blood samples treated with 1% acetone, both irradiated with 4 Gy, served as the controls to indicate the maximum of radiation-induced SSBs. With

both types of irradiated controls, straight elution curves were obtained. The interstrand crosslinks induced by the treatment with mustard gas mask part of the SSBs induced by the 4 Gy irradiation, which results in curved elution graphs. From the graphs the amount of interstrand crosslinks in DNA can be calculated as described in II.12.4 (see also ref. 91 and Appendix A).

II.13. Immunochemical methods for the detection of mustard gas adducts to DNA

II.13.1. Preparation of a polyclonal antiserum against DNA treated with mustard gas

For raising an antiserum against DNA treated with mustard gas, an immunogen was prepared by the treatment of double-stranded calf-thymus DNA (ds-ct-DNA; 1 mg/ml) or single-stranded calf-thymus DNA (ss-ct-DNA; 1 mg/ml; prepared as described in II.11.1) with 1 mM mustard gas in 1% acetone for 45 min at 37 °C. The DNA was precipitated with 0.1 volume of 3 M sodium acetate, 1 mM Na_2EDTA, pH 5.5, and 2 volumes of 100% ethanol cooled at -20 °C. After collection of the DNA with a glass pipet and washing in 80% ethanol, the DNA was dissolved in PBS to a concentration of 1 mg/ml and sonicated for 30 s. The ss-ct-DNA and ds-ct-DNA with mustard gas (ss-ct-DNA-HD and ds-ct-DNA-HD) were coupled to a carrier protein, i.e., methylated bovine serum albumin (1 mg complex/ml PBS). Rabbits were immunized intracutaneously with 250 µg complex of ds-ct-DNA-HD in complete Freund's Adjuvant and boostered once with ds-ct-DNA-HD in complete Freund's Adjuvant (after 4 weeks), once with ds-ct-DNA-HD in incomplete Freund's Adjuvant (after 8 weeks) and once with ss-ct-DNA-HD in incomplete Freund's Adjuvant (after 14 weeks). Three weeks after the last booster, the blood of the rabbits was collected and the serum was isolated, which was stored at -70 °C.

II.13.2. Immunoassay with a polyclonal antiserum: Enzyme-Linked Immunosorbent Assay (ELISA)

Direct and competitive ELISA

Microtiter plates (96 wells; polyvinyl chloride; Costar) were precoated with poly-L-lysine (50 µl of 10 µg/ml PBS per well; overnight at 4 °C) and washed once with PBS. The wells were coated overnight at 37 °C with ss-ct-DNA (50 µl of 1 µg/ml in PBS per well), treated with 10 µM mustard gas (1 h; 37 °C) or with untreated ss-ct-DNA (the latter only in controls for aspecific binding in the direct ELISA) and washed three times with 0.05% Tween 20. After the coating with DNA, the plates were incubated with 50 µl (in later experiments 100 µl) PBS containing 0.5% gelatin/well for 60 min at 37 °C and washed again with 0.05% Tween 20. Antiserum dilutions (direct ELISA) or competition mixtures (competitive ELISA) in PBS containing 0.05% Tween 20 and 0.1% gelatin were incubated in the wells in duplicate for 40 min at 37 °C. The competition mixtures contained various amounts of inhibitor DNA (ss-ct-DNA-HD or ss-ct-DNA) in the range of 0.1-10,000 ng/ml and the appropriate amount of polyclonal serum (final dilution 1:40,000). As controls, incubations without both the

antiserum and the competitor DNA were done, and for the maximal response incubations were done with the antiserum but without competitor DNA (100% point). The competition mixtures were preincubated for 30 min at room temperature. The plates were washed 3 times with PBS containing 0.5% Tween 20 and 50 µl of conjugated second antibody (goat-anti-rabbit-IgG-alkaline phosphatase; 1:1000 diluted in PBS containing 0.5% gelatin, 5% FCS and 0.05% Tween 20) was added, followed by incubation for 40 min at 37 °C. The wells were washed three times with PBS containing 0.05% Tween 20 and once with 0.1 M diethanolamine, pH 9.8. As substrate for alkaline phosphatase a solution of 4-methylumbelliferyl phosphate (MUP; 0.2 mM in 10 mM diethanolamine, pH 9.8, 1 mM $MgCl_2$) or 4-nitrophenyl phosphate (PNP; 1 mg/ml in 10 mM diethanolamine, pH 9.8, 1 mM $MgCl_2$) was added. With MUP, a fluorescent product is formed, and with PNP, a colored product. In the competitive ELISA, incubation was continued until 30% of the highest detectable level of the fluorescence in the sample without inhibitor DNA (the 100% point) was reached. In the direct ELISA the absorbance or fluorescence was read after 1-2 h of incubation. The fluorescence (excitation: 355 nm; emission: 480 nm) was recorded with a Fluoroskan (Eflab, Finland) and the absorbance at 405 nm with a Titertek Multiskan (Flow). The data of the competitive ELISA (only the fluorometric detection was applied) were recorded on a tape deck and the amount of inhibition was calculated by computer (VAX) according to

$$\% \text{ inhibition} = \left(1 - \frac{\text{fluorescence}_{\text{sample}} - \text{fluorescence}_{\text{background}}}{\text{fluorescence}_{100\% \text{ point}} - \text{fluorescence}_{\text{background}}}\right) \times 100\%$$

II.13.3. Preparation of DNA from blood treated with mustard gas for the competitive ELISA

Various isolation procedures were carried out to obtain DNA from mustard gas-treated blood cells with an optimal accessibility of the DNA-damage for the antibodies. All procedures were applied on WBC isolated from blood (as described in II.11.3) which had been treated with mustard gas (0.1 or 1 mM in 1% acetone) for 45 min at 37 °C.

Treatment with alkali

A WBC suspension (0.15 ml; 3.10^6 cells) in PBS was added to 0.8 ml "alkali" solution (1.3 M NaCl, adjusted to pH 12.1 with 1 M NaOH) and the mixture was incubated for 30 min at room temperature. The solution was sonicated for 20 s to prevent annealing of the DNA-strands, and, subsequently, neutralized with 0.15 ml 250 mM KH_2PO_4. Finally, 0.1 ml 0.2% SDS in PBS was added to prevent adsorption to the walls of the tubes. These samples were used in a competitive ELISA.

Treatment with 2x SSC/70% formamide

WBC suspension (0.1 ml; 2.10^6 cells) in PBS was treated with 0.3 ml SSC/formamide solution, resulting in a final concentration of 2x SSC (0.3 M NaCl, 0.03 M sodium citrate) and 70% formamide. The solution was incubated for 30 min at 56 °C and then directly sonicated for

10 s. A 0.05% SDS solution (0.1 ml) was added. The samples were used directly in the competitive ELISA on the same day.

Isolation of DNA by phenol-extraction and ethanol-precipitation
DNA was isolated from the WBC as described in II.11.4 and finally dissolved in either 0.01 M Tris and 1 mM EDTA or PBS. The DNA concentration was measured spectrophotometrically or fluorometrically as described II.11.5. Parallel to these treatments to isolate DNA from WBC, ss- and ds-ct-DNA-HD were treated in the same way to study the influence of the treatment on the outcome of the ELISA. Also DNA was isolated from WBC and afterwards treated with mustard gas to compare these samples with ds- and ss-ct-DNA-HD.

II.13.4. Detection of single-strandedness in a competitive ELISA

The DNA from WBC treated with mustard gas, which had been isolated with any of the procedures described in II.13.3, was tested on single-strandedness in a competitive ELISA (93). The 96-well microtiter plates (polyvinyl chloride; Costar) were precoated with 50 µl poly-L-lysine (1 µg/ml; overnight at 4 °C) in PBS and washed once with PBS. The wells were coated overnight at 37 °C with 50 µl of a 1 µg/ml ds-ct-DNA solution which had been treated with 0.8% OsO_4 for 10 min at 55 °C and washed three times with 0.05% Tween 20. The modification of the single-stranded coating-DNA by OsO_4 was required in order to decrease the affinity of the D1B antibodies for the coating material, thus preventing transfer of D1B antibodies originally bound to the competitor DNA to the immobilized antigen (93). After the coating with DNA, the plates were incubated with PBS containing 1% FCS (1 h, 37 °C) and washed three times with 0.05% Tween 20. The competitor DNA (DNA from the WBC treated with mustard gas) was heated for 3 min at 56 °C diluted in PBS containing 0.01% SDS (0.01-10 ng/well) and preincubated (1:1) with monoclonal antibody D1B (1:1000 diluted in PBS containing 0.01% SDS and 0.1% FCS), which recognizes single-stranded DNA, for 30 min at room temperature. A portion of this competition mixture (50 µl) was transferred to the microtiter plate and incubated for 40 min at 37 °C. The plates were washed three times with 0.05% Tween 20. The conjugated second antibody (50 µl), i.e., goat-anti-mouse-Ig-alkaline phosphatase (diluted 1:1000 in PBS containing 0.05% Tween 20 and 5% FCS), was added and incubated for 40 min at 37 °C. The wells were washed three times with PBS containing 0.05% Tween 20 and once with 0.1 M diethanolamine, pH 9.8. As substrate for alkaline phosphatase a solution of 4-methylumbelliferyl phosphate (0.2 mM in 10 mM diethanolamine, pH 9.8, 1 mM $MgCl_2$) was added and incubated for 2 h at 37 °C. The fluorescence was recorded and the percentage inhibition was calculated, using the background fluorescence and the fluorescence of the sample without competitor DNA (100% point), as described in II.13.2.

II.13.5. Coupling of N7-(2"-hydroxyethylthioethyl)-guanosine-5'-phosphate and guanosine-5'-phosphate to carrier protein with periodate

Keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA) were dialyzed against PBS (4 °C, 16 h), and UV spectra were taken. N7-(2"-hydroxyethylthioethyl)-5'-monophosphate-guanosine (GMP-7-HD; 10 µmol; see II.6.1) or guanosine 5'-monophosphate (GMP; 10 µmol) were dissolved in water, containing 10 µmol $NaIO_4$. The final volume was 300 µl. The solution was shaken for 20 min in the dark at room temperature. Ethylene glycol (100%; 10 µl) was added to inactivate $NaIO_4$. The solution was incubated for 10 min. The GMP-7-HD- and the GMP-solutions were added to the proteins, either KLH or BSA, in the ratio 1:1, 1:10, 1:25 and 1:100 (w/w, nucleotide/protein). Sodium carbonate (0.2 M) was added to adjust the pH to 8. The solutions were incubated for 2 h in the dark at room temperature. In this way the carrier protein was coupled to the adduct. The resulting instable sugar ring structure was stabilized by reduction with 0.1 M aqueous $NaBH_4$, in equimolar amounts with respect to GMP-7-HD or GMP. The solutions were again incubated for 2 h at room temperature and finally dialyzed against PBS (4 °C; 16 h). UV spectra were taken to check the coupling.

II.13.6. Coupling of N7-(2"-hydroxyethylthioethyl)-guanosine-5'-phosphate (GMP-7-HD) to KLH with EDC

KLH was dialyzed against 0.125 M N-methylimidazole, pH 6. KLH was added to 3 samples of GMP-7-HD in 0.5 M N-methylimidazole, pH 6 (100 mol GMP-7-HD/mol KLH, i.e., 0.67 µmol GMP-7-HD/mg KLH). The final volume of each sample was 413 µl. While the sample was slowly vortexed, a solution of EDC [1-(3-dimethyl-aminopropyl)-3-ethylcarbodiimide] in 0.5 M N-methylimidazole, pH 6, was dropwise added to each of the samples up to a ratio of 50, 100 or 250 mol EDC/mol GMP-7-HD. The solutions were shaken slowly for 45 min at room temperature and dialyzed against PBS (4 °C; 16 h). UV spectra were taken to check the coupling.

II.13.7. Monoclonal antibodies

Eight mice (female BALB/c, 14 weeks old) were immunized intraperitoneally (ip) with 50 µg of immunogen (GMP-7-HD-KLH, described in II.13.6) precipitated onto alum, and also subcutaneously (sc) with 50 µg of the same immunogen in complete Freund's Adjuvant in the foot pad sole of the foot (25 µg in both soles of the hind legs). Four mice were immunized with the immunogen of KLH coupled with 50 mol EDC/mol GMP-7-HD and four mice with KLH reacted with 100 mol EDC/mol GMP-7-HD. After eight days blood samples of all the mice were taken to test the serum for antibody response against ss-ct-DNA treated with 10 µM mustard gas in a direct ELISA. The mouse with the serum showing the best response against mustard gas-treated DNA was chosen for isolation of the cells which should be used for fusion. At four weeks after the immunization all mice were boostered, ip, with 50 µg of immunogen without alum and sc in the sole of the foot with

50 μg of immunogen in incomplete Freund's Adjuvant. On the fourth day after the booster injection, both spleen cells and lymph-node cells of one mouse were isolated for fusion with SP2/0 plasmacytoma cells. Also a blood sample was taken from this mouse to test the serum for antibody response. The SP2/0 plasmacytoma cells were grown in RPMI1640-medium (GIBCO), supplemented with 10% FCS, sodium pyruvate (final concentration: 1 mM), glutamine (final concentration: 1 mM), penicillin (final concentration: 100 U/ml), streptomycin (final concentration: 0.1 mg/ml) and β-mercaptoethanol (final concentration: 50 mM). Spleen cells, lymph-node cells and SP2/0 cells were washed twice in RPMI-medium without serum. Then, 1.10^8 spleen cells were added to 2.10^7 SP2/0 cells and 4.10^7 lymph-node cells were added to 9.10^6 SP2/0 cells and centrifuged (20 min at 10-20 g). The supernatant was removed and the cells were exposed to fusion conditions by brief consecutive incubations of a mixture of these cells in 41% and 25% poly(ethylene glycol) (PEG 4000) as follows. The cell pellets were resuspended for 11 min in 0.5 ml of a 41% PEG solution, then 0.5 ml of a 25% PEG solution was added and shaken slowly for 1 min. RPMI medium without serum (4 ml) was added twice and the cell suspension was shaken slowly for 2 min. The cell suspension was incubated for 15-30 min at room temperature and then centrifuged (20 min at 10-20 g). The supernatant was removed and the pellet resuspended in RPMI medium with 10% FCS. The cells were seeded in 75-cm^2 culture flasks and incubated overnight. After 24 h of incubation the cells were centrifuged (20 min at 10 g) and the cells were resuspended in 30 ml of complete RPMI medium with 10% FCS (the same medium as used for SP2/0 cells) supplemented with HAT medium, i.e.: hypoxanthine (final concentration: 0.1 mM), thymidine (final concentration: 16 μM) and aminopterine (final concentration: 0.4 μM). In this HAT medium hybridomas are selected because they can grow in this medium whereas SP2/0 cells do not survive, and lymph-node cells and spleen cells cannot be cultured. The cells were seeded in 96-well polystyrene plates (COSTAR) in which macrophages of mice had been seeded as feeder layer, two days before the fusion (5.10^3 macrophages per well in HAT medium). Hybrid cells were cultured and refreshed in this selective HAT medium and their supernatants were screened for specific antibody production in a direct ELISA and in a cell-ELISA (as described in II.13.9). Cells producing specific antibodies against ss-ct-DNA treated with mustard gas were recloned twice by limiting dilution as described in II.13.8.

II.13.8. Cloning of hybridomas by limiting dilution

Cells of the fusion mixture producing specific antibodies against ss-ct-DNA treated with mustard gas were counted by light microscopy and diluted in HAT medium to a concentration of 50, 10 and 5 cells/ml. Per well of 96-well plates which already contained macrophages, 0.1 ml of one of these solutions was added resulting in 5, 1 and 0.5 cell/well. The plates were incubated for eight days without refreshing the medium. Then the amount of clones per well was counted. The supernatants of wells with only one clone were tested for specific antibody activity against ss-ct-DNA treated with mustard gas. Clones showing a positive result were recloned once again by

limiting dilution to make sure that monoclonal antibodies would be obtained.

II.13.9. Screening of the hybridoma supernatants

II.13.9.1. ELISA

The supernatants of the hybridomas were screened in a direct ELISA (see section II.13.2). There were a few modifications. As coating DNA, ss-ct-DNA treated with 10 μM mustard gas was used next to untreated ss-ct-DNA. During the first screening of the 96-well plates, an aliquot of 50 μl of the undiluted supernatant was transferred to the ELISA-plates. In later stages, the cells were seeded in 24- or 6-well plates or in culture flasks, and the supernatants were diluted 1:10 for screening. Goat-anti-mouse-Ig-alkaline phosphatase (1:500 diluted) was used as a second antibody with 4-nitrophenyl phosphate as a substrate for this enzyme.

II.13.9.2. Competitive ELISA with monoclonal antibodies

Antibody-saturated supernatants of the hybridomas or purified monoclonal antibodies were tested in a competitive ELISA as described in section II.13.2. There were a few modifications. The supernatants of the hybridomas were diluted 1:1,000 in PBS containing 0.05% Tween 20 and 0.1% gelatin. The purified monoclonal antibodies were diluted in PBS containing 0.05% Tween 20 and 0.1% gelatin; the extent of dilution was chosen such that 30% of the highest detectable level of the fluorescence was reached after 2 h of incubation for the sample without inhibitor DNA (the 100% point).

II.13.9.3. Cell-ELISA

For immunofluorescence microscopy specific antibodies are needed that perform well under the conditions of this type of microscopy; the cell-ELISA was developed to screen monoclonal antibodies for this use. The principles of this test are that the wells of a 96-well microtiter plate are coated with WBC that have been treated with mustard gas. Next, these cells are treated in the same way as should occur during preparation for immunofluorescence microscopy. The test is performed as follows. The 96-well microtiter plates are precoated with poly-L-lysine (10 μg/ml, overnight at 4 °C) and washed once with PBS. The cells used for the coating are WBC, isolated from human blood which was treated with 1 mM mustard gas (45 min at 37 °C), or with untreated WBC. After washing of the WBC, they are resuspended in freshly prepared 70% ethanol. The wells are coated with 40 μl WBC (1.10^6 cells/ml in 70% ethanol). The plates are dried overnight at room temperature. When the plates are dry, 50 μl of hydration buffer (50 mM Tris-HCl, 1 M KCl, pH 7.2, and 3 ml Triton X-100/l) is added and the plates are gently shaken for 30 min at room temperature. After washing three times with TBS (20 mM Tris-HCl, 150 mM NaCl, pH 7.4), per well 50 μl RNAse A (0.1 mg/ml in TBS) are added and the plates are incubated for 60 min at 37 °C. After washing with TBS the cells are incubated with 50 μl 2xSSC/70% formamide (as described in

II.13.3) for 15 min at 56 °C. Next, the plates are washed for 2 min with 70%, 90% and 96% ethanol, and are air-dried for 60 min at 37 °C. Proteins are digested by incubating the preparations with 50 µl proteinase K (1 µg/ml in 20 mM Tris-HCl, 2 mM $CaCl_2$, pH 7.4; 10 min; 37 °C) and afterwards the plates are washed with TBS. From here on, the ELISA is carried out exactly as described for the direct ELISA (see II.13.9.1), except for TBS instead of PBS.

II.13.9.4. Ig-subclass identification of the monoclonal antibodies

The Ig-subclass is determined with an ELISA that is almost the same as the direct ELISA described in II.13.9.1. The main variations relate to the second antibodies that are used. These are rabbit-anti-mouse antibodies which are (sub)class-specific (IgM, IgG1, IgG2a, IgG2b, IgG3, κ and λ; 1:500 diluted). These antibodies are detected with a third antiserum, viz., goat-anti-rabbit-IgG-alkaline phosphatase as described in II.13.2.

II.13.10. Purification of monoclonal antibodies with a protein A column

After cloning twice by limiting dilution (see section II.13.8) ten monoclonal cell cultures were obtained that produced antibodies. Cells were grown for two weeks without refreshing the medium, and 100 ml of the antibody-saturated supernatant was collected. The supernatants were centrifuged (20 min; 700 g) to remove cell debris. The supernatants were placed on ice and stirred, while a 100% saturated $(NH4)_2SO_4$ solution was dripped slowly into the solution to precipitate proteins. The solutions were placed overnight at 4 °C for complete precipitation and the next day they were centrifuged (30 min; 13,600 g; 4 °C). The supernatants were discarded and the pellets were dissolved in a small volume (4-6 ml) PBS and dialyzed overnight against PBS at 4 °C. The dialysates were centrifuged (20 min; 400 g; 4 °C) to remove precipitates. The monoclonal antibodies in the supernatant were purified by chromatography over a protein A column (Pharmacia; 7.5 x 1 cm). The columns were washed with 30 ml binding buffer (1.5 M glycine, 3 M NaCl, pH 8.9). The monoclonal antibody solution was diluted 1:1 with this buffer and the column was loaded (6 drops/min). After loading, the column was washed with the binding buffer until $A_{280\ nm}$ was less than 0.1. The eluate was collected in 1-ml fractions. The protein-containing fractions were pooled to be tested for anti-HD-adduct activity. Then, the antibodies were eluted from the column with 0.1 M sodium citrate buffer, pH 4.7, until $A_{280\ nm}$ was below 0.1. Afterwards, the column was regenerated with 30 ml of 0.1 M Tris-HCl, 0.5 M NaCl, pH 8.5, and with 30 ml 0.1 M sodium acetate, 0.5 M NaCl, pH 4.5. The fractions containing the monoclonal antibodies were pooled and dialyzed overnight against PBS (4 °C). The volume of the monoclonal antibody solution was reduced by dialysing against a solution of PEG 20,000 (3:7, w/v in PBS) for 4-5 h and then the solution was dialyzed against PBS for 48 h at 4 °C. Samples were taken apart at each purification step for testing antibody activity.

The protein content of the monoclonal antibodies was determined by a Bio-Rad protein assay, as described in II.13.11.

II.13.11. Bio-Rad assay for protein content

Bovine serum albumin (BSA) was diluted in distilled water to a concentration of 100, 75, 50, 25, 10, 5 and 1 μg/ml to be used for a calibration curve. The protein solutions to be tested were diluted in distilled water to fall within this range. Wells of a 96-well polystyrene plate were filled with 100 μl BSA- or protein solution in duplicate. The Bio-Rad agent (Bio-Rad protein assay; dry reagent concentrate; Bio-Rad Laboratories GmbH) was diluted in distilled water (4:6, v/v) and 100 μl was added to the wells. The plate was incubated for 20-30 min at room temperature and the absorbance was measured with a Titertek Multiskan (Flow) at 580 nm. The protein concentration of the monoclonal antibody solutions was calculated by using the BSA-calibration curve.

II.13.12. Immunofluorescence microscopy for the detection of mustard gas-induced adducts to DNA in human skin

Human skin resulting from cosmetic surgery was exposed to air saturated with mustard gas vapor at 30 °C for 0, 2, 4, 6 or 10 min and cryostat sections were subjected to an immunostaining procedure. Sections (5 μm thickness) were fixed on aminoalkylsilane-precoated slides, stored at -20 °C and thawed just before immunochemical staining. They were washed with TBS (20 mM Tris-HCl, 150 mM NaCl, pH 7.4) and treated with RNase, followed by exposure to 70% formamide in 0.3 M NaCl, 0.02 M sodium citrate for 15 min at 52 °C to denature the DNA, and to 10% formaldehyde to prevent rewinding. After treatment with proteinase K, washing and incubation with TBS containing 0.5% gelatin (to prevent aspecific antibody binding), slides were incubated with 2F8 antibodies followed by the fluorescent "second" antibody, i.e., fluorecein-labelled "goat-anti-mouse" IgG (FITC-GaM), counterstained with propidium iodide and mounted. The equipment used to examine the fluorescence of the preparations comprised a laser scanning microscope (LSM-41, Zeiss, FRG) interfaced to a Microdutch 100 workstation (Schreiner, Netherlands). The overall procedure requires the recording of twin images, viz., first the fluorescence of FITC-GaM, then the fluorescence from propidium iodide, which serves to localize the nuclei in the image.

II.14. Distribution of radioactivity in blood treated with [^{35}S]mustard gas

Blood of human volunteers (10 ml) was collected in evacuated glass tubes containing 15 mg Na_2EDTA. The blood was treated with 0.1 or 1 mM [^{35}S]mustard gas (same batch as described in section II.11.2) for 45 min at 37 °C. The serum was collected as described in II.11.3 and the blood cells were washed three times with PBS. The supernatants of these washing steps were added to the serum fraction. The hemoglobin was collected after lysis of the erythrocytes as described in II.11.3

and the WBC were washed three times with PBS. The supernatants of these washing steps were added to the hemoglobin fraction. The serum proteins were collected by precipitation in 10 mM HCl in 99% acetone (-20 °C) and centrifugation (15 min, 400 g). The precipitate was dissolved in distilled water and the precipitation step was repeated. The supernatants of both precipitations were combined. The proteins were dissolved in distilled water. The globin was precipitated from the hemoglobin-solution according to the same procedure as used to isolate the serum proteins. The DNA was isolated from the WBC as described in II.11.4. The radioactivity was determined in a scintillation counter as described in II.11.7 in 100 µl samples of each of the fractions obtained. The radioactivity present in the different fractions was expressed as the percentage of the radioactivity measured in whole blood.

II.15. Identification of reaction products of mustard gas in proteins of erythrocytes

II.15.1. Isolation of hemoglobin from human blood

Blood of human volunteers (10 ml) was collected in evacuated glass tubes, containing 15 mg Na_2EDTA. The blood was centrifuged (15 min; 400 g; 4 °C) and the serum was discarded. The cells were washed twice with PBS and finally resuspended in PBS. The erythrocytes were lysed with three volumes of lysis buffer as described in II.11.3. The solution was centrifuged (15 min; 400 g) and the supernatant containing the hemoglobin (Hb) was collected. The Hb-solution was ultracentrifuged for 45 min at 50 kg at 20 °C to remove cell debris and non-lysed erythrocytes. The supernatant was dialyzed against water (48 h; 4 °C) and stored at -70 °C.

II.15.2. Isolation of globin from hemoglobin

Isolated Hb (2 ml) from human blood or commercially obtained Hb, dissolved in water (10 mg/ml), was added dropwise to 20 ml of ice-cold 10 mM HCl in 99% acetone, which was stirred and was placed in a bath of solid CO_2/ethanol. Hydrochloric acid in 99% acetone (10 mM) was added to a final volume of 30 ml. The suspensions were cooled for some time (2-16 h) at -20 °C to precipitate the globin completely and then centrifuged (15 min; 700 g; 4 °C). The supernatant containing the heme-fraction was discarded and the globin was dissolved in distilled water after air drying (10 min at 37 °C). The precipitation procedure was repeated. To remove traces of HCl and acetone the globin was freeze-dried. The preparations were stored at -20 °C.

II.15.3. Trypsin hydrolysis of globin

Isolated globin (10 mg) was dissolved in 3 ml of water. An aqueous solution of trypsin (2 mg/ml; 100 µl) was added followed by 600 µl of 0.5 M NH_4HCO_3. The pH was adjusted to pH 8.5 ± 0.2. The solution was incubated for 2 h at 37 °C. Next, the pH was adjusted to 6.4 with 1 M HCl and the volume was brought to 5 ml. Acetonitrile (10 ml) was added and the solution was left overnight at 4 °C to precipitate the

large fragments. The solution containing the smaller peptides was centrifuged (15 min; 700 g). The supernatant was collected. To reduce the volume, acetonitrile was removed by flushing with a gentle stream of nitrogen. The solution was used for chromatographic separation of the peptides; it was directly injected onto HPLC.

In an improved procedure globin was digested with immobilized trypsin, which can easily be removed from the peptide solution by centrifugation. To this end, globin (3 mg) was dissolved in 1 ml distilled water. The immobilized trypsin suspension (121 μl; Immobilized TPCK-Trypsin, Pierce, USA; 14 units/ml gel) was washed twice with 1 ml 0.05 M NH_4HCO_3 and then suspended in 60 μl 0.05 M NH_4HCO_3 and added to the globin solution. Aqueous 0.5 M NH_4HCO_3 (200 μl) was added to adjust the pH to 6.4. The mixture was incubated for 48 h at 37 ^{o}C under gentle shaking and then centrifuged (15 min; 500 g) to remove the trypsin. The supernatant was directly injected onto HPLC. The HPLC profile of globin fragmented by immobilized trypsin was identical with that of the globin digested by soluble trypsin.

II.15.4. Treatment of hemoglobin and chicken gammaglobulin with mustard gas

Hemoglobin (commercially obtained and isolated Hb from human blood) was dissolved in water (1-5 mg/ml) and was treated with [^{35}S]mustard gas (0.1-1 mM in 1% acetonitrile) or unlabelled mustard gas (1-5 mM) for 2 h. The pH was checked with pH paper and adjusted with 5 N NaOH to pH 7. After the treatment, globin was isolated, digested with trypsin (II.15.3) and analyzed on HPLC. Chicken gammaglobulin was treated with 5 mM mustard gas in a similar way as hemoglobin.

II.15.5. HPLC condition for trypsinized globin and small peptides

Solutions of synthetic peptides or the trypsin fragments of globin were injected onto a HPLC system as described previously (column: RP18, corasil, 250 x 16 mm; 5 μm particle size). Gradient elution was applied: buffer A contained 0.1% trifluoroacetic acid (TFA) in water wheras buffer B consisted of 0.1% trifluoroacetic acid in 70% acetonitrile. A linear gradient was applied, which varied depending on the type of analysis. The best conditions for the identification of the N-terminal heptapeptide of α-hemoglobin, alkylated with mustard gas at the valine residue, appeared to be 0-30 min: 0-50% B, 30-40 min: 50-90% B, and 40-45 min: 90% B. The flow rate was 1 ml/min and the absorbance was detected at wavelength 220 nm. In experiments with ^{35}S-labelled proteins, fractions were collected and the radioactivity was determined as described in II.11.7.

II.15.6. Amino acid analysis

Amino acid analysis of the peptides was performed as described by Janssen et al. (79,80). The peptide (1-1.5 nmol) was gas-phase hydrolyzed in 6 N HCl-1% phenol medium for 24 h at 110 ^{o}C and the amino acids liberated were reacted for 20 min at room temperature with 20 μl of a 10% phenyl isothiocyanate (PITC) solution

[acetonitrile-water-TEA (triethylamine)-PITC = 6:2:1:1 v/v]. Subsequently, the samples were dried in vacuo to remove excess reagent. The dried samples were dissolved in 250 µl of the starting HPLC buffer solution and 100 µl was injected onto HPLC. The PTC-amino acids (phenylthiocarbamyl amino acids) were analyzed by HPLC on a Supelcosil LC-18DB (250 x 4.6 mm) column. Elution was with a gradient of buffer A containing 0.7 M NaOAc + 2.5 ml TEA /l and HOAc to adjust pH to 6.4, and buffer B containing acetonitrile-water (80:20, v/v). The flow rate was 1 ml/min. The column was kept at 45 °C and the amino acids were detected at 254 nm. The calculation of the amino acid ratios was performed versus a calibration mixture of 23 amino acids run under identical conditions.

II.15.7. Stability of mustard gas adducts to hemoglobin and globin under acidic, alkaline and neutral circumstances

Human blood was treated with 1 mM [^{35}S]mustard gas in 1% acetonitrile (final concentration) for 60 min at 37 °C and pH 8, and Hb was isolated as described in II.11.3. A part of the Hb was precipitated and globin (Gb) was isolated (II.15.2). Hb and Gb were incubated for several hours at 37 °C with 1 and 5 N of, respectively, NaOH, methanesulfonic acid (MSA) and HCl. Treatment of Hb with the 5 N solutions led to precipitation of the protein. Hence, only the incubations with the 1 N solutions were continued. In addition, Hb isolated from blood treated with [^{35}S]mustard gas was incubated at pH 7 for several days to study its stability under this condition. The treatments were terminated by neutralizing the solutions with NaOH or HCl and/or by precipitation of the proteins with ice-cold 10 mM HCl in 99% acetone. The radioactivity was determined in the supernatants (containing the heme group -if present- and the alkali- and acid-labile adducts) and in the precipitated "globin fraction" after it had been dissolved in water. The percentage of alkali- and acid-labile adducts was calculated.

II.16. Immunochemical methods for the detection of mustard gas adducts to proteins of erythrocytes

II.16.1. Preparation of a polyclonal antiserum against hemoglobin treated with mustard gas and N-(2'-hydroxyethylthio-ethyl)-D,L-valine coupled to KLH

Two immunogens were prepared to immunize rabbits. Hb (2 mg/ml) was treated with 1 mM mustard gas (60 min at 37 °C) in 1% acetonitrile (final concentration). This protein was not coupled to a carrier protein. N-(2'-hydroxyethylthioethyl)-D,L-valine (val-HD) was coupled to KLH with EDC [1-(3-dimethylaminopropyl)-3-ethylcarbodiimide] as described in II.13.6. With each immunogen, one rabbit was immunized intracutaneously, with 250 µg of the immunogen in complete Freund's Adjuvant and boostered once with 250 µg of the immunogen in complete Freund's Adjuvant after 4 weeks and once with 250 µg of the immunogen in incomplete Freund's Adjuvant after another 4 weeks. Two weeks after every immunization, blood samples were taken to test antibody

activities. Two weeks after the last booster, the rabbits were sacrificed and the serum was collected.

II.16.2. Immunoassays with the polyclonal antisera: ELISA

The polyclonal antisera were tested in a direct ELISA against Hb treated with mustard gas (0, 0.1, 1 mM), globin isolated from mustard gas-treated Hb, human serum albumin (HSA) and chicken gammaglobulin (CγG) treated with mustard gas (0, 0.1, 1 mM) and the N-terminal heptapeptide of Hb, untreated and treated with 5 mM mustard gas (pH 8.5; 60 min; 37 °C). The ELISA was performed as follows. The 96-well microtiter plates (polyvinyl chloride; Costar) were precoated with poly-L-lysine (10 μg/ml; overnight at 4 °C) and washed once with PBS. The proteins and peptides were diluted in PBS (0.5-5 μg/ml) and 50 μl per well was added and incubated for 30 min at room temperature. The plates were washed three times with PBS with 0.05% Tween 20. Next, the plates were incubated with PBS containing 0.5% gelatin for 60 min at room temperature and again washed three times. The polyclonal antisera were diluted (10-100,000 times in PBS with 0.05% Tween 20 and 0.1% gelatin) and 50 μl was added per well and incubated for 60 min at room temperature. After washing, the second antibody, viz., goat-anti-rabbit-IgG-alkaline phosphatase (1:1000 diluted in PBS with 0.05% Tween 20, 0.5% gelatin and 5% FCS), was added (50 μl/well) and the plates were incubated for 60 min at room temperature. After three washings with PBS containing 0.05% Tween 20, the plates were washed once with 0.1 M diethanolamine, pH 9.8. As substrate for alkaline phosphatase, 50 μl of a solution of 4-methylumbelliferyl phosphate (0.2 mM in 10 mM diethanolamine, pH 9.8, 1 mM $MgCl_2$) was added and incubated for 2 h.

II.16.3. Monoclonal antibodies against mustard gas-protein adducts

II.16.3.1. Peptide-immunogens and peptides for use in ELISA

The synthetic peptide corresponding to N-terminal heptapeptide of α-hemoglobin, mustard gas-alkylated at the amino group of valine (see II.10.1) and glycylglycylglycyl-glutamic acid-5-(2'-hydroxyethyl-thioethyl) ester-1-amide hydrochloride [$(gly)_3$-glu-TDG](see II.10.3) were coupled to KLH with the EDC-procedure (see II.13.6).

The pentapeptide val-leu-ser-glu-gly-OH (PP) is commercially available in large amounts and is suitable for the screening of sera and supernatants of hybridomas because of its partially identical amino acid sequence (val-leu-ser) as compared with the N-terminal heptapeptide of hemoglobin. The alkylated peptide was obtained by treatment of the pentapeptide with 10 mM mustard gas at pH 8.5 in distilled water (room temperature; 16 h; final concentration acetonitrile: 1%), while the pH was controlled by a pH-stat, and subsequent purification by HPLC on a semi-preparative column, analogous to that described for the monoadduct of the heptapeptide (see III.16.2). The identity was checked by amino acid analysis.

This alkylated pentapeptide, $(gly)_3$-glu-TDG, and the two nonalkylated peptides were coupled to BSA to serve as control compounds to check antibodies for cross-reactivity against the native peptides and to test sera in a direct ELISA. To prevent the coupling to BSA of the two last-mentioned peptides via their amino groups, the free amino groups were blocked with 2,4,6-trinitrophenyl sulfonic acid (TNP). The peptides were incubated with equimolar quantities of TNP overnight in the dark at room temperature. The next day 6 peptides (the alkylated pentapeptide, $(Gly)_3$-Glu-TDG, the pentapeptide and the $(Gly)_3$-Glu treated with TNP, and the pentapeptide and $(Gly)_3$-Glu not treated with TNP) were coupled to BSA via EDC as described in II.13.6.

II.16.3.2. Monoclonal antibodies against the mustard gas-treated N-terminal heptapeptide of hemoglobin and against mustard gas-treated chicken gammaglobulin

Four mice were immunized with the N-terminal heptapeptide of α-hemoglobin treated with mustard gas and coupled to KLH. The sera of the mice, obtained after the first and second booster, were tested on antibody activity towards the coupling product of the N-(2'-hydroxyethylthioethyl)-val-leu-ser-glu-gly to bovine serum albumin (BSA). After the third immunization, cells of the spleen of one mouse were fused with mice SP2/0 cells as described in II.13.7. The supernatants of the resulting hybridomas were screened in a direct ELISA on mustard gas-alkylated hemoglobin (5 mM) and on native hemoglobin. Cells producing specific antibodies against mustard gas-alkylated hemoglobin were recloned by limiting dilution as described in II.13.8.

Also, a mouse was immunized with mustard gas-alkylated chicken gammaglobulin and its spleen cells were used for a fusion experiment. The supernatants of the resulting hybridomas were also screened on mustard gas-alkylated hemoglobin and on native hemoglobin. Cells producing specific antibodies against mustard gas-alkylated hemoglobin were recloned by limiting dilution as described in II.13.8.

III. RESULTS

III.1. Synthesis of [^{35}S]-mustard gas

Radioactively labelled mustard gas was needed to study its in vitro and in vivo binding to DNA and proteins. Therefore it was essential to obtain material with a high specific activity and radiochemical purity. For economical reasons ^{35}S was chosen as the most appropriate radioactive isotope, rather than ^{14}C. ^{3}H was considered to be less suitable because of possible exchange reactions during the investigations. The synthesis involved the reaction between hydrogen [^{35}S]sulfide at the highest specific activity which was commercially available with ethylene oxide to give thiodiglycol, followed by conversion to mustard gas. This reaction sequence was carried out according to the method of Boursnell et al. (50) with slight modifications (51), according to the reaction scheme in Figure 3.

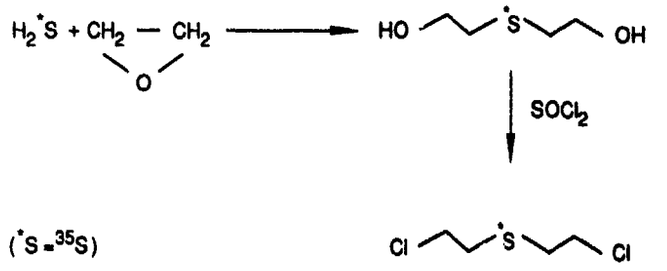

Figure 3. Reaction scheme for the synthesis of [^{35}S]mustard gas

An alternative for the first reaction step is the reaction of 2-bromoethanol with sodium sulfide (94). Apart from the higher cost of sodium [^{35}S]sulfide, the method involves the laborious removal of water from thiodiglycol, which makes it somewhat less attractive. Prior to the high specific activity syntheses, several cold runs of the second reaction step using thionyl chloride were carried out, which gave yields of 90% or more of mustard gas with a gas chromatographic purity exceeding 98%. The first reaction step had been evaluated and was found to give quantitative yields of pure product during previous work on several syntheses of low specific activity [^{35}S]mustard gas (51), as well as in the 1 mmol scale synthesis of deuterated thiodiglycol, which was prepared analogously.

Unexpectedly, the radiochemical yields in the present two synthesis runs (21-27%) were much lower than previously (>60%; 51). As shown in Figure 4, thin layer radiochromatography of the crude thiodiglycol revealed the presence of a series of radioactive contaminants. Mustard gas obtained from this material contained ca. 15% (GLC) of a radioactive contaminant which was identified as 1-(2-chloroethoxy)-2-(2'-chloroethylthio)ethane, Cl-C_2H_4-S-C_2H_4-O-C_2H_4-Cl. Therefore, we ascribe this decrease in yield tentatively to the formation in the first reaction step of higher homologues of thiodiglycol containing

extra oxyethylene groups. This may be due to the use of a halogen photo lamp for heating of the gaseous reaction components in the first reaction step, instead of the 500 W incandescent lamp used in previous runs.

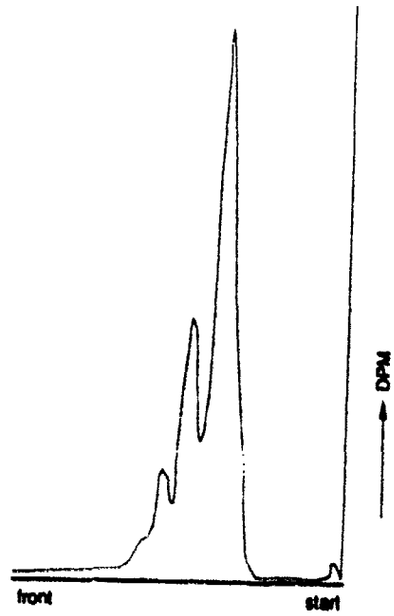

Figure 4. Thin layer radiochromatogram of [^{35}S]thiodiglycol (crude reaction product)

A final distillation of the labelled mustard gas gave satisfactory products with radiochemical purities of 98% and chemical purities of ca. 95%, and with specific activities of 855 and 877 MBq/mmol, i.e., 23.1 mCi/mmol and 23.7 mCi/mmol, respectively.

Recently, a procedure was developed for the synthesis of mustard gas in which hydrogen [^{35}S]sulfide was prepared from sodium [^{35}S]sulphate by reduction with hydrogen iodide, formic acid, and sodium phosphite (95). The starting sodium [^{35}S]sulphate is generally available as a commercial product. According to this procedure mustard gas was obtained in an overall yield of 63%.

III.2. Synthesis of protected derivatives of thiodiglycol and of semi-mustard gas

In the course of our synthetic work for this project, we felt the need for derivatives of mustard gas and thiodiglycol which allow their use for the specific purpose of obtaining products which result from a reaction with only one of the two functional groups in these molecules. The instability of semi-mustard gas, $HO\text{-}C_2H_4\text{-}S\text{-}C_2H_4\text{-}Cl$,

almost prohibits its use in a reasonably pure state. Therefore, we developed two types of protective groups which block a 2-hydroxyethyl moiety of thiodiglycol or semi-mustard gas in a reversible way (Figure 5). The trialkylsilyl group in the first type of derivative can be removed under acidic conditions, whereas the hydroxy group in the acyl-protected derivative is regenerated under basic conditions.

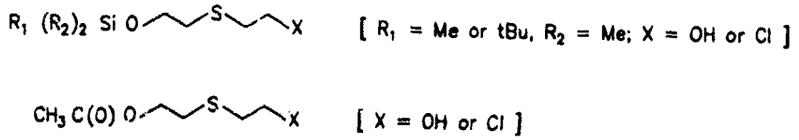

Figure 5. Chemical structures of reversibly protected derivatives of thiodiglycol and of semi-mustard gas

III.2.1. Trialkylsilyl derivatives

2-Trimethylsilyloxyethyl 2'-hydroxyethyl sulfide was readily obtained from the reaction in acetone at -40 °C of thiodiglycol with equimolar trimethylsilyl chloride and triethylamine, according to the reaction scheme in Figure 6. However, the product could not be purified due to disproportion/decomposition reactions during distillation, which left substantial amounts of thiodiglycol and of the di-trimethylsilyl derivative in all fractions. The t-butyldimethyl-silyl monoether, prepared in the same way as the trimethylsilyl derivative, is more stable towards hydrolysis than the latter derivative. Therefore, the t-butyldimethylsilyl derivative could be washed with water prior to distillation in order to remove thiodiglycol and acidic impurities. Subsequent fractional distillation gave the desired product in > 38% yield with a purity (GLC) of 98%. The new product has been fully characterized by means of MS (EI), ^{1}H- and ^{13}C-NMR spectroscopy and is stable for months at -20 °C.

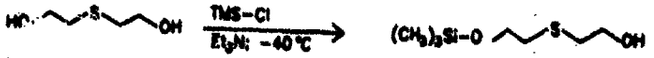

Figure 6. Reaction scheme for the synthesis of 2-trimethylsilyloxyethyl 2'-hydroxyethyl sulfide

The hitherto undescribed compound 2-trimethylsilyloxyethyl 2'-chloroethyl sulfide was prepared from reaction of crude semi-mustard gas, trimethylsilyl bromide, and triethylamine in ether at -60 to -40 °C according to the reaction scheme in Figure 7. Work-up and distillation of the reaction mixture gave the desired product in 19% yield and a purity of 93% (GLC), with semi-mustard gas as a major impurity. The product was fully characterized by means of MS (EI), ^{1}H- and ^{13}C-NMR spectroscopy, and elemental analysis. It appeared to be stable for months at -20 °C.

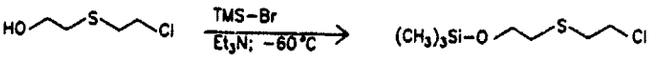

Figure 7. Reaction scheme for the synthesis of 2-trimethylsilyloxyethyl 2'-chloroethyl sulfide

III.2.2. Acyl derivatives

2-Acetoxyethyl 2'-hydroxyethyl sulfide was obtained from the reaction at room temperature of equimolar acetyl chloride and pyridine with a tenfold excess of thiodiglycol in dichloromethane, according to the reaction scheme in Figure 8. Excess of thiodiglycol was conveniently removed by washing with water. The desired product was obtained in 49% yield and a purity of 95% (GLC) by means of distillation. The new product is stable upon storage at -20 °C and was fully characterized by means of MS (thermospray), ^{1}H- and ^{13}C-NMR spectroscopy.

2-Acetoxyethyl 2'-chloroethyl sulfide has been reported by Seligman et al. (54). These authors obtained the product from the reaction of semi-mustard gas with acetic anhydride. No analytical data were presented, other than the refractive index. We have obtained the desired product by conversion of 2-acetoxyethyl 2'-hydroxyethyl sulfide with thionyl chloride at 0-20 °C, according to the reaction scheme in Figure 8. After two distillations the product was obtained in 79% yield and with a purity of 99.7% (GLC). It was fully characterized by means of MS (EI), ^{1}H- and ^{13}C-NMR spectroscopy. The product is stable for months at -20°C.

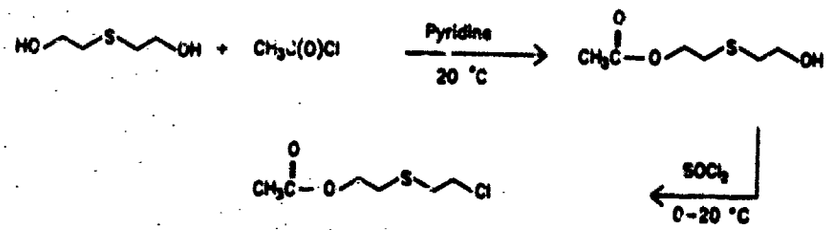

Figure 8. Reaction scheme for the synthesis of 2-acetoxyethyl 2'-hydroxyethyl sulfide and subsequent conversion into 2-acetoxyethyl 2'-chloroethyl sulfide

III.3. Synthesis of mustard gas adducts with guanine

Early investigations by Lawley et al. (30-35) have suggested that primarily the N7 nitrogen in guanine bases of DNA and RNA is alkylated by mustard gas, leading to N7-(2'-hydroxyethylthioethyl)-guanine, as well as to the corresponding intrastrand and interstrand di-adduct di-(2-guanin-7'-yl-ethyl) sulfide, whereas Ludlum et al. (37) suggest that traces of O6-(2'-hydroxyethylthioethyl)-guanine are also formed. So far, these adducts were only characterized on the

basis of similarity of their UV spectra with those of analogous alkyl-substituted purines, and only the isolation of the N7 guanine monoadduct has been explicitly described by Brookes and Lawley (30). In order to identify the adducts of mustard gas with bases in DNA in a definitive way, we have resynthesized these adducts and have characterized them fully by way of spectroscopic and chromatographic techniques.

III. 3.1. N7-(2'-Hydroxyethylthioethyl)-guanine

In our early attempts to prepare the desired N7 guanine monoadduct by the reaction of mustard gas with guanosine in glacial acetic acid at 100 °C (30), followed by hydrolysis in aqueous hydrochloric acid according to the reaction scheme in Figure 9, we found (thermospray-LC-MS) that the desired product was indeed present in the reaction mixture (MH^+, m/z = 256). In spite of increasing the molar excess of mustard gas and prolongation of the reaction time, the ratio between reaction products and unreacted guanine did not increase.

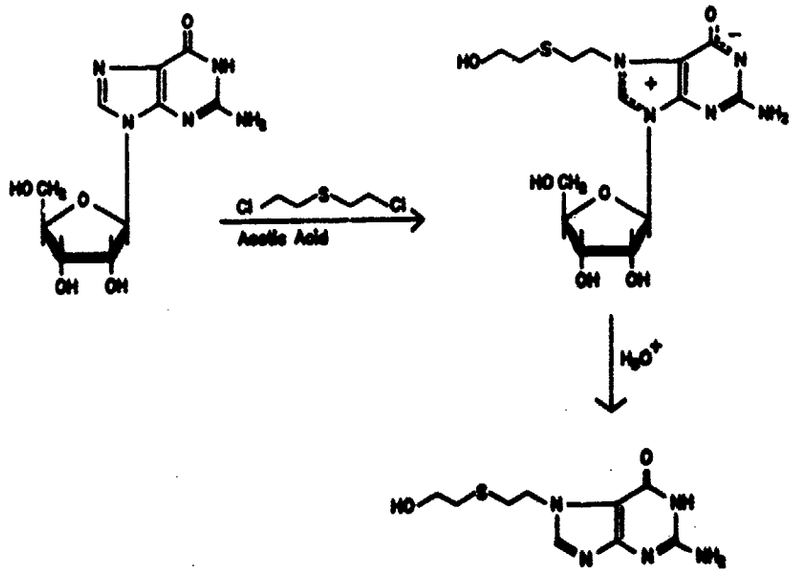

Figure 9. Reaction scheme for the synthesis of N7-(2'-hydroxy-ethylthioethyl)-guanine from guanosine

The reversed phase HPLC chromatogram of the crude reaction mixture with diode array detection (Figure 10) showed, in addition to guanine (peak 3), two reaction products with identical UV spectra. We suspected that these were the N7 monoadduct (peak 4) and the corresponding di-adduct (peak 5), respectively. Reversed phase HPLC on a semi-preparative scale yielded enough material to positively identify both the N7 mono- and di-adducts. These experiments learned also that this method was too time consuming to isolate sufficient amounts of product for full characterization. Similar problems were encountered with ion exchange HPLC on SAX-type columns. Attempts to purify the product by means of repeated recrystallizations and extractions of the crude material with various solvents and dilute

aqueous hydrochloric acid gave a product with a purity < 60%. Finally, the best results were obtained with a commercial medium pressure liquid chromatography system (Lobar, Merck) using the largest glass column available (44x3.7 cm) packed with reversed phase silica gel (RP 18). This system allowed sample loads of up to 20 mg crude material, containing ca. 34% of the desired monoadduct. In this way, 100 mg of crude material gave a yield of 35 mg of the product as monohydrate after one additional recrystallization from water.

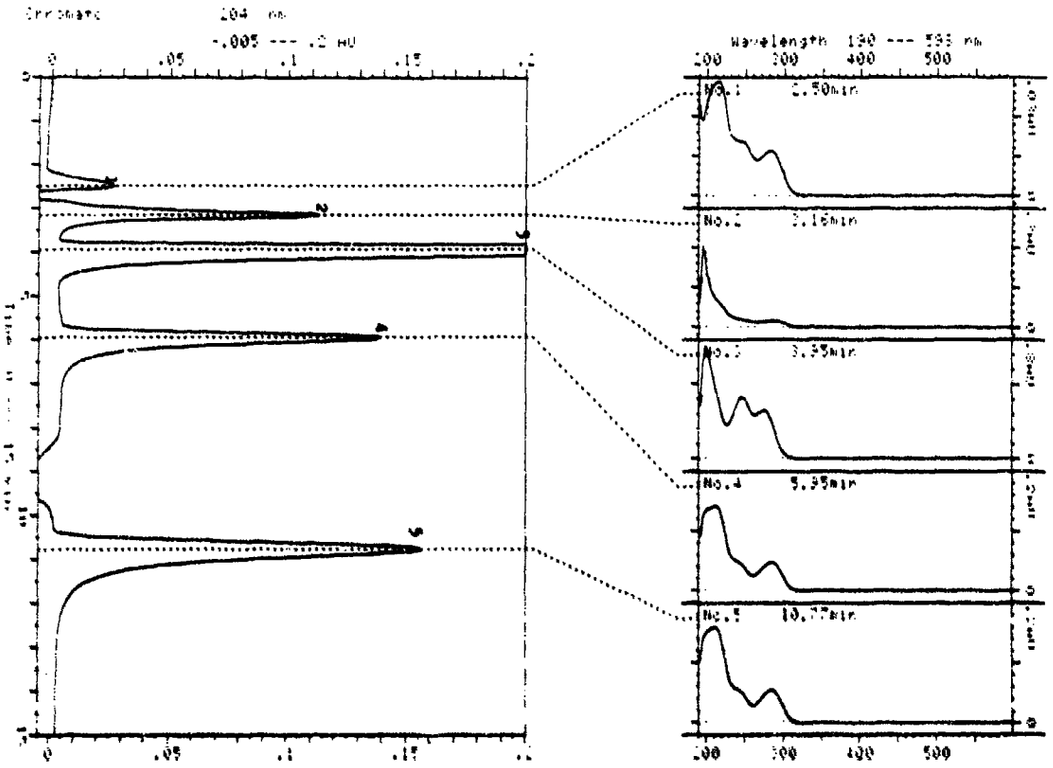

Figure 10. Reversed phase HPLC chromatogram with diode array detection after hydrolysis of the crude reaction mixture resulting from the alkylation of guanosine with a 100% molar excess of mustard gas in glacial acetic acid. The chromatogram was measured at a set wavelength of 204 nm; the UV spectra of the major peaks, measured on-line, are also given. The column (250x5 mm) was packed with LiChrosorb RP18 (particle size 5 µm). Eluent: 25 mM ammonium bicarbonate in water/methanol (3/1, v/v)

According to reversed phase HPLC (UV detection at 254 nm) the purity of the product was 96-97%. The UV spectra (λ_{max} 284 nm at pH 7.0; 249 nm at pH 1.0) and melting point (dec. > 280 °C) were identical with those reported by Brookes and Lawley (30). As mentioned above thermospray MS of the product showed MH^+ at m/z 256, whereas electron impact MS showed a small peak at m/z 255 (M^+) and major peaks at m/z

237 (M-H_2O, loss of water from 2'-hydroxyethylthioethyl group) and at m/z 151 [M-(CH=CH-S-CH_2-CH_2-OH).

The structure of the product followed unequivocally from ^1H- and ^{13}C-NMR spectroscopy (Figure 11). The long range couplings found between N-CH_2 and C-5/C-8 clearly show that the 2'-hydroxyethylthioethyl moiety is attached to N7.

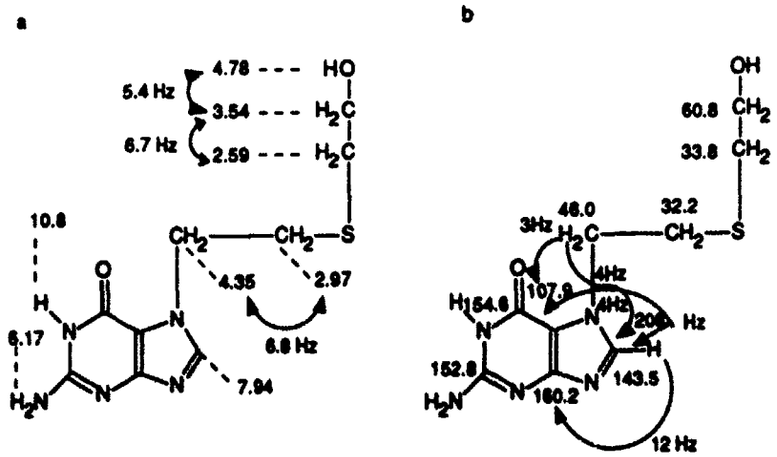

Figure 11. Chemical shift assignments and coupling constants for the hydrogen (400 MHz; a) and carbon atoms (100.6 MHz; b) of N7-(2'-hydroxyethylthioethyl)-guanine in DMSO-d_6. C-H couplings within the 2'-hydroxyethylthioethyl group are not given

III.3.2. Di-(2-guanin-7'-yl-ethyl) sulfide

When the above-mentioned reaction conditions for the synthesis of the N7 guanine monoadduct of mustard were changed to equimolar amounts of reactants in order to optimize the yield of the corresponding di-adduct, a maximal conversion to ca. 10% of di-adduct was observed with reversed phase HPLC. No further improvements were achieved by varying the reaction conditions. An alternative route of synthesis was sought in the conversion of crude monoadduct with thionyl chloride to the corresponding N7-(2'-chloroethylthioethyl)-guanine, followed by conversion of this product to di-adduct with remaining guanine in the reaction mixture, according to the reaction scheme given in Figure 12. Although the chloro-compound was probably formed, as observed by the formation of the corresponding acetoxy derivative in thermospray-LC-MS of the reaction mixture in acetate buffer, the formation of the desired di-adduct was not observed.

A third method of synthesis was evaluated, according to Brookes and Lawley (96), based on the reaction of equimolar mustard gas and guanosine-5'-phosphate (guanilic acid) in water, followed by acid

hydrolysis to obtain the free base, as shown in the reaction scheme given in Figure 13.

Although a non-homogeneous reaction mixture is obtained due to the slight solubility of mustard gas in water, the reaction proceeds smoothly at room temperature. However, again a maximum conversion to

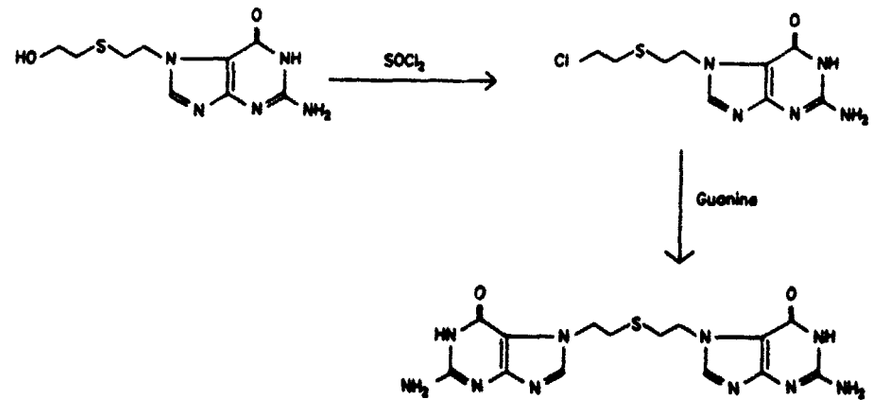

Figure 12. Reaction scheme for the attempted synthesis of di-(2-guan-7'-yl-ethyl) sulfide via chlorination of the 2'-hydroxyl-ethylthioethyl moiety of N7-(2'-hydroxyethylthioethyl)-guanine and subsequent alkylation of guanine

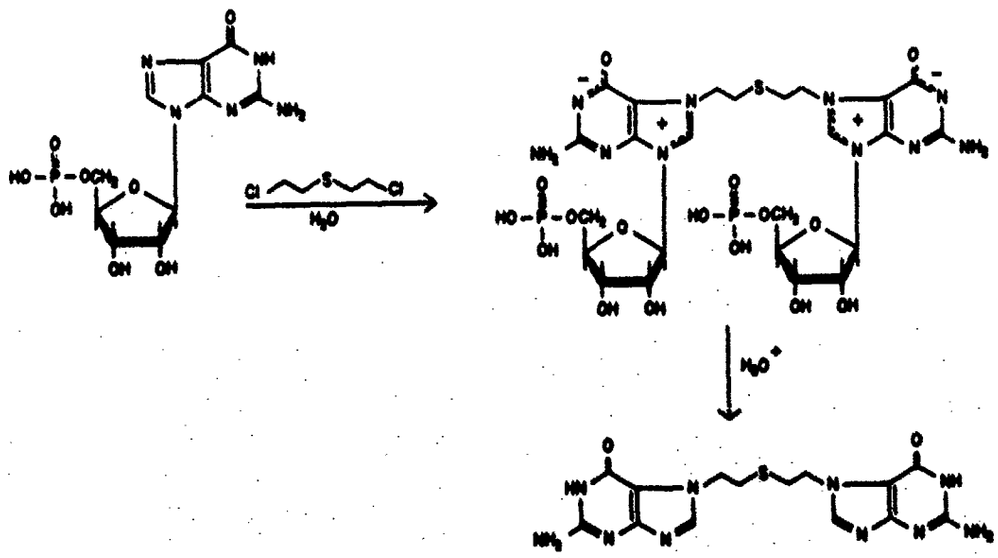

Figure 13. Reaction scheme for the synthesis of di-(2-guan-7'-yl-ethyl) sulfide via alkylation of guanosine-5'-phosphate with mustard gas and subsequent hydrolysis

only 10% of di-adduct was observed. Initial attempts to purify the di-adduct from the crude reaction mixture by means of chromatographic

techniques were poorly reproducible. Separations were inadequate and collected eluates were sometimes found to contain no product. When we found out that these problems were due to the extreme low solubility of the di-adduct in water, these complications were used to our advantage. Repeated extractions of crude material with dilute aqueous hydrochloric acid removed virtually all contaminants and starting material. The residual di-adduct was obtained as analytically pure hydrochloride salt by means of recrystallization from boiling aqueous hydrochloric acid (pH 2). The UV spectrum showed maxima at 284 nm (pH 7.0), and at 249 nm (pH 1.0), i.e., identical with the spectrum of the monoadduct (vide supra). Under optimal conditions with regard to the temperature of the tip of the probe (ca. 250 °C), thermospray MS of the product showed only MH^+ at m/z = 389 and MNa^+ at m/z = 411.

The structure of the product followed unequivocally from ^1H- and ^{13}C-NMR (Figure 14), using CF_3COOD as solvent. The long range couplings between N-CH_2 and C-5/C-8 indicate that the side chain is attached to N7.

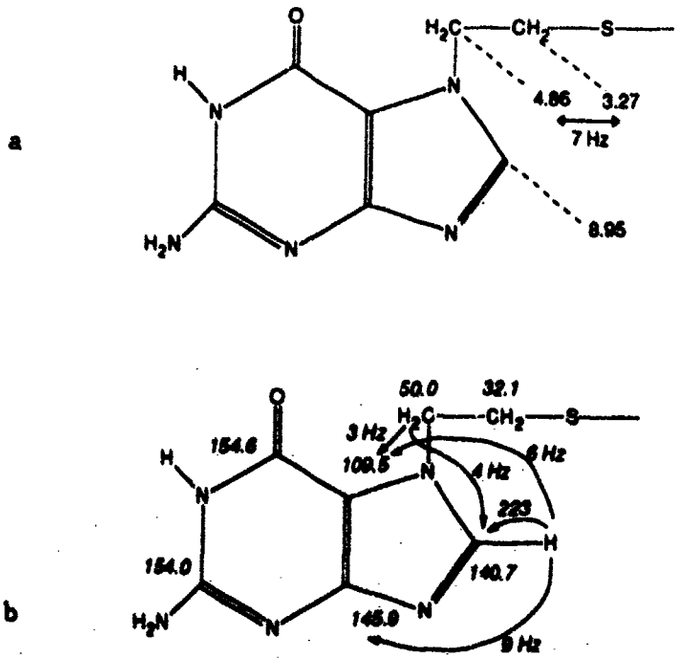

Figure 14. Chemical shift assignments and coupling constants for the hydrogen (400 MHz; a) and carbon atoms (100.6 MHz; b) of di-(2-guan-7'-yl-ethyl) sulfide in CF_3COOD

III.3.3. O6-(2"-Hydroxyethylthioethyl)-2'-deoxyguanosine

Our first efforts to obtain this product were based on the work of Ludlum et al. (37,97,98). These authors reported the synthesis of the desired product, and of the corresponding (2'-ethylthioethyl) derivative, in unqualified yields from the reaction of 6-chloro-3',5'-di-O-acetyl-2'-deoxyguanosine with the (mono)sodium salt of

thiodiglycol and of ethyl 2-hydroxyethyl sulfide, respectively. In our hands this route of synthesis did not give the desired O6-derivative of mustard gas. Only when we used the sodium salt of 2-trimethylsilyloxyethyl 2'-hydroxy-ethyl sulfide instead of thiodiglycol, mass spectrometry (positive CI,isobutane) of the crude reaction mixture showed peaks at m/z = 256 and 238, corresponding with MH^+ of O6-(2'-hydroxyethylthioethyl)-guanine and loss of water from the latter product. Attempts to isolate the O6-derivative from the reaction mixture by means of reversed phase HPLC were unsuccessful.

Since the direct reaction of 2'-deoxyguanosine with mustard gas is expected to give only traces of alkylation at O6 due to the soft character of the episulfonium ion electrophile, we concentrated our efforts on a route of synthesis of O6-derivatives of 2'-deoxy-guanosines as developed by Gaffney and Jones (55,56), and modified by Van Boom (57). The sequence of reactions as shown in Figure 15 involves reaction of 3',5',N2-triacetyl-2'-deoxyguanosine (1) with 2,4,6-triisopropylbenzenesulfonyl chloride to yield the O6-sulfonylated derivative (2), in which the sulfonyl ester moiety is

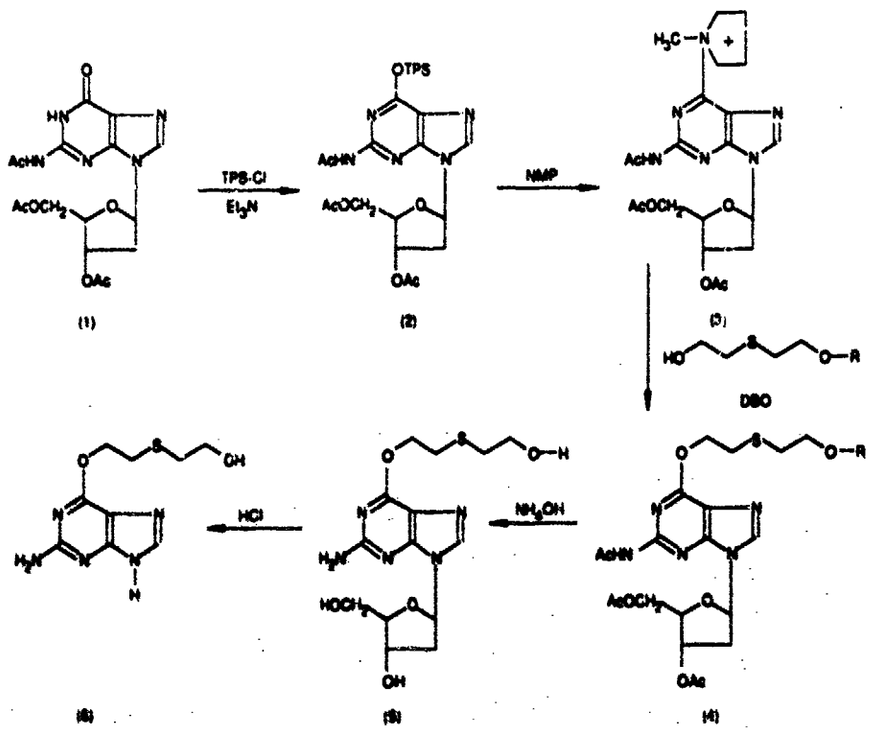

Figure 15. Reaction scheme for the O6-alkylation of 2'-deoxy-guanosine. TPS-Cl = 2,4,6-triisopropylbenzenesulfonyl chloride; NMP = N-methylpyrrolidine; R = acetyl or t-Bu(Me)$_2$Si; DBU = 1,8-diazabicyclo-(5.4.0)-undec-7-ene

readily replaced by a 1-methyl-pyrrolidinium group to give (3). The latter group is displaced in turn by the appropriate alcohol in the presence of the strong base 1,8-diazabicyclo-(5.4.0)-undec-7-ene (DBU) to yield (4), which is deprotected with aqueous ammonia to give the desired product (5). First, we explored this route of synthesis using the t-butyldimethylsilyl derivative of thiodiglycol [R = $tBu(Me)_2Si$-; see III.2.1]. It was concluded that this route was viable, but the insolubility of the silylated derivative of 2'-deoxyguanosine in water complicated the purification of the end product. Therefore we used subsequently the monoacetylated derivative of thiodiglycol [R = MeC(O)-; see III.2.2]. This turned out to provide a smooth synthesis, in which a one-step deprotection removed all four acetyl groups to give 5) in ca. 12% overall yield. According to reversed phase HPLC (UV detection at 214 nm) the purity of the product was 98.6%, whereas ^{1}H-NMR confirmed that the overall purity was > 95%.

The structure of the product follows from the route of synthesis and was confirmed in several ways. The UV spectra at pH 7 and 14 show maxima at 247 and 281 nm. These maxima shift to 243 and 288 nm at pH 1, in accordance with the UV spectra of other O6-alkylated 2'-deoxyguanosine derivatives (58). The thermospray mass spectrum of the product shows peaks at m/z = 372 (MH^+), 256 [MH^+ of O6-(2'-hydroxyethylthioethyl) guanine], and at 152 (MH^+ of guanine). See experimental part for a full assignment of the ^{1}H- and ^{13}C-NMR spectra of the product in D_2O.

III.3.4. <u>O6-(2'-Hydroxyethylthioethyl)-guanine</u>

According to Ludlum et al. (37,97,98) it is impossible to depurinate O6-(2'-hydroxyethylthioethyl)-2"-deoxyguanosine by means of acidic hydrolysis since this results in simultaneous loss of the 2'-hydroxyethylthioethyl group at O6. We have found, however, that depurination in 0.1 N aqueous hydrochloric acid for 100 min at room temperature leads to almost complete depurination and only to partial dealkylation at O6 (Figure 15). A one-step purification step on a Sephadex G-10 column gave the desired product in 39% yield with a purity > 95% (^{1}H-NMR). The thermospray mass spectrum of the product shows peaks at m/z = 278 (MNa^+), 256 (MH^+), and 152 (MH^+, guanine). See experimental part for a full assignment of the ^{1}H- and ^{13}C-NMR spectra of the product in DMSO-d_6.

III.3.5. <u>Kinetics of dealkylation of O6-alkylated guaninas</u>

The lability in acidic aqueous solution of O6-(2'-hydroxyethylthioethyl)-guanine was further investigated by measuring the rate of dealkylation at pH 0.5 and 25 °C. For comparison we also measured the dealkylation rate of the corresponding O6-ethyl compound. The reactions were followed spectrophotometrically at ca. 0.1 mM in a 0.05 M KCl solution. UV spectra taken in the course of the reactions show sharp isobestic points over a time course of several half times, which indicates that a well-defined reaction of a sufficiently pure substrate is being measured (see Figure 16 for an example). The

results were plotted as the logarithm of the difference between the absorbance of the reaction mixture and the absorbance of the dealkylated compound versus reaction time. We measured this difference at 248 nm, i.e., at the wavelength of maximal difference between the absorbances of the dealkylated product and the starting compound (Figure 16). A representative example of such a semilogarithmic plot, pertaining to run 1 in Table 2, is given in Figure 17. The measured absorbances (A_t) at 248 nm and the calculated differences (A_∞ - A_t) between this absorbance and the absorbance for complete dealkylation (A_∞) of the two O6 adducts as a function of reaction time are given in Tables 2 and 3, for duplicate runs.

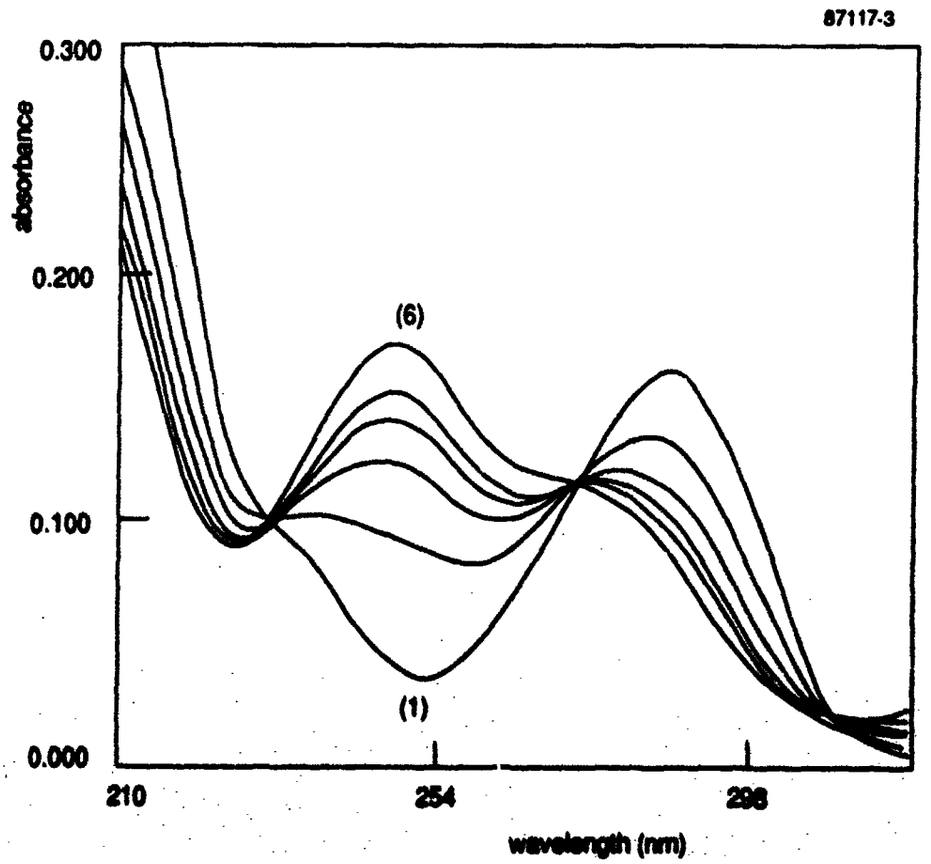

Figure 16. UV spectra of O6-(2'-hydroxyethylthioethyl)-guanine (0.1 mM) in the course of dealkylation in 0.05 M KCl, pH 0.5, 25 °C. The spectra were taken after 0 (1), 30, 60, 90, 120, and 360 (6) min

The semilogarithmic plots for dealkylation of the O6-(2'-hydroxyethylthioethyl) compound show deviation from first-order kinetics after 1 - 2 half lives of the reaction. First-order rate constants were therefore evaluated from data points measured within the first two half-life times, as the slope of the plots. Plots for dealkylation of the O6-ethyl compound were linear for at least three half lives. The calculated rate constants and half lives are summarized in Table 4.

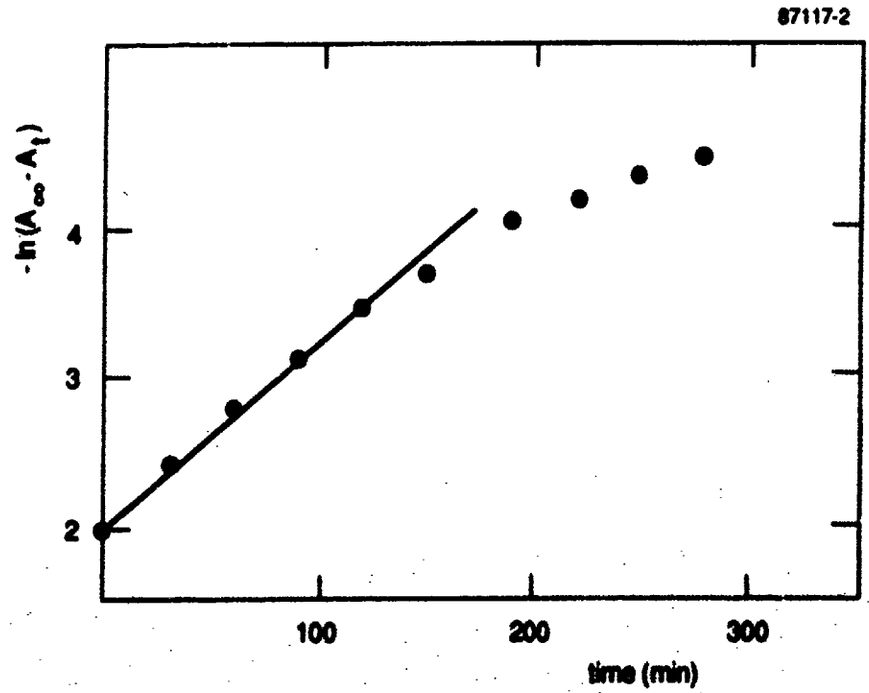

Figure 17. Plot of natural logarithm of the difference between the the absorbance (248 nm) of O6-(2'-hydroxyethylthioethyl)-guanine in 0.05 M KCl, pH 0.5, 25 °C, at a given time (A_t) and the absorbance of the dealkylated compound (A_∞) versus reaction time. Data taken from Table 2, run 1

The rate of dealkylation of O6-alkyl-guanine increases at least two orders of magnitude upon substitution of the ethyl group by the 2'-hydroxyethylthioethyl group. As shown in the reaction scheme in Figure 18, it is suggested that anchimeric assistance by the formation of an episulfonium ion provides the extra driving force for the highly accelerated hydrolysis of the 2'-hydroxyethylthioethyl adduct.

Table 2. Absorbances at 248 nm (A_t) measured in the course of dealkylation of O6-(2'-hydroxyethylthioethyl)-guanine (0.1 mM) in 0.05 M KCl, pH 0.5, 25 °C, and calculated differences ($A_\infty - A_t$) between this absorbance and the absorbance after complete reaction (A_∞)

Time (min)	A_t (248 nm) Run 1	A_t (248 nm) Run 2	$-\ln (A_\infty - A_t)$ Run 1	$-\ln (A_\infty - A_t)$ Run 2
0	0.038	0.042	1.92	1.75
30	0.091	0.099	2.37	2.15
60	0.123	0.135	2.77	2.53
90	0.141	0.157	3.13	2.85
120	0.153	0.171	3.45	3.12
150	0.160	0.181	3.70	3.37
191	0.167		3.99	
213		0.193		3.81
221	0.170		4.18	
243		0.196		3.98
251	0.172		4.33	
273		0.199		4.14
281	0.173		4.46	
303		0.201		4.25
311	0.175	0.202	4.56	4.32
341	0.176	0.204	4.71	4.48
371		0.206		4.69
401		0.208		4.92
431		0.209		5.13
end	0.185	0.215		

Table 3. Absorbances at 248 nm (A_t) measured in the course of dealkylation of O6-ethyl-guanine (0.1 mM) in 0.05 M KCl, pH 0.5, 25 °C, and calculated differences ($A_\infty - A_t$) between this absorbance and the absorbance after complete reaction (A_∞)

Time (h)	A_t (248 nm) Run 3	A_t (248 nm) Run 4	$-\ln (A_\infty - A_t)$ Run 3	$-\ln (A_\infty - A_t)$ Run 4
1	0.477		-0.36	
163		0.124		1.857
167	0.987		0.08	
336	1.319		0.53	
503	1.510		0.92	
523		0.217		2.757
8[illegible]		0.260		3.912
839	1.741		1.77	
954		0.268		4.423
1169		0.269		4.528
end	1.910	0.280		

Table 4. Calculated (pseudo) first-order rate constants (k; min^{-1}) and half lives (min) for dealkylation of two O6-alkyl-guanines in 0.05 M KCl, pH 0.5, 25 °C

Run	k (min^{-1})	Half life (min)
1[a]	1.27×10^{-2}	55
2[a]	1.15×10^{-2}	60
Averaged[a]	1.21×10^{-2}	57
3[b]	4.2×10^{-5}	16500
4[b]	4.8×10^{-5}	14400
Averaged[b]	4.5×10^{-5}	15400

[a] Alkyl = 2'-hydroxyethylthioethyl.
[b] Alkyl = ethyl.

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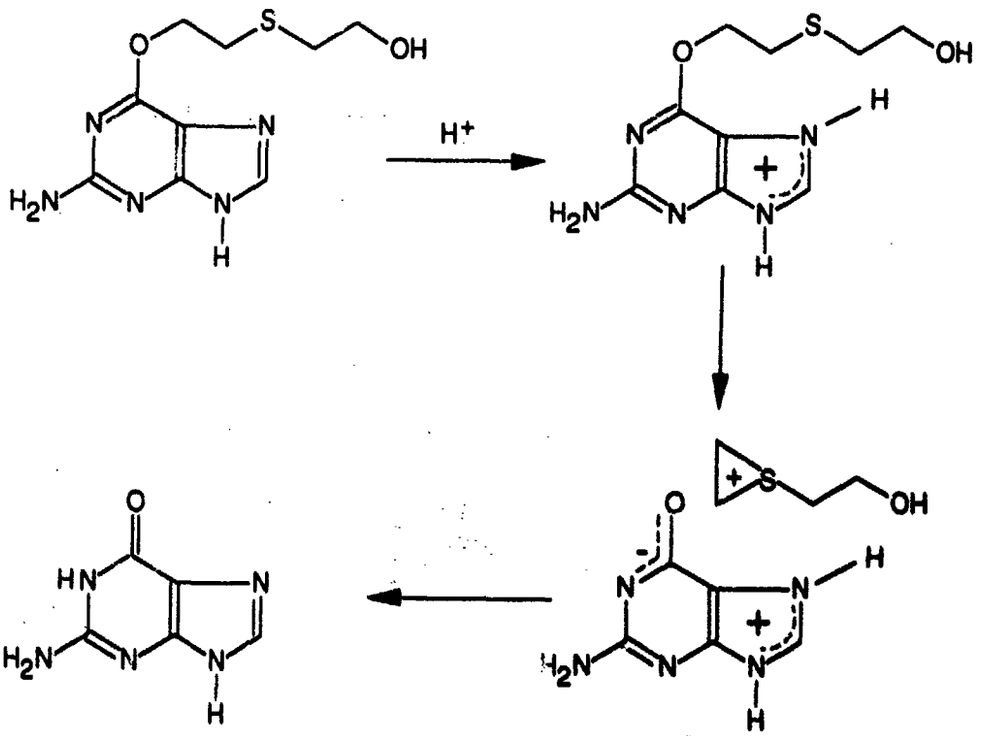

Figure 18. Suggested reaction mechanism for anchimeric assistance in the dealkylation of O6-(2'-hydroxyethylthioethyl)-guanine in 0.05 M KCl, pH 0.5, at 25 °C.

III.4. Synthesis of mustard gas adducts with adenine

Alkylation of adenosine with excess of mustard gas in glacial acetic acid at 100 °C, by analogy with the alkylation of guanosine (see III.3.1) was followed by hydrolysis of the reaction mixture in 1 N aqueous hydrochloric acid. Reversed phase HPLC analysis with photodiode array detection of this mixture (Figure 19) led to the

identification of peak 4 as adenine, based on retention time and uv maximum (λ_{max} 259 nm). Peak 6 was tentatively assigned as N3-(2'-hydroxyethylthioethyl)-adenine (λ_{max} 274 nm), and peak N7 as N9-(2'-hydroxyethylthioethyl)-adenine (λ_{max} 262 nm). The tentative

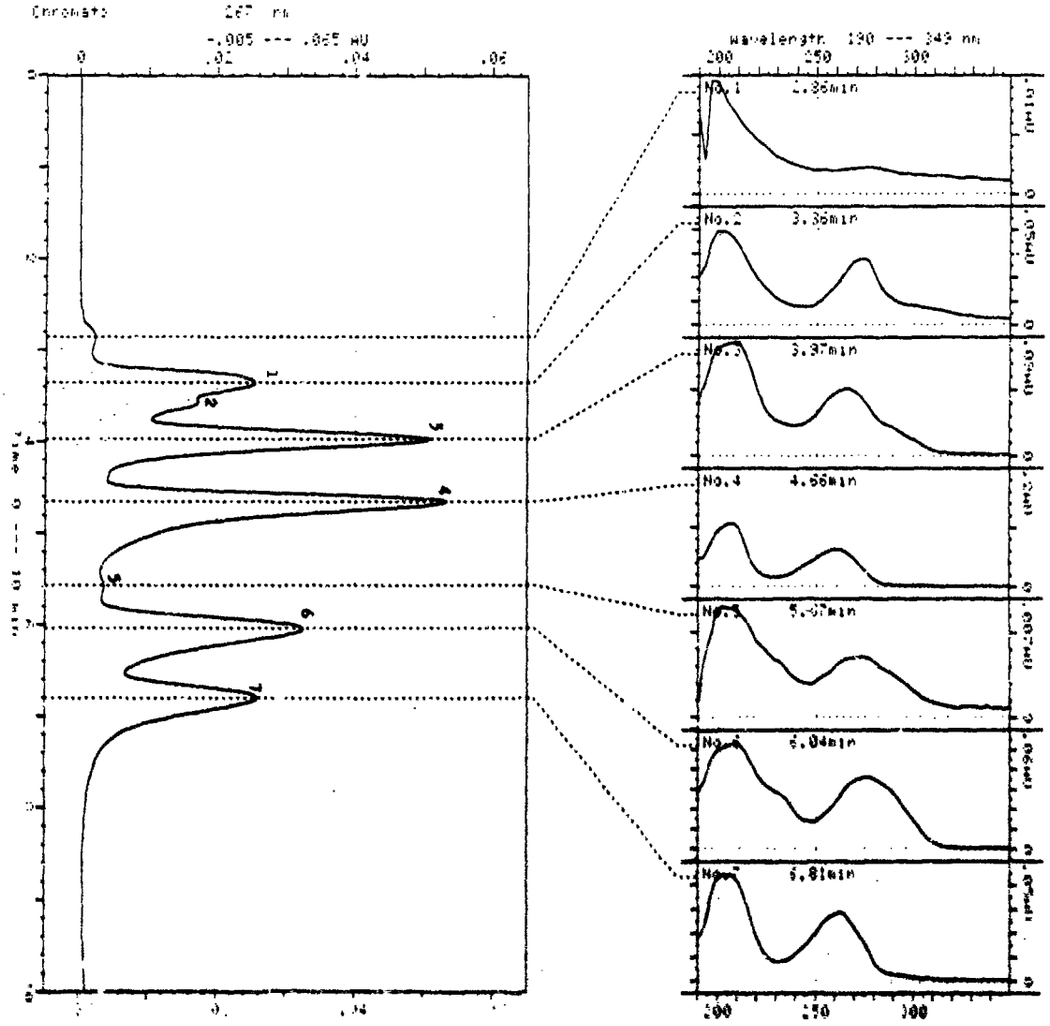

Figure 19. Reversed phase HPLC chromatogram with diode array detection after hydrolysis of the crude reaction mixture resulting from the alkylation of adenosine with mustard gas in glacial acetic acid at 100 °C. The chromatogram was measured at a set wavelenght of 267 nm; the UV spectra of the major peaks, measured on-line, are also given. The column (250x5 mm) was packed with LiChrosorb RP18 (particle size 7 μm). Eluent: 25 mM ammonium bicarbonate in water/methanol (2/1, v/v)

assignments of the latter two peaks were corroborated by means of thermospray-LC-MS, which showed protonated parent ions at m/z = 240, as expected for 2'-hydroxyethylthioethyl monoadducts of adenine. The product corresponding with peak 6 was isolated by means of two

successive purifications on a reversed phase medium pressure Lobar column, which gave the product in ca. 1% overall yield. A final proof of the structure was obtained from ^{1}H- and ^{13}C-NMR spectroscopy (Figure 20). The assignments of the carbon signals are based on single frequency decoupling. The long range couplings between N-CH_2 and C-2/C-4 indicate that the 2'-hydroxyethylthioethyl moiety is attached to N3.

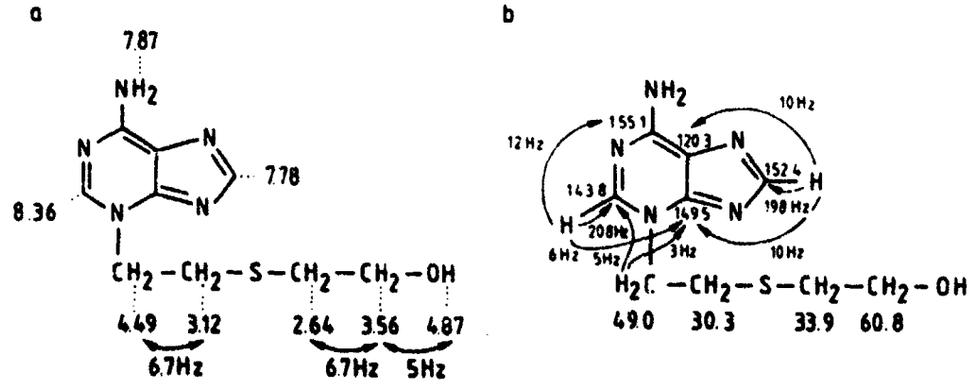

Figure 20. Chemical shift assignments and coupling constants for the hydrogen (400 MHz; a) and carbon atoms (100.6 MHz; b) of N3-(2'-hydroxyethylthioethyl)-adenine in DMSO-d_6, excluding C-H couplings within the 2'-hydroxyethylthioethyl group

Simultaneously with the N3 adduct, a small quantity of the supposed N9 adduct was isolated from the Lobar column. The structure of this product was tentatively assigned by means of ^{1}H-NMR as the N9 adduct (data not given). We assume that this adduct has been formed due to partial depurination during the alkylation reaction in glacial acetic acid, which makes the N9 position available for alkylation. We found no evidence for the formation of an N1 adduct of adenine, while this product has been isolated from the reaction of 2'-deoxyguanosine-5'-phosphate (35) and of adenine (34) with semi-mustard gas in aqueous solution.

III.5. Attempted synthesis of N7-adducts of 2'-deoxyguanosine-5'-phosphate

One of the most straightforward approaches to obtain haptens for the generation of monoclonal antibodies against adducts of mustard gas with DNA is the synthesis of nucleotide adducts corresponding with the major adducts which have been identified, followed by coupling to a protein of such nucleotide adducts via the phosphate moiety in order to raise antibodies. In this context we have started our efforts to obtain haptens with attempts to synthesize the N7 monoadduct of mustard gas with 2'-deoxyguanosine-5'-phosphate, since the N7 monoadduct of guanine appears to be the most abundant adduct upon alkylation of human and calf-thymus DNA with mustard gas (confer III.15).

Reaction of 2'-deoxyguanosine-5'-phosphate with a 50% molar excess of mustard gas in a 0.1 M aqueous triethylammonium bicarbonate/-acetonitrile mixture (2/1, v/v; pH 7.5) at room temperature for 16 h gave conversion to ca. 40% of one major reaction product, according to anion exchange HPLC analysis. This product was isolated by means of anion exchange chromatography on a Sepharose Q column, using a gradient increasing from 0.1 to 1.0 M aqueous triethylammonium bicarbonate. This gave the purified reaction product in ca. 16% overall yield. Attempts to obtain thermospray or EI mass spectra of the product were unsuccessful. The UV maxima of the adduct in aqueous solution at pH 1, 7, and 13 were at 255, 252, and at 267 nm, respectively. The similarity of these spectra with those of unsubstituted 2'-deoxyguanosine (58) and the absence of a rapidly exchanging hydrogen atom at C8 in the ^{1}H-NMR spectrum of the product in D_2O indicated that the desired N7 adduct had not been obtained. Chemical shifts and coupling constants in the ^{1}H- and ^{13}C-NMR spectra of the product in D_2O are summarized in Figure 21. The splittings in

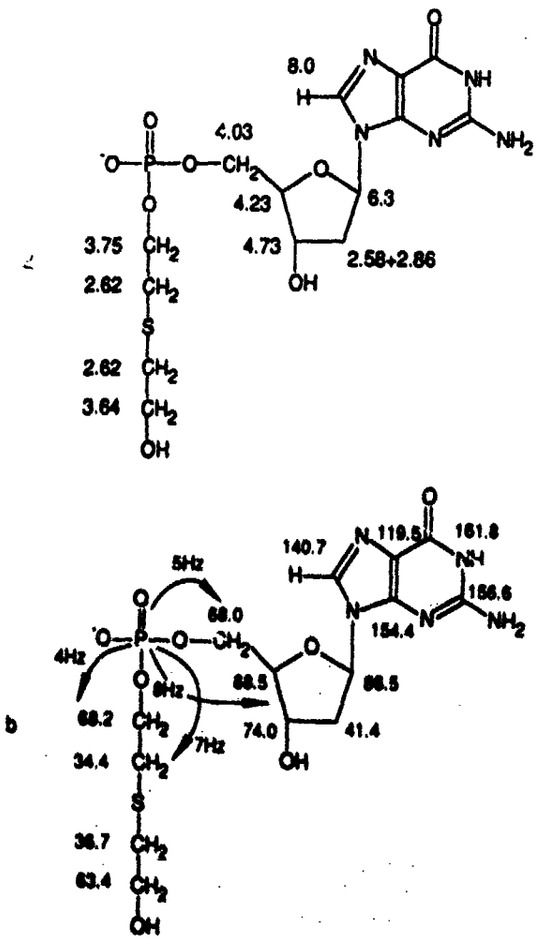

Figure 21. Chemical shift assignments and P-C coupling constants for the hydrogen (400 MHz; a) and carbon atoms (100.6 MHz; b) of 5'-[O-(2"-hydroxyethylthioethyl) phosphate]-2'-deoxy-guanosine in D_2O

the ^{1}H-decoupled ^{13}C-NMR spectrum, which are due to ^{31}P-^{13}C-couplings, clearly indicate that the 2'-hydroxyethylthioethyl moiety is attached to phosphate. It follows that alkylation of the phosphate moiety of 2'-deoxyguanosine-5'-phosphate by mustard gas has taken place almost exclusively with formation of 5'-[O-(2"-hydroxyethyl-thioethyl) phosphate]-2'-deoxyguanosine.

We assume that this preference for alkylation of phosphate instead of the N7 position of the guanine base is caused by the ionization of the weakly acidic (pK_a 6.4) and strongly nucleophilic secondary hydroxyl group of the phosphate moiety at the pH (7.5) of the reaction (59). Reaction at acidic pH in order to protonate the hydroxyl group is not feasible in view of the rapid depurination of 2'-deoxyguanosine derivatives at acidic pH (99). Therefore, we attempted the alkylation at N7 of 2'-deoxyguanosine-3',5'-cyclic phosphate, which has only a strongly acidic, weakly nucleophilic group at the phosphate moiety. If successful, the reaction product can be hydrolyzed with phosphodiesterase, 3',5'-cyclic nucleotide (E.C. 3.1.4.37) (100), to give the desired end product, according to the reaction scheme given in Figure 22. The reaction was performed under the same conditions as those described above for 2'-deoxyguanosine 5'-phosphate. Anion exchange HPLC as well as ^{1}H-NMR analysis of the crude reaction mixture after replacement of solvents by D_2O revealed that a mixture of at least 6 reaction products had been formed. No attempts were made to isolate individual products. As

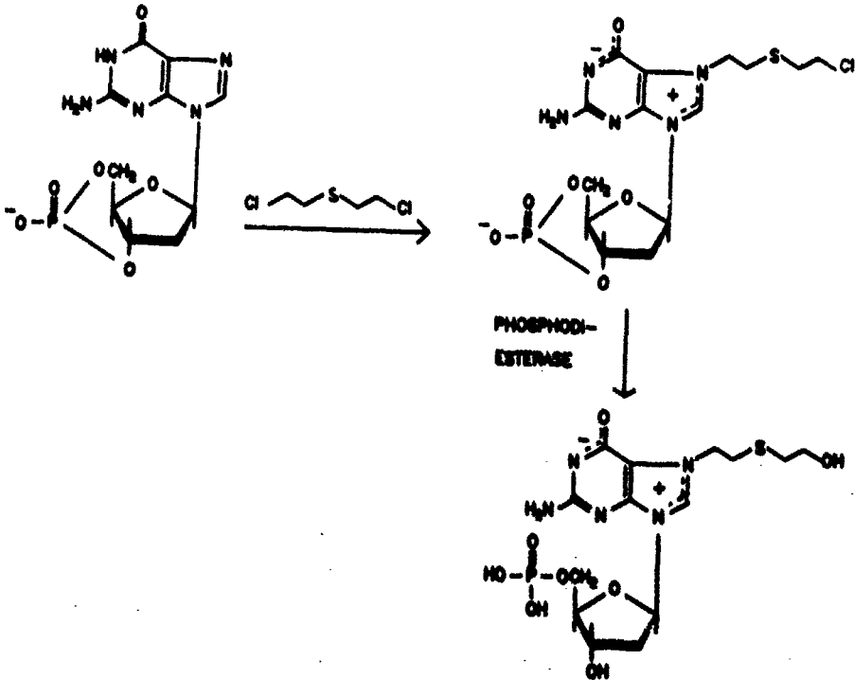

Figure 22. Reaction scheme for the attempted synthesis of N7-(2"-hydroxyethyl-thioethyl)-2'-deoxyguanosine-5'-phosphate via alkylation of 2'-deoxy-guanosine-3',5'-cyclic phosphate with mustard gas and subsequent enzymatic hydrolysis of the cyclic phosphate moiety

expected, depurination occurred when it was attempted to alkylate the cyclic phosphate derivative at acidic pH (pH 4.5).

A final attempt was made to obtain the desired N7 monoadduct of mustard gas with 2'-deoxynucleoside-5'-phosphate by means of alkylation at N7 of 2'-deoxy-guanosine, followed by phosphorylation of the 5'-position with nucleoside phosphotransferase by analogy with the published procedure for the N7 methyl adduct (101). The reaction scheme is given in Figure 23.

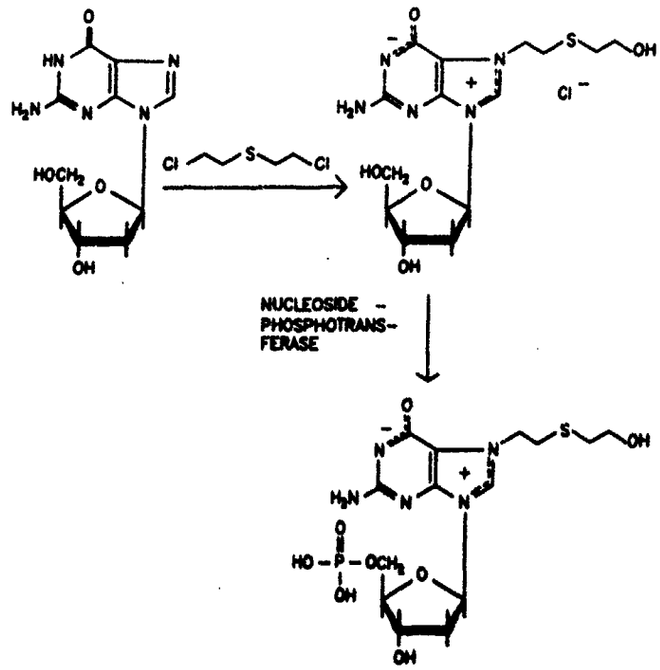

Figure 23. Reaction scheme for the attempted synthesis of N7-(2"-hydroxyethylthioethyl)-2'-deoxyguanosine-5'-phosphate via alkylation of 2'-deoxy-guanosine with mustard gas and subsequent enzymatic phosphorylation at the 5'-position

2'-Deoxyguanosine was reacted with mustard gas as described above for 2'-deoxy-guanosine-5'-phosphate. Cation exchange HPLC of the crude reaction mixture shows (Figure 24), in addition to the peak of unreacted starting material, two peaks of reaction products. Small amounts of these products were isolated by means of preparative reversed phase HPLC on an RP18 column. ^{1}H-NMR of the first reaction product indicated that this was N7-(2'-hydroxyethylthioethyl)-guanine, contaminated with other unidentified products. We assume that N7-(2"-hydroxyethylthioethyl)-2'-deoxyguanosine has been formed during the reaction, but that depurination has proceeded during work-up.

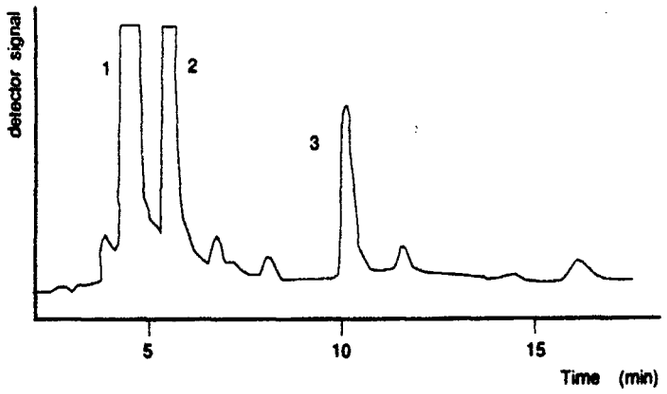

Figure 24. Cation exchange HPLC chromatogram of the crude reaction mixture resulting from alkylation with mustard gas of 2'-deoxyguanosine in 0.1 M aqueous triethylammonium bicarbonate (pH 7.5)/acetonitrile (2/1, v/v). The column (300x2 mm) was packed with Partisil-10 SAX. Eluent: aqueous KH_2PO_4 with a linear gradient increasing from 0.001 M to 0.3 M in 30 min. UV detection at 254 nm. Peak 1: 2'-deoxy-guanosine; peak 2: N7-(2"-hydroxyethyl-thioethyl)-2'-deoxyguanosine(?) and other products; peak 3: N1-(2"-hydroxyethylthioethyl)-2'-deoxyguanosine

Analysis of the ^1H- and ^{13}C-NMR spectra in DMSO-d_6 of the last eluting minor product revealed unequivocally that a hitherto undescribed adduct of mustard gas had been isolated, i.e., N1-(2"-hydroxyethylthioethyl)-2'-deoxyguanosine. The assignment of the carbon signals is based on single frequency decoupling experiments and on a two-dimensional heteronuclear chemical shift correlated spectrum (HETCOR; see Figure 25). The assignment of C2 and the hydroxylated carbons was concluded from the observed splittings after addition of D_2O (ratio H_2O/D_2O ca. 1/1). The long range couplings of approximately 3 Hz found between N-CH_2 and both C6 and C2 indicate that the 2'-hydroxyethylthioethyl moiety is attached to N1. Chemical shift assignments and some coupling constants for the hydrogen and carbon atoms of the N1 adduct are summarized in Figure 26. The thermospray MS spectrum of the N1-adduct had major peaks at m/z = 372 (MH^+) and at m/z = 256 [MH^+ of N1-(2'-hydroxyethylthioethyl)-guanine], in accordance with the assigned structure.

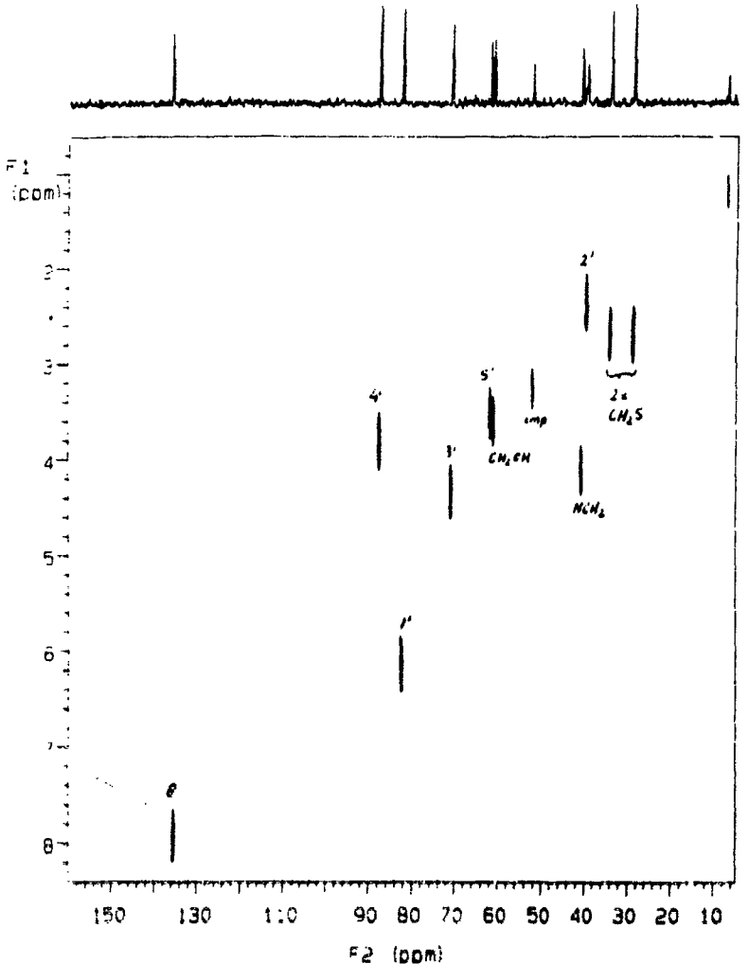

Figure 25. Heteronuclear chemical shift correlated (HETCOR) spectrum of N1-(2"-hydroxyethylthioethyl)-2'-deoxyguanosine in DMSO-d_6

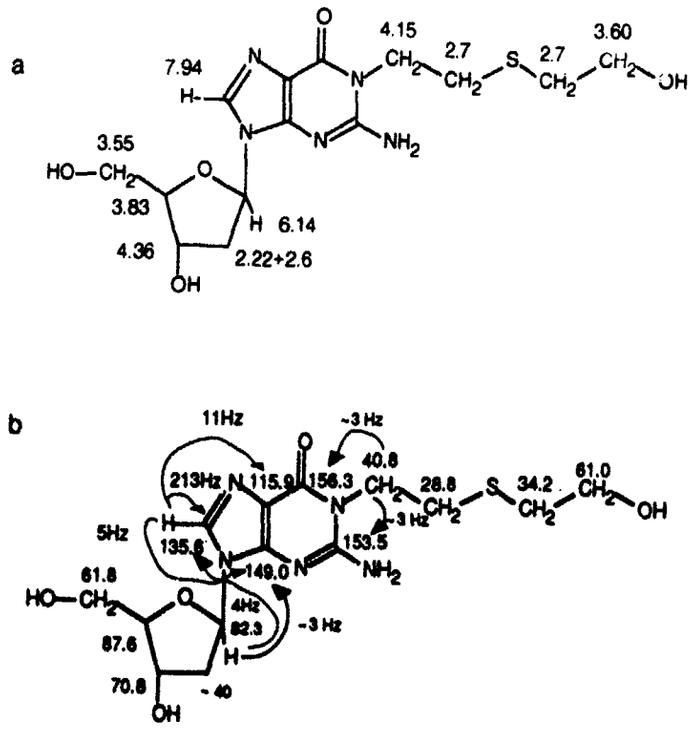

Figure 26. Chemical shift assignments and coupling constants for the hydrogen (400 MHz; a) and carbon atoms (100.6 MHz; b) of N1-(2"-hydroxyethylthioethyl)-2'-deoxyguanosine in DMSO-d_6

III.6. Synthesis of N7 mono- and di-adducts of guanosine derivatives

Experience with the attempted synthesis of N7 mustard gas-adducts of 2'-deoxy-guanosine-5'-phosphate had learned that alkylation at neutral pH leads to almost exclusive alkylation of the weakly acidic phosphate moiety, whereas attempts to perform the synthesis under conditions of complete protonation of the secondary hydroxyl moiety at phosphate lead to depurination of starting material and of reaction products. Since guanosine-5'-phosphate depurinates more slowly than the corresponding deoxyribose derivative under acidic conditions and since haptens derived from guanosine adducts for generation of antibodies against deoxy-guanosine adducts have a similar or even better affinity than antibodies raised against the actual adducts of 2'-deoxyguanosine (101), we decided to attempt the synthesis of mustard gas-adducts of guanosine-5'-phosphate at pH 4.5, i.e., at full protonation of the weakly acidic phosphate moiety (pK_a 6.4).

III.6.1. Synthesis of N7-(2"-hydroxyethylthioethyl)-guanosine-5'-phosphate and of di-[2{(guanosine-5'-phosphate)-7-yl}-ethyl] sulfide

A 10 mM aqueous solution of guanosine-5'-phosphate was alkylated with 20 mM mustard gas for 16 h at room temperature, while the pH was kept at 4.5 by means of automatic titration with a pH-stat apparatus. Reversed phase HPLC of the crude reaction mixture (Figure 27) showed, in addition to starting material and thiodiglycol, three peaks presumably corresponding with mono- and di-adducts of mustard gas. A pre-separation of reaction products from unreacted starting material was obtained by means of anion exchange chromatography on a Sepharose Q column. By using water as an eluens, the reaction products and thiodiglycol are eluted whereas starting material remains on the column. A separation between peaks 3 and 5 (confer Figure 27), corresponding with N7 monoadduct and di-adduct, respectively (vide infra), was obtained by re-chromatography on the same type of column using a 0-1 M gradient of aqueous sodium chloride. Finally, gel

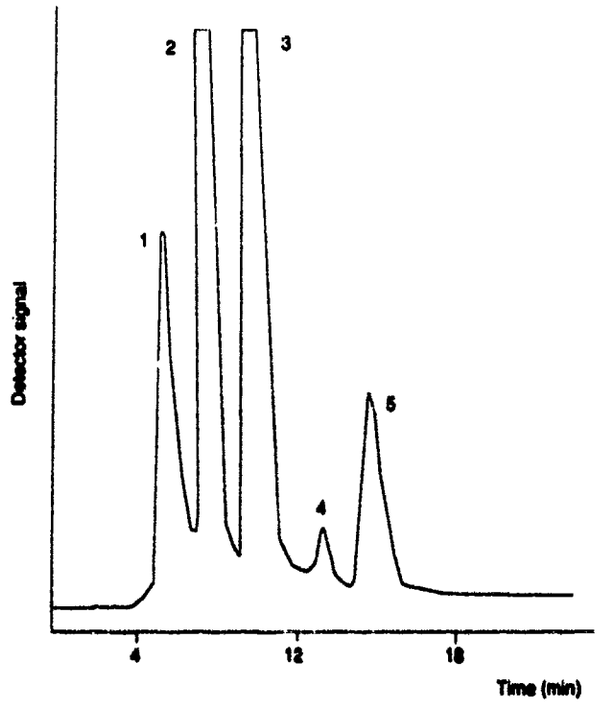

Figure 27. HPLC chromatogram of the crude reaction mixture obtained after reaction of guanosine-5'-phosphate with a 100% molar excess of mustard gas in aqueous solution at room temperature, pH 4.5. Reversed phase chromatography on an RP 18 column (300x2 mm). Eluent: 4 mM $(n\text{-}Bu)_4NHSO_4$ and 0.3 M KH_2PO_4 in water/methanol (3/1, v/v). UV detection at 260 nm. Peak 1, thiodiglycol; peak 2, guanosine-5'-phosphate; peak 3, N7-(2"-hydroxyethylthioethyl)-guanosine-5'-phosphate; peak 4, unknown; peak 5, di-[2{(guanosine-5'-phosphate)-7-yl}-ethyl] sulfide

chromatography on a Sephadex G-10 column removed thiodiglycol and inorganic salts from the two adducts. The monoadduct was obtained in ca. 23% yield with a purity of ca. 94% (estimated from ^{1}H-NMR), whereas the di-adduct was isolated in ca. 4.5% yield, purity ca. 90% (estimated from ^{1}H-NMR). The UV spectra of the monoadduct in aqueous solution have maxima at 258, 258, and 266 nm at pH 1, 7, and 13, respectively, whereas the corresponding maxima of the di-adduct were found at 266, 260, and 266 nm, respectively.

The FAB-MS spectrum of the monoadduct (Figure 28) shows peaks at m/z 490 (MNa^+) and at 468 (MH^+), whereas additional peaks are probably related to the glycerol/thioglycerol matrix. Several attempts to obtain a FAB-MS spectrum of the di-adduct were unsuccessful.

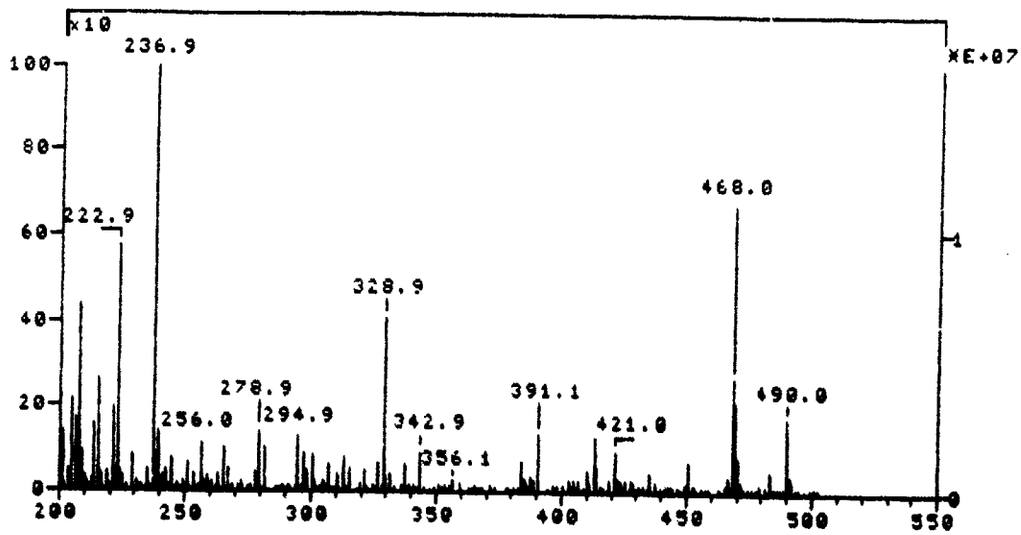

Figure 28. Fast atom bombardment (FAB) mass spectrum of N7-(2"-hydroxyethylthioethyl)-guanosine-5'-phosphate. The product was ionized from a glycerol/thioglycerol matrix with Xenon atoms (7-8 kV acceleration voltage)

Analysis of the ^{1}H- and ^{13}C-NMR spectra of the monoadduct (Figure 29) shows unequivocally that N7-(2"-hydroxyethylthioethyl)-guanosine-5'-phosphate has been obtained. The assignment of the carbon atom signals in DMSO-d_6 is based on single frequency decoupling experiments. Long range H-C couplings found between N-CH_2 and both C8 and C5 indicate that the 2'-hydroxyethylthioethyl moiety is attached to N7. Moreover, the ^{1}H-NMR spectrum in D_2O shows exchange of the hydrogen atom at C8 with deuterium. This exchange of the acidic hydrogen atom at C8 is characteristic for N7 adducts of guanosine and guanosine-5'-phosphate adducts (102).

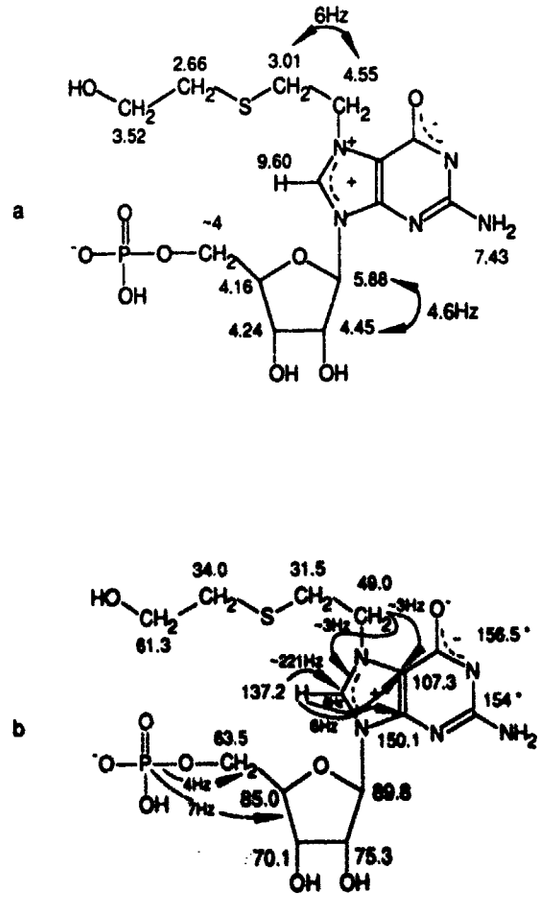

Figure 29. Chemical shift assignments and coupling constants for the hydrogen (400 MHz; a) and carbon atoms (100.6 MHz; b) of N7-(2"-hydroxyethylthioethyl)-guanosine-5'-phosphate in DMSO-d_6. Assignments marked with an asterisk may be interchanged

The ^{1}H-NMR spectrum of the di-adduct gives little characteristic information. An analysis of the ^{13}C-NMR spectrum of the di-adduct in H_2O/D_2O is given in Figure 30. The assignment of the carbon atom signals is tentative. The assigned structure, i.e., di-[2((guanosine-5'-phosphate)-7-yl)-ethyl] sulfide, was concluded from the close similarity of the chemical shifts to those of the N7 monoadduct. In order to facilitate comparison, the ^{13}C-NMR spectrum of the monoadduct in H_2O/D_2O is also given in Figure 30. The ^{1}H-NMR spectrum of the di-adduct in D_2O shows also the exchange of the acidic hydrogen atoms at C8, similar to that in the monoadduct.

It should be noticed that the di-adduct decomposed within a few months upon storage of the neat solid material at -80 °C, whereas the monoadduct was stable under such conditions.

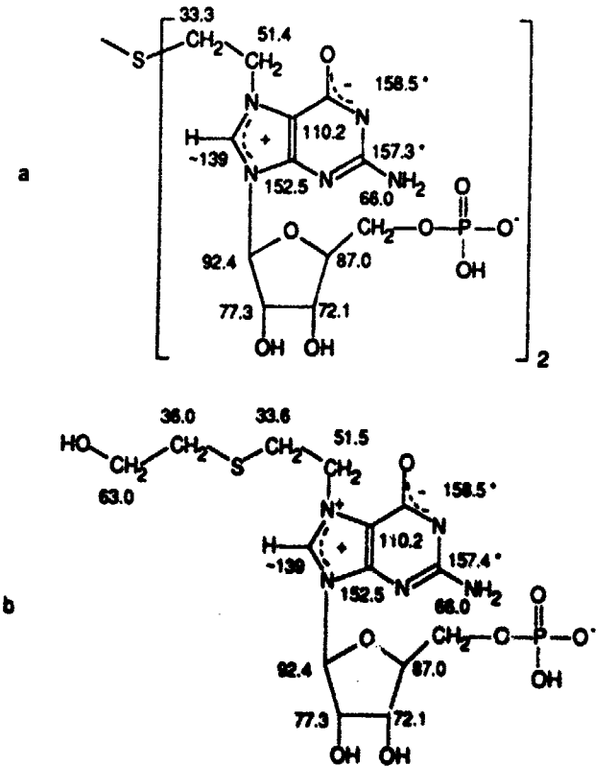

Figure 30. Tentative chemical shift assignments (a) for the carbon atoms (100.6 MHz) of di-[2{(guanosine-5'-phosphate)-7-yl}-ethyl] sulfide in H_2O/D_2O. For comparison the corresponding data for N7-(2"-hydroxyethylthioethyl)-guanosine-5'-phosphate in H_2O/D_2O are also given (b). Assignments marked by an asterisk may be interchanged

III.6.2. Attempted synthesis of N7 mustard gas-adducts of ethyl levulinate-(O-2',3'-guanosine-acetal)

Rupprecht et al. (103) have synthesized 2',3'-O-[1-(2-carboxyethyl) ethylidene]-N7-methyl-guanosine 5'-diphosphate (see Figure 31) for coupling of its carboxylic function to AH-Sepharose 4B, in order to obtain an affinity column for the purification of eukaryotic messenger ribonucleic acid cap binding protein. Since coupling of carboxylic acid functions to proteins is supposed to be an efficient method of coupling (104), we have attempted (see reaction scheme in Figure 32) to obtain the analogous hapten 2',3'-O-[1-(2-carboxyethyl) ethylidene]-N7-(2"-hydroxyethylthioethyl)-guanosine via alkylation of 2',3'-O-[1-(3-ethoxy-3-oxo-propyl)ethylidene] guanosine with mustard gas. Due to insolubility of the latter derivative in water, the alkylation was attempted in DMSO-d_6 and in glacial acetic acid. Even if traces of water were added to these solvents and even if the solutions were heated to 80 °C for a week, no reaction could be observed according to ^{1}H-NMR spectroscopy. It was concluded that the

reactivity of mustard gas in nonaqueous solvents was insufficient to alkylate the N7 position. This reaction route was abandoned.

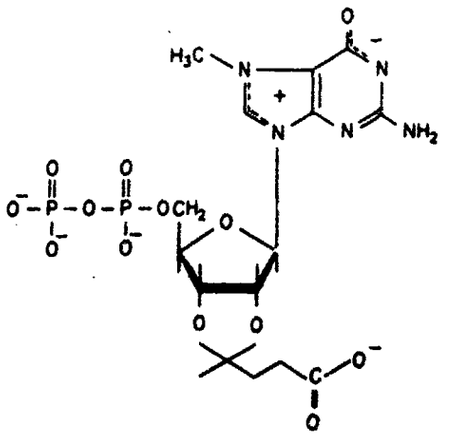

Figure 31. Chemical structure of 2',3'-O-[1-(2-carboxyethyl)-ethylidene]-N7-methyl-guanosine 5'-diphosphate

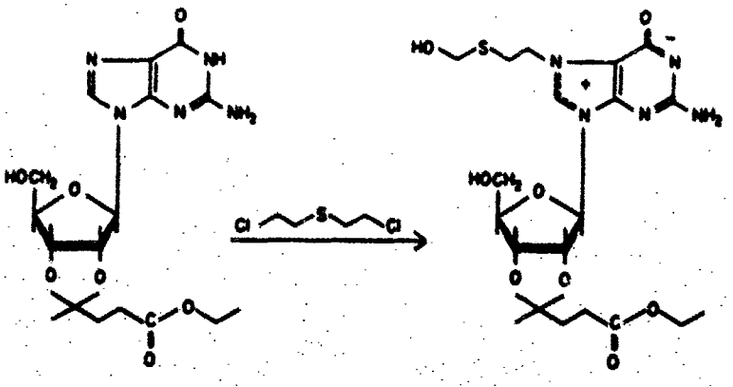

Figure 32. Attempted reaction scheme for the synthesis of 2',3'-O-[1-(2-carboxyethyl)ethylidene]-N7-(2"-hydroxyethylthioethyl)-guanosine

III.7. Kinetics of imidazolium ring opening of N7-(2"-hydroxyethylthioethyl)-guanosine 5'-phosphate

Adducts at N7 of guanosine and their 5'-phosphate derivatives are reasonably stable in neutral aqueous solution. However, as shown in the reaction scheme in Figure 33, such compounds depurinate under acidic conditions whereas alkaline reaction conditions cause ring opening of the imidazolium ring. Especially the latter type of reaction may complicate the immunochemical detection of N7 adducts of guanine in DNA, for example when alkaline conditions are used to obtain single-stranded DNA (confer III.14.2).

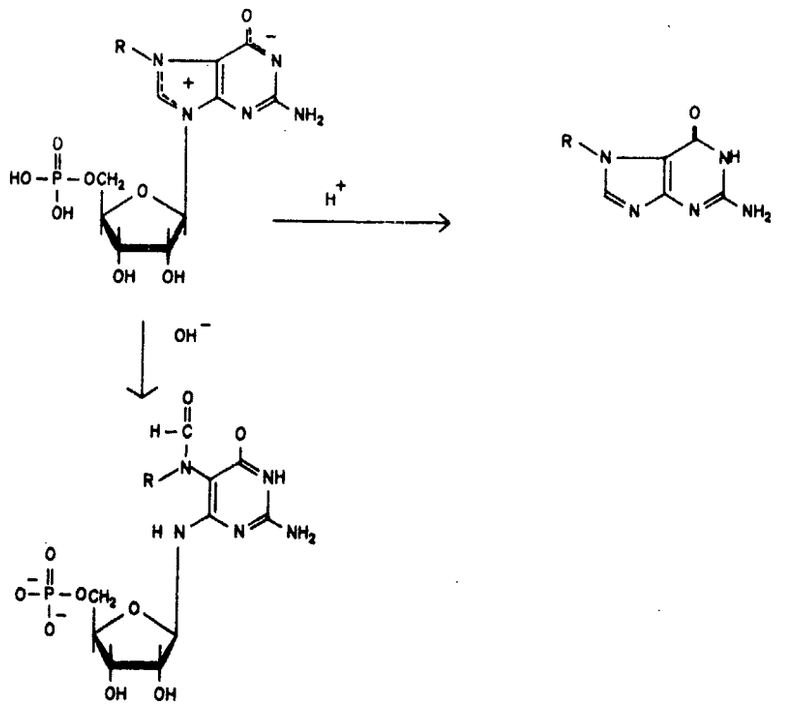

Figure 33. Decomposition reactions of N7-alkyl-guanosine-5'-phosphates (R = alkyl) in acidic aqueous medium (depurination) and in alkaline aqueous medium (imidazolium ring opening)

For this reason, we have measured the rate of ring opening of 7-(2"-hydroxyethylthioethyl)-guanosine-5'-phosphate under alkaline conditions. For comparison we have also measured the reaction rate of the corresponding N7-methyl derivative. All reactions were measured spectrophotometrically in ca. 86 μM aqueous solution at pH 11.2, 25 °C, which appeared to be convenient conditions to measure the reaction rates. As shown in Figure 34, the UV spectra in the course of the reaction show sharp isosbestic points over a time course of several half lives, which indicates that a well-defined reaction of a sufficiently pure substrate is being measured. Very similar spectra are obtained with the corresponding N7-methyl derivative under the same reaction conditions.

(Pseudo) first order rate constants were calculated from a plot of the log of the difference between the absorbance at a given time and the absorbance of the imidazolium ring opened compound. We measured this difference at 266 nm, i.e., at the wavelength of maximal difference between the absorbances of the ring opened product and the starting compound (Figure 34). A representative example of such a plot, pertaining to run 1 in Table 5, is given in Figure 35.

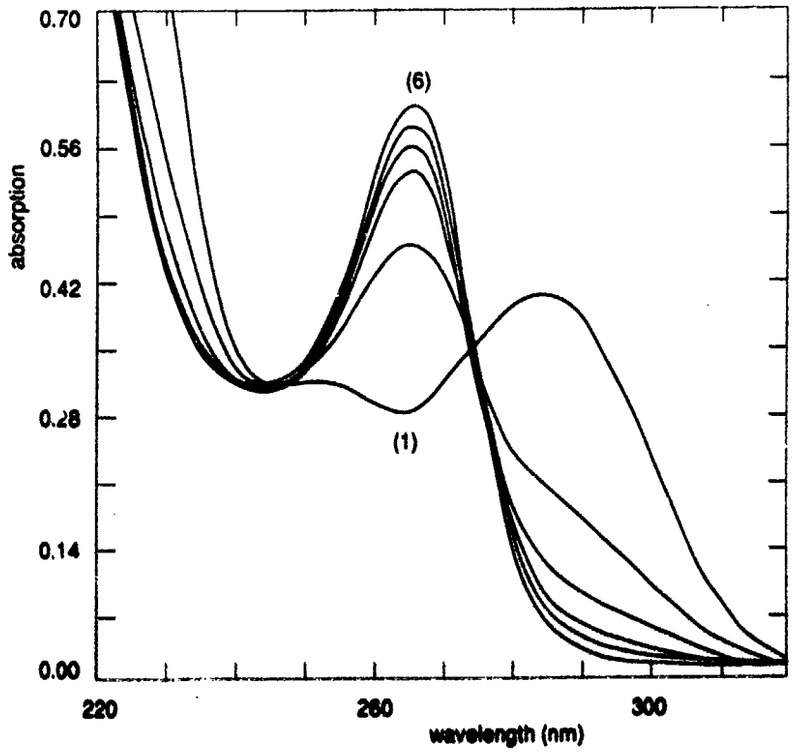

Figure 34. UV spectra of N7-(2"-hydroxyethylthioethyl)-guanosine-5'-phosphate (86 μM) in the course of imidazolium ring opening in an aqueous sodium carbonate buffer, pH 11.2, 25 °C. The spectra were taken after 0 (1), 30, 60, 90, 120, and 360 (6) min

The measured absorbances (A_t) at 266 nm and the calculated differences ($A_\infty - A_t$) between this absorbance and the absorbance A_∞ for complete ring opening of the two N7 adducts as a function of reaction time are given in Tables 5 and 6, for two duplicate runs. Rate constants calculated from data points measured within the first half life time of the reaction were not significantly different from those calculated over several half life times. The calculated rate constants and half lives are summarized in Table 7.

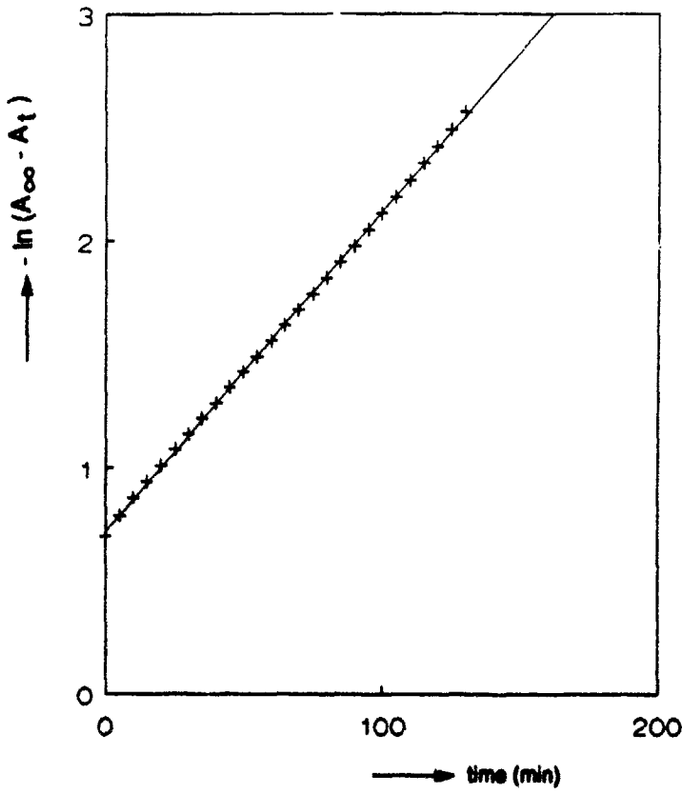

Figure 35. Plot of reaction time versus natural logarithm of the difference between the absorbance (266 nm) at a given time (A_t) and the absorbance of the completely imidazolium ring opened N7-(2"-hydroxyethylthioethyl)-guanosine-5'-phosphate (A_∞) in aqueous solution, pH 11.2, 25 °C. Data taken from Table 5, run 1

The reproducibility of the measurements, as evidenced by the data in Table 7 suggest that it may be concluded that ring opening of the N7-(2'-hydroxyethylthioethyl)-derivative is slightly but significantly faster than that of the corresponding N7-methyl derivative. Such an order of reactivity should be expected (61-64) since the electron withdrawing character of the (2'-hydroxy-ethylthioethyl) moiety is presumably slightly larger than that of the methyl group.

Table 5. Absorbances at 266 nm (A_t) measured in the course of ring opening of N7-(2"-hydroxyethylthioethyl)-guanosine-5'-phosphate (86 μM) in a buffered aqueous solution, pH 11.2, 25 °C, and calculated differences (A_∞ - A_t) between this absorbance and the absorbance after complete reaction (A_∞)

Time (min)	A_t (266 nm) Run 1	A_t (266 nm) Run 2	-ln (A_∞-A_t) Run 1	-ln (A_∞-A_t) Run 2
0	0.4731	0.5685	0.6990	0.5416
5	0.5154	0.6158	0.7879	0.6264
10	0.5501	0.6552	0.8673	0.7030
15	0.5795	0.6897	0.9398	0.7752
20	0.6062	0.7205	1.0106	0.8444
25	0.6314	0.7491	1.0823	0.9133
30	0.6538	0.7751	1.1507	0.9803
35	0.6745	0.7992	1.2184	1.0467
40	0.6940	0.8224	1.2866	1.1150
45	0.7122	0.8440	1.3548	1.1832
50	0.7294	0.8632	1.4238	1.2479
55	0.7448	0.8822	1.4899	1.3164
60	0.7602	0.8995	1.5606	1.3831
65	0.7742	0.9164	1.6296	1.4529
70	0.7870	0.9315	1.6972	1.5196
75	0.7994	0.9470	1.7673	1.5931
80	0.8101	0.9612	1.8363	1.6655
85	0.8220	0.9740	1.9092	1.7356
90	0.8318	0.9864	1.9776	1.8085
95	0.8414	0.9968	2.0495	1.8741
100	0.8504	1.0082	2.1219	1.9512
105	0.8588	1.0185	2.1946	2.0265
110	0.8667	1.0280	2.2682	2.1013
115	0.8742	1.0371	2.3434	2.1786
120	0.8811	1.0457	2.4180	2.2576
125	0.8875	1.0538	2.4925	2.3382
130	0.8938	1.0614	2.5718	2.4202
end	0.9702	1.1503		

Table 6. Absorbances at 266 nm (A_t) measured in the course of ring opening of N7-methyl-guanosine-5'-phosphate (86 μM) in a buffered aqueous solution, pH 11.2, 25 °C, and calculated differences (A_∞ - A_t) between this absorbance and the absorbance after complete reaction (A_∞)

Time (min)	A_t (266 nm) Run 3	A_t (266 nm) Run 4	-ln (A_∞-A_t) Run 3	-ln (A_∞-A_t) Run 4
0	0.8495	0.8064	0.3279	0.9054
5	0.8894	0.8251	0.3849	0.9527
10	0.9280	0.8440	0.4433	1.0029
15	0.9650	0.8621	0.5027	1.0535
20	1.0001	0.8794	0.5625	1.1044
25	1.0327	0.8960	0.6214	1.1558
30	1.0634	0.9120	0.6802	1.2080
35	1.0931	0.9280	0.7407	1.2630
40	1.1208	0.9441	0.8005	1.3216
45	1.1471	0.9588	0.8609	1.3783
50	1.1720	0.9732	0.9216	1.4372
55	1.1952	0.9874	0.9816	1.4988
60	1.2174	1.0004	1.0427	1.5587
65	1.2390	1.0134	1.1059	1.6225
70	1.2594	1.0260	1.1696	1.6885
75	1.2782	1.0377	1.2320	1.7539
80	1.2965	1.0495	1.2968	1.8245
85	1.3138	1.0607	1.3622	1.8965
90	1.3298	1.0711	1.4267	1.9683
95	1.3464	1.0818	1.4983	2.0479
100	1.3611	1.0920	1.5664	2.1303
105	1.3751	1.1024	1.6358	2.2219
110	1.3884	1.1115	1.7065	2.3096
115	1.4008	1.1208	1.7773	2.4079
120	1.4124	1.1300	1.8483	2.5158
125	1.4244	1.1394	1.9276	2.6395
130	1.4354	1.1481	2.0062	2.7694
end	1.5699	1.2108		

Table 7. Calculated (pseudo) first-order rate constants (k; min^{-1}) and half lives (min) for imidazolium ring opening of two N7-alkyl-guanosine-5'-phosphates, in aqueous buffer at pH 11.2, 25 °C

Run	k (min^{-1})	Half life (min)
1[a]	0.01410	49.16
2[a]	0.01413	49.05
Averaged[a]	0.01411	49.10
3[b]	0.01277	54.30
4[b]	0.01279	54.19
Averaged[b]	0.01278	54.25

[a] Alkyl = 2'-hydroxyethylthioethyl
[b] Alkyl = methyl

III.8. Synthesis of model peptides*

In order to find out which type of products can be expected upon reaction of mustard gas with proteins, we have synthesized several simple model peptides of single amino acids having functional groups in the side chain which may be prone to alkylation. Based upon early investigations on the reactions of mustard gas and other alkylating agents with proteins and amino acids (40-43,49), valine, aspartic acid, glutamic acid, cysteine, methionine and histidine were selected. The structures of the peptide derivatives are shown in Figure 36.

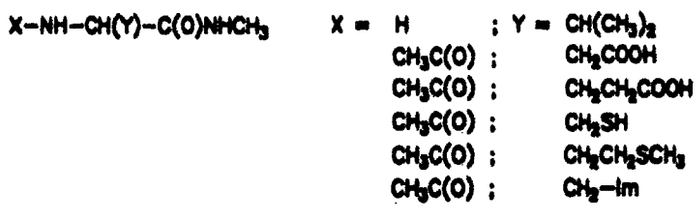

Figure 36. Chemical structures of model peptides synthesized for investigation of the alkylation of various side chains of amino acids

In the case of valine, the α-amino group was left unprotected in order to find out which products can be expected upon reaction with mustard gas of the terminal amino group of a protein, e.g., the amino group of valine in the α-chain of hemoglobin (65). In all other amino acids both the α-amino and the α-carboxylic acid group were protected as amides by means of acetylation and derivatization with methylamine, respectively. In this way only the nucleophilic groups in the side chains are available to react with mustard gas (105).

* In this report it is assumed that all amino acids have the natural L-configuration, unless mentioned otherwise.

The synthesis of the model peptides was straightforward. Valine-methylamide was obtained via trans-amidation of N-benzyloxycarbonyl-valine-methyl ester with methylamine, followed by hydrogenolysis of the benzyloxycarbonyl group, according to the reaction scheme in Figure 37. Both the histidine and methionine peptides were synthesized from the corresponding N-α-acetyl derivatives via esterification by means of the mixed anhydride method with acetyl chloride, followed by trans-amidation of the ester with methylamine as shown in Figure 38.

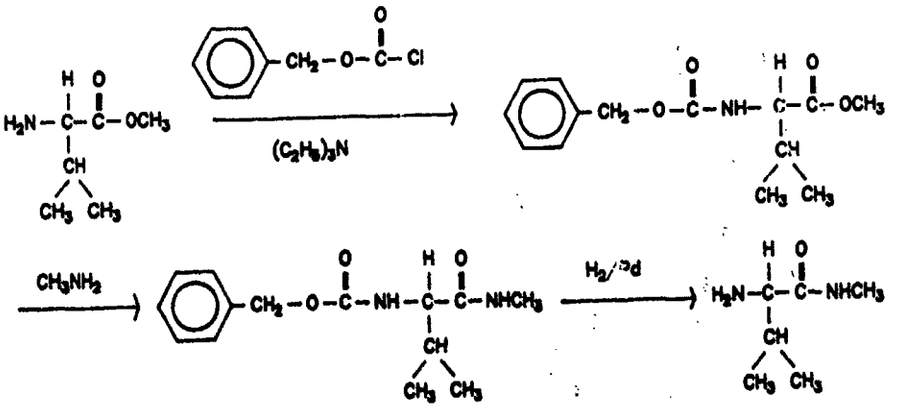

Figure 37. Reaction equation for the synthesis of valine-methylamide

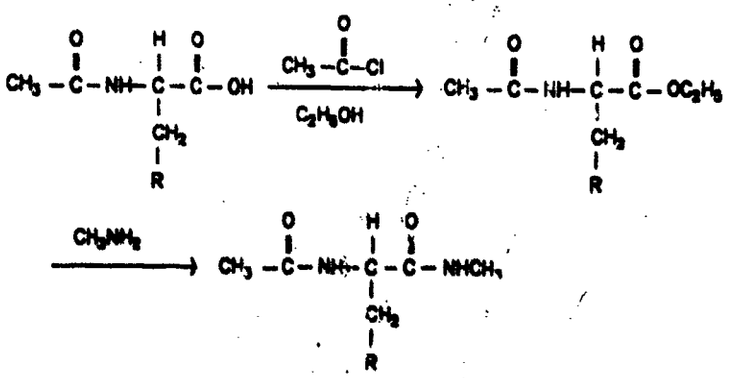

Figure 38. Reaction scheme for the synthesis of N-α-acetyl α-methylamide derivatives of methionine ($R=CH_2SCH_3$) and histidine (R=1..)

The peptide derivatives of aspartic acid and glutamic acid were obtained by means of the reaction sequence given in Figure 39. The amino acid in which the side chain carboxylic acid function is protected by means of a benzyl ester is N-acetylated with acetic anhydride. Next, the α-carboxylic acid function is esterified with ethanol by means of the mixed anhydride method with ethyl chloroformate, and is trans-amidated with methylamine. Finally hydrogenolysis, catalyzed by palladium on charcoal, removes the protective benzyl group.

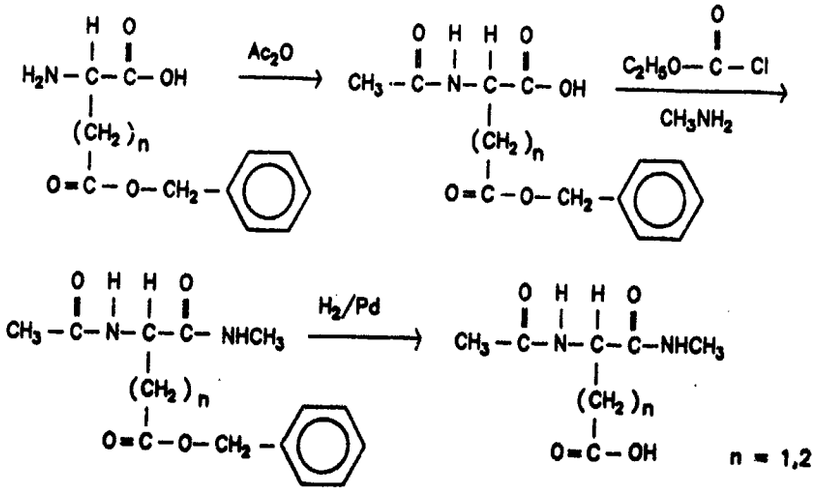

Figure 39. Reaction scheme for the synthesis of N-α-acetyl α-methylamide derivatives of aspartic (n=1) and glutamic (n=2) acid

The N-acetyl-cysteine-methylamide was obtained according to the reaction scheme in Figure 40 starting from S-benzyl-cysteine-methyl

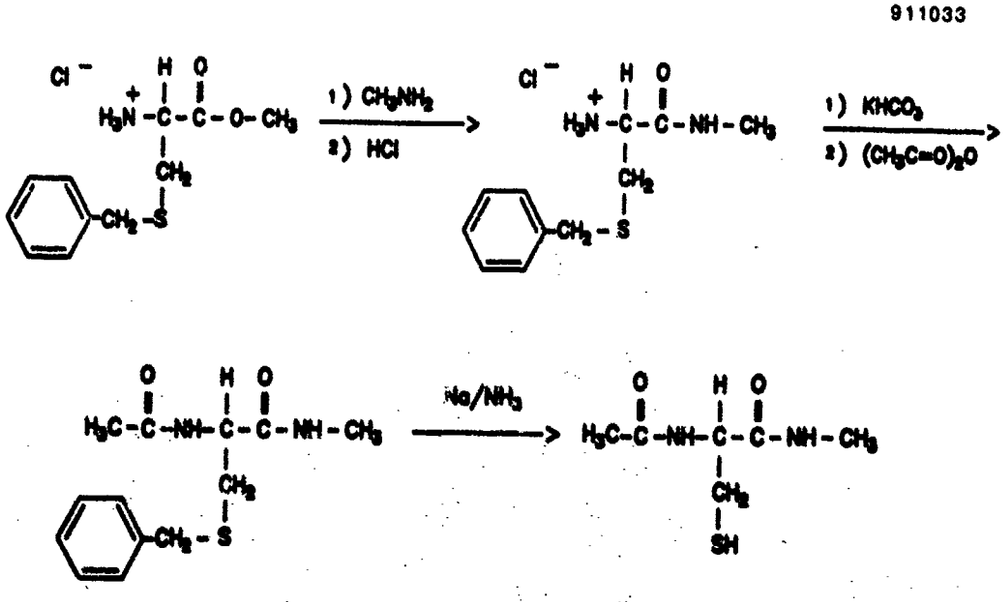

Figure 40. Reaction scheme for the synthesis of N-acetyl-cysteine-methylamide

ester. After trans-amidation with methylamide followed by acetylation, the protective group was removed by hydrogenolysis with sodium in liquid ammonia.

III.9. Synthesis of the major adducts of mustard gas with model peptides

The products of the alkylation reactions of model peptides with mustard gas in aqueous solution at pH 7.5 were identified tentatively with thermospray MS-detected HPLC of the reaction mixtures, as will be described in III.10. In order to identify the major products in a definitive way, these were synthesized by independent routes, purified, and fully identified with spectroscopic techniques. Co-chromatography with the reaction mixture obtained from alkylation with mustard gas in aqueous solution served to establish that the reaction products were identical with the independently synthesized products.

It will also be essential to have available the pure reaction products in order to perform competition experiments on a quantitative basis, in which excess of a mixture of the various model peptides will be reacted in aqueous solution with mustard gas.

III.9.1. N-(2'-Hydroxyethylthioethyl)-valine-methylamide

In a first attempt to prepare N-(2'-hydroxyethylthioethyl)-valine-methylamide (confer III.10.1), we have used a variant of the procedure of Grant and Kinsey (72), who obtained N-(2'-hydroxyethylthioethyl)-valine from the reaction of valine with semi-mustard gas. According to the reaction scheme in Figure 41, we alkylated valine-methylamide with a 50% molar excess of 2-acetoxyethyl 2'-chloroethyl sulfide (confer III.2.2) in aqueous solution at pH 9-10. Analysis of the reaction products with ^{1}H-NMR indicated that, in addition to the desired product, substantial amounts of the bis N-(2'-hydroxyethyl-thioethyl)-substituted valine-methylamide derivative were formed. The protective acetyl group was hydrolyzed during alkylation at alkaline pH. Attempts to isolate the mono-substituted product by means of

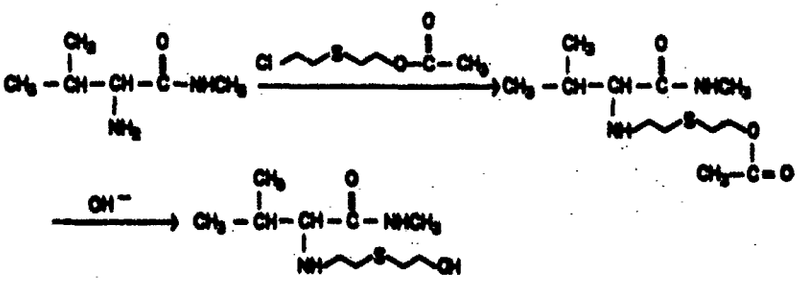

Figure 41. Reaction scheme for the synthesis of N-(2'-hydroxyethyl-thioethyl)-valine-methylamide via alkylation of valine-

cation exchange chromatography with Sepharose S, or by means of preparative TLC were unsuccesful. Finally, reversed phase HPLC gave ca. 1 mg of product, which was pure according to thermospray MS analysis (MH^+, m/z = 235).

A better route of synthesis of the desired N-(2'-hydroxyethylthio-ethyl)-valine-methylamide is outlined in Figure 42. Reaction of

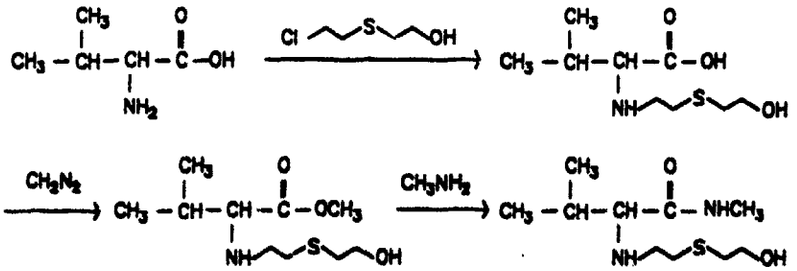

Figure 42. Reaction scheme for the synthesis of N-(2'-hydroxyethyl-thioethyl)-valine-methylamide via alkylation of valine with semi-mustard gas, followed by esterification and trans-amidation of the carboxylic acid function

valine with semi-mustard gas according to the procedure of Grant and Kinsey (72) yielded N-(2'-hydroxy-ethylthioethyl)-valine in 31% yield with a purity of ca. 80% (^{1}H-NMR). This crude product was converted into the corresponding methyl ester with diazomethane (yield 60%), whereas ca 12% N-(2'-hydroxyethylthioethyl)-N-methylvaline-methyl ester was formed as a byproduct (^{1}H-NMR). Subsequent trans-amidation with aqueous methylamine converted both esters into the corresponding methylamides in ca. 80% yield. A final purification by means of medium pressure preparative chromatography on a Lobar column packed with Lichrosorb RP 18, with methanol/water (1/1,v/v) as eluent, gave the desired product in 11% yield.

III.9.2. <u>1-Methyl-3-acetamido-succinimide</u>

1-Methyl-3-acetamido-succinimide, the major product resulting from the alkylation of N-acetyl-aspartic acid-1-methylamide with mustard gas in aqueous solution and subsequent cyclization (confer III.10.2), was synthesized in 64% yield by means of cyclization of N-acetyl-aspartic acid-4-methyl ester-1-methyl-amide in methanol/triethyl-amine, as shown in Figure 43. The 4-methyl ester was obtained by esterification of N-acetyl-aspartic acid-1-methylamide in methanol in the presence of acetyl chloride.

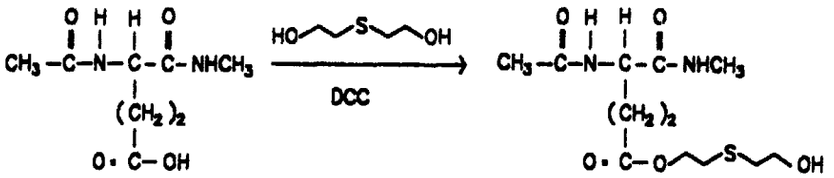

Figure 43. Reaction scheme for the synthesis of 1-methyl-3-acetamido-succinimide via cyclization of N-acetyl-aspartic acid-methyl ester-1-methylamide

III.9.3. N-acetyl-glutamic acid-5-(2'-hydroxyethylthioethyl) ester-1-methylamide

Esterification of N-acetyl-glutamic acid-1-methylamide with a slight molar excess of thiodiglycol in N,N-dimethylformamide at room temperature in the presence of dicyclohexylcarbodiimide and of a catalytic amount of 4-dimethyl-aminopyridine (75), gave an almost quantitative conversion to the desired N-acetyl-glutamic acid-5-(2'-hydroxyethylthioethyl) ester-1-methylamide according to the reaction scheme in Figure 44. Trituration with diethyl ether removed a small amount of the corresponding diester derivative of thiodiglycol. According to ^{1}H-NMR and thermospray MS, the oily product (MH^+, m/z = 185) contains only traces of this diester (MH^+, m/z = 491).

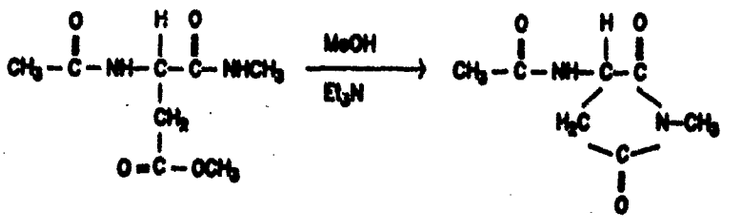

Figure 44. Reaction scheme for the synthesis of N-acetyl-glutamic acid-5-(2'-hydroxyethylthioethyl) ester-1-methylamide via esterification of N-acetyl-glutamic acid-1-methylamide with thiodiglycol in the presence of dicyclohexylcarbodiimide (DCC)

III.9.4. N-α-acetyl-Nl-(2'-hydroxyethylthioethyl)-histidine-methylamide

It appeared that N-α-acetyl-histidine-methylamide reacts rather sluggishly with mustard gas and with derivatives of semi-mustard gas. After several preliminary experiments we found that alkylations in methanol as solvent with semi-mustard gas in which the hydroxyl group is protected (confer III.2), in the presence of anhydrous sodium carbonate for neutralization of acids produced during the reaction (76), gives the least unsatisfactory results.

2-Trimethylsilyloxyethyl 2'-chloroethyl sulfide was refluxed in methanol for 7 days with a 4-fold molar excess of N-α-acetyl-histidine-methylamide and anhydrous sodium carbonate. The large excess of histidine derivative was used in order to suppress the formation of di-substituted adducts. Analysis of the reaction mixture with thermospray MS-detected reversed phase LC showed that two mono-substituted products were formed, as well as a late eluting di-adduct with unelucidated structure (confer III.10.5). Apparently, the adducts had lost their protective trimethylsilyl groups during the alkylation reaction. Preparative medium pressure chromatography of the reaction mixture on a reversed phase Lobar column gave two fractions, each containing one of the monoadducts. Subsequent medium pressure chromatography of the first eluting monoadduct on a straight phase Lobar column gave a mono-substituted adduct in ca. 0.5% overall yield, with a purity of 95% (HPLC, UV detection at 220 nm). Thermospray MS analysis of the product showed MH^+ at m/z = 315. The structure of the adduct was derived from an analysis of the ^1H- and ^{13}C-NMR spectra. The assignment of the carbon signals was based on a two-dimensional heteronuclear chemical shift correlated (HETCOR) spectrum, as shown in Figure 45. The point of attachment of the (2'-hydroxyethylthioethyl) moiety was concluded from the results of single frequency decoupling experiments in conjunction with a comparison of the shifts of the ring carbon atoms with those of the isomeric N3(τ) compound (see III.9.5).

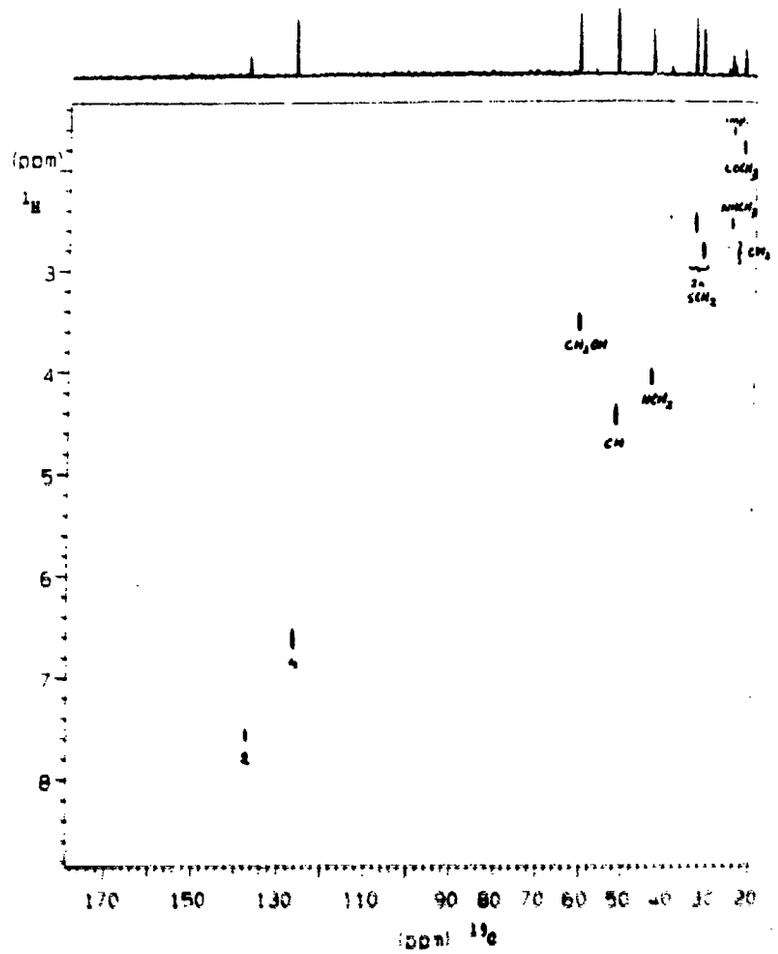

Figure 45. Heteronuclear chemical shift correlated (HETCOR) spectrum of N-α-acetyl-N-1-(2'-hydroxyethylthioethyl)-histidine-methylamide in DMSO-d_6 (1 bond C-H couplings)

III.9.5. <u>N-α-acetyl-N3-(2'-hydroxyethylthioethyl)-histidine-methylamide</u>

The fraction containing the second adduct, as obtained from reversed phase Lobar chromatography of the reaction mixture described in III.9.4, was further purified by semi-preparative reversed phase HPLC. This gave the second monoadduct with a purity of ca. 95% (HPLC, UV detection at 220 nm) in 0.5% yield (thermospray MS: MH^+ at m/z = 315). The structure of the adduct followed from an analysis of the ^{1}H- and ^{13}C-NMR spectra. As in the case of the N1 adduct, the carbon signals are derived from a two-dimensional heteronuclear chemical shift correlated spectrum (HETCOR), as shown in Figure 46. The long range couplings of 3-4 Hz between hydrogen in NCH_2 and C2/C4, as found by a single frequency decoupling experiment, indicate that the (2'-hydroxyethylthioethyl) moiety is attached to N3.

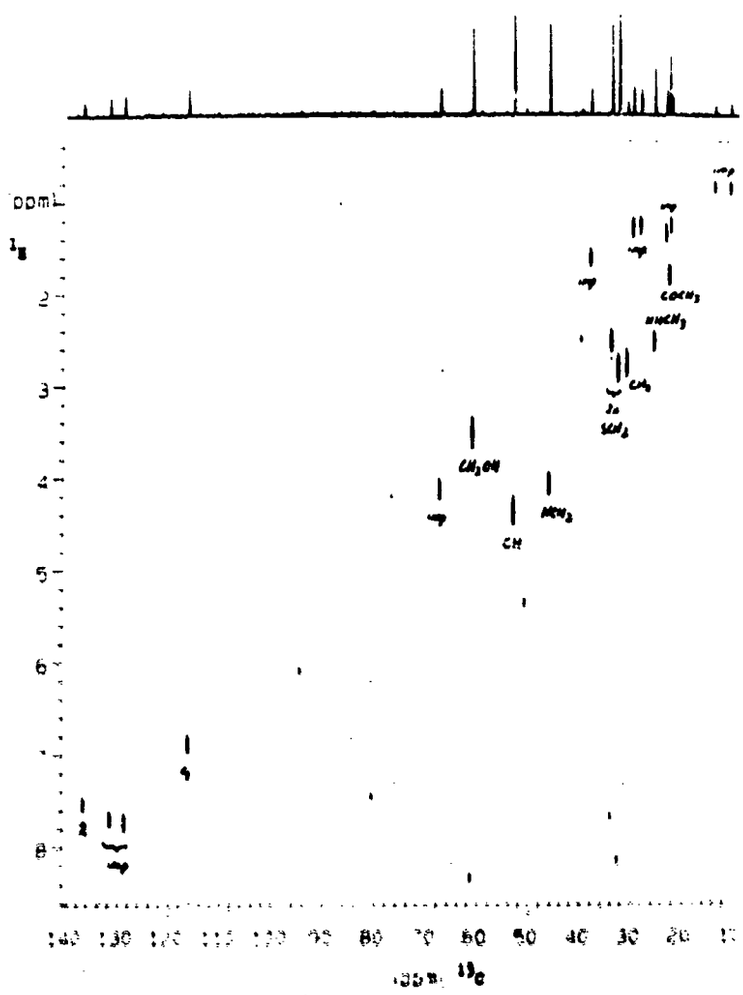

Figure 46. Heteronuclear chemical shift correlated (HETCOR) spectrum of N-α-acetyl-N3-(2'-hydroxyethylthioethyl)-histidine-methylamide in DMSO-d_6 (1 bond C-H couplings)

The N1 and N3 monoadducts were also obtained from alkylation of N-α-acetyl histidine methylamide under the same reaction conditions as described in III.9.4, but using equimolar 2-acetoxyethyl 2'-chloroethyl sulfide (confer III.2.3) as alkylating agent instead of the trimethylsilyl protected derivative of semi-mustard gas. The acetyl derivative of semi-mustard gas is more reactive than the trimethylsilyl derivative since a reaction period of 7 h was sufficient to obtain a maximal conversion. Also in this case, the protective acetyl group had hydrolyzed from the alkylation products at the end of the reaction period. Separation of the two mono-substituted adducts was achieved by means of preparative medium pressure chromatography on a straight phase Lobar column, with the N3 adduct now eluting ahead of the N1 adduct. The N3 adduct was recrystallized from diethyl ether containing 1% (v/v) of methanol without further chromatographic clean-up, which gave a product with 95% purity (HPLC, UV detection at 220 nm) in 0.8% yield. All analyses of

the product were the same as those of the N3 adduct resulting from alkylation with the trimethylsilyl derivative of semi-mustard gas.

For practical reasons, no attempts were made to obtain the N1 adduct from the reaction mixture.

III.10. Reactions of model peptides with mustard gas

Reactions of the model peptides with varying molar ratios of mustard gas were performed in aqueous solution at 25 °C. Depending on the reactivity of the model peptide relative to water, hydrochloric acid is produced from mustard gas due to alkylation of the peptide and hydrolysis to thiodiglycol. In order to maintain an approximately physiological pH, the solution was titrated automatically during the reaction with 0.1 N NaOH to pH 7.5, by means of a pH-stat apparatus. This procedure is preferred over the use of a buffer since this would involve the complication of alkylation of the buffer components in competition with the model peptide (compare III.11.2).

When the reaction had subsided after 4-24 h or after 0.5 h for the cysteine derivative, the aqueous solution (10-50 ml) was concentrated to a small volume. Addition of ethanol dissolved the organic reaction products while sodium chloride precipitated. The filtered solution was used for analysis with reversed phase LC and thermospray-LC-MS detection. The usual eluent was 0.1 M aqueous ammonium acetate/methanol (1/1, v/v). It was observed in several cases that chlorine in reactive 2-chloroethyl-thioethyl moieties was replaced by acetate due to reaction in the thermospray MS detector.

III.10.1. Valine-methylamide (65)

An aqueous solution of valine-methylamide (0.38 M) was reacted with equimolar mustard gas for 24 h. The thermospray-LC-MS chromatogram is given in Figure 47, together with the thermospray mass spectra of the various peaks.

As mentioned above, it is assumed that 2-acetoxyethyl 2'-hydroxyethyl sulfide (peak 1) is formed by replacement of chlorine in semi-mustard gas. In a similar way, peak 5 corresponding with N-(2'-acetoxyethyl-thioethyl)-valine-methylamide (MH^+, m/z = 277), may have been formed from N-(2'-chloroethylthioethyl)-valine-methylamide. The latter product is the obvious intermediate needed for the subsequent formation of the di-adduct bis[2-(isopropyl-N-methylcarbamoylmethyl-amino)ethyl] sulfide corresponding with peak 7 (MH^+; m/z = 347), whereas hydrolysis of this intermediate would lead to the formation of N-(2'-hydroxyethylthioethyl)-valine-methylamide (peak 6; MH^+ at m/z = 235). Obviously, the latter product may also have been formed by alkylation of valine-methylamide with semi-mustard gas. The product corresponding with peak 4 (MH^+, m/z = 217) has been tentatively assigned the structure N-(vinylthioethyl)-valine-methyl-amide. It may have been formed either by elimination of hydrogen chloride from N-(2'-chloroethylthioethyl)-valine-methylamide or by elimination of water from N-(2'-hydroxyethylthioethyl)-valine-

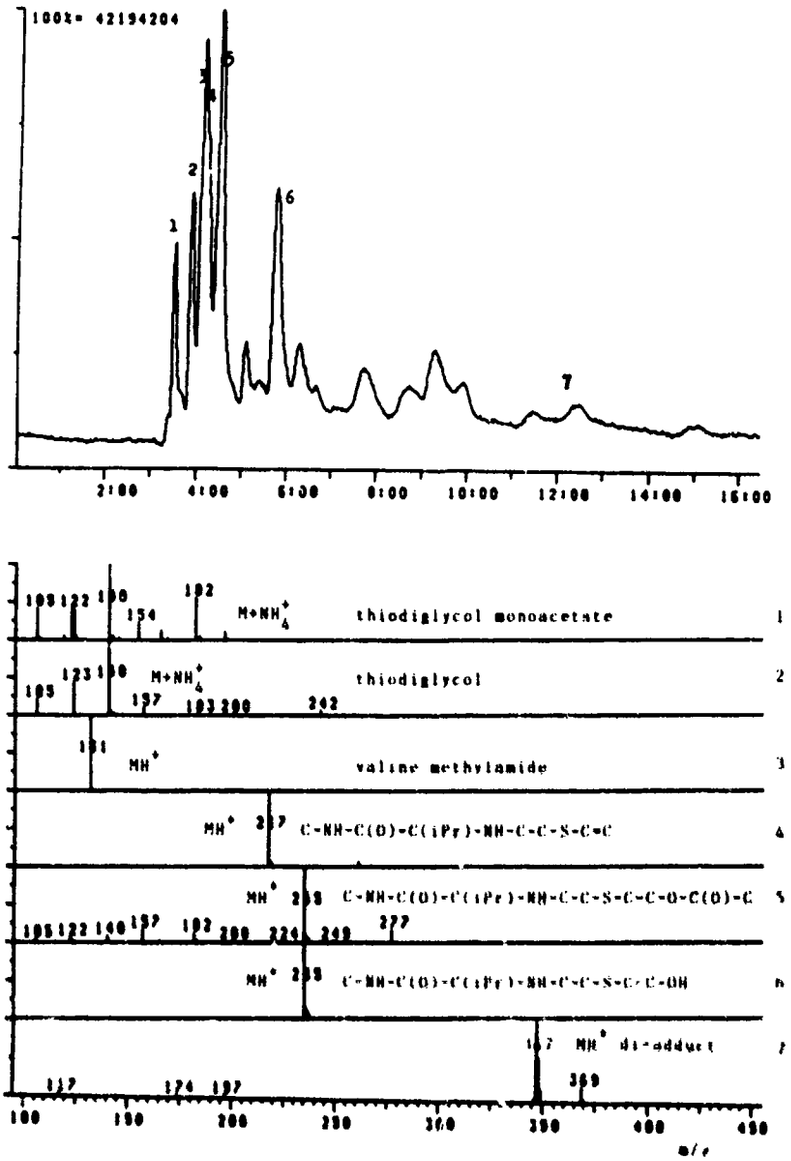

Figure 47. Thermospray-LC-MS chromatogram and mass spectra of the various peaks after reaction of valine-methylamide (0.38 M) with equimolar mustard gas in aqueous solution, pH 7.5, 25 °C

methylamide. Without isolation and subsequent NMR-analysis of the product corresponding with peak 4, the possibility cannot be excluded that it has a cyclic structure instead of a vinyl group. The cyclic product, i.e., 4-(isopropyl-N-methylcarbamoyl)methyl-tetrahydro-1,4-thiazine may have been formed by an internal alkylation reaction of N-(2-chloroethylthioethyl)-valine-methylamide, according to the reaction scheme shown in Figure 48.

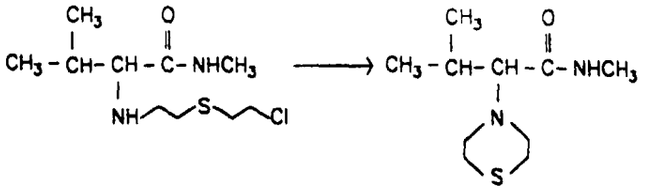

Figure 48. Proposed reaction scheme for the tentative formation of 4-(isopropyl-N-methylcarbamoyl)methyl-tetrahydro-1,4-thiazine during the alkylation of valine-methylamide with mustard gas in aqueous solution, pH 7.5, 25 °C

The formation of a large number of reaction products under the reaction conditions as described above does not permit to conclude which reaction products are of primary importance. Therefore the experiment was repeated with a 10-fold lower concentration of mustard gas, i.e., with a large excess of valine-methylamide. As shown in Figure 49, the thermospray-LC-MS chromatogram now clearly shows that N-(2'-hydroxyethylthioethyl)-valine-methylamide is the major reaction product, with formation of a small amount of di-adduct. We consider the formation of the di-adduct as nonrelevant for the in vivo situation since it is highly improbable that two valine moieties are close enough together in the tertiary structure of a protein to enable the formation of a di-valine adduct.

The structure of the major reaction product was confirmed by independant synthesis (cf III.9.1) and co-chromatography of this synthetic product with the above-mentioned product in reversed phase HPLC.

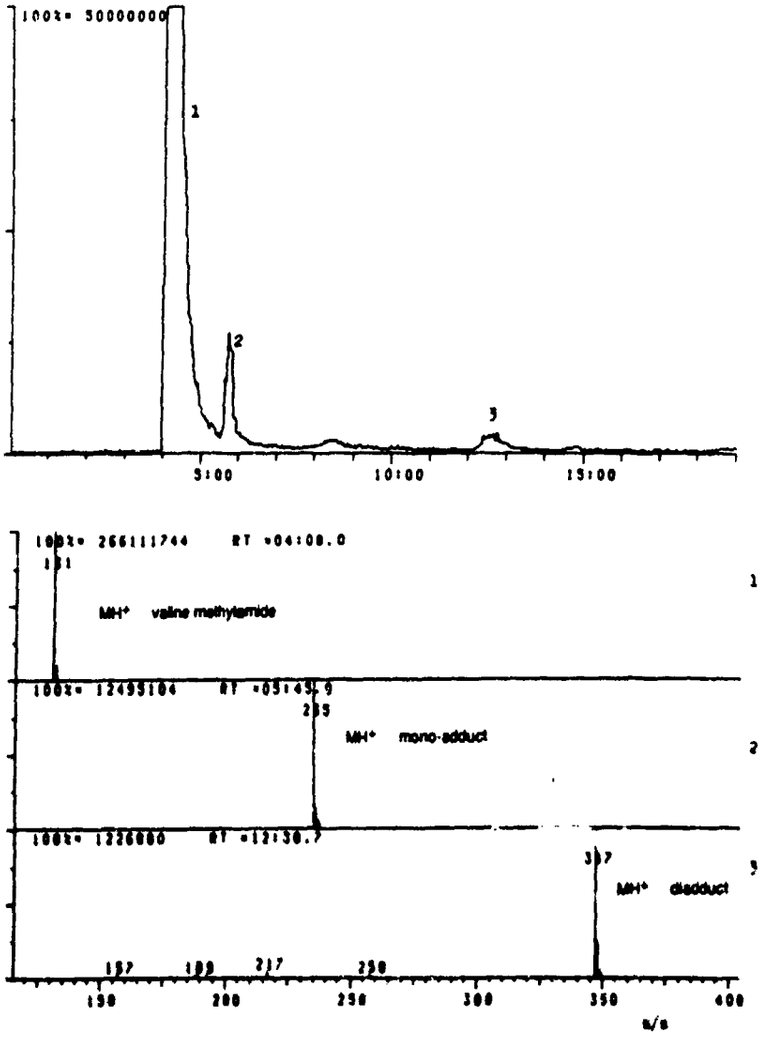

Figure 49. Thermospray-LC-MS chromatogram and mass spectra of the various peaks after reaction of valine-methylamide (0.38 M) in tenfold molar excess with mustard gas in aqueous solution, pH 7.5, 25 °C

III.10.2 N-Acetyl-aspartic acid-1-methylamide

An aqueous solution of N-acetyl-aspartic acid-1-methylamide (28 mM) was reacted with a 15% molar excess of mustard gas. A reaction ensued, which was complete within 4 h. Analysis of the reaction mixture with thermospray-LC-MS as summarized in Figure 50, showed two reaction products (peaks 2 and 4) in addition to starting material (peak 1) and thiodiglycol (peak 3). Peak 2 was identified as 1-methyl-3-acetamido-succinimide (MH^+, m/z = 171), whereas the small peak 4 (MH^+, m/z = 293) was identified as the expected reaction product N-acetyl-aspartic acid-4-(2'-hydroxyethylthioethyl) ester-1-

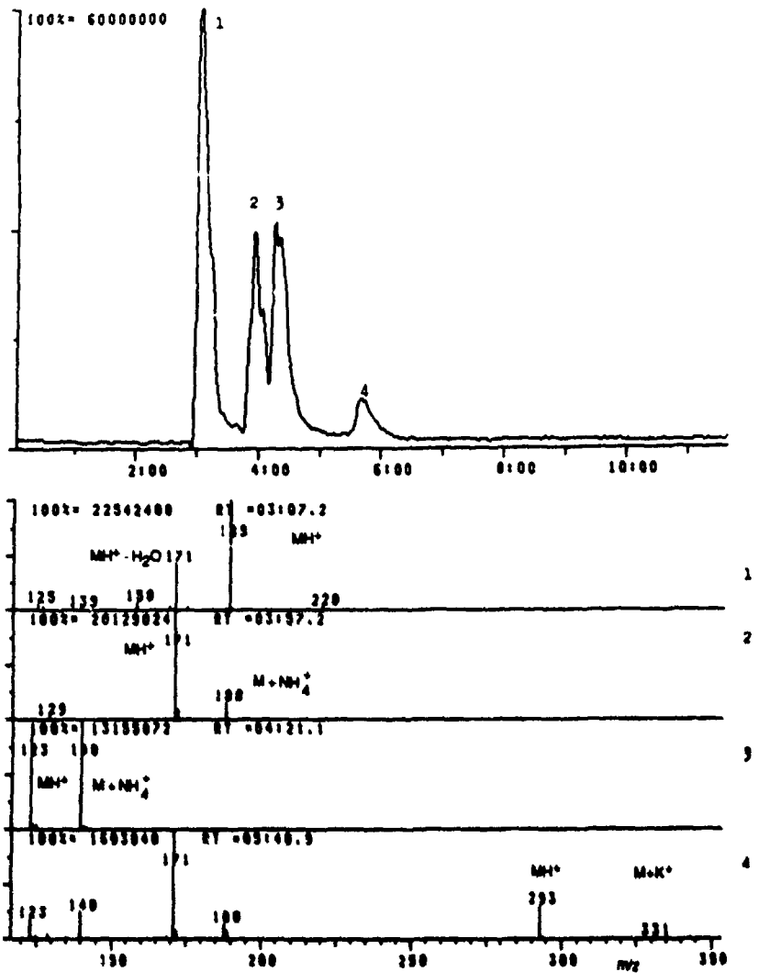

Figure 50. Thermospray-LC-MS chromatogram and mass spectra of the various peaks after reaction of aqueous N-acetyl-aspartic acid-1-methylamide (28 mM) with a 15% molar excess of mustard gas, pH 7.5, 25 °C

methylamide. The formation of a large amount of the succinimide derivative and the presence of only a small amount of the expected ester suggests that the latter product is formed primarily but is rapidly transformed in aqueous solution to the succinimide by means of nucleophilic displacement of the thiodiglycol moiety by amide with ring closure, according to the reaction scheme shown in Figure 51.

Such cyclization reactions of 4-carboxylic acid esters of aspartic acid have been described before (74). In a separate experiment, it was shown that the ring closure was not due to the work-up of the reaction mixture before thermospray-LC-MS analysis. A reaction run similar to the one described except that a fourfold molar excess of

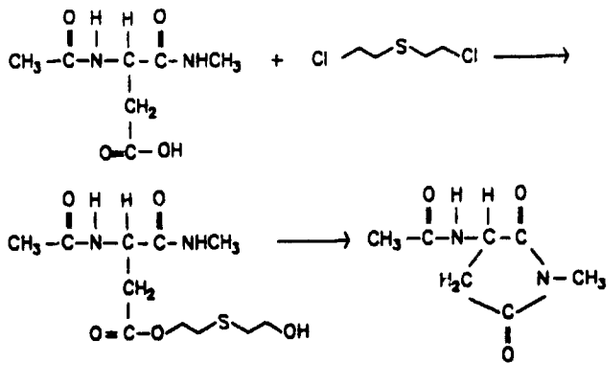

Figure 51. Reaction scheme for the formation of 1-methyl-3-acetamido-succinimide upon alkylation of N-acetyl-aspartic acid-1-methylamide with mustard gas in aqueous solution, pH 7.5, 25 °C

mustard gas was used, was analyzed directly with thermospray-LC-MS. The results were very similar to those described for the first experiment. 1-Methyl-3-acetamido-succinimide was obtained by independant synthesis (confer III.9.2). Co-chromatography of this product with the reaction mixture obtained from the alkylation of N-acetyl-aspartic acid-1-methylamide with mustard gas showed that the major reaction product was indeed the above-mentioned succinimide derivative.

III.10.3. N-Acetyl-glutamic acid-1-methylamide

N-acetyl-glutamic acid-1-methylamide in aqueous solution (9.5 mM) was reacted with a fivefold molar excess of mustard gas for 24 h. According to the thermospray mass chromatogram of the reaction mixture (Figure 52), the starting material had almost disappeared (peak 1) and two alkylation products had been formed. Peak 5 was tentatively identified as the major reaction product N-acetyl-glutamic acid-5-(2'-hydroxyethylthioethyl) ester-1-methylamide (MH^+, m/z = 307). Peak 4 was assigned the structure N-acetyl-glutamic acid-5-(2'-acetoxyethylthioethyl) ester-1-methylamide (MH^+, m/z = 349). As in previously described reactions (vide supra), it is assumed that the latter product is formed during thermospray MS analysis via replacement of chlorine from the corresponding 5-(2'-chloroethyl-thioethyl) ester by acetate ions from the buffer solution. Evidently, and as remarkable as in earlier mentioned cases, the 2'-chloroethyl-thioethyl moiety in the alkylated product is sufficiently resistant to hydrolysis to survive several hours in aqueous solution. The reactions are summarized in Figure 53. No evidence was found for the formation of secondary cyclic products as was the case with the aspartic acid model peptide. This is in accordance with literature data which indicate that such cyclization reactions are much less pronounced with glutamic acid derivatives than with aspartic acid derivatives (74).

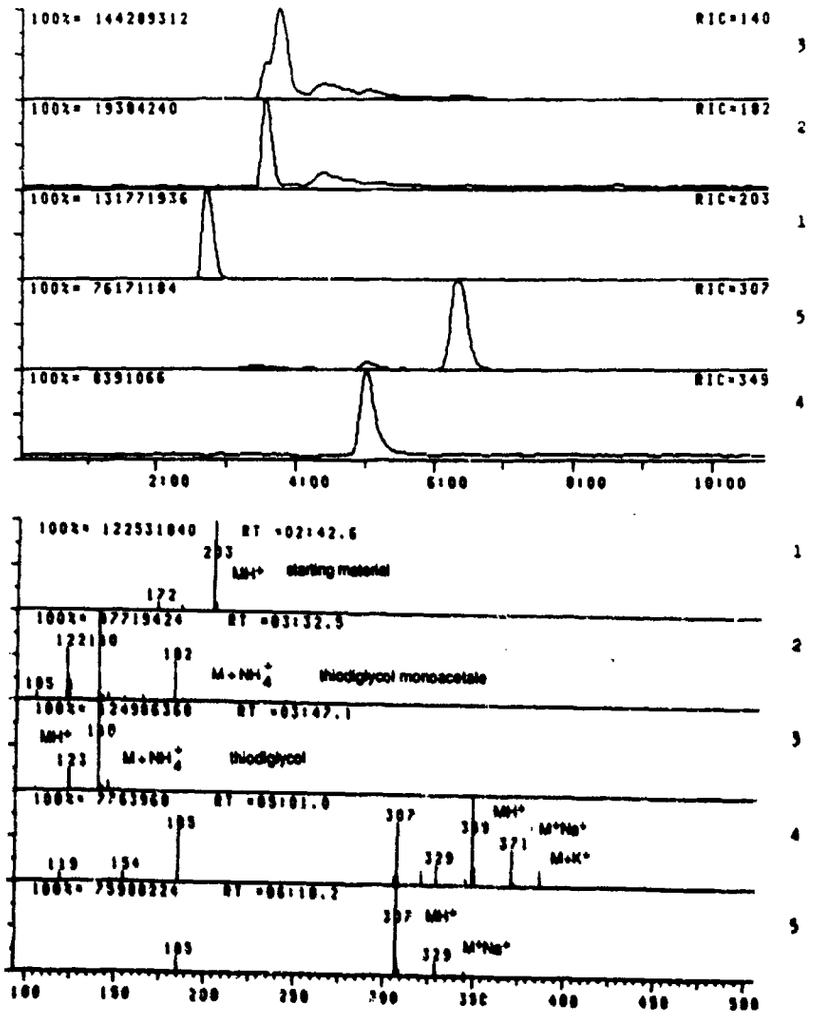

Figure 52. LC-thermospray mass chromatogram and mass spectra of the products after reaction of aqueous N-acetyl-glutamic acid-1-methylamide (9.5 mM) with a fivefold molar excess of mustard gas, pH 7.5, 25 °C

The structure of the major reaction product (peak 4) was confirmed by independant synthesis (confer III.9.3) and co-chromatography (HPLC) of the synthetic product with the reaction mixture as described above.

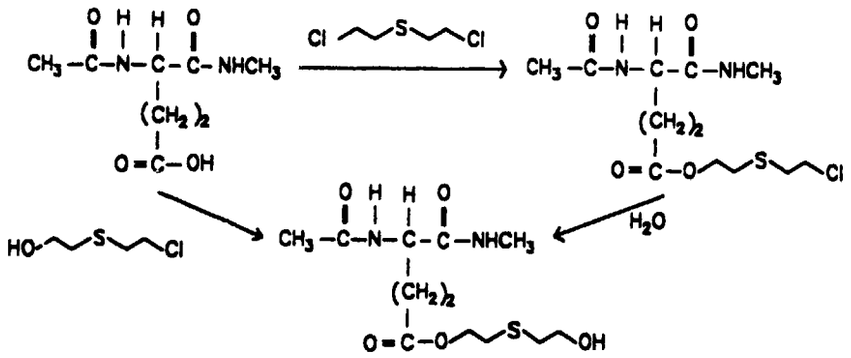

Figure 53. Proposed reaction scheme for the formation of N-acetyl-glutamic acid- 5-(2'-hydroxyethylthioethyl) ester-1-methylamide upon alkylation of aqueous N-acetyl glutamic acid-1-methylamide (9.5 mM) with a fivefold molar excess of mustard gas, pH 7.5, 25 °C

III.10.4. N-Acetyl-methionine-methylamide

An aqueous solution of N-acetyl-methionine-methylamine (17.5 mM) was reacted with a 10% molar excess of mustard gas for 42 h. Reversed phase HPLC of the reaction mixture as shown in Figure 54 showed a peak presumably belonging to an alkylation product (peak 3), in addition to peaks of thiodiglycol (peak 1) and starting material (peak 2). Thermospray-LC-MS confirmed the assignment of peaks 1 and 2. The thermospray mass spectrum of peak 3 showed the major peak at m/z = 205, i.e., corresponding with MH^+ of starting material, and additional minor peaks at m/z = 174, 157, 140 and 122, which are not present in the corresponding thermospray mass spectrum of starting material. Direct thermospray MS of the reaction mixture shows major peaks at m/z = 295 and 205, in addition to minor peaks at m/z = 157 and at various other m/z values. The peak at m/z = 295 is tentatively assigned to MH^+ of N-acetyl-S-(2'-hydroxyethylthioethyl)-homocysteine-methylamide.

Tentatively, we assume that the latter product is a decomposition product of the major alkylation product (peak 3 in Figure 54), i.e., the S-(2'-hydroxy-ethylthioethyl)-sulfonium ion of N-acetyl-methionine-methylamide. The latter ion may decompose during thermospray MS with loss of any of the three ligands bound to ternary sulfur, as shown in Figure 55. Loss of methyl leads to the above-mentioned homocysteine derivative with m/z = 295 (MH^+). Loss of the 2'-hydroxy-ethylthioethyl moiety would lead to reformation of starting material (MH^+, m/z = 205), whereas loss of (2'-hydroxy-ethylthioethyl) methyl sulfide would explain the formation of α-(acetamido)-vinylacetic acid-methylamide (MH^+, m/z = 157). Earlier investigations (49, 106-108) have shown that such sulfonium ions decompose thermally, but also with acid or base catalysis, with loss of any of the three moieties bound to sulfur.

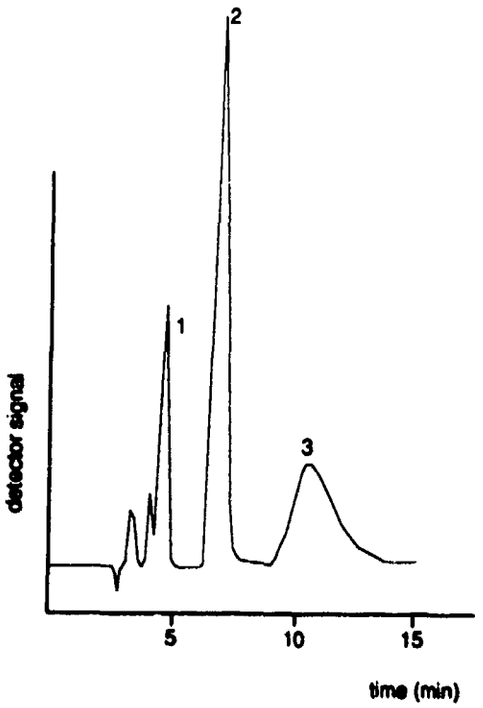

Figure 54. Chromatogram of the crude reaction mixture obtained after reaction of aqueous N-acetyl-methionine-methylamide (17.5 mM) with a 10% molar excess of mustard gas, pH 7.5, 25 °C. Reversed phase HPLC on an RP 18 column (300x2 mm). Eluent: ammonium acetate (10 mM, pH 6.0) in water/methanol (7/1, v/v). UV detection at 214 nm

^{1}H- and ^{13}C-NMR analysis of the crude reaction mixture indicated that ca. 60% of starting material was left. Additional aspects in the spectra (data not given) can be explained on the assumption that equal amounts of two reaction products are present, which are diastereoisomeric. This would be in accordance with the suggested structure of the sulfonium product, since both the α-carbon of the amino acid and the sulfonium sulfur are chiral.

The proposed structure of the sulfonium compounds needs further confirmation after isolation of the pure product from the reaction mixture and full analysis.

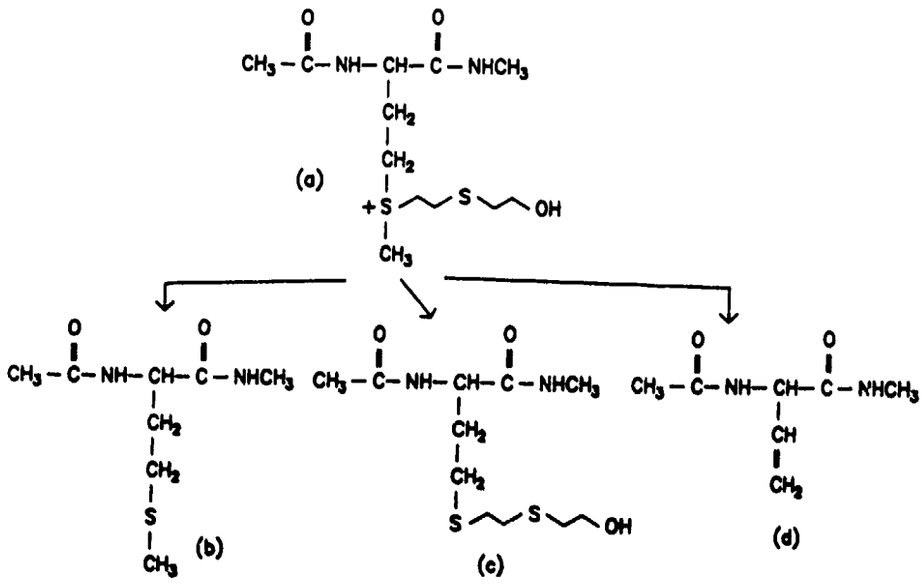

Figure 55. Tentative decomposition scheme during thermospray MS of the S-(2'-hydroxyethylthioethyl)-sulfonium derivative of N-acetyl-methionine-methylamide (a); (b) N-acetyl-methionine-methylamide; (c) N-acetyl-S-(2'-hydroxyethyl-thioethyl)-homocysteine-methylamide; (d) α-(acetamido)-vinylacetic acid-methylamide

III.10.5. N-α-Acetyl-histidine-methylamide

An aqueous solution of N-α-acetyl-histidine-methylamide (7.6 mM) was reacted for 24 h with an equimolar amount of mustard gas at pH 7.5. Reversed phase HPLC with UV detection at 214 nm of the reaction mixture gave the chromatogram as shown in Figure 56. Peaks 1 and 2 correspond with unreacted starting material and thiodiglycol, respectively. Presumably, peaks 3 and 4 pertain to alkylation products. Thermospray MS-detected chromatography of the reaction mixture showed a molecular ion at m/z = 315 for peak 4, corresponding with MH^+ of starting material in which one (2'-hydroxyethylthioethyl) moiety has been introduced. Peak 3 gave two major ions at m/z = 315 and 419. Single ion monitoring at the latter two values of m/z confirmed that peak 3 consists of an unresolved mixture of two reaction products presumably corresponding with MH^+ of an isomer of peak 4 (m/z = 315), and of a product in which two (2'-hydroxy-ethylthioethyl) moieties have been introduced (MH^+ at m/z = 419).

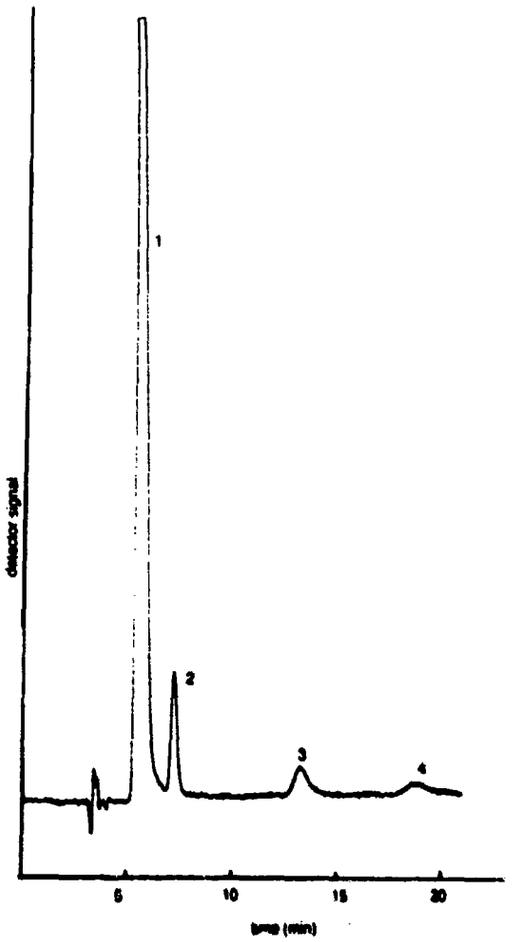

Figure 56. Chromatogram of the reaction mixture obtained after reaction of aqueous N-acetyl-histidine-methylamide (7.6 mM) with an equimolar amount of mustard gas, pH 7.5, 25 °C. Reversed phase HPLC on an RP 18 column (300x2 mm). Eluent: ammonium acetate (10 mM, pH 5.0) in water/methanol (9/1, v/v). UV detection at 214 nm

Our results obtained with these reactions can be explained on the basis of the assumption that two singly substituted alkylation products are obtained by alkylation of the N1(π) and N3(τ) positions of the histidine group (70,105), according to the reaction scheme given in Figure 57. The two singly substituted reaction products were finally identified as the N1(π)-substituted product (peak 3) and as the N3(τ)-substituted adduct (peak 4) by means of independant synthesis, full characterization of the two products (confer III.9.4) and co-chromatography with the products in the reaction mixture as described above.

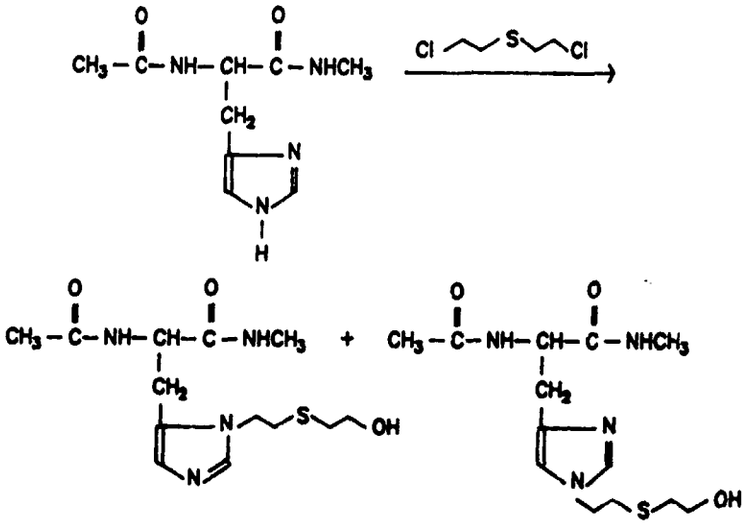

Figure 57. Reaction scheme for the formation of N1(π)- and N3(τ)-(2'-hydroxy-ethylthioethyl)-substituted N-acetyl-histidine-methylamides by means of alkylation of aqueous N-acetyl-histidine-methylamide with mustard gas, pH 7.5

No further attempts were made to synthesize or further identify the di-substituted reaction product. Presumably, both the 1- and 3-positions of imidazole in the starting material are substituted by (2'-hydroxyethylthioethyl) moieties, leading to a quaternary imidazolium structure. Thermospray MS of the pure 1- and 3-(2'-hydroxyethylthioethyl)-substituted histidine derivatives, obtained by independant synthesis, showed that the ion at m/z = 419 is not caused by an artifact due to thermospray ionization of the singly substituted products.

III.10.6. N-Acetyl-cysteine-methylamide

An aqueous solution of N-acetyl-cysteine-methylamide (0.5 mM) was reacted at a tenfold molar excess with mustard gas. The reaction was completed within 30 min. Analysis of the reaction mixture with thermospray-LC-MS (Figure 58) showed three products in addition to thiodiglycol (peak 1). The analysis shows that the expected reaction product N-acetyl-S-(2'-hydroxyethylthioethyl)-cysteine-methylamide was formed (peak 3) in addition to the diadduct of the starting compound (peak 4). Remaining starting compound was not detected, but was apparently oxidized into the N,N'-diacetyl-cystine-dimethylamide (peak 2).

The monoadduct was isolated from the reaction mixture by HPLC separation. The thermospray-LC-MS spectrum of the product was identical to that of peak 3 (Figure 58). ^{1}H- and ^{13}C-NMR analyses (see II.9.6) of the product confirm the structure of the monoadduct.

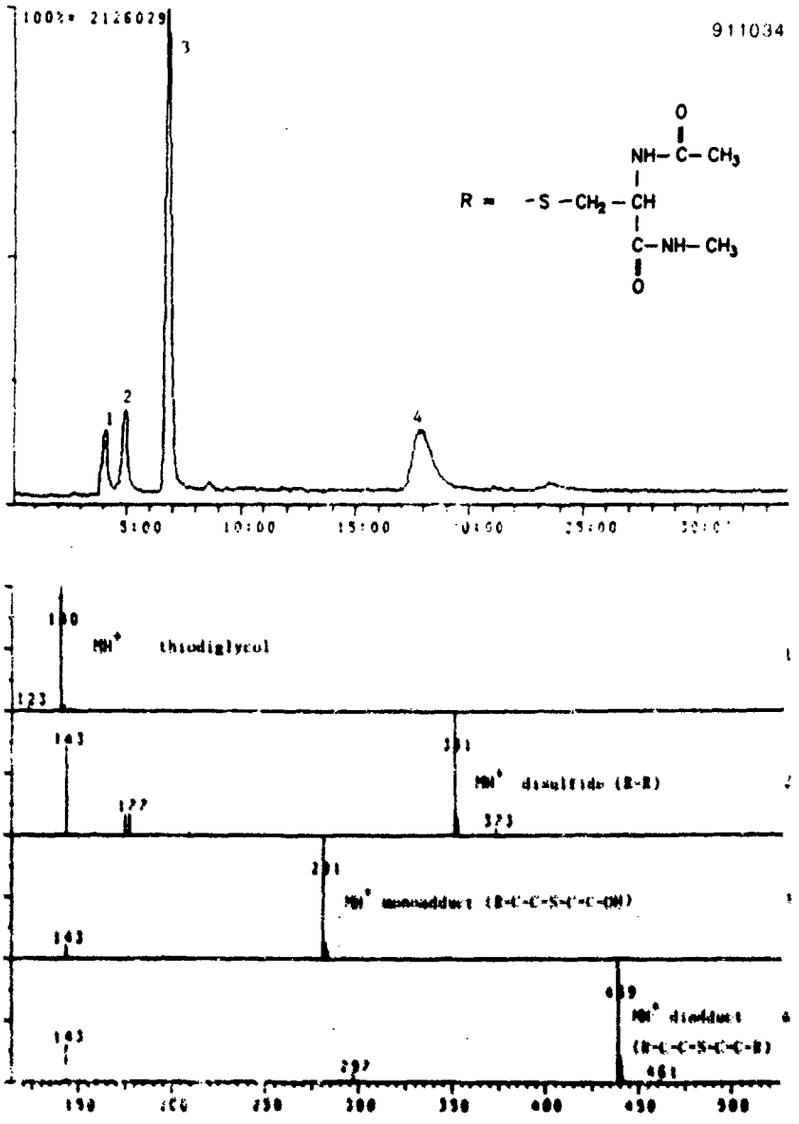

Figure 58. Thermospray-LC-MS chromatogram and mass spectra of the various peaks after reaction of N-acetyl-cysteine-methylamide (0.5 mM) at a tenfold molar excess with mustard gas in aqueous solution, pH 7.5, 25 °C. R = $CH_3C(O)NHCH[C(O)NHCH_3]CH_2S$-

III.10.7. Competition reactions of model peptides with mustard gas

The results presented in the preceding subsections show that the functional groups in the side chains of the model peptides and the amino group of valine form monoadducts with mustard gas. In order to get more insight into the preferred alkylation sites by mustard gas in proteins, the reaction of mustard gas with the model peptides was studied in the following competition experiments.

In the first series of experiments mustard gas was allowed to react with all six model peptides at pH 7.5 and 25 °C in a pH-stat equipment. The reaction mixture was analyzed with micro-LC after the alkali consumption had subsided. In this analysis system the monoadducts that can be formed from the six model peptides are resolved and are also separated from the starting model peptides. N-acetyl-S-(2'-hydroxyethylthioethyl)-cysteine-methylamide was the sole product formed both after incubation of the model peptides (1.2 mM of each) with twice the molar concentration of mustard gas (2.7 mM) and after incubation of the model peptides (0.1 mM of each) with an excess of mustard gas (0.86 mM) relative to the total concentration of the nucleophilic sites in the model compounds. The results clearly show a high preference of mustard gas for reaction with the thiol moiety in cysteine.

The relative reactivities of the model peptides other than the cysteine derivative were studied in a second series of experiments. The model peptides (0.02 mM of each) were allow to react with a large molar excess of mustard gas (4 mM). Samples of the reaction mixture were analyzed with micro-LC after 75 and 105 min of reaction time (Figure 59). The results indicate that the valine monoadduct (Figure 59, peak 7) was formed and, to a lesser extent, the N1(π)- and

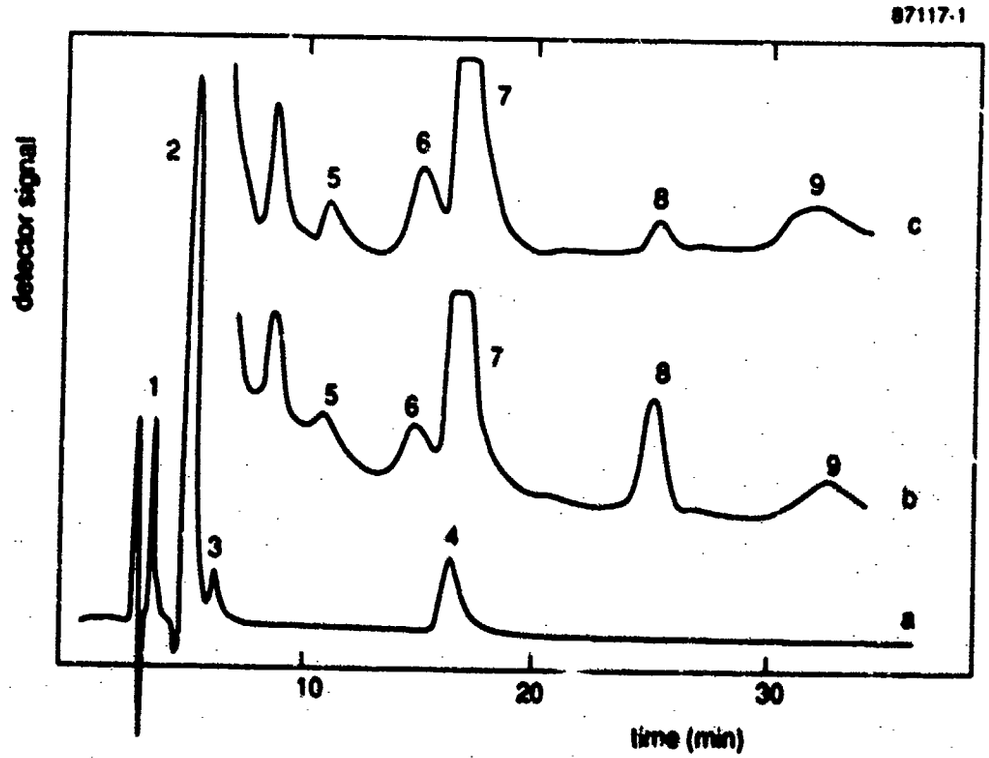

Figure 59. Micro-LC chromatogram of the reaction mixture obtained after reaction of mustard gas with valine-methylamide (3) and with the N-acetyl-methylamides of aspartic and glutamic acid (1), of histidine (2), and of methionine (4) for 0 (a), 75 (b) and 105 (c) min at pH 7.5 and 25 °C

N3(τ)-substituted histidine adducts (Figure 59, peaks 5 and 6, respectively) and the glutamic acid adduct (Figure 59, peak 9). Peak 8 in Figure 59, which decreased after longer time of incubation, may be ascribed to N-acetyl-aspartic acid-4-(2'-hydroxyethylthioethyl) ester-1-methylamide, which is initially formed but is subsequently transformed into the 1-methyl-3-acetamido-succinimide (see also III.9.2).

III.11. Synthesis of mustard gas-adducts of peptides as haptens

In order to generate cells which produce antibodies against amino acids alkylated with mustard gas, we wished to synthesize haptens consisting of various alkylated amino acids as a terminal moiety in a tri- or higher peptide. Such a hapten should be coupled to a protein, which is then used to generate the antibody producing cells. In principle two routes of synthesis of the haptens can be followed: (i) use of the alkylated amino acid in the synthesis of the hapten, and (ii) synthesis of the non-alkylated hapten and subsequent alkylation with mustard gas of the target amino acid in the peptide. In the following, examples of both approaches will be given.

In view of the many examples in literature (22,24,25) on the successful analysis of alkylation by various alkylating agents of the amino group of terminal valine in the α-chain of hemoglobin, we have selected to attempt the synthesis of a tri- and heptapeptide peptide based on the actual sequence of amino acids bound to this terminal valine. Moreover we have synthesized a tetrapeptide with a terminal glutamic acid, alkylated at the 5-carboxylic acid function by mustard gas, since data in literature indicate that in vivo alkylation at carboxylic acid functions by mustard gas, recognized by their instability towards alkali, is prominent (40-43).

III.11.1. Attempted synthesis of N-(2'-hydroxyethylthioethyl)-val-leu-ser

According to the reaction scheme shown in Figure 60, N-benzyloxycarbonyl-leu-ser was converted into the corresponding ethyl ester with ethanol, catalyzed by concentrated sulfuric acid (68% yield). Next, the benzyloxycarbonyl group was removed in 55% yield by hydrogenolysis, catalyzed by palladium on charcoal. Subsequent attempts to couple N-(2'-hydroxyethylthioethyl)-valine (see III.9.1) to the dipeptide ethyl ester with dicyclohexylcarbodiimide or with 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide in N,N-dimethylformamide failed. Thermospray MS analysis of the reaction mixtures showed that the carbodiimides added readily to the N-alkylated valine to give products with MH^+ at m/z = 428 for the dicyclohexylcarbodiimide derivative and at m/z = 247 ($MH^+ - H_2O$) for the other carbodiimide derivative. However, the subsequent reaction with the amino function of the dipeptide-ethyl ester did hardly proceed, since only traces of the desired tripeptide were observed. We assume that the primarily formed O-acyl isourea derivative of N-(2'-hydroxyethylthioethyl)-valine lacks reactivity towards the carboxylic acid function of the dipeptide, possibly due to the bulk of the isopropyl

moiety of the valine derivative. This may lead to an internal O --> N shift in the O-acyl isourea derivative (109), which yields a stable N-acylurea compound, as shown in Figure 61.

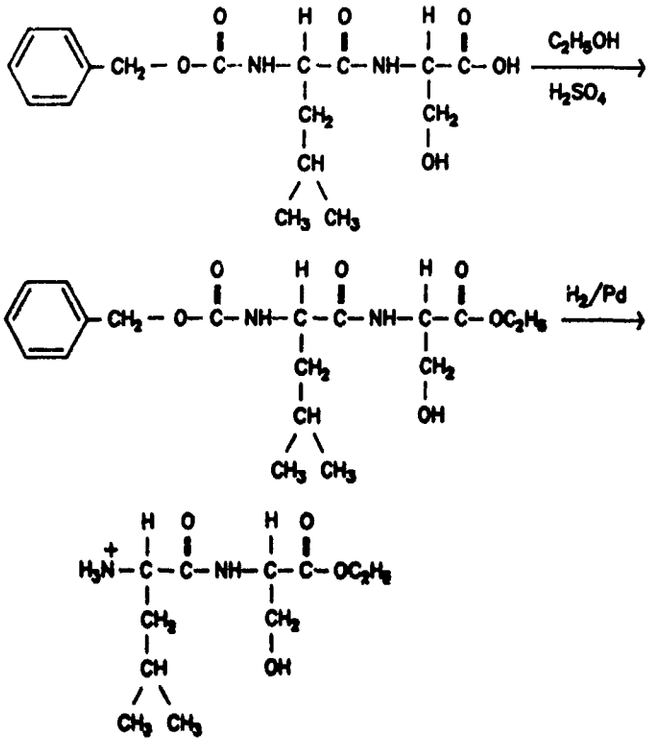

Figure 60. Reaction scheme for the synthesis of leu-ser-ethyl ester

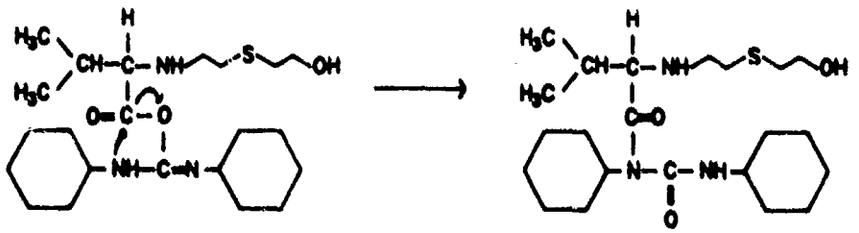

Figure 61. Rearrangement of the primarily formed O-acyl isourea derivative of N-(2'-hydroxyethylthioethyl)-valine with dicyclohexylcarbodiimide to a stable N-acylurea derivative

III.11.2. Synthesis of N-(2'-hydroxyethylthioethyl)-val-leu-ser-pro-ala-asp-lys

The N-terminal heptapeptide of the α-chain of hemoglobin (val-leu-ser-pro-ala-asp-lys) was synthesized as described by van Denderen et al. (77) using a Bioscarch Sam II automatic peptide synthesizer according to the solid phase synthesis method essentially as

described by Merrifield (78) with t-butyloxy-carbonyl-amino acids. Final deblocking and cleavage from the resin was performed by treatment with trifluoromethanesulfonic acid/thioanisole/m-cresol in trifluoroacetic acid. The product was purified using liquid chromatography on a Sephadex G-15 column. The structure of the peptide was confirmed by amino acid analysis of the hydrolyzed peptide (vide infra).

For the synthesis of the heptapeptide alkylated with mustard gas at the N-terminal valine, two portions of the heptapeptide were dissolved in PBS and treated with 1 mM mustard gas (for amino acid analysis) and 1 mM [^{35}]mustard gas (for HPLC) in dry acetone (final concentration 1%) for 45 min at 37 ^{o}C. The solutions were directly injected onto the HPLC column; from the heptapeptide treated with [^{35}S]mustard gas, fractions eluted in 0.5 min intervals were collected and the radioactivity was determined. The radioactivity profile showed two components, one being thiodiglycol and one which appeared to be the reaction product of mustard gas with phosphate in the PBS buffer. These two peaks were also collected upon analysis of the sample treated with 1 mM mustard gas. By amino acid analysis it was shown that no amino acids were present in these two fractions. The UV-profile showed two extra peaks which were identified as contaminants of acetone. In further experiments, mustard gas was dissolved in dry acetonitrile and the heptapeptide in distilled water, while the pH was controlled by a pH-stat. No adduct formation could be realized at pH 7, possibly because of insufficient deprotonation of the NH_3^+-group of valine. Treatment of the heptapeptide with 5 mM mustard gas dissolved in dry acetonitrile (final concentration 1%) at pH 8.5, resulted in the formation of one prominent new peak (peak 3 in Figure 62) and some smaller peaks. In the HPLC pattern, 52% of the total peak area represented the original heptapeptide (peak 2) and 30% the most prominent adduct (peak 3). Peak 1 represented thiodiglycol. Peaks 2 and 3 were collected separately by means of HPLC and the amino acid composition was assessed. The amount of amino acids (nmol) in the samples was expressed as the ratio of nmol amino acid:nmol ala. Peak 2 is the original heptapeptide [val(0.9), leu(0.9), ser(1.2), pro(1.0), ala(1.0),asp(0.9) and lys(1.2)]. The adduct peak 3 contained the following amino acids: val(0.1),leu(0.7),ser (1.3), pro(1.2), ala(1.0), asp(1.0) and lys(1.0). This peak contained all amino acids of the heptapeptide but for the low content of valine. Probably, val in the heptapeptide was alkylated at the amino group, blocking the reaction with phenyl isothiocyanate, which is used in the amino acid analysis. Valine alkylated with mustard gas was eluted in the void volume. Also, the amount of leu was somewhat decreased. A possible explanation could be incomplete hydrolysis of the bond between val and leu, as a result of the alkylation by mustard gas at the amino group of valine.

Peaks 2 and 3 were analyzed with thermospray mass spectrometry, whereas peak 3 was also analyzed with NMR.

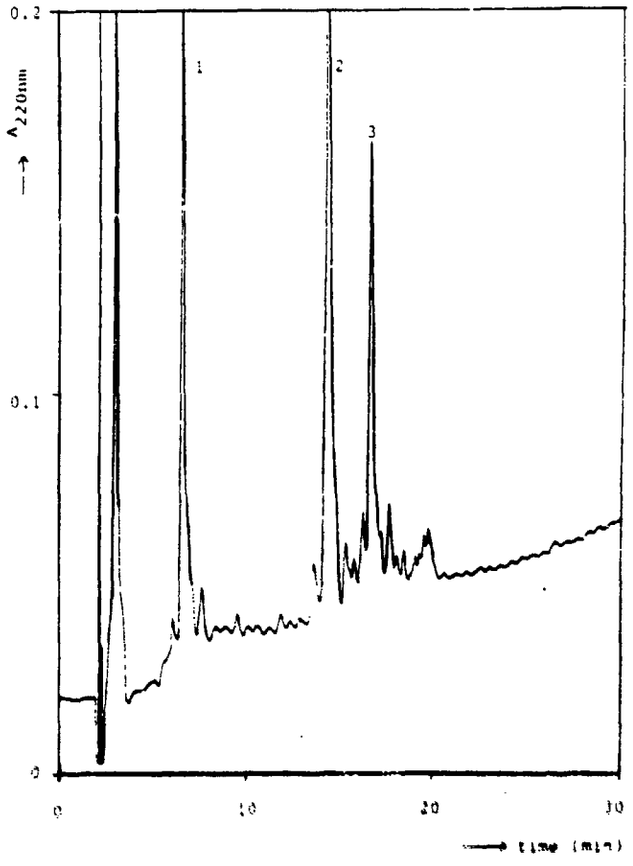

Figure 62. HPLC-chromatogram (ODS-Sephadex reversed-phase column) of the N-terminal heptapeptide of the α-chain of hemoglobin treated with 5 mM mustard gas at pH 8.3. The absorbance was recorded at 220 nm. To elute the various peptides an acetonitrile gradient in 0.1% trifluoroacetic acid was used. Peak 1: thiodiglycol: peak 2: N-terminal heptapeptide of the α-chain of hemoglobin; peak 3: N-(2'-hydroxyethylthioethyl)-val-leu-ser-pro-ala-asp-lys

Thermospray MS analysis of peak 2 showed MH^+ of the intact heptapeptide at m/z = 729 and fragments at m/z = 711 (MH^+-H_2O), 414 (MH^+ of val-leu-ser-pro) and at m/z = 485 (MH^+ of val-leu-ser-pro-ala). It can be concluded that peak 2 represents the intact heptapeptide. The thermospray MS analysis of the adduct peak 3 showed MH^+ of the expected monoadduct of the heptapeptide at m/z = 833, as well as MH^+ at m/z = 334 of a fragment, i.e., N-(2'-hydroxyethyl-thioethyl)-val-leu.

NMR was used to confirm the structure of peak 3. Because of the small amount of product available, only ^{1}H-NMR spectra could be taken. In order to avoid problems of overlapping resonances due to the many resonances and complicated coupling patterns of the modified heptapeptide, two-dimensional NMR techniques were applied. The peptide was dissolved at a concentration of 3.5 mg/ml in a buffer containing H_2O and D_2O in a ratio of 9:1 (v/v) and 10 mM of perdeuterated sodium acetate, pH 4.30, 30 °C. Under these conditions the amides of the peptide backbone are mainly protonated and exchange slowly with water. Since these measurements require saturation of the water resonance of the solvent, the slow exchange is essential to prevent transfer of saturation to the amide protons. The proton resonances of the various amino acid residue side chains were assigned using double quantum filtered COSY (two-dimensional correlated spectroscopy) NMR, which shows scalar couplings, and chemical shift information (110).

Especially the assignments of the resonances of the α-carbon proton in the valine residue and the protons in the sulfur mustard group were needed to show the modification of valine. Although the measurements were carried out in a H_2O containing buffer, the valine amine proton was not visible, probably due to an unfavorable pK-value. NOESY (two-dimensional nuclear Overhauser enhancement spectroscopy) and ROESY (two-dimensional rotating frame Overhauser enhancement spectroscopy) were used to show dipolar couplings (through space) between protons in the sulfur mustard group and protons in the valine residue. These couplings are only visible if the distance between the involved protons is less than ca. 0.4 nm. Because of the unfavorable rotational correlation time of the modified peptide, the NOESY spectrum showed only very weak cross peaks. This problem was avoided using the ROESY technique. As shown in Figure 63 (cross peaks A and B), clearly dipolar couplings between the α-carbon proton of thevaline residue (3.80 ppm) and the neighbouring protons of the (2'-hydroxyethylthioethyl) moiety (3.14 and 3.24 ppm) were visible. These couplings are visualized in Figure 64. Dipolar couplings between the latter protons and protons of other amino acid residues are not observed.

Modified valine, i.e., N-(2'-hydroxyethylthioethyl)-valine (confer II.10.2.5) was studied with one- and two-dimensional NMR techniques. A great resemblance with the modified heptapeptide was observed. Chemical shift assignments for the (2'-hydroxyethylthioethyl)-valine moiety of the modified heptapeptide are shown in Figure 65, whereas some additional assignments are summarized in Table 8.

It is concluded that ^{1}H-NMR and thermospray-LC-MS analysis of the alkylated heptapeptide (peak 3 in Figure 62) fully support the assigned structure, i.e., the heptapeptide is mono-substituted with a (2'-hydroxyethylthioethyl) moiety at the amino group of valine.

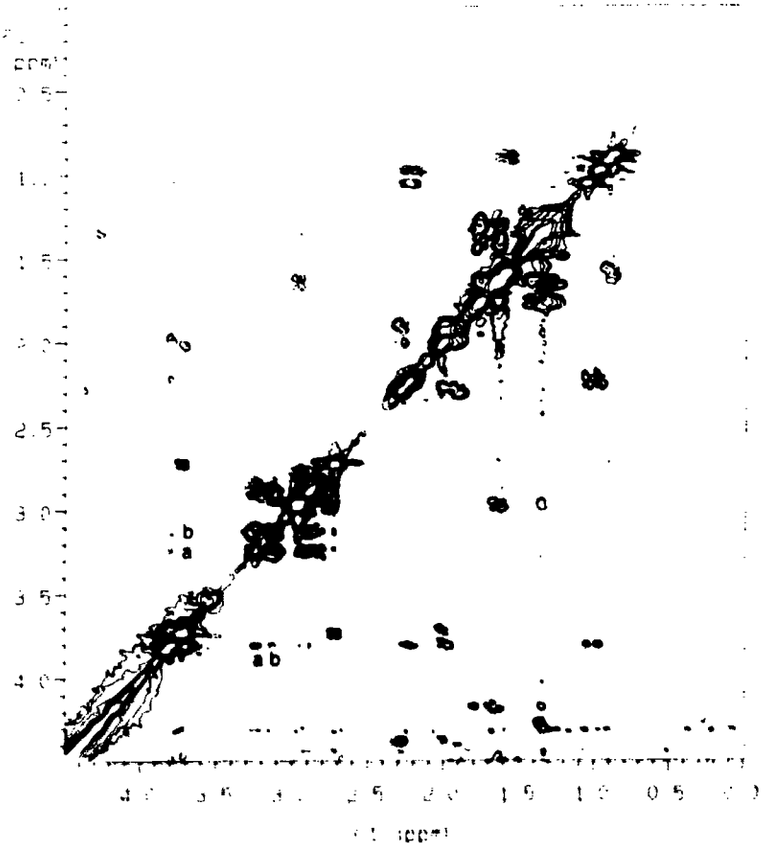

Figure 63. Part of the ROESY (two-dimensional rotating frame Overhauser enhancement spectroscopy) spectrum (400 MHz) of N-(2'-hydroxyethylthioethyl)-val-leu-ser-pro-ala-asp-lys in H_2O/D_2O (9/1, v/v), containing 10 mM perdeuterated sodium acetate, pH 4.30. Mixing time 0.200 s. Cross-peaks indicated with a and b are due to dipolar C-H of the valine residue (3.80 ppm) and the neighbouring protons of the (2'-hydroxyethylthioethyl) moiety

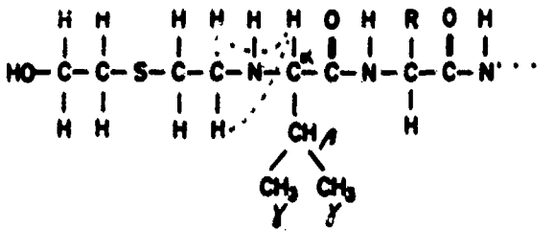

Figure 64. Observed dipolar couplings in the N-(2'-hydroxyethyl-thioethyl)-valyl moiety in the ROESY (two-dimensional rotating frame Overhauser enhancement spectroscopy) spectrum of N-(2'-hydroxyethylthioethyl)-val-leu-ser-pro-ala-asp-lys

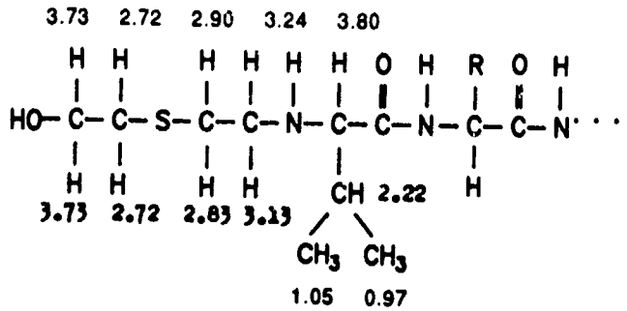

Figure 65. Chemical shift assignments for the N-(2'-hydroxyethylthioethyl)-valyl moiety in the ^{1}H-NMR spectrum (400 MHz) of N-(2'-hydroxyethylthioethyl)-val-leu-ser-pro-ala-asp-lys in H_2O/D_2O (9/1, v/v), containing 10 mM perdeuterated sodium acetate, pH 4.30

Table 8. Some chemical shift assignments in the ^{1}H-NMR spectrum of N-(2'-hydroxyethylthioethyl)-val-leu-ser-pro-ala-asp-lys

	NH	Cα-H	Cβ-H	Cγ-H	Cδ-H	Cε-H
Val	-	3.80	2.22	1.05 0.97		
Leu	8.81	4.52	1.59	1.59	0.92 0.88	
Ser	8.42	4.76	3.83			
Pro	-	4.38	2.28 1.90	2.00	3.80 3.69	
Ala	8.16	4.28	1.35			
Asp	8.24	4.75	2.96 2.79			
Lys	7.76	4.18	1.80 1.70	1.35	1.65	2.95

III.11.3. Synthesis of gly-gly-gly-glutamic acid-5-(2'-hydroxyethylthioethyl) ester-1-amide hydrochloride

The title peptide was obtained according to the reaction scheme shown in Figure 66. N-Benzyloxycarbonyl-gly-gly-gly-glutamic acid-5-t-butyl ester-1-amide was obtained in 73% yield by coupling N-benzyloxycarbonyl-gly-gly-gly with glutamic acid-5-t-butyl ester-1-amide via

the mixed anhydride method with ethyl chloroformate. Next, the benzyloxycarbonyl moiety was removed by hydrogenolysis, catalyzed by palladium on charcoal.

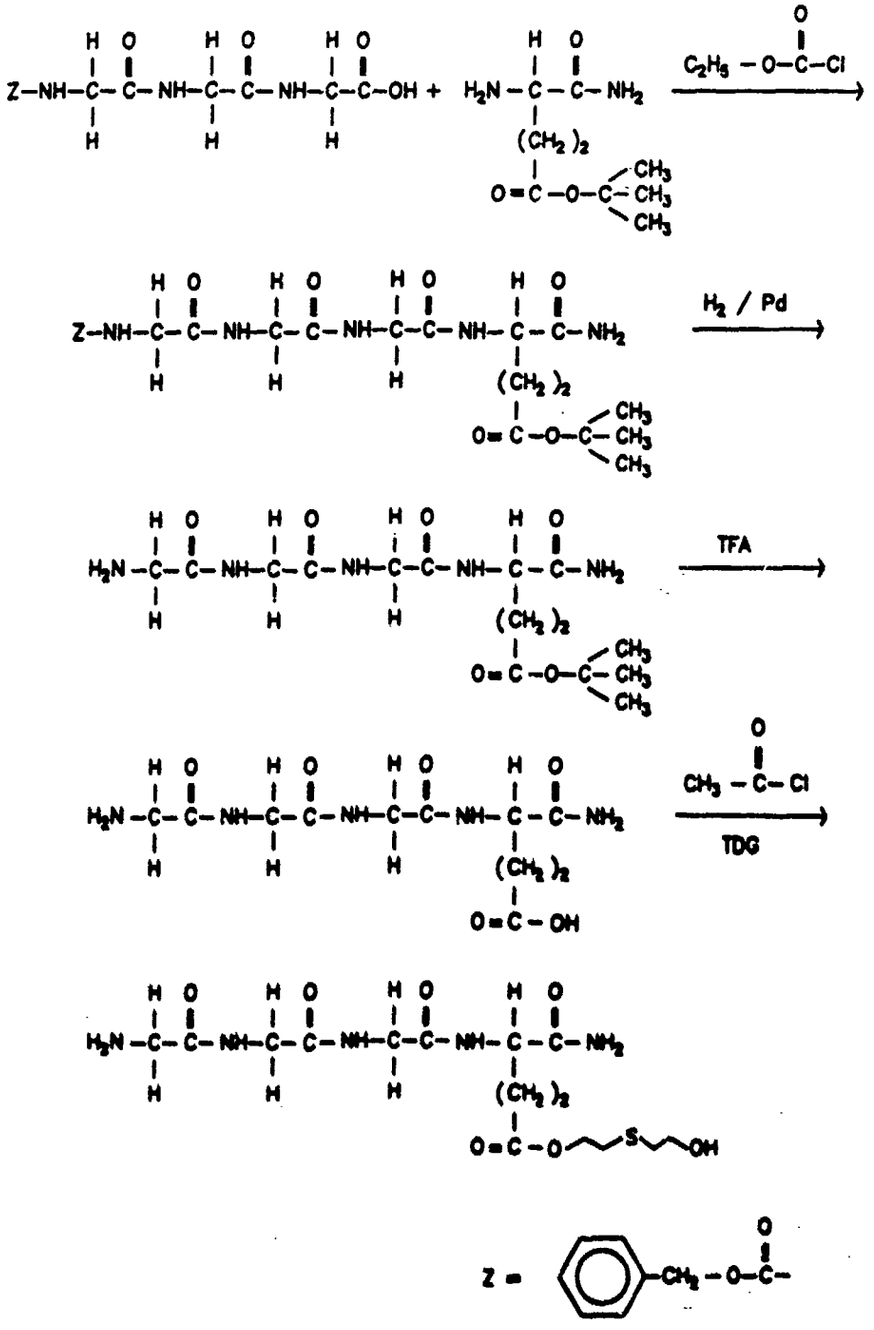

Figure 66. Reaction scheme for the synthesis of gly-gly-gly-glutamic acid-5-(2'-hydroxyethylthioethyl) ester-1-amide hydrochloride

Without isolation of the peptide with deprotected amino group, the t-butyl group was removed by treatment with trifluoroacetic acid. This gave gly-gly-gly-glutamic acid-1-amide trifluoroacetate in quantitative yield after the combined deprotection steps. Thermospray MS showed MH^+ at m/z = 318, as expected. Finally, the 5-carboxylic acid function of the peptide was esterified in thiodiglycol at room temperature, in the presence of a catalytic amount of acetyl chloride (69). Removal of excess thiodiglycol left the desired tetrapeptide in almost quantitative yield. Thermospray MS showed major signals at m/z = 422 (MH^+) and at m/z = 300 (MH^+ -thiodiglycol).

The ^1H- and ^{13}C-NMR spectral data of the end product (confer II.10.3.3) were in good agreement with the structure. In order to provide evidence of the point of attachment of the (2'-hydroxyethylthioethyl) moiety, several attempts were made to establish a connectivity or proximity between this group and the rest of the molecule. Although these attempts failed, the proposed structure seems to be the best explanation of the spectral data which were obtained.

III.11.4. Attempted synthesis of cys-gly-gly-gly

The reaction scheme shown in Figure 67 was followed in an attempt to synthesize the tetrapeptide cys-gly-gly-gly. The tripeptide t-butyloxycarbonyl-gly-gly-gly-ethyl ester was obtained in 60% yield by coupling t-butyloxycarbonyl-glycine with triglycyl-ethyl ester via dicyclohexylcarbodiimide. After deprotection of the terminal amino group the tripeptide was coupled with the activated cyanomethyl ester of benzyloxycarbonyl-cysteine. The crude product obtained in 84% yield consisted of the desired tetrapeptide (72%) and presumably the S -> O analog of this tetrapeptide (22%) according to thermospray MS analysis. In a preliminary experiment the two protective groups were removed and the ester group was split off with sodium in liquid ammonia, using the crude product without further purification. ^1H-NMR and IR spectra of the product obtained indicated that the desired tetrapeptide was formed in addition to the disulfide triglycyl-cystine-triglycine. Further experiments to obtain the tetrapeptide were not carried out.

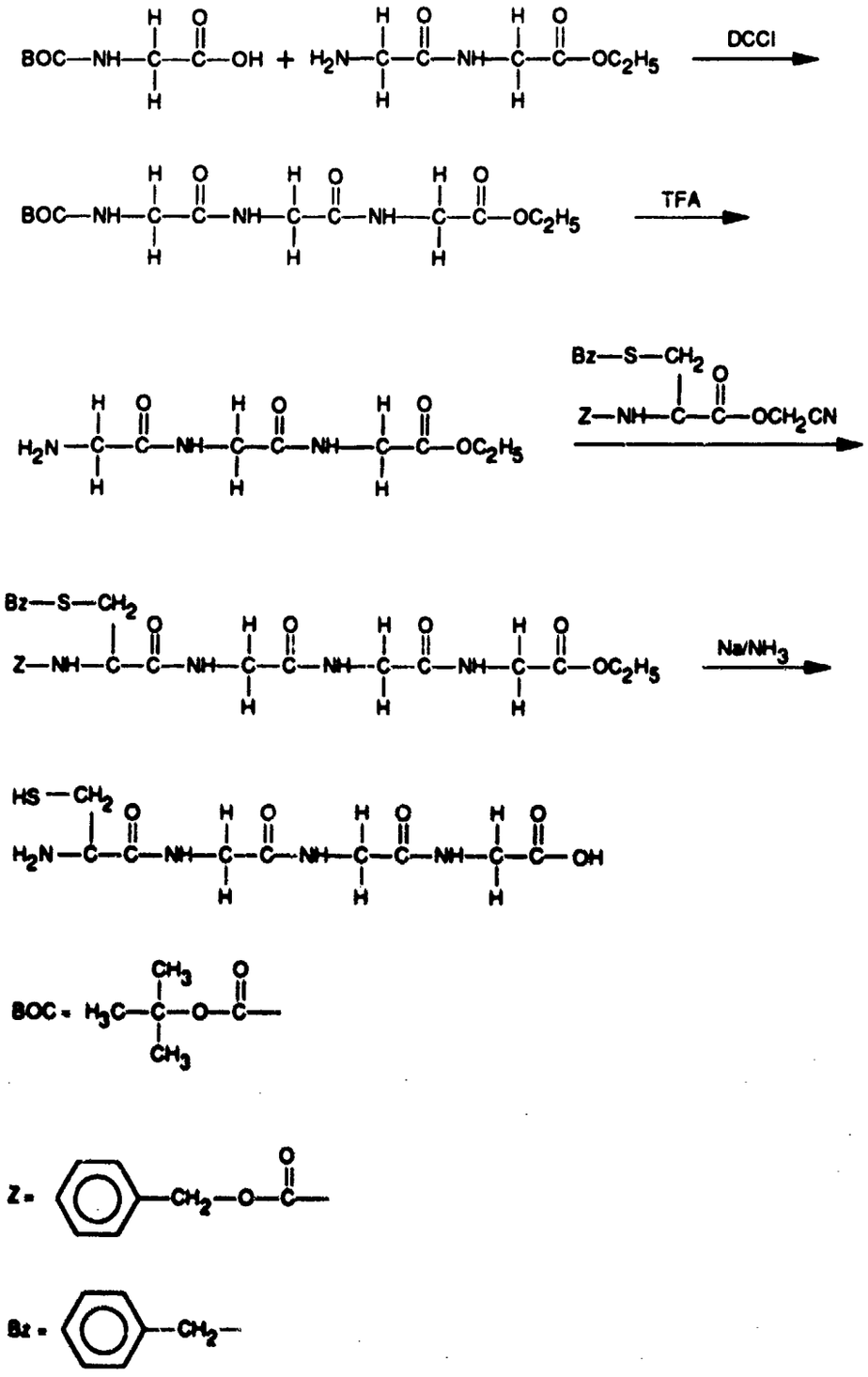

Figure 67. Reaction scheme for attempted synthesis of cys-gly-gly-gly

III.12. Identification and quantitation of mustard gas adducts to calf-thymus DNA and DNA of human white blood cells

Various degradation procedures are known for the detection of DNA modifications by means of liquid chromatography: (i) the enzymatic breakdown of the treated DNA into nucleosides and the separation by Fast Performance Liquid Chromatography (FPLC) on a cation-exchange column (111), (ii) the release of purines and alkylated purines (depurination) at a low or neutral pH and high temperature followed by separation of these bases by HPLC on a reversed-phase column (112), (iii) combination of DNA breakdown and depurination, and the use of various columns (reversed-phase and ion-exchange) for the subsequent purification steps (113).

We chose for an enzymatic breakdown of the DNA into nucleosides, followed by a mild depurination to bring about the selective release of the modified guanines and adenines, and a separation by HPLC on a reversed-phase column. First, ammonium formate buffers were used for the gradient elution. However, the ammonium formate was not readily removed from the collected peaks by freeze-drying. Therefore, the NH_4HCO_3 buffers as described in II.11.7 were used in later experiments.

Both the isolation of DNA from WBC and the breakdown of DNA into nucleosides are standard techniques in this laboratory (111,114). Commercially available nucleosides were used as markers (dG: 2'-deoxyguanosine; dA: 2'-deoxyadenosine; T: thymidine; dC: 2'-deoxycytidine) to develop an HPLC procedure for the analysis of calf-thymus DNA and DNA isolated from WBC after the degradation into nucleosides. The marker nucleosides were co-injected with the degraded DNA and retention times were compared. Also UV spectra were taken of the collected nucleoside peaks and compared with the spectra of the marker nucleosides. Figure 68 shows the HPLC profile of untreated calf-thymus DNA degraded into the nucleosides dC, dG, T and dA. A smaller peak, 2'-deoxy-5'-methylcytidine, was also detected, with a retention time between those of dC and dG. This compound is a modification which always is present in DNA. The profile of degraded DNA isolated from WBC corresponds to the profile shown in Figure 68.

After the development of this HPLC-procedure, resulting in the separation of the various unmodified nucleosides, calf-thymus DNA and DNA from whole blood of human volunteers treated with various concentrations of [^{35}S]mustard gas, were degraded into nucleosides and analyzed by HPLC. Figure 69 shows the HPLC profile of the degradation products of double-stranded calf-thymus DNA (ds-ct-DNA) exposed to 1 mM [^{35}S]mustard gas.

In addition to the four nucleosides, four major radioactivity peaks were detected. In this figure, only the fractions containing more than 1% of the total radioactivity detected are shown, since in the present project merely the identification of the major adducts is of importance. In a small aliquot of the mixture of nucleosides the

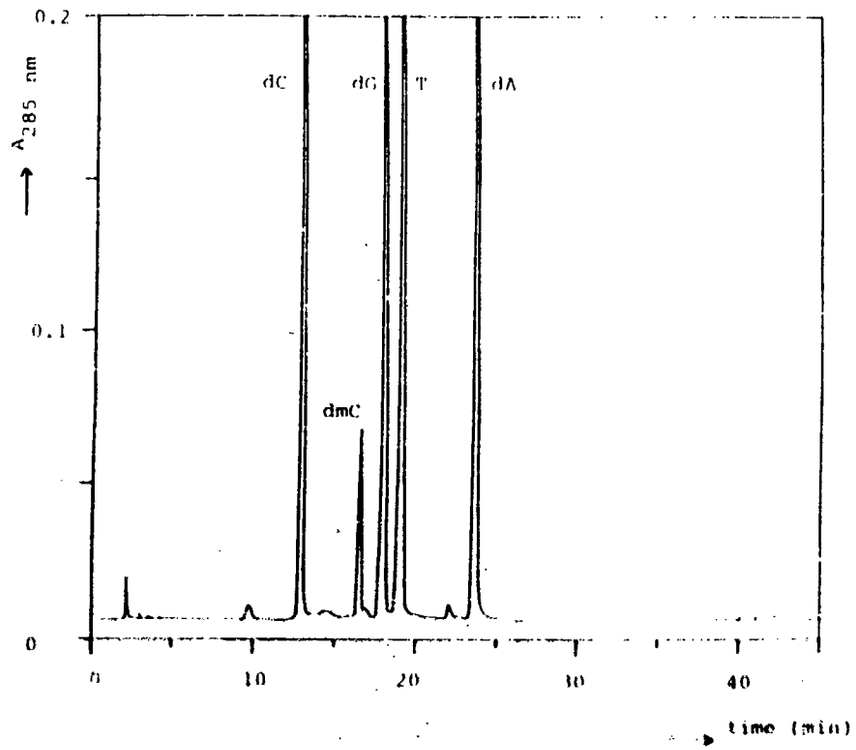

Figure 68. HPLC-chromatogram (ODS-Sephadex, reversed-phase column) of calf-thymus DNA after enzymatic degradation into nucleosides. The UV-absorbance (285 nm) was recorded. dC: 2'-deoxycytidine; dmC: 2'deoxy-5'methylcytidine; dG: 2'-deoxyguanosine; T: thymidine; dA: 2'-deoxy-adenosine

radioactivity was directly counted without HPLC, to check the loss of radioactivity during HPLC. In all experiments the loss of radioactivity was not more than 5-10%. The adducts in Figure 69 were also detectable by UV, and their retention times corresponded with the radioactive adduct peaks. One radioactive peak (retention time 11 min) did not correspond with an UV peak. This peak could be ascribed to [^{35}S]thiodiglycol, the hydrolysis product of mustard gas, which does not show UV absorbance at 285 nm. The presence of thiodiglycol was unexpected since after treatment of DNA or whole blood with mustard gas, the DNA is isolated by ethanol precipitation which was supposed to remove free thiodiglycol from the samples. However, the thiodiglycol still present in the samples suggests an incomplete removal of this hydrolysis product during the DNA-isolation step (the peak corresponds to 1.3% of the amount of mustard gas used). The other three ^{35}S-peaks were co-eluted with the synthetic N7-(2'-hydroxyethylthioethyl)-guanine (N7-G-HD; confer III.3.1) marker, the N3-(2'-hydroxyethylthioethyl)-adenine (N3-A-HD; confer III.4) marker and the di-[(2-guanin-7'-yl-ethyl] sulfide (N7-G-HD-G; confer III.3.2) marker, with retention times of 24, 28 and 39 min, respectively. The three adduct peaks derived from ds-ct-DNA treated with mustard gas were also characterized by their UV spectra obtained

with a diode-array detector (Appendix B) as well by thermospray-LC-MS (Appendix C), with the markers used as references.

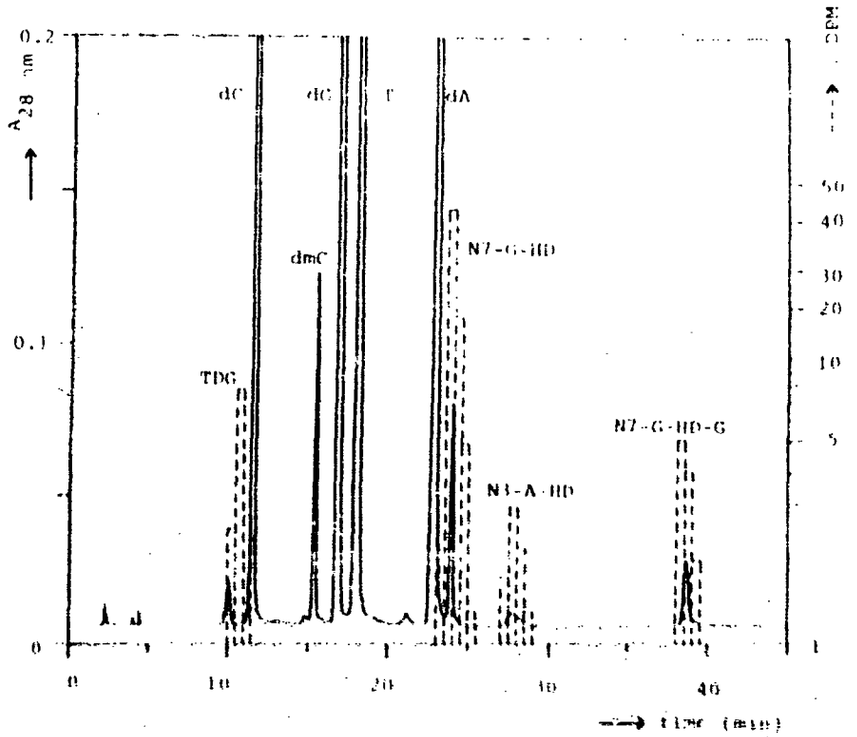

Figure 69. HPLC-chromatogram of hydrolyzed calf thymus DNA exposed to 1 mM [^{35}S]mustard gas (30 min; 37 °C). The DNA hydrolysate was analyzed on an ODS-Sephadex column; UV absorbance (285 nm) and radioactivity were monitored. The radioactivity of the fractions (0.5 min) was counted for 10 min in a Mark III liquid scintillation counter. dC: 2'-deoxycytidine; dmC: 2'-deoxy-5'-methylcytidine; dG: 2'-deoxyguanosine; T: thymidine; dA: 2'-deoxyadenosine; N7-G-HD: N7-(2'-hydroxyethylthioethyl)-guanine; N3-A-HD: N3-(2'-hydroxyethylthioethyl- adenine; N7-G-HD-G: di-(2-guanin-7'-yl-ethyl) sulfide; TDG: thiodiglycol

DNA which was isolated from whole human blood after treatment with 1 mM [^{35}S]mustard gas and was degraded into nucleosides, showed the same radioactivity profile as double-stranded calf-thymus DNA treated with mustard gas. Three radioactive adduct peaks were detected (Figure 70). Thiodiglycol was not detectable, confirming that the presence of thiodiglycol in digested calf-thymus DNA probably was due to insufficient removal during the DNA-isolation step. UV peaks could not be detected at the position of the adducts. Hence characterization by diode array detection or thermospray-LC-MS was not attempted. However, the radioactive adduct peaks again were co-eluted with the three adduct markers as described before. It could be concluded, therefore, that the same three major adducts were formed in whole blood as in calf-thymus DNA treated with mustard gas.

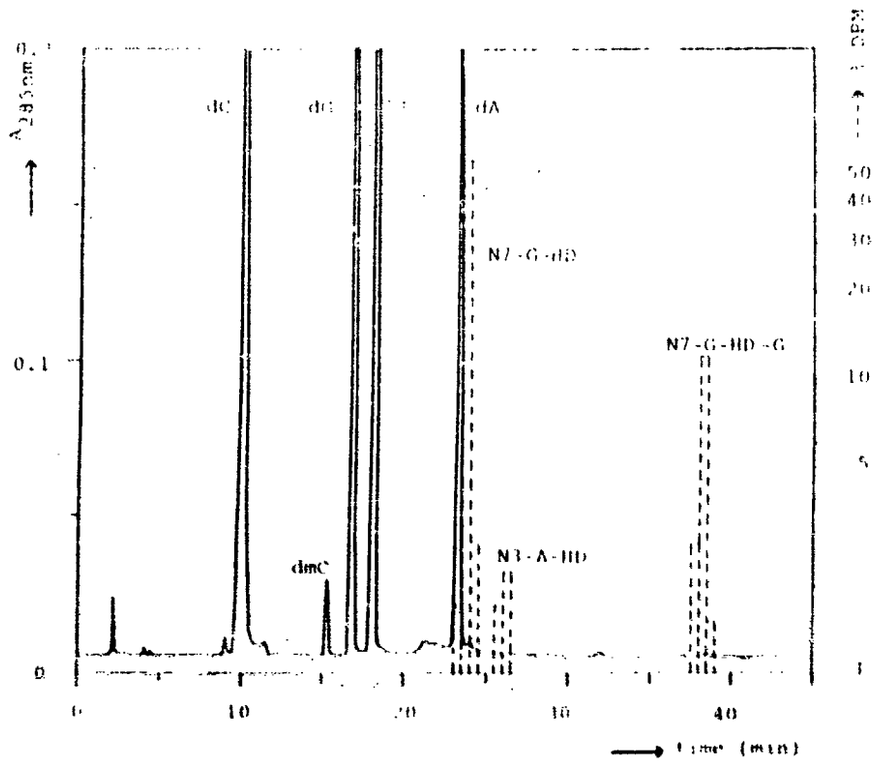

Figure 70. HPLC-chromatogram of DNA from human whole blood exposed to 1 mM [^{35}S]mustard gas (30 min; 37 °C). The DNA hydrolysate was analyzed on an ODS-Sephadex column; UV absorbance (285 nm) and radioactivity were monitored.The radioactivity of the fractions (0.5 min) was counted for 10 min in a liquid scintillation counter. dC: 2'-deoxycytidine; dmC: 2'-deoxy-5'methylcytidine; dG; 2'-deoxyguanosine; T: thymidine; dA: 2'-deoxyadenosine; N7-G-HD: N7-(2'-hydroxyethylthioethyl)-guanine; N3-A-HD: N3-(2'-hydroxyethylthioethyl)-adenine; N7-G-HD-G: di-(2-guanin-7'-yl-ethyl) sulfide

In addition to the adducts with mustard gas mentioned above the O6-(2'-hydroxyethylthioethyl)-guanine (O6-G-HD) adduct also might be formed. Attempts were made to locate this adduct. It was unknown whether this compound would be released from the deoxyribose during the heating-step in the procedure. Therefore, both the markers O6-G-HD (confer III.3.4) and O6-(2'-hydroxyethylthioethyl)-2'-deoxyguanosine (O6-dGuo-HD; confer III.3.3) were synthesized and used for the identification. On HPLC, both markers had a retention time longer than that of the di-adduct. Figure 71 shows the HPLC profile of the four markers N7-G-HD, N3-A-HD, N7-G-HD-G and O6-G-HD. A different column and elution gradient was used. Consequently, the retention times of the markers in Figure 71 are not comparable with the retention times of the markers in Figures 69 and 70. The O6-dGuo-HD marker had a retention time longer than that of the O6-G-HD (profile not shown). It was questionable whether significant amounts of radioactivity above the background value were present at the position of the O6-adduct. Therefore, if any O6-G-HD adduct was formed, it was

a very small amount (less than 0.5 % of total detected radioactivity).

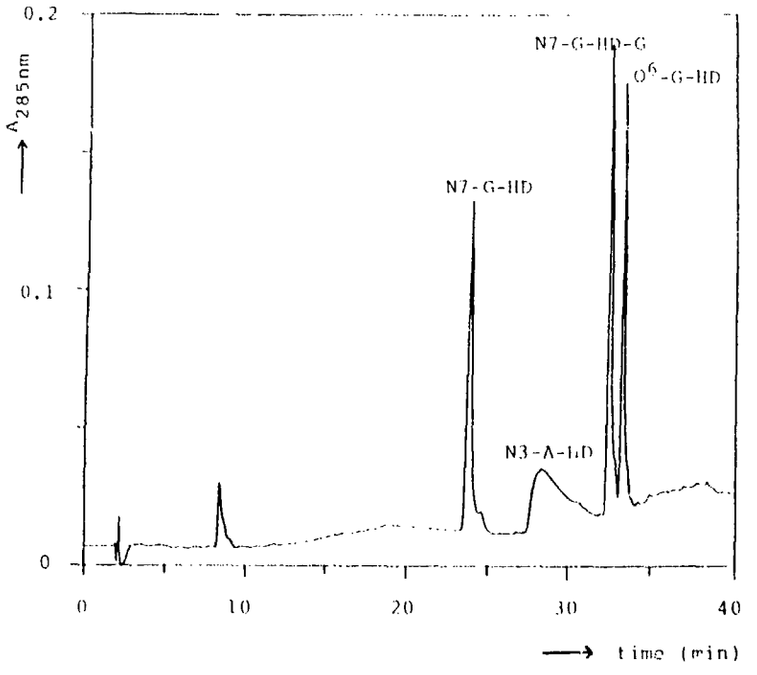

Figure 71. HPLC-chromatogram (ODS-Sephadex column) of synthetic adducts of mustard gas with DNA, used as markers. UV absorbance (285 nm) was recorded. N7-G-HD: N7-(2'-hydroxyethylthioethyl)-guanine; N3-A-HD: N3-(2'-hydroxyethylthioethyl)-adenine; N7-G-HD-G: di-(2-guanin-7'-yl-ethyl) sulfide; O6-G-HD: O6-(2'-hydroxyethylthioethyl)-guanine

The three major adducts were also quantified in two completely quantitative experiments. The same batch of [^{35}S]mustard gas (specific activity at the day of preparation 850 MBq/mmol) was used in both experiments. The specific activities of the [^{35}S]mustard gas on the days of counting were 261 and 245 MBq/mmol, respectively. The amount of DNA in the injected samples was determined spectrophotometrically. In both experiments 10 ml of whole blood was treated with 1 and 0.1 mM [^{35}S]mustard gas, and 0.5 ml ds-ct-DNA (0.5 mg) was treated with 1, 0.1 and 0.05 mM [^{35}S]mustard gas The amount of DNA isolated from the blood was 200-250 µg DNA/10 ml blood. In one experiment 0.5 ml ss-ct-DNA (0.5 mg) was treated with 1 and 0.1 mM [^{35}S]mustard gas. However, in this experiment a different batch of [^{35}S]mustard gas was used (specific activity on the day of use 353 MBq/mmol) and also a different reversed phase column. The amount of radioactivity connected to the DNA relative to the amount of radioactivity applied is shown in Table 9.

Table 9. Percentage of radioactivity bound to calf-thymus DNA and to DNA from white blood cells in whole blood, after treatment with [^{35}S] mustard gas

Sample	Percentage of radioactivity bound to DNA[a]		
	Concentration of mustard gas (mM)		
	1	0.1	0.05
Ds-ct-DNA[b]	9.6	20.0	19.5
	11.7	12.4	nd[c]
Ss-ct-DNA[d]	7.8	11.1	nd
Blood[b]	0.014	0.022	0.032
	0.023	0.021	nd

[a] The percentage of radioactivity bound to DNA is expressed relative to the amount of radioactivity applied
[b] Data are shown from two experiments
[c] Not determined
[d] Data are shown from one experiment performed at a later date with a different batch of [^{35}S]mustard gas and a different reversed phase column

Much more radioactivity had reacted with naked calf-thymus DNA than with the DNA in the WBC of whole blood. This suggests that in whole blood there is interference with regard to the reaction of mustard gas with nuclear DNA, probably because of the presence of serum components, red blood cells and cell walls, cytoplasm components and nuclear walls of the WBC. Mustard gas also reacts with these constituents. The molar ratio of the amount of [^{35}S]mustard gas (treatment with 1 mM mustard gas) which had reacted with ds-ct-DNA (0.5 mg/0.5 ml) and DNA in WBC (250 μg DNA/10 ml blood) has been calculated, which amounted to 3.5×10^{-2} and 2.1×10^{-3} mol [^{35}S]mustard gas/mol nucleotides in DNA, respectively (mean of the two experiments). Per mol nucleotide 17 times as much [^{35}S]mustard gas had reacted with double-stranded calf-thymus DNA compared to DNA in WBC. The amounts of adducts formed during the treatment with 0.1 mM [^{35}S]mustard gas were, respectively, 5.4×10^{-3} and 3.0×10^{-4} mol [^{35}S]mustard gas/mol nucleotides in DNA. Treatment with a tenfold higher concentration of [^{35}S]mustard gas (1 mM versus 0.1 mM) did not result in a proportionally increased level of alkylation. Evidently, the concentration of mustard gas is not the only rate-determining factor in the formation of DNA adducts.

The [^{35}S]-DNA preparations obtained were processed further to allow the HPLC separation of the modified nucleosides. The resulting three major adduct peaks of N7-G-HD, N3-A-HD and N7-G-HD-G, together with thiodiglycol, were responsible for most of the radioactivity observed in the HPLC fractions (only fractions containing more than 1% of the radioactivity were taken into account). A background of radioactivity (0-0.5% per fraction) was found in each of the fractions eluted

beyond the thymidine peak. Table 10 shows the amounts of adducts and thiodiglycol found after exposure of ct-DNA and whole blood to 1, 0.1 and 0.05 mM [^{35}S]mustard gas.

Table 10. DNA-adducts found after exposure of calf-thymus DNA and whole blood to [^{35}S]mustard gas

Sample	Concentration mustard gas (mM)	Percentage radioactivity of HPLC input[a]		
		N7-G-HD	N3-A-HD	N7-G-HD-GD
s-ct-DNA[b]	1	66.3	10.1	14.2
	0.1	54.8	11.1	14.7
	0.05	54.4	8.7	21.4
Ss-ct-DNA[c]	1	49.5	4.3	10.5
	0.1	54.6	3.8	9.5
Blood[b]	1	61.4	4.8[d]	15.2
	0.1	60.9	6.5[d]	19.6

[a] Only fractions containing more than 1% of the radioactivity were taken into account; the percentages have been corrected for the amount of thiodiglycol recovered
[b] Data from two experiments (averaged)
[c] Data from one experiment
[d] Data from one experiment; in the other experiment N3-A-HD was not formed

Table 10 shows that N7-G-HD monoadduct is the major adduct formed in ct-DNA and in DNA from WBC. Higher concentrations of mustard gas resulted in relatively more N7-G-HD monoadducts and less di-adducts, suggesting a certain saturation. The proportion of N3-A-HD adducts in ct-DNA is higher than in WBC. As was observed before, the relative amount of thiodiglycol was high in the experiment with ds-ct-DNA, probably because during the DNA-isolation by a single ethanol precipitation not all of it had been removed. The percentages of the adducts, therefore, have been corrected for the amount of thiodiglycol present. In one experiment with human blood N3-A-HD was not found. For ss-ct-DNA, the three major adduct peaks represent only 66% of all radioactivity. There were two additional large peaks (containing about 11 and 16% of total radioactivity) which had longer retention times than the three major peaks; one appeared close to the elution position of O6-G-HD. The other one may pertain to the N1-monoadduct of adenine. The N1-position in adenine is more reactive towards mustard gas than the N3-position, but in double-stranded DNA the N1-position is shielded. These peaks have not been identified yet. Steric hindrance may also be a reason for the absence of O6-G-HD adduct in ds-DNA. The formation of the di-adduct of N7-guanine in ss-ct-DNA in roughly the same proportion as in ds-ct-DNA suggests that the majority of these adducts do not result from interstrand crosslinks. Probably, in most cases it is formed via the reaction of

one molecule of mustard gas with two different guanines belonging to the same DNA-strand (36).

From the total data the degree of alkylation of DNA, that is the ratio of alkylated bases versus non-modified bases, has been calculated. The results are presented in Table 11. This table shows that mustard gas is a very effective alkylating agent. Even in blood treated with mustard gas (1 mM), where so many other reactive constituents are present, 1 out of every 124 guanine bases is alkylated to form the N7-G-HD monoadduct. It should be mentioned that the peaks corresponding to adducts with a low degree of labelling (< 1:1,000) contained small amounts of radioactivity, which resulted in data that are not very accurate. This prohibited the determination of the adducts formed in WBC at lower concentration of mustard gas. Also, extrapolation from the data of Table 11 to lower doses of mustard gas results in uncertain values.

Table 11. Degree of alkylation with mustard gas in calf-thymus DNA and DNA from white blood cells

Sample	Concentration mustard gas (mM)	Ratio alkylated bases/unmodified bases		
		N7-G-HD/G	N3-A-HD/A	N7-G-HD-G/G[a]
Ds-ct-DNA	1	1:10	1:64	1:47
	0.1	1:75	1:378	1:286
	0.05	1:151	1:946	1:390
Ss-ct-DNA	1	1:18	1:283	1:61
	0.1	1:129	1:1,550	1:844
Blood	1	1:124	1:1,640	1:502
	0.1	1:1,000	1:12,550	1:3,280

[a] Alkylation expressed as mol crosslink/mol G

In all circumstances of treatment of DNA with mustard gas, the N7-G-HD monoadduct was shown to be the major adduct; for that reason it was decided to aim the development of an immunochemical detection method at this adduct. A suitable derivative of N7-G-HD (confer III.6.1), therefore, was used for the immunization of mice in the attempts to isolate hybridomas that produce specific antibodies against mustard gas-damage in DNA.

III.13. Detection of interstrand crosslinks in mammalian cells

The development of methods to detect mustard gas-induced DNA-DNA interstrand crosslinks by means of the method of "alkaline elution" was started with experiments using Chinese Hamster Ovary cells (CHO), because of the experience with such cells at TNO-MBL. To ensure that concentrations of mustard gas were chosen which permit the cells to maintain their integrity, the so-called biologically relevant doses, experiments were carried out to determine the cell survival, i.e., colony-forming ability, of CHO cells after exposure to 0.5-2.5 μM mustard gas. Figure 72 shows that exposure in this concentration range resulted in cell survival (and cell division) gradually decreasing from 100% to 5%.

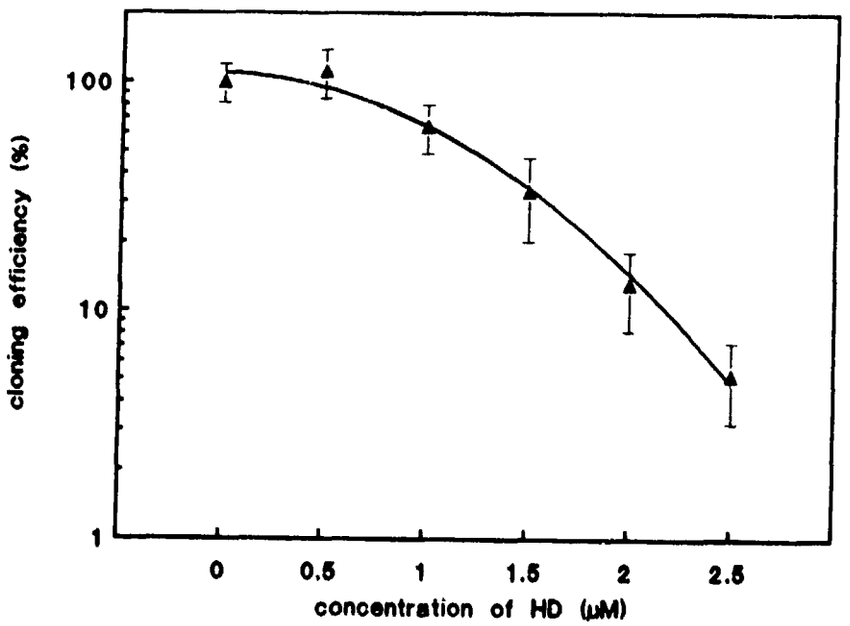

Figure 72. Survival (colony-forming ability) of CHO-cells exposed to mustard gas (0.5-2.5 μM mustard gas; 30 min; 37 °C). After six days of incubation, colonies of 50 or more cells were counted and cell survival was calculated. The data are mean values of six samples in one experiment with SEM

On the basis of these results the same concentrations of mustard gas were applied in experiments performed for the detection of crosslinks in CHO cells by alkaline elution. Crosslink induction increased linearly with dose (Figure 73). The exposure induced about 0.4 crosslink/10^6 guanines per μM mustard gas. From this data it was concluded that the method is suitable for the detection of interstrand crosslinks in CHO cells.

Also experiments were carried out to study removal of the crosslinks. After exposure of CHO cells to 1 or 2 μM mustard gas and removal of

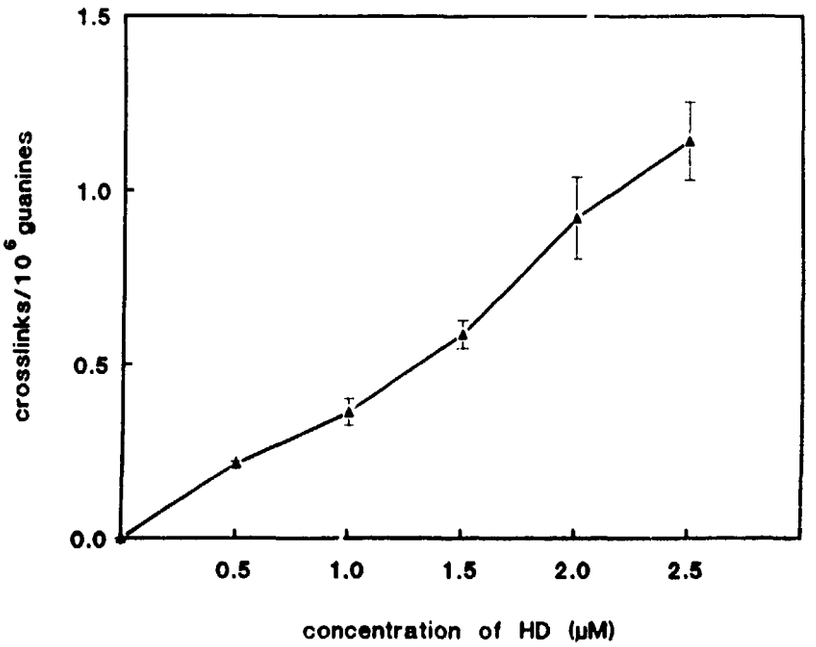

Figure 73. Crosslink detection in CHO-cells exposed to mustard gas (0.5-2.5 µM mustard gas; 30 min; 37 °C) by alkaline elution. The data are mean values of four samples in one experiment with SEM

the mustard gas solutions, the cells were incubated in fresh medium at 37 °C for 0-4 h and at intervals the amount of crosslinks remaining was measured. Figure 74 shows the repair curves. After both concentrations the highest amount of crosslinks was detected after 1 h of post-treatment incubation. Evidently, the formation of crosslinks continues after termination of the exposure, probably by through-reaction of mustard gas which is initially bound mono-functionally. After 4 h almost all interstrand crosslinks had been removed. With this technique only the disappearance of crosslinks can be detected. It is not possible to tell whether the crosslink-forming diethyl thioether chain is removed properly or that the crosslinks are simply broken, resulting in other "DNA-damages".

The method for the detection of crosslinks was developed with CHO cells, which could be radioactively labelled. Radio-labelled thymidines were incorporated in the DNA-strands during replication. Consequently, the DNA determination in the alkaline elution could be based on radioactivity measurements. However, white blood cells (WBC) are resting cells, so no radioactivity could be incorporated via DNA-synthesis during culturing. Therefore, for the detection of crosslinks in WBC, the DNA in the eluted fractions was detected fluorometrically.

It was attempted to separate different types of WBC (lymphocytes and granulocytes) by Percoll-gradient centrifugation. It was thought of interest to isolate both types of cells and to examine the induction

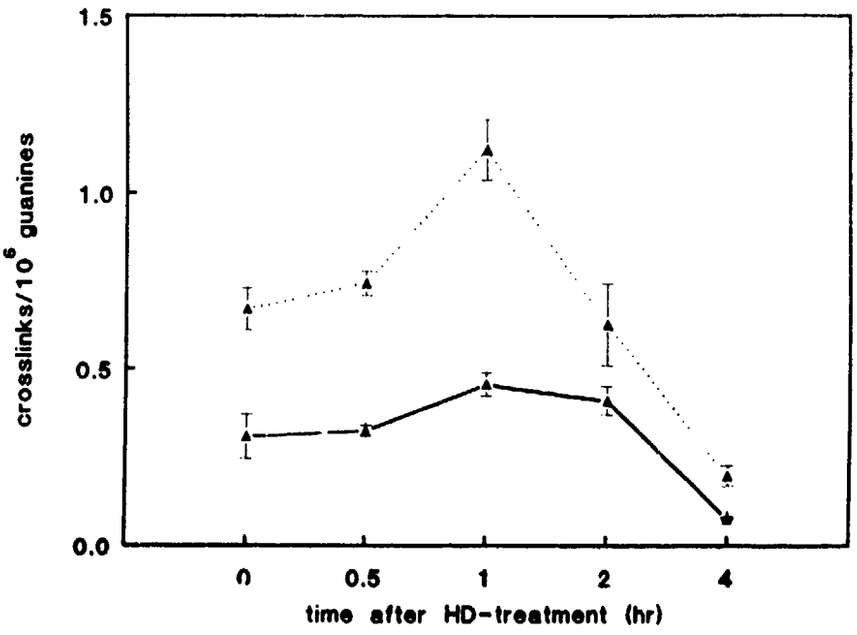

Figure 74. DNA-repair of crosslinks in CHO-cells exposed to 1 (——) and 2 (···) μM mustard gas (20 min; 37 °C) as studied with alkaline elution. After the incubation with mustard gas, the medium was replaced with fresh medium and the plates were incubated at 37 °C (0-4 h). The data are mean values of four samples in one experiment with SEM

of crosslinks in each cell type, because the reaction of mustard gas with various types of WBC, their DNA-repair systems and the amount of background single-strand breaks might differ in the various cell types. However, problems were encountered with the Percoll-gradient method, in particular in the isolation of the required amount of granulocytes. Therefore, most experiments were carried out with a lysis buffer which lyses the red blood cells while the total WBC fraction could be isolated. This mixed cell population was used for the detection of interstrand crosslinks. The results are presented in Figure 75, showing a dose dependent increase of the amount of crosslinks in the dose-range covered.

By means of HPLC of enzymatically degraded DNA of whole blood treated with 0.1 mM [^{35}S]mustard gas, it was found that 1 N7-G-HD-G (crosslink) was formed per 3,280 unmodified guanines, that is 1 such crosslink per 328,000 guanines when extrapolated to an exposure to 1 μM mustard gas. As can be derived from Figure 75, with alkaline elution of cells treated with 1 μM mustard gas, 0.2 interstrand crosslink per 1,000,000 unmodified guanines was found (that is 1 crosslink per 5,000,000 guanines). This is 15-fold difference. However, with alkaline elution only the interstrand crosslinks are detected, whereas with HPLC, both interstrand and intrastrand crosslinks are determined, suggesting that many more intrastrand crosslinks are induced compared to interstrand crosslinks (36).

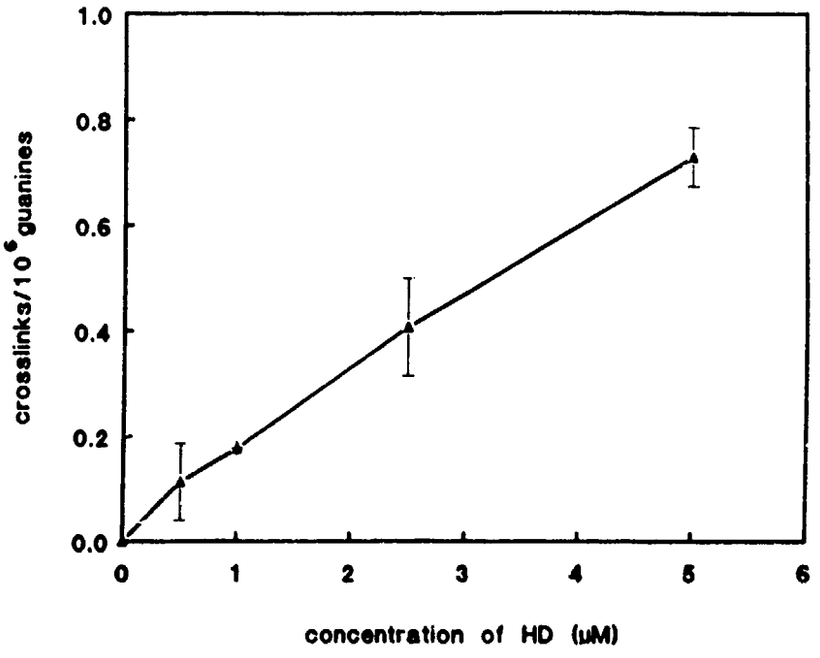

Figure 75. Crosslink detection by alkaline elution in white blood cells from whole human blood exposed to 0.5-5 µM mustard gas (30 min; 37 °C). The white cells were isolated by treatment of the blood with a buffer that induces lysis of the red blood cells. The data are mean values of four samples in one experiment with SEM

We had the opportunity to analyze blood samples from four victims of the Iran-Iraq War, who were presumably exposed to mustard gas 22-28 days earlier. Blood of European volunteers was collected to use as controls. This "alkaline elution" method as described, is suitable for the detection of both single-strand breaks (SSB) and interstrand crosslinks which mask SSB's, so the results obtained from the experiments with blood of the Iran-Iraq War victims represent an accumulation of SSB's due to alkali-labile sites and interstrand crosslinks. In these experiments, blood of the alleged victims was irradiated with 4 Gy and the number of SSB's in the DNA of WBC's as a result of alkali-labile sites, irradiation and crosslinks (masking the SSB's), was compared with the control cells of European volunteers, irradiated with the same dose. Also unirradiated cells of the victims and the European volunteers were examined. A ratio of 1 indicates, that no alkali-labile sites or interstrand crosslinks are detectable, whereas a ratio > 1 indicates an induction of alkali-labile sites and a ratio < 1 an induction of crosslinks. In these experiments both the lymphocytes and the granulocytes were isolated by Percoll-gradient centrifugation. Table 12 shows the results of three experiments (22, 26 and 28 days after exposure), concerning the blood samples of the four alleged victims.

The results show a slight induction of SSB's in the DNA of blood cells of the patients 02 and 03 (both only suffering from lung injury), possibly due to the formation of alkali-labile sites by mustard gas. This is even more pronounced if the amount of SSB's in unirradiated WBC of the victims is compared with the amount of SSB's in unirradiated control cells. The damage in lymphocytes is more severe than in granulocytes. The experiment with blood of these two patients was, however, carried out only once. No more blood was available to repeat the experiment. It is not possible to detect a significant increase of SSBs in the DNA of patients 01 and 04 (with the medical diagnosis: no lung injury, only skin blisters in the pelvis area). Crosslinks are not detected in any patient.

Table 12. The relative number of single-strand breaks (SSB's) in the DNA of lymphocytes and granulocytes from alleged victims of mustard gas exposure during the Gulf War[a]

Patient	Number of days after exposure[b]	Relative amount of SSB's after 4 Gy		unirradiated	
		Lym[c]	Gran[d]	Lym[c]	Gran[d]
01	22	0.99	0.96	0.62	1.21
	26	0.79	0.85	0.75	1.07
	28	1.21	1.22	1.18	1.80
02	22	1.77	1.21	3.04	1.75
03	22	1.23	1.15	2.75	1.94
04	26	0.94	0.83	0.75	0.69
	28	1.15	0.86	2.77	0.78

[a] The amount of SSB's in cells of alleged victims, after 4 Gy ^{60}Co-c-irradiation and the amount in unirradiated cells relative to the amount of SSB's in control cells, not exposed to mustard gas, but irradiated with 4 Gy ^{60}Co-c-irradiation and unirradiated control cells, respectively

[b] The data shown are averages of three independent experiments at each day indicated

[c] Lymphocytes

[d] Granulocytes

The conclusions have to be regarded as very preliminary about the induction of alkali-labile sites or crosslinks by exposure to mustard gas in vivo, or about the persistence of adducts in these experiments, 3-4 weeks after exposure. Without blood of control volunteers, also living in Iran, it is difficult to evaluate a possible induction of SSB's (patients 02 and 03) by mustard gas, because factors such as sunshine, food or exposure to other DNA-damaging agents present in the environment, could influence the outcome of the experiments. However, it may be possible that

alkylation of DNA of blood cells in patients with lung injury is more severe than in patients with skin damage, as a result of a higher diffusion rate of mustard gas into the blood circulation via the respiratory system, than via the skin (patients with blisters).

III.14. Immunological detection of mustard gas adducts to DNA

III.14.1. Competitive Enzyme-Linked Immunosorbent Assay (ELISA)

For the development of immunochemical detection methods of exposure to DNA-damaging agents, an antiserum raised against a suitable DNA-damage is needed. To this end, calf-thymus DNA was treated with 1 mM mustard gas and used as immunogen for immunization of rabbits to obtain a polyclonal antiserum. As immunogen for the last booster immunization, ss-ct-DNA treated with 1 mM mustard gas was used (instead of ds-ct-DNA), which was thought to stimulate, especially, the production of antibodies against the N7-G-HD monoadduct. Two rabbits were immunized and both polyclonal antisera were tested on activity against untreated and mustard gas-treated ss-ct-DNA and in a direct ELISA.

The difference in response against these two DNA-samples reflects the specific affinity for DNA adducts with mustard gas. After the first immunization, the sera of both rabbits were tested and one (W7/10) showed a specific response against DNA treated with mustard gas (Figure 76). With this serum it was possible to start the optimalization of the ELISA. In the ELISA, small reaction vessels, i.e., wells in a plastic microtiter plate, are coated with poly-L-lysine followed by control or mustard gas-treated DNA. Next, the antiserum is added in various dilutions. The binding of antibodies to the immobilized DNA is assayed via attachment of a so-called second antibody that carries a detection enzyme. The aim of the optimalization was to reduce aspecific binding of the antiserum and the conjugated second antibodies to the DNA and the polystyrene wells. The aspecific binding was reduced by blocking free binding places of poly-L-lysine and DNA with gelatine instead of foetal calf serum (FCS), which is normally used in this laboratory, and by using Tween 20 in the various washing steps. FCS could not be used because it increased the background signal. The responses of the antisera against ss-ct-DNA and ss-ct-DNA treated with 1 mM mustard gas after the first, second, and third immunization are shown in Figures 76, 77, and 78, respectively. The results show that the antiserum activity of W7/10 against HD-DNA increased after each immunization, while the response against untreated ss-ct-DNA remained very low, a criterion for the selectivity of the antiserum. In contrast, the antibody response of W6/5 was not increased after the second immunization. Therefore, only rabbit W7/10 was bled.

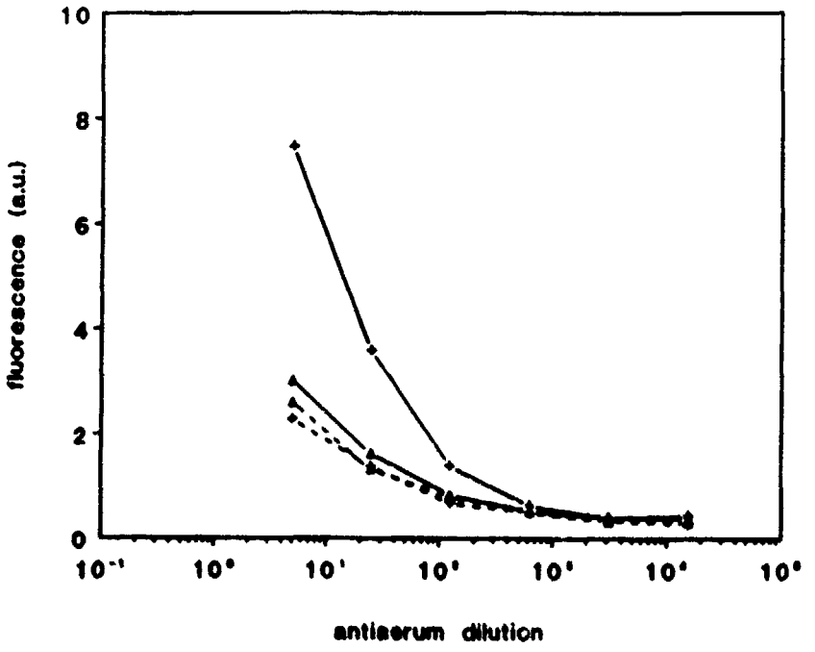

Figure 76. Antibody response observed with the rabbit sera W7/10 and W6/5 in a direct ELISA, two weeks after the first immunization with double-stranded calf-thymus DNA treated with 1 mM mustard gas. The wells were coated with excess single-stranded calf-thymus DNA treated with 1 mM mustard gas (+——+: W7/10; ▲——▲: W6/5) or with untreated single-stranded calf thymus DNA (+---+: W7/10; ▲---▲: W6/5). The antibody molecules bound to the walls were detected with enzyme-conjugated second antibodies on the basis of the enzyme activity

With the W7/10 serum collected after the bleeding, the ELISA was further improved (e.g., by estimating the optimal concentration of mustard gas for treatment of the coating-DNA, the optimal amount of conjugated second antibodies, and the optimal amount of DNA/well).

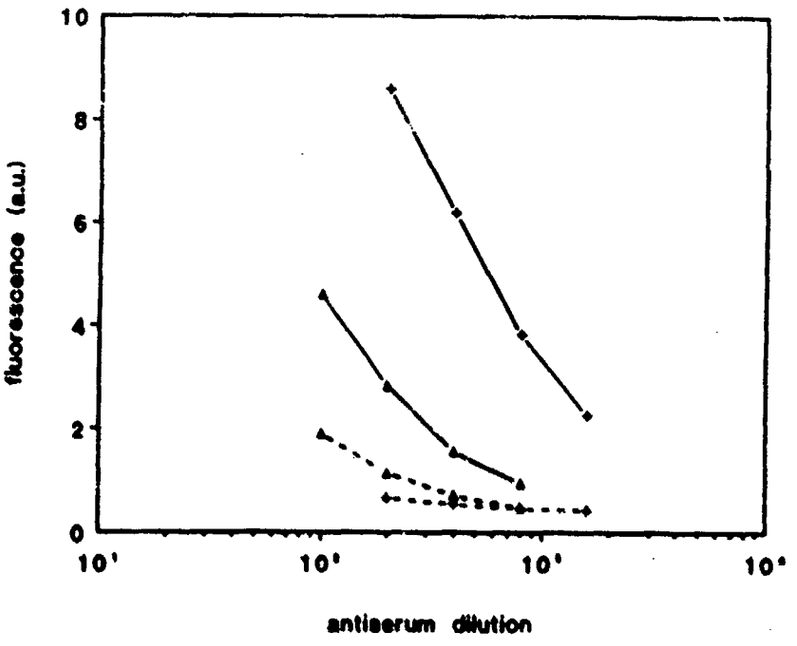

Figure 77. Antibody response observed with the rabbit sera W7/10 and W6/5 in a direct ELISA, two weeks after the second immunization with double-stranded calf-thymus DNA treated with 1 mM mustard gas. The wells were coated with excess single-stranded calf-thymus DNA treated with 1 mM mustard gas (+——+: W7/10; ▲——▲: W6/5) or with untreated single-stranded calf-thymus DNA (+---+: W7/10; ▲---▲: W6/5)

Ss-ct-DNA was treated with 1-1000 μM mustard gas and used to coat to the wells of the 96-well microtiter plates and the serum was tested in three concentrations (1:40,000, 1:80,000 and 1:160,000, Figure 79). In the preceding assays, the serum was only tested on ss-ct-DNA treated with 1 mM mustard gas, but Figure 79 shows that treatment with 100 μM mustard gas resulted in the highest response.

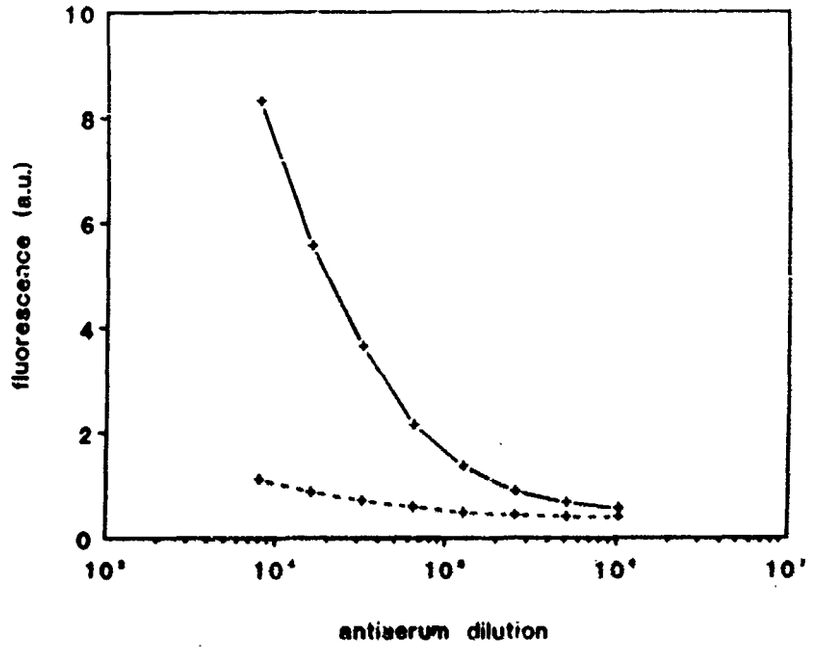

Figure 78. Antibody response observed with rabbit serum W7/10 in a direct ELISA, two weeks after the third immunization, with single-stranded calf-thymus DNA treated with 1 mM mustard gas. The wells were coated with excess single-stranded calf-thymus DNA treated with 1 mM mustard gas (+——+) or with untreated single-stranded calf-thymus DNA (+···+)

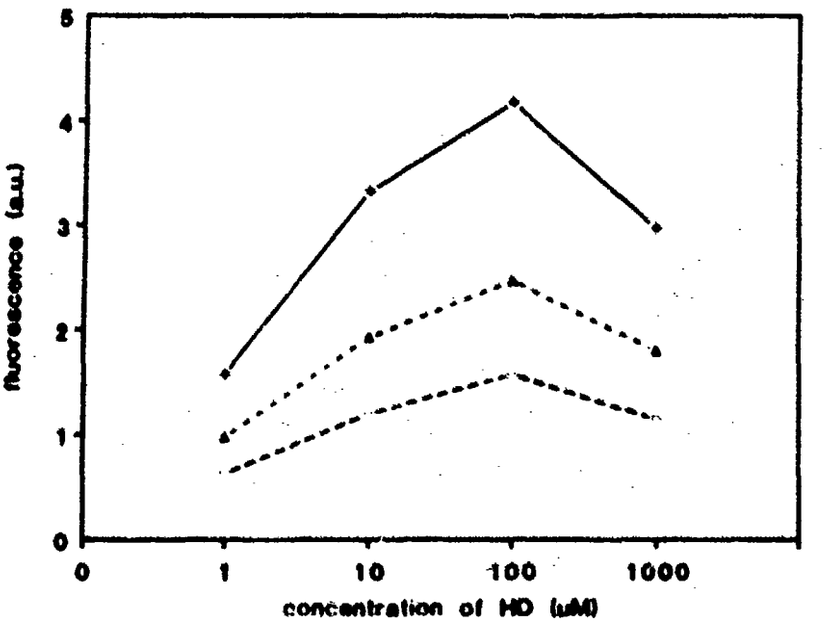

Figure 79. Antibody response observed with rabbit serum W7/10 in a direct ELISA. The wells were coated with excess single-stranded calf-thymus DNA treated with 1-1000 μM mustard gas. Three different serum concentrations were tested: 1:40,000 (+——+), 1:80,000 (▲···▲) and 1:160,000 (o···o)

Another important factor for an optimal detection of the DNA-damage, is the amount of conjugated second antibodies (goat-anti-rabbit-IgG-alkaline phosphatase conjugated: GAR-IgG-AP), which should be in excess over the amount of bound first antibodies. Figure 80 shows the results of different dilutions of GAR-IgG-AP tested on ss-ct-DNA treated with 1 mM mustard gas. Three different dilutions of W7/10 serum were tested (1:8,000, 1:16,000, and 1:32,000). The ELISA signal (fluorescence) increased with the concentration of GAR-IgG-AP, but beyond 1:1,000 hardly any further increase was observed. Therefore, in subsequent experiments the concentration used was 1:1,000

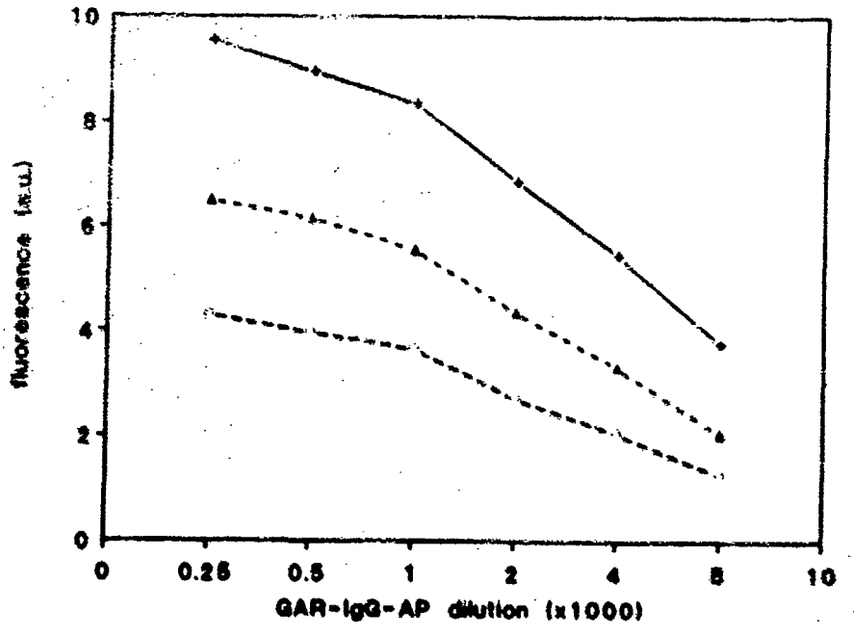

Figure 80. Antibody response observed with rabbit serum W7/10 in a direct ELISA, in which various dilutions were tested of the second antibody, i.e., goat-anti-rabbit-IgG-alkaline phosphatase conjugate (1:250 - 1:8,000). The wells were coated with excess single-stranded calf-thymus DNA treated with 1 mM mustard gas. Three different W7/10 serum concentrations were tested: 1:8,000 (+——+), 1:16,000 (▲- -▲) and 1:32,000 (o···o)

The amount of DNA adducts present in the coating of the wells should be in excess over the amount of antibodies that can bind to these adducts. DNA treated with 100 μM mustard gas was used for the coating at various concentrations (0.1-10 μg/ml) and three different W7/10 serum dilutions were tested (1:20,000, 1,40,000 and 1:80,000), as is shown in Figure 81. At a DNA-concentration of 1 μg/ml (50 ng/well) the maximum fluorescence was reached. When more DNA was added the fluorescence even showed a slight decrease.

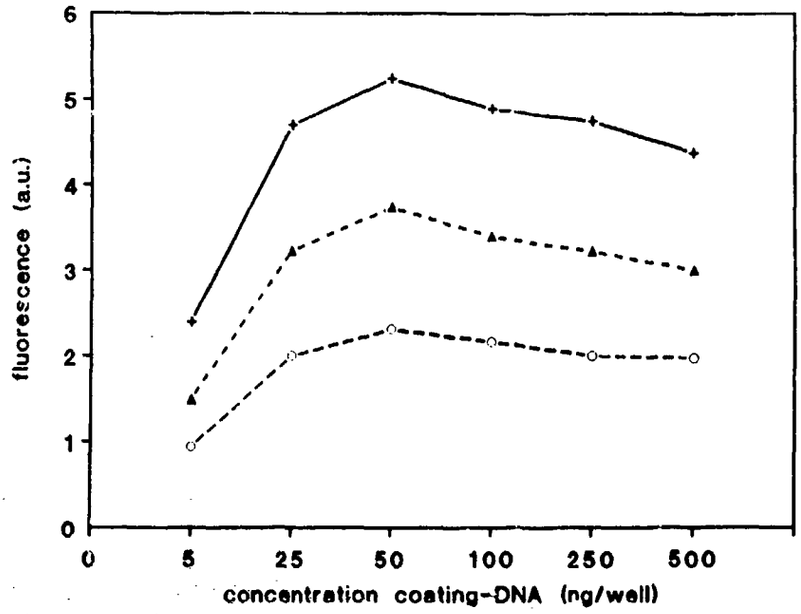

Figure 81. Antibody response observed with rabbit serum W7/10 in a direct ELISA, in which various concentrations of single-stranded calf-thymus DNA treated with 0.1 M mustard gas were used as coating-DNA in the wells. Three different serum concentrations were tested: 1:20,000 (+——+), 1:40,000 (▲---▲) and 1:80,000 (o---o)

With the optimalization of the ELISA, also parameters were determined for the competitive ELISA. In the competitive mode of this assay, a fixed amount of antibodies is incubated with various concentrations of competitor DNA before the solutions are added to the wells. Then, only the antibody molecules not occupied in attachment to the competitor can bind to the coating DNA. These are "back titrated" under the conditions of the direct ELISA. The fixed amount of W7/10 antiserum was selected such that without any competitor material added the ELISA reading would reach a fluorescence level of 30% of the highest attainable level after 2 h of incubation with the substrate. This is the 100% value of the method. A 1:40,000 dilution was chosen.

In the development of the competitive ELISA, untreated ss-ct-DNA and ss-ct-DNA treated with 10 and 100 μM mustard gas were used as competitor. For the coating of the wells, ss-ct-DNA treated with 10 and 100 μM mustard gas was used (50 ng/well). The competition curves are shown in Figure 82. The amounts of competitor DNA needed for a reduction of 50% of the maximal fluorescence signal ("50% inhibition point"), with ss-ct-DNA treated with 10 μM mustard gas as competitor, are 3.1 and 12.5 ng DNA/well on coatings consisting of DNA treated with 10 and 100 μM mustard gas, respectively. With ss-ct-DNA treated with 100 μM mustard gas as competitor, the 50% inhibition points are

0.15 and 0.52 ng DNA/well on coating DNA treated with 10 and 100 μM mustard gas, respectively. These results show that DNA treated with 10 μM mustard gas leads to a higher sensitivity in the competitive ELISA, although DNA treated with 100 μM mustard gas gives a better signal in the direct ELISA. For this reason, ss-ct-DNA treated with 10 μM mustard gas has been selected as coating DNA for the competitive ELISA.

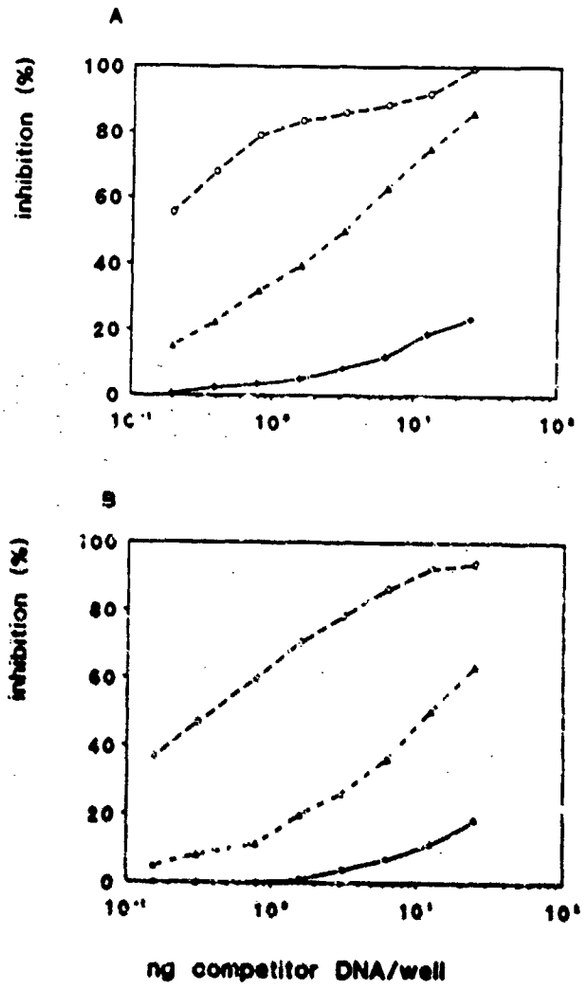

Figure 82. Competitive ELISA with rabbit serum W7/10 and single-stranded calf-thymus DNA treated with 100 μM mustard gas (o---o), 10 μM mustard gas (▲---▲) or untreated DNA (+——+) as the competitor. The wells were coated with an excess of single-stranded calf-thymus DNA treated with 10 μM mustard gas (panel A) or 100 μM mustard gas (panel B)

After optimalization of all parameters, the sensitivity of the competitive ELISA (the amount of mustard gas-adducts detectable per well) could be estimated for this polyclonal antiserum, by using

untreated and mustard gas-treated ss-ct-DNA (0.1, 1 and 10 μM mustard gas) as competitor. The competition curves are shown in Figure 83. The sensitivity derived from these curves is shown in Table 13.

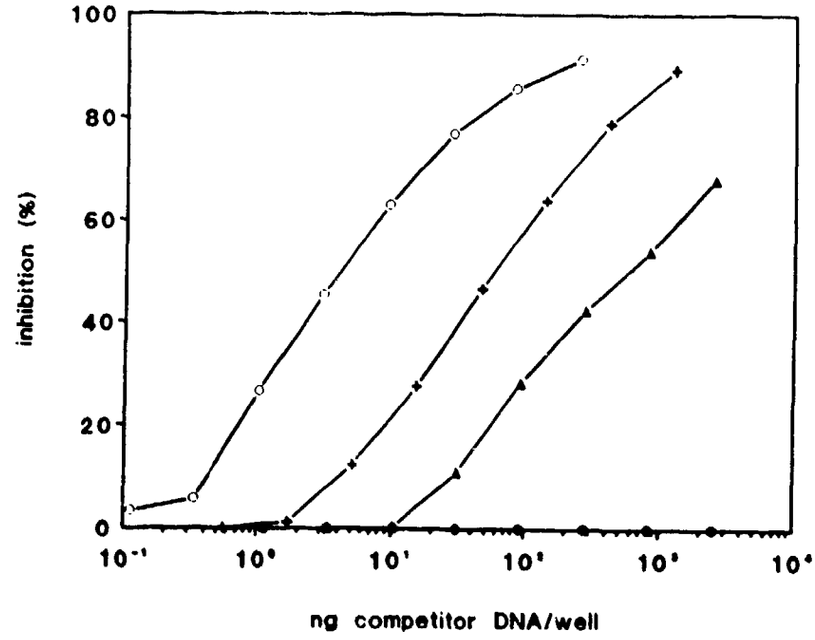

Figure 83. Competitive ELISA with rabbit serum W7/10 and single-stranded calf-thymus DNA as competitor. The competitor DNA was treated with 10 μM (o—o), 1 μM (+—+) or 0.1 μM (▲—▲) mustard gas or was untreated (●—●). The wells were coated with an excess of single-stranded calf-thymus DNA treated with 10 μM mustard gas

As Figure 83 indicates, untreated DNA does not give any inhibition, not even at high doses. The three DNA's show the expected dependence of the amounts needed to obtain 50% inhibition on the adduct content in the DNA. The quantity required increases in proportion to the decrease in adduct content. When expressed as the amount of adducts detected, all three DNA's indicate a sensitivity of a few femtomoles of adduct at the 50% inhibition points. This conclusion is based on the results of the experiments with [^{35}S]mustard gas (confer III.12), which indicate that the major adduct, i.e., the monoadduct N7-G-HD, is expected to be induced in single-stranded DNA to a level of one adduct per 51600 nucleotides per μM of mustard gas (extrapolation from the 100 μM data in Table 11), or 0.065 fmol N7-G-HD per ng DNA. Thus, at the 50% inhibition point 2.4 (10 μM) to 3.8 (0.1 and 1 μM) fmol of this adduct should have been present per well. In practice, when the ELISA is used as a detection assay, often the 20% inhibition point is applied, which increases the sensitivity. This would mean that a positive detection appears possible of an amount of mustard gas-exposed DNA that contains as little as 0.4 fmol N7-G-HD per well.

According to a similar reasoning, the specificity of the method was calculated, i.e., the ratio between the number of N7-G-HD adducts to the number of unmodified nucleotides. Again, these values were estimated by linear extrapolation of the degree of alkylation detected by HPLC in ss-ct-DNA that had been treated with 0.1 mM [^{35}S]mustard gas. This DNA contained 1 N7-G-HD monoadduct per 129 unmodified guanines (Table 13). For competitor ss-ct-DNA treated with 0.01 μM mustard gas, this would mean that 1 N7-G-HD monoadduct can be detected amongst 1,290,000 unmodified guanines or ca. 5,160,000 unmodified nucleotides.

Table 13. Competitive ELISA for the detection of mustard gas adducts in calf-thymus DNA treated with mustard gas, with polyclonal antiserum W7/10

Conc. mustard gas (μM)	Sensitivity N7-G-HD monoadduct at 50% inhi-ition point (fmol/well)	Guanines at 50% inhibition point (pmol/well)	Specificity (N7-G-HD monoadduct/ guanine)
0	-	-	0
0.1	3.8	492	1:129,000
1	3.8	49.2	1: 12,900
10	2.4	3.1	1: 1,290

III.14.2. The detection of mustard gas adducts in calf-thymus DNA and WBC with the competitive ELISA

Effects of alkali treatment and of variations in DNA batches

To detect mustard gas adducts to DNA of white blood cells (WBC), it is necessary to disrupt the cell wall and nuclear membrane to release the DNA, and to optimize the accessibility of the DNA for the antibodies. In a first approach, the DNA was released by treatment of the cells at high pH. In this way the DNA is made single-stranded as well. Since the antiserum was raised against mustard gas-treated single-stranded DNA, it was thought that adducts in single-stranded DNA would be recognized better by the antiserum than those in double-stranded DNA. The WBC from mustard gas-treated blood were incubated in a NaCl-solution, adjusted to pH 12.1, for 30 min at 20 ^{o}C. After sonication, the solution was neutralized. The sonication is performed to procure extensive fragmentation of the DNA while single-stranded, yielding relatively small fragments that will be unable to retrieve the complementary counterparts to re-form double strands. This is particularly relevant in case interstrand crosslinks are expected which are starting places for renaturation after neutralization. In control experiments, mustard gas-treated ss- and ds-ct-DNA was run through the same procedures and tested in the competitive ELISA together with the WBC samples.

Figure 84 shows the competition curves for whole blood treated with 1, 0.1 and 0.01 mM mustard gas (panel A) and ss-ct-DNA treated with

10 and 1 μM mustard gas or untreated (B). Panel B also shows results obtained with alkali-treated and untreated DNA, tested to observe the influence of alkali on the outcome of the assay. However, the ELISA results did not indicate the presence of mustard gas adducts that were recognized by the antibodies, neither with the WBC-DNA (Figure 84A) nor with the mustard gas-exposed ss-ct-DNA treated with alkali

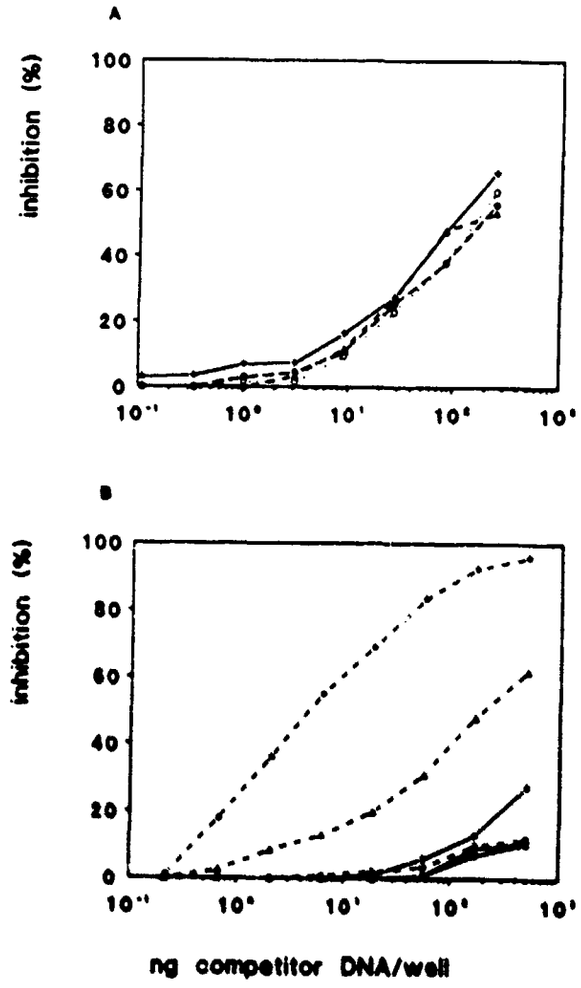

Figure 84. Competitive ELISA with rabbit serum W7/10 and DNA as competitor. Panel A: DNA from human blood; whole blood was treated with 1 mM (+—+), 0.1 mM (●···●) or 0.01 mM (▲···▲) mustard gas or untreated (o···o) and then brought at alkaline pH to release DNA and to induce single-strandedness. Panel B: single-stranded calf-thymus DNA, treated with 10 μM (+···+) or 1 μM (▲···▲) mustard gas and untreated (o···o), before and after exposure to alkaline pH (10 μM mustard gas: +—+; 1 μM mustard gas: ▲—▲; untreated: o—o). The wells were coated with an excess of single-stranded calf-thymus DNA with 10 μM mustard gas

(Figure 84B). In contrast, ss-ct-DNA treated with mustard gas, but not with alkali, did show the expected competition curves (Figure 84B). A possible explanation could be that during the alkali treatment in all N7-G-HD adducts the imidazole ring of the guanine was opened and, as a consequence, the adducts are no longer recognized by the antibodies which were raised with mustard gas-treated DNA having intact guanine ring systems. This ring opening is known to occur with N7-alkylated guanines in alkaline medium. Therefore, methods using alkali probably are unsuitable for the isolation of WBC-DNA for an ELISA to test mustard gas damage with these antibodies. Another remarkable fact is the high response of DNA of untreated WBC and untreated ss-ct-DNA in this experiment. In several other experiments, it appeared that different batches of DNA could lead to different outcomes, so it is important to perform control experiments with the same DNA-batch.

DNA batch from another species

Because of poor reproducibility and varying high background levels in the competitive ELISA when different batches of mustard gas-treated calf-thymus DNA (ct-DNA) were used, we investigated whether these problems could be overcome by using salmon-sperm DNA. Salmon-sperm DNA was purified by phenol extraction and ethanol-precipitation and treated with mustard gas (0.001-10 μM; 37 ^{o}C; 45 min) as described for calf-thymus DNA (see II.11.2). The samples were tested in the competitive ELISA. Disappointingly, the results obtained with this material showed that the sensitivity did not exceed the level reached with the competitive ELISA using the ct-DNA and, again, the reproducibility was poor.

Since no improvement was obtained, it was decided to continue the experiments with ct-DNA and to produce a standard batch of mustard gas-treated ct-DNA (with a known degree of alkylation) to be used as a standard in the competitive ELISA. It was decided to include in each assay the standard batch for calibration purposes. In this way, the amount of alkylation can be calculated in samples exposed to unknown concentrations of mustard gas, and the reproducibility of the method can be checked.

Heating at low ionic strength

In another experiment we tested whether heating at low ionic strength, just above the melting temperature at which the DNA unwinds, will increase the single-strandedness without too much loss of N7-G-HD monoadduct from the DNA by depurination. Therefore, we heated both ss-ct-DNA and ds-ct-DNA at 70, 75 and 80 ^{o}C for 10 min. A significant increase in inhibition in the competitive ELISA was obtained with ds-ct-DNA, particularly after heating at 75 ^{o}C. However, the experiments with ss-ct-DNA revealed about a 50% loss of N7-G-HD at the lowest temperature applied (70 ^{o}C).

Treatment with formamide

Another method to disrupt cell walls and membranes, and to make the DNA single-stranded is to treat the cells in a citrate buffer with 70% formamide at 56 ^{o}C. However, WBC treated with mustard gas

followed by release and denaturation of DNA with formamide, showed results comparable with those of cells treated with alkali (Figure 85). Interference of the medium with the ELISA could be excluded as the explanation for this result on the basis of control studies with mustard gas-treated calf-thymus DNA (Figure 86), where the responses of ss-ct-DNA untreated and treated with the formamide solution were practically the same. It appears possible that the formamide treatment is not capable to degrade the cells completely and to remove the histones from the DNA, which is necessary to make the adducts inside the protein-DNA cluster accessible. Furthermore, it appeared that concentrations of formamide exceeding 2.5% resulted in high background values in the ELISA. Lowering the concentration beneath this level simply by dilution was possible with the treated ct-DNA, but not with the biological samples containing low amounts of DNA.

Attempts failed to precipitate the DNA in the formamide solutions with a high-salt buffer and ice-cold absolute ethanol. In another approach the DNA samples were dialyzed in very small dialysis cups either against demineralized water or PBS. The concentration of DNA was measured and the samples were tested in the ELISA. The experiment showed that formamide can be removed in this way to such an extent that it no longer interferes in the ELISA. In preliminary experiments with various DNA samples, a remarkably high background response of untreated DNA in the ELISA was observed when the samples were dialyzed against PBS, while the background response was low when demineralized water was used. Renewed attempts to remove the formamide by dialysis resulted again in a high background response of untreated DNA, or in poorly reproducible results.

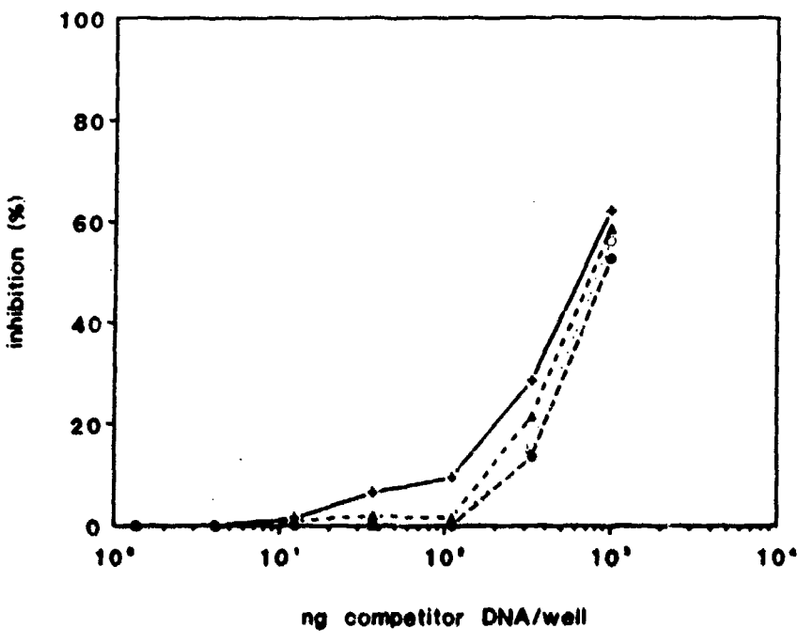

Figure 85. Competitive ELISA with rabbit serum W7/10 and DNA from human blood as competitor. Whole blood was treated with 1 mM (+——+), 0.1 mM (▲···▲) or 0.01 mM (o···o) mustard gas or untreated (●···●). Next, DNA was released and denatured with the 70% formamide buffer. The wells were coated with an excess of single-stranded calf-thymus DNA treated with 10 µM mustard gas

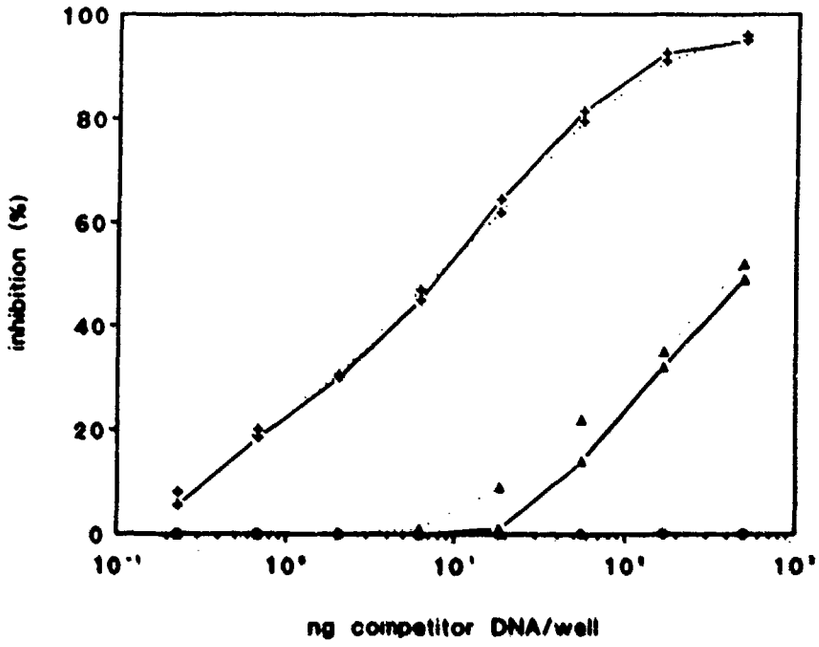

Figure 86. Competitive ELISA with rabbit serum W7/10 and single-stranded calf-thymus DNA as competitor. The DNA was treated with 10 µM (+) or 1 µM (▲) mustard gas or untreated (●) and then tested in the competitive ELISA before (···) or after (——) treatment with the 70% formamide buffer. The wells were coated with an excess of single-stranded calf-thymus DNA treated with 10 µM mustard gas

DNA isolation after lysis with SDS

The fourth procedure attempted was more successful. In this approach the mustard gas-treated blood cells were lysed overnight with 1% SDS (to degrade the cell wall) and proteinase K to degrade the proteins. Next day, the DNA was purified by phenol extraction and ethanol precipitation and then dissolved in PBS. The DNA (still double-stranded) was sonicated and directly thereafter tested in the ELISA. With this double-stranded material, significant competition was obtained.Using this procedure several (control) experiments were carried out. Ss-ct-DNA was used that had been treated with 1 and 10 μM mustard gas (Figure 87). DNA was first isolated from WBC and then treated, either in single-stranded (Figure 88) or in double-stranded form (Figure 89), with 0.1, 1 and 10 μM mustard gas. Also isolated WBC from whole blood (Figure 90) and whole blood (Figure 91) were treated with 1 and 0.1 mM mustard gas and then the DNA was isolated and assayed in the ELISA.

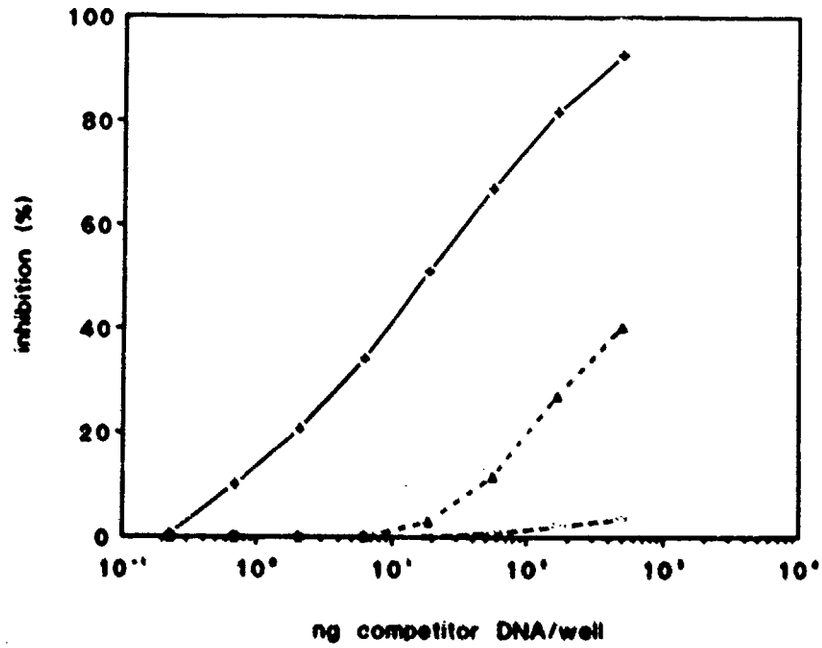

Figure 87. Competitive ELISA with rabbit serum W7/10 and single-stranded calf-thymus DNA as competitor. The DNA was treated with 10 μM (+——+) or 1 μM (▲---▲) mustard gas or untreated (o---o) and then tested in the competitive ELISA. The wells were coated with an excess of single-stranded calf-thymus DNA treated with 10 μM mustard gas

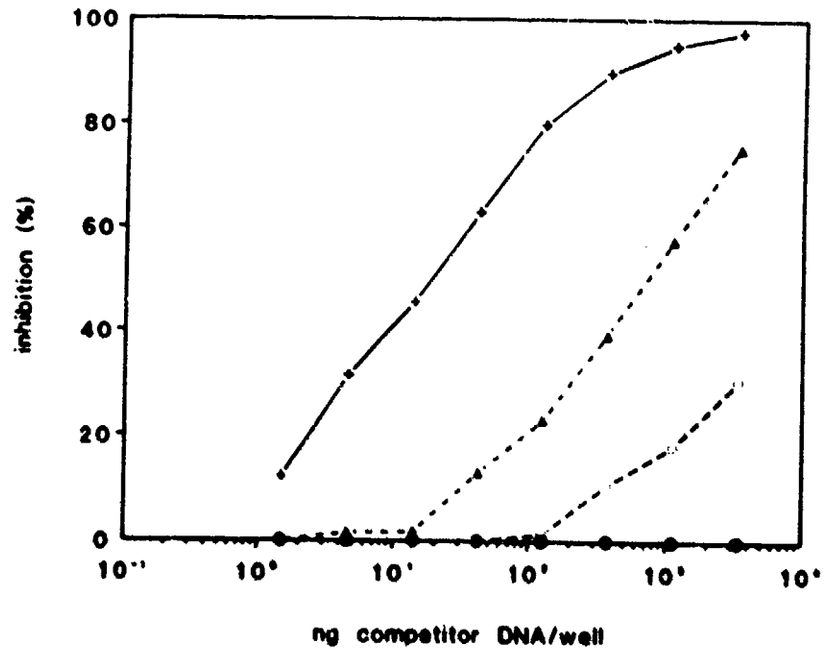

Figure 88. Competitive ELISA with rabbit serum W7/10 and DNA isolated from human white blood cells as competitor. The DNA was isolated by phenol extraction and ethanol precipitation and then, after heating (10 min; 100 °C) to induce single-strandedness, treated with 10 μM (+—+), 1 μM (▲···▲) or 0.1 μM (o···o) mustard gas or untreated (●···●). The wells were coated with an excess of single-stranded calf thymus DNA treated with 10 μM mustard gas

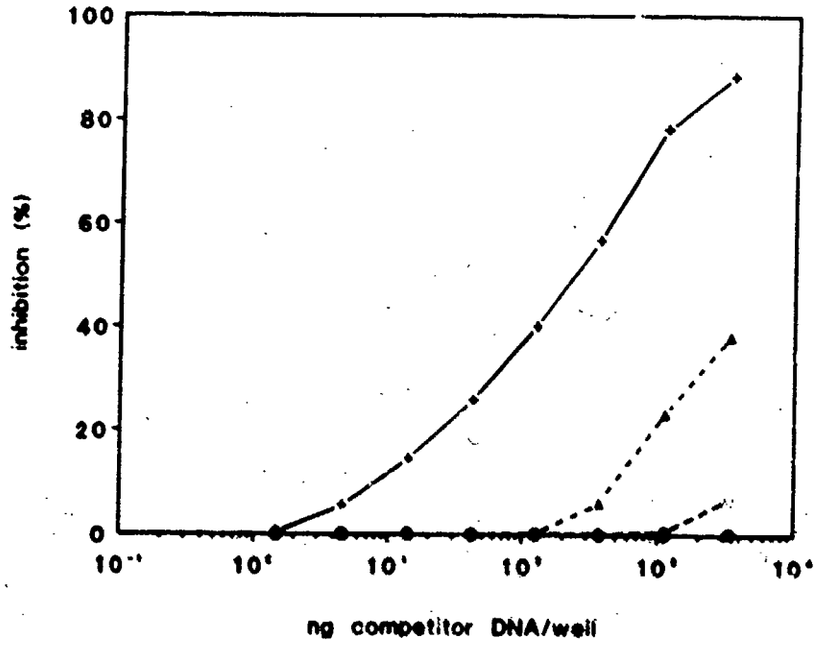

Figure 89. Competitive ELISA with rabbit serum W//10 and DNA isolated from human white blood cells as competitor. The DNA was isolated by phenol extraction and ethanol precipitation and then treated with 10 μM (+—+), 1 μM (▲···▲) or 0.1 μM (o···o) mustard gas or untreated (●···●). The wells were coated with an excess of single-stranded calf-thymus DNA treated with 10 μM mustard gas

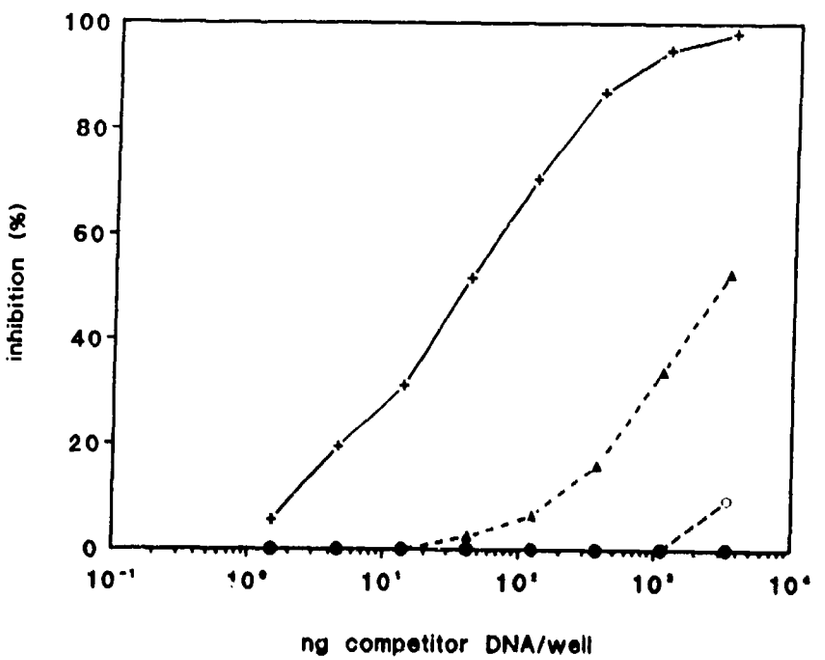

Figure 90. Competitive ELISA with rabbit serum W7/10 and DNA isolated from human white blood cells in as competitor. White blood cells were isolated from whole blood and treated with 1 mM (+——+), 100 μM (▲---▲) or 10 μM (o---o) mustard gas or untreated (●···●). The DNA was isolated by phenol extraction and ethanol precipitation and tested as double-stranded DNA. The wells were coated with an excess of single-stranded calf-thymus DNA treated with 10 μM mustard gas

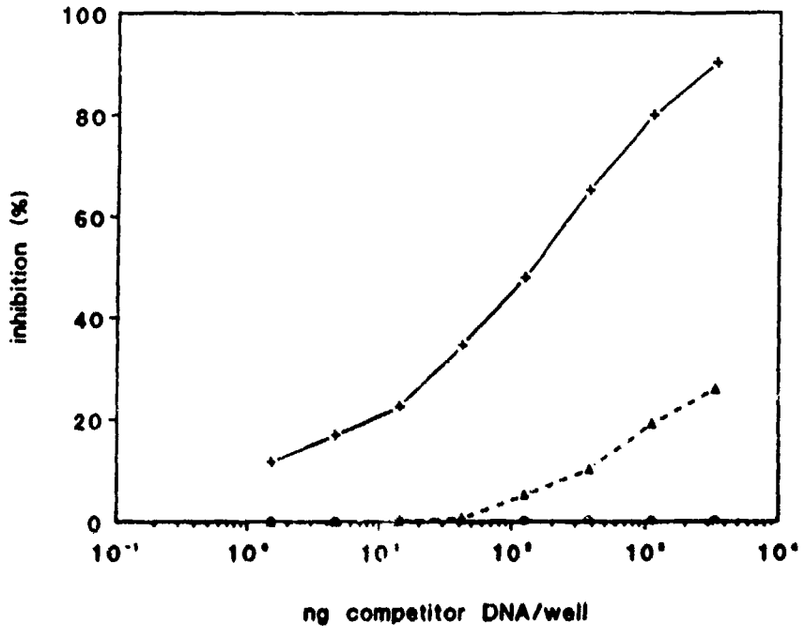

Figure 91. Competitive ELISA with rabbit serum W7/10 and DNA isolated from human white blood cells as competitor. Whole blood was treated with 1 mM (+——+) and 100 μM (▲---▲) mustard gas or untreated (o---o). After isolation of the white cells, DNA was isolated by phenol extraction and ethanol precipitation and tested as double-stranded DNA. The wells were coated with an excess of single-stranded calf-thymus DNA treated with 10 μM mustard gas

The 50% inhibition points of all these samples are shown in Table 14. The amount of N7-G-HD monoadduct present with competitor DNA at the 50% inhibition point for ss-ct-DNA, ds-ct-DNA and DNA from whole blood, has been derived by extrapolation from the data shown in Table 11 for ss- and ds-ct-DNA treated with 0.1 mM [^{35}S]mustard gas and whole blood treated with 1 mM [^{35}S]mustard gas (1 adduct per 129, 75 and 124 unmodified guanines, respectively).

Table 14. N7-(2'-Hydroxyethylthioethyl)-guanine monoadducts (N7-G-HD) after treatment of calf-thymus DNA, DNA of white blood cells, white blood cells and whole blood with mustard gas detected in a competitive ELISA

Sample	Single-/ double-stranded (ss/ds)	Mustard gas concentration (μM)	Amount of N7-G-HD N7-G-HD at 50% inhibition point (fmol/well)	Amount of competitor DNA at 50% inhibition point (ng/well)
Ct-DNA	ss	1	51.7	880[a]
	ss	10	10.5	18
Blood DNA[b]	ss	1	43.2	735
	ss	10	10.5	18
	ds	1	959	9500[a]
	ds	10	234	232
WBC[b]	ds	100	n.m.[c]	3000[a]
	ds	1000	n.m.[c]	38
Whole blood[b]	ds	1000	892	146

[a] Extrapolated
[b] DNA was isolated by phenol extraction and ethanol precipitation, and made single-stranded (if applicable) by heating at 100 °C for 10 min
[c] Not measured

Evidently, when DNA of WBC was isolated, made single-stranded and then treated with 10 μM mustard gas, the amount of competitor DNA at the 50% inhibition point was the same as the amount of similarly treated ss-ct-DNA at its 50% inhibition point. However, the competition curves differ from those pertaining to data shown in Table 13. These differences appear to be related to differences in the DNA batches, which complicates this detection method.

In these experiments, the shift in the inhibition curve when the treatment concentration of mustard gas was varied, was not always in proportion to the concentration change. For ss-DNA treated with 10 and 1 μM mustard gas the curve shifted by a factor of about 40, i.e., 40 x more DNA was needed after exposure to 1 μM mustard gas to obtain a comparable extent of inhibition. This was found for ss-ct-DNA as well as for ss-WBC-DNA (Figures 87 and 88). With ss-WBC-DNA a tenfold lower concentration of mustard gas (0.1 μM) was also tested, which resulted in a still detectable inhibition (Figure 88) although 50% inhibition was not reached. In this case, however, the shift appeared to correspond rather well to the concentration differences.

With the double-stranded DNA a similar phenomenon was observed (Figure 89). After exposure to 10 μM mustard gas, the amount of

competitor DNA in the 50% inhibition point was 232 ng/well, whereas at the tenfold lower concentration about fortyfold more DNA was needed to achieve the same extent of competition. A comparison of the data in Table 14 demonstrates that after exposure to the same concentration of mustard gas much more double-stranded DNA is required for effective competition than mustard gas-treated single-stranded DNA. At the 50% inhibition point the difference amounts to a factor of 13. The DNA isolated from WBC and whole blood treated with mustard gas, was tested in the ELISA as double-stranded material. The amount of competitor DNA at the 50% inhibition point of DNA derived from samples of WBC treated with 1 mM mustard gas was 38 ng/well, while the amount of competitor DNA from samples of whole blood treated with 1 mM was 146 ng/well (Figures 90, 91 and Table 14). Assuming that 1 out of 124 guanines is alkylated to form a N7-G-HD monoadduct when blood is exposed to 1 mM mustard gas (based on HPLC analyses on [^{35}S]mustard gas treated whole blood), 892 fmol N7-G-HD monoadduct/well is detected in the DNA present in the double-stranded form at the 50% inhibition point.

Test on single-strandedness after various denaturation treatments

Since adducts of mustard gas appeared to be better recognized in single- than in double-stranded DNA, the poor results obtained with the material from cells treated with alkali or formamide to liberate DNA, might be due to insufficient single-strand character. For that reason, a[illegible] ct-DNA samples treated with alkali and formamide were tested on single-strandedness. In these tests, monoclonal antibodies which specifically recognize single-stranded DNA (D1B) were used. The amount of competitor DNA/well needed to reach the 50% inhibition point is summarized in Table 15. Untreated, single-stranded ct-DNA was taken as the 100% point and double-stranded ct-DNA, treated with 1 µM mustard gas, as the 0% point, assuming that no single-stranded DNA is present (arbitrary 0% point).

These results show that in ss-ct-DNA the percentage single-strandedness ranges from 95-100. The three ds-ct-DNA samples range from 0 to 15%. It can be concluded that alkali treatment of double-stranded DNA induced 100% single-strandedness, while formamide did so for about 90%. Evidently, the failure to detect with the competitive ELISA mustard gas-DNA adducts in DNA treated with alkali or formamide cannot be attributed to lack of single-strandedness. When alkali is used to disrupt the cells, the opening of the imidazolium ring in the N7-guanine adducts is induced, which destroys the affinity of the W7/10 antibodies for the mustard gas adduct. The reason for the absence of a response in the ELISA in case of formamide treatment is not clear. Possible explanations have been suggested above. This question has not been pursued further since the fourth method appeared to give good results.

Table 15. The amount of single-strandedness in single- and double-stranded calf-thymus DNA exposed to mustard gas and treated with alkali or formamide

Sample	Concentration mustard gas (μM)	Treatment alkali/ formamide	DNA at 50% inhibition point (ng/well)	Single-strandedness (percentage)
Ss-ct-DNA	0	-	0.24	100[a]
	1	-	0.30	98
	10	-	0.24	100
	0	alkali	0.45	95
	1	alkali	0.34	97
	10	alkali	0.30	98
	0	formamide	0.36	97
	1	formamide	0.27	99
	10	formamide	0.36	97
Ds-ct-DNA	0	-	3.6	15
	1	-	4.2	0[a]
	10	-	3.6	15
	0	alkali	0.23	100
	1	alkali	0.31	98
	10	alkali	0.31	98
	0	formamide	0.77	87
	1	formamide	0.62	90
	10	formamide	0.53	93

[a] By definition

Optimalization of the immunochemical assay to detect N7-guanine monoadducts in DNA

In the attempts to optimize the ELISA on DNA from mustard gas-treated blood, the effect of unwinding the DNA was studied further. Treatment of mustard gas-exposed ds- and ss-ct-DNA at low concentration of formamide (4.1%) plus 0.2% formaldehyde in combination with a low ionic strength (0.01 M Tris, 1 mM EDTA) to induce single-strandedness resulted in an even stronger inhibition with the ds-ct-DNA than with ss-ct-DNA (Table 16). This is in agreement with our observation that, after treatment at the same concentration of mustard gas, the amount of N7-G-HD monoadduct induced in ds-ct-DNA is about twice as high as that in ss-ct-DNA (see III.12, Table 11). Evidently, this procedure is very effective without significant interference in the ELISA.

Table 16. Effect of treatment with formamide (fd) with or without formaldehyde (f), at low ionic strength, on the inhibition with mustard gas-treated double- and single-stranded calf-thymus DNA in the competitive ELISA. The 50% inhibition points (ng DNA/well) are presented[a]. The wells were coated with poly-L-lysine and ss-ct-DNA treated with 10 μM mustard gas; rabbit antiserum W7/10 was used.

Concentration mustard gas (μM)	ds-ct-DNA			ss-ct-DNA		
	4.1% fd + 0.2% f	4.1% fd	without treatment	4.1% fd + 0.2% f	4.1% fd	without treatment
0	(46%)	(43%)	(46%)	(42%)	(26%)	(26%)
0.01	nd[b]	nd	nd	(47%)	(28%)	(34%)
0.1	146	256	(48%)	294	(41%)	824
1	26	83	408	113	211	139
10	15	43	120	56	82	57

[a] When 50% inhibition was not reached, the % inhibition at 2500 ng DNA/well is presented in parentheses.
[b] nd: not done.

The sensitivity of the assay could be further increased by a fivefold decrease in the concentration of the antibodies, combined with a subsequent longer incubation period with substrate (24 h at 20 °C). Figure 92 shows the results of this experiment. It is evident that exposure of ss-ct-DNA to 0.01 μM mustard gas is detectable. Figure 93 shows the effect of the antiserum dilution on the 50% inhibition point when ss-ct-DNA treated with various concentrations of mustard gas is used as competitor. It is clearly shown that less DNA/well is required to obtain 50% inhibition at higher antiserum dilutions.

When the problem to make the ds-DNA single-stranded without destroying the N7-guanine monoadduct or interfering with the detection appeared to have been solved, the improvements introduced in the immunochemical assay for ds-ct-DNA were applied on DNA isolated from white blood cells in blood exposed to mustard gas. Whereas in earlier experiments only exposure of whole blood to ≥ 100 μM mustard gas was detectable (see Figure 91), now the lower detection limit was found to be ca. 2 μM mustard gas.

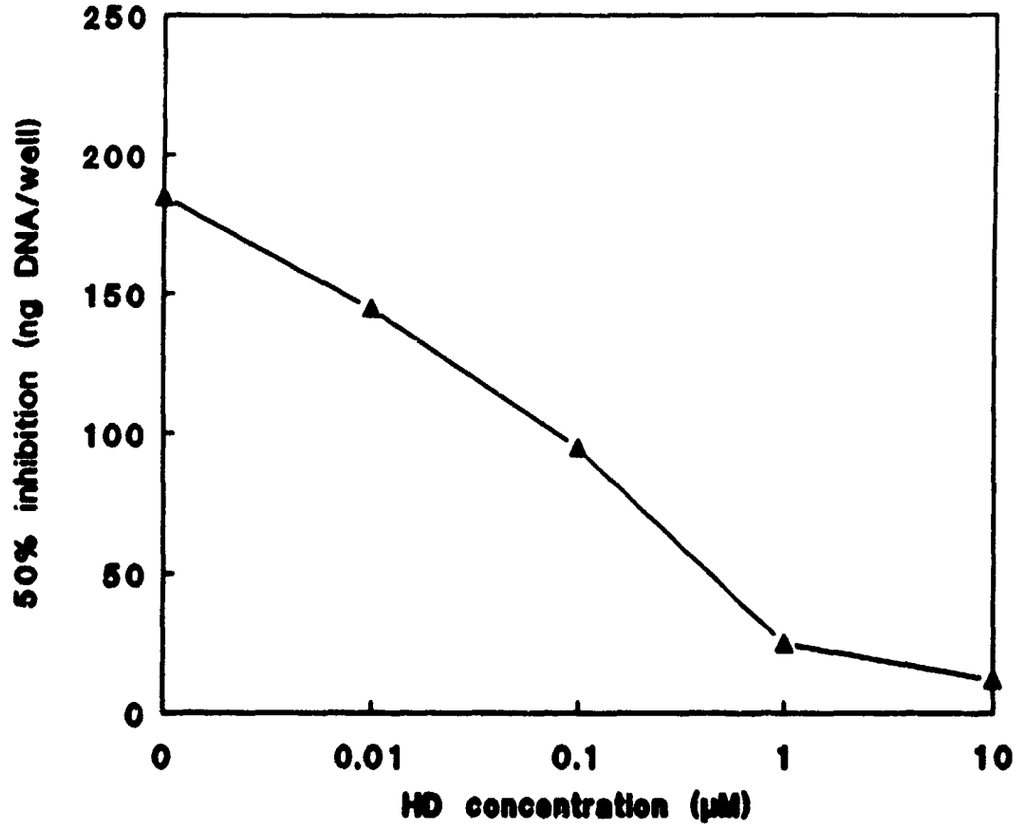

Figure 92. The effect of the concentration of mustard gas to which single-stranded calf-thymus DNA had been exposed on the 50% inhibition point (ng DNA/well) in a competitive ELISA with W7/10 rabbit serum (200,000x diluted). The wells were coated with poly-L-lysine and ss-ct-DNA treated with 10 μM mustard gas

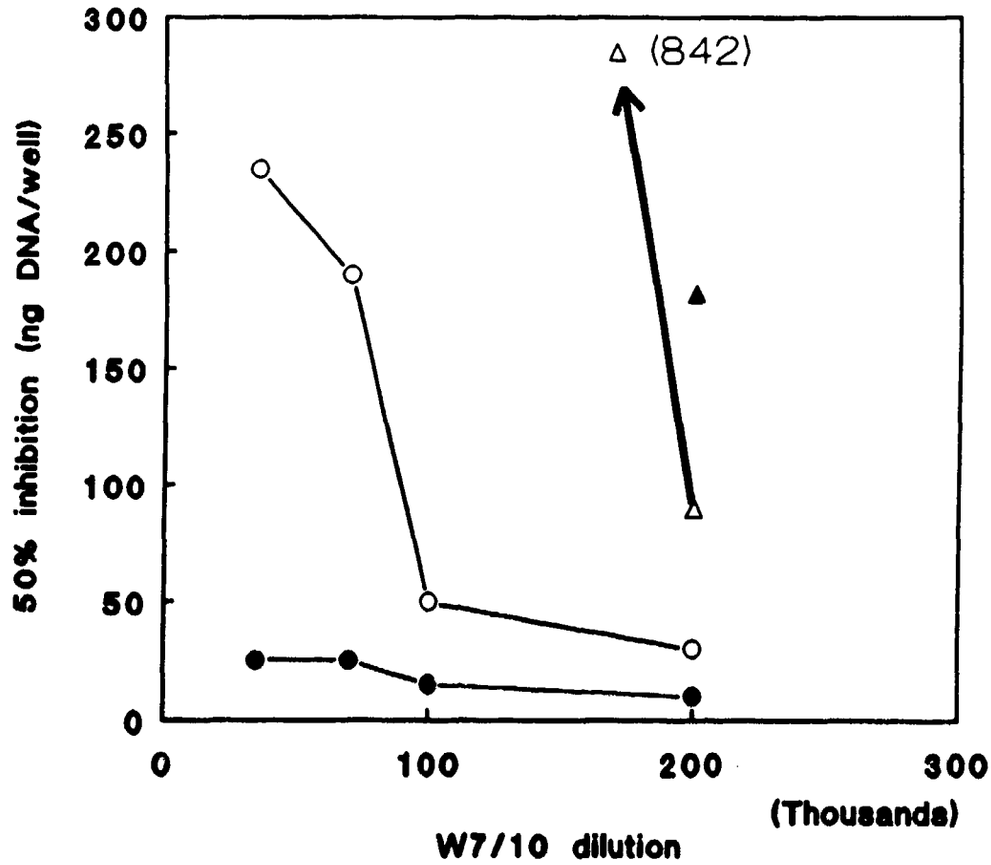

Figure 93. The effect of W7/10 rabbit serum dilution on the 50% inhibition point (ng DNA/well) in a competitive ELISA with single-stranded calf-thymus DNA treated with 0 (▲), 0.1 (△), 1 (o) or 10 (●) μM mustard gas. The wells were coated with poly-L-lysine and ss-ct-DNA treated with 10 μM mustard gas

<u>Calibration of the immunochemical assay for the detection of N7-guanine monoadducts in DNA</u>

One additional control was introduced to check whether certain variations in the results obtained might be attributed to the use of different mustard gas preparations. We have prepared a batch of ct-DNA treated with newly synthesized ^{35}S-labeled mustard gas at various concentrations in order to obtain absolute standards for the level of mustard gas modifications in a treated DNA sample. These samples, of which the amount of bound radioactivity per μg DNA had been determined, were used for calibration of the competitive ELISA on the basis of the known specific radioactivity of the ^{35}S used. Reassuringly, with this new batch of [^{35}S]mustard gas the same amount of N7-G-HD monoadduct was induced in ds-ct-DNA per μg DNA (Table 17) as with the same concentration of mustard gas from the [^{35}S]mustard gas batch mentioned in Table 11.

Table 17. Amount of N7-guanine monoadduct present in double-stranded calf-thymus DNA after treatment with [^{35}S]mustard gas at 37 °C, pH 7.4, for 1 h

Mustard gas concentration (μM)	N7-guanine monoadduct[a] (mmol/mmol guanine)
1	1:6,250
10	1: 787
100	1: 79

[a] Values based on the specific radioactivity of the ^{35}S used and the amount of ^{35}S present in the relevant peak of N7-guanine monoadduct in the HPLC chromatogram of a hydrolysate of the mustard gas-treated DNA (see Table 10).

When applied in the competitive ELISA, these samples gave results comparable to those of ct-DNA samples treated with equal concentrations of unlabelled mustard gas (Table 18). It was decided that the batch of ds-ct-DNA treated with unlabelled mustard gas was suitable to be used in future experiments for calibration.

Table 18. Competitive ELISA for the detection of N7-guanine monoadducts (N7-G-HD) in unwound double-stranded calf-thymus DNA treated with either [^{35}S]mustard gas or unlabelled mustard gas[a]. The 50% inhibition points (ng DNA/well) are presented[b]

Concentration mustard gas (μM)	N7-G-HD at 50% inhibition point (fmol/well)	Amount of competitor DNA at 50% inhibition (ng/well)	
		^{35}S-HD-DNA	HD-DNA
0		(20%)	(10%)
0.01			(30%)
0.1	19	1290	1100
1	13	96	180
10	7	7	9
100		< 1[c]	

[a] Just before the ELISA the DNA was made single-stranded by heating for 25 min at 52 °C in 0.01 M Tris, 1 mM EDTA, 4.1% formamide and 0.2% formaldehyde.

[b] When 50% inhibition was not reached, the % inhibition at 2500 ng DNA/well is presented in parentheses.

[c] 70% inhibition at 1 ng DNA/well.

Comparison with [^{14}C]mustard gas-treated DNA samples

In another experiment we used for calibration ct-DNA treated with ^{14}C-labelled mustard gas at various concentrations, made available by Dr Yaverbaum (USAMRICD). These samples contain ds- or ss-ct-DNA

treated with 0, 1.42, 14.2 or 142 μM [^{14}C]mustard gas (calculated on the basis of Dr Yaverbaum's experimental data). We determined the radioactivity per mg DNA of the various samples and compared these values with those given in the report that accompanied the samples (Table 19).

Table 19. Specific radioactivity and amount of N7-guanine monoadduct (N7-G-HD) for double- and single-stranded calf-thymus DNA samples treated with [^{14}C]mustard gas

Preparation code[a]	DNA (dpm/mg) (report)	(our data)	N7-G-HD (mmol/mmol guanine)
ds-DNA-142	49,809	128,000	36 x10^{-4}
ds-DNA-14.2	3,226	10,800	3.1 x10^{-4}
ds-DNA-1.42	95	1,000	0.28x10^{-4}
ss-DNA-142	146,292	162,000	46 x10^{-4}
ss-DNA-14.2	14,902	16,800	4.8 x10^{-4}
ss-DNA-1.42	2,007	2,900	0.8 x10^{-4}

[a] The numbers refer to the concentration mustard gas (μM) by which DNA was treated.

From the ^{14}C-content per mg DNA according to our determinations and the specific radioactivity of the [^{14}C]mustard gas used (423 MBq/mmol mustard gas, according to Dr Yaverbaum's report), the number of N7-G-HD monoadduct per milligram of DNA was calculated and subsequently expressed as the molar ratio to total guanine. In this calculation it was assumed that 60% of the DNA-bound radioactivity represented this monoadduct, in accordance with our results presented in Table 10. When we compare the resulting adduct levels (Table 19) with the data in Table 17 obtained with [^{35}S]-mustard gas, after correction for the differences in exposure concentration, a substantial discrepancy is seen, in particular for the ds-DNA. For this DNA, the ^{14}C-data are ca. 6- to 7-fold lower than the ^{35}S-results. For the ss-DNA the difference is only 2-fold lower. (For the latter comparison, the values of Table 17 were halved to obtain the data for ss-DNA, in view of its lower reactivity.) The reason for this discrepancy is as yet unknown.

The samples were also tested in the competitive ELISA. In Table 20, the results are presented. From these data it is clear that adducts can be detected in the [^{14}C]mustard gas-treated DNA samples. The amounts of N7-G-HD monoadduct present at the 50% inhibition point appear rather high, however, when compared with the corresponding data obtained with our [^{35}S]mustard gas-treated DNA samples.

Table 20. Competitive ELISA for the detection of N7-guanine monoadducts (N7-G-HD) in single-stranded and unwound double-stranded calf-thymus DNA treated with [^{14}C]mustard gas[a]. The 50% inhibition points (ng DNA/well) are presented[b]. As a control, ss-ct-DNA treated with unlabelled mustard gas (0, 0.1, 1 and 10 μM has been included in the assay

Preparation code[c]	Amount of competitor DNA at 50% inhibition (ng/well)	N7-G-HD monoadduct[d] at 50% inhibition point (fmol/well)
ds-DNA-142	21	63
ds-DNA-14.2	249	64
ds-DNA-1.42	(40%)	
ds-DNA-A	(0%)	
ss-DNA-142	10.5	40
ss-DNA-14.2	832	333
ss-DNA-1.42	(38%)	
ss-DNA-A	(0%)	
ss-ct-DNA-10	10	
ss-ct-DNA-1	160	
ss-ct-DNA-0.1	1600	
ss-ct-DNA-0	(15%)	

[a] Just before the ELISA double-stranded DNA was made single-stranded by heating for 25 min at 52 °C in 0.01 M Tris, 1 mM EDTA, 4.1% formamide and 0.2% formaldehyde.

[b] When 50% inhibition was not reached, the % inhibition at 2500 ng DNA/well is presented.

[c] The numbers refer to the concentration mustard gas (μM) by which DNA was treated. DNA-A refers to Dr Yaverbaum's untreated control ct-DNA.

[d] According to the values in Table 19.

Since it was uncertain whether the DNA had suffered some degradation before it reached us, we also tested these samples after an additional purification by precipitation with alcohol (Tables 21 and 22).

The purification greatly reduced the specific radioactivity of the DNA preparations. Apparently, the additional purification resulted in a considerable loss of ^{14}C and a proportionally much smaller loss of nucleic acid. In the ds-ct-DNA samples, the reduction in specific radioactivity was by ca. 80%, while the ss-ct-DNA showed some 60% reduction. This means that the adduct content of these purified DNA samples, which dropped accordingly, disagreed even more strongly with the results obtained from DNA treated with [^{35}S]mustard gas. For the ds-ct-DNA the discrepancy in the molar ratio of monoadduct to guanine increased ca. 30-fold, for the ss-ct-DNA 4- to 5-fold. However, when

the purified DNA's were tested in the competitive ELISA (Table 22). the resulting amounts of adduct at the 50% inhibition point were in the same range as those obtained with our ^{35}S-labeled samples (Table 18).

Table 21. Specific radioactivity and amount of N7-guanine monoadduct/mmol guanine for double- and single-stranded calf-thymus DNA samples treated with [^{14}C]mustard gas, followed by an additional precipitation with alcohol

Preparation code[a]	dpm/mg DNA	N7-G-HD (mmol/mmol guanine)
ds-DNA-142	23,600	6.7 $x10^{-4}$
ds-DNA-14.2	1,900	0.54 $x10^{-4}$
ds-DNA-1.42	300	0.08 $x10^{-4}$
ss-DNA-142	70,000	20 $x10^{-4}$
ss-DNA-14.2	8,200	2.3 $x10^{-4}$
ss-DNA-1.42	800	0.23 $x10^{-4}$

[a] The numbers refer to the concentration mustard gas (μM) by which DNA was treated.

Table 22. Competitive ELISA for the detection of N7-guanine monoadducts (N7-G-HD) in single-stranded and unwound double-stranded calf-thymus DNA treated with [^{14}C]mustard gas, after an additional precipitation with alcohol[a]. The 50% inhibition points (ng DNA/well) are presented[b]

Preparation code[c]	Amount of competitor DNA at 50% inhibition (ng/well)	N7-G-HD at 50% inhibition point[d] (fmol/well)
ds-DNA-142	7	4
ds-DNA-14.2	250	11
ds-DNA-1.42	(45%)	
ds-DNA-A	(0%)	
ss-DNA-142	6.7	11
ss-DNA-14.2	280	54
ss-DNA-1.42	1520	29
ss-DNA-A	(10%)	

[a] Just before the ELISA assay double-stranded DNA was made single-stranded by heating for 25 min at 52 °C in 0.01 M Tris, 1 mM EDTA, 4.1% formamide and 0.2% formaldehyde.
[b] When 50% inhibition was not reached, the % inhibition at 2500 ng DNA/well is presented.
[c] The numbers refer to the concentration mustard gas (μM) by which DNA was treated.
[d] According to the values in Table 21.

III.14.3. Cell-ELISA

One of the goals of this project is the development of an immunochemical method to detect damage due to exposure to mustard gas in DNA of WBC or skin biopsies on the single-cell level, by immunofluorescence microscopy. In the ultimate procedure aimed at, monoclonal antibodies will be used for this application. The development of such antibodies implies the screening of hybridoma clones with respect to the properties of the antibody molecules they produce. In order to select the proper clones, it is important to use screening procedures that resemble as much as possible the conditions of the eventual practical application of the antibodies. For that reason, a screening method had to be developed mimicking the single-cell detection conditions, a so-called cell-ELISA. In the developmental stage, the polyclonal antiserum W7/10 was used.

WBC were processed in the same way as they should be for immunofluorescence microscopy, but they were attached to wells of microtiter plates instead of on object glasses (as described in II.3.9.3). WBC isolated from blood exposed for 1 h to 1 or 0.1 mM mustard gas, were applied ($4x10^4$ per well) and treated with RNAse followed by 70% formamide (at 56 ^oC), or in NaOH in 70% ethanol (at room temperature) and washed with ethanol. As a control, mustard gas-exposed cells without any treatment were used. After a subsequent proteinase K incubation (in order to digest proteins) the antiserum W7/10 was added in various dilutions (50 to 1.10^6 x). Detection of the antibody molecules attached to the DNA was performed with the second antibody goat-anti-rabbit-IgG-alkaline phosphatase. The substrate was 4-nitrophenyl phosphate (PNP) or 4-methylumbelliferyl phosphate (MUP). The results of the cell-ELISA are shown in Figures 94, 95, and 96.

As expected, untreated cells (Figure 94) and cells treated with alkali (Figure 95) did not show mustard gas-damage specific binding of W7/10. The damage due to mustard gas is not accessible for antibodies when the cells are not treated with cell-disrupting agents, such as alkali or formamide. Though alkali disrupts the cell-wall, it also induces ring-opening of the guanine adducts, so these adducts will not be recognized by the antibodies. When the cells were treated with formamide (Figure 96) a high response was found, but only for cells treated with 1 mM mustard gas. In this application, the W7/10 serum is not as sensitive as in the competitive ELISA, because at a dilution factor of 10,000, only a tenfold higher response can be seen with WBC from blood treated with 1 mM mustard gas than with WBC from untreated blood. At a dilution factor of 40,000 (dilution factor of the W7/10 serum used in competitive ELISA's) no significant difference in response could be detected, whereas in the competitive ELISA with the corresponding blood samples no inhibition was found with untreated blood, while 50% inhibition was found at 0.15 µg DNA/well with treated blood (1 mM mustard gas). It was concluded that this system would be suitable for the screening of monoclonal antibodies, by using WBC from whole blood treated with 1 mM mustard gas and disruption of the cell-walls with formamide.

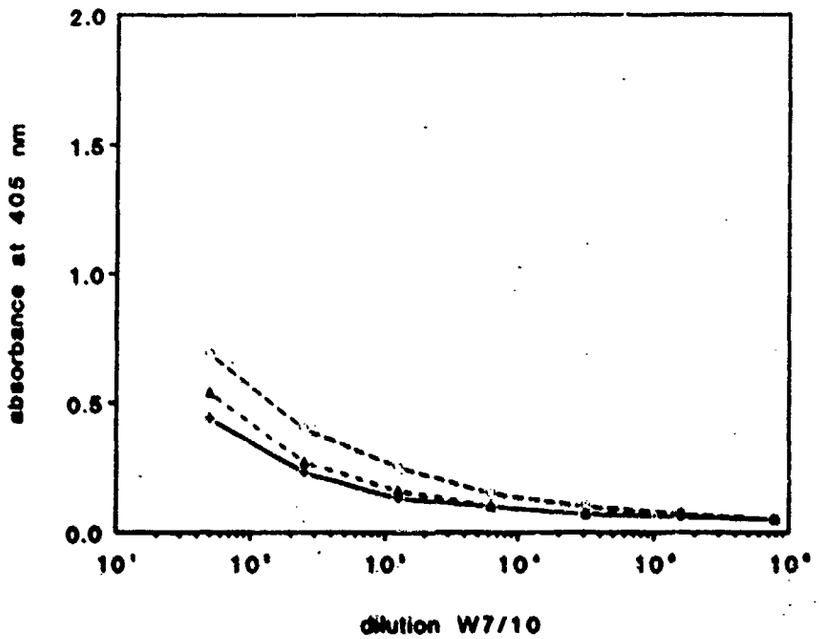

Figure 94. Antibody response of rabbit serum W7/10 against mustard gas-treated human white blood cells in a cell-ELISA. Human blood was treated with 1 mM (o---o) or 0.1 mM (▲---▲) mustard gas or untreated (+——+). The white blood cells were isolated and used to coat the wells (40.000 cells/well). After RNA-digestion and protein degradation, various W7/10 dilutions were tested in the same way as in a direct ELISA

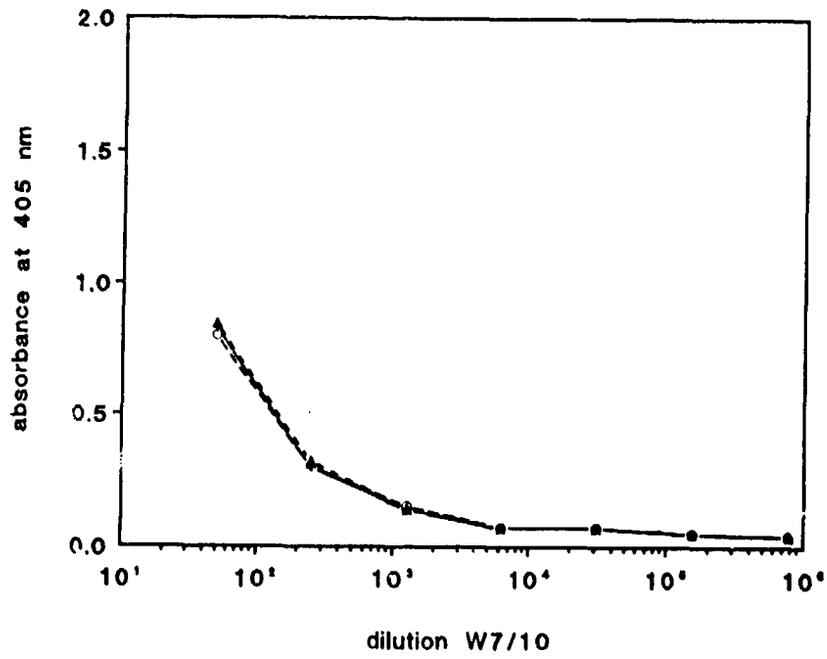

Figure 95. Antibody response of rabbit serum W7/10 against mustard gas-treated human white blood cells in a cell-ELISA. Human blood was treated with 1 mM (o---o) or 0.1 mM (▲---▲) HD or untreated (+——+). The white blood cells were isolated and used to coat the wells (40,000 cells/well). After RNA-digestion the cells were treated with alkali to disrupt the cells and to denature the DNA. After subsequent protein degradation, various W7/10 dilutions were tested in the same way as in a direct ELISA

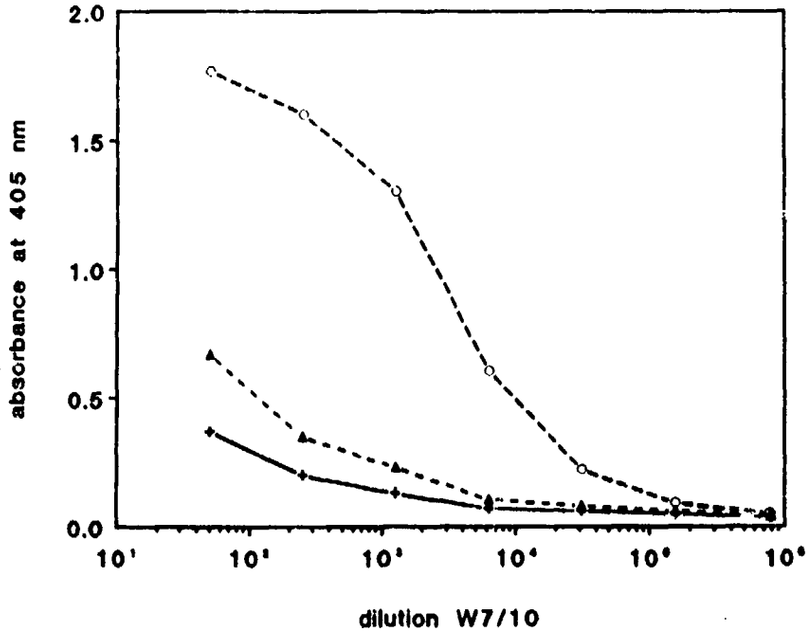

Figure 96. Antibody response of rabbit serum W7/10 against mustard gas-treated human white blood cells in a cell-ELISA. Human blood was treated wiht 1 mM (o---o) or 0.1 mM (▲---▲) mustard gas or untreated (+——+). The white blood cells were isolated and used to coat the wells (40,000 cells/well). After RNA-digestion the cells were treated with a 70% formamide buffer to disrupt the cells to denature the DNA. After subsequent protein degradation, different W7/10 dilutions were tested in the same way as in a direct ELISA

III.14.4. Monoclonal antibodies against N7-(2'-hydroxyethylthio-ethyl)-guanosine

For the development of monoclonal antibodies with specificity for the major mustard gas-adduct in DNA, mice have to be immunized with a proper antigen. The immunogen prepared for this purpose was the product of the reaction of mustard gas with guanosine-5'-phosphate (GMP) coupled to a carrier protein. The use of such a protein was required since in general small molecules will not elicit an efficient immune response. After synthesis and characterization of N7-(2"-hydroxyethylthioethyl)-guanosine 5'-phosphate (GMP-7-HD; see III.6), this adduct was coupled to the protein Keyhole Limpet

Hemocyanine (KLH), with periodate or 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC). As a control for the coupling reaction and in order to screen the antisera for the presence of anti-adduct activity, coupling products of GMP and GMP-7-HD to bovine serum albumin (BSA) were also prepared, which were used in the direct ELISA. BSA was used instead of KLH to prevent a high anti-KLH background in the assay due to antibodies raised against determinants of that protein.

Periodate coupling

In this procedure, the carbon atoms bearing two vicinal hydroxyl groups in the sugar moiety of guanosine are oxidized to aldehyde functions with rupture of the C-C bond, followed by condensation with amino groups in the protein. UV spectra were taken of KLH, BSA, GMP- and GMP-7-HD and of the protein products after the coupling (Figure 97). Four different ratios adduct:protein (w/w; 1:1, 1:10, 1:25 and 1:100) were applied for the coupling of GMP or GMP-7-HD to KLH or BSA. As can be derived from Figure 97D, GMP was coupled to BSA. However, GMP-7-HD was not coupled to either KLH (Figure 97C) or BSA (Figure 97D). We presume that periodate had oxidized the sulfur atom of the (2'-hydroxyethylthioethyl) residue instead of the sugar moiety of guanosine.

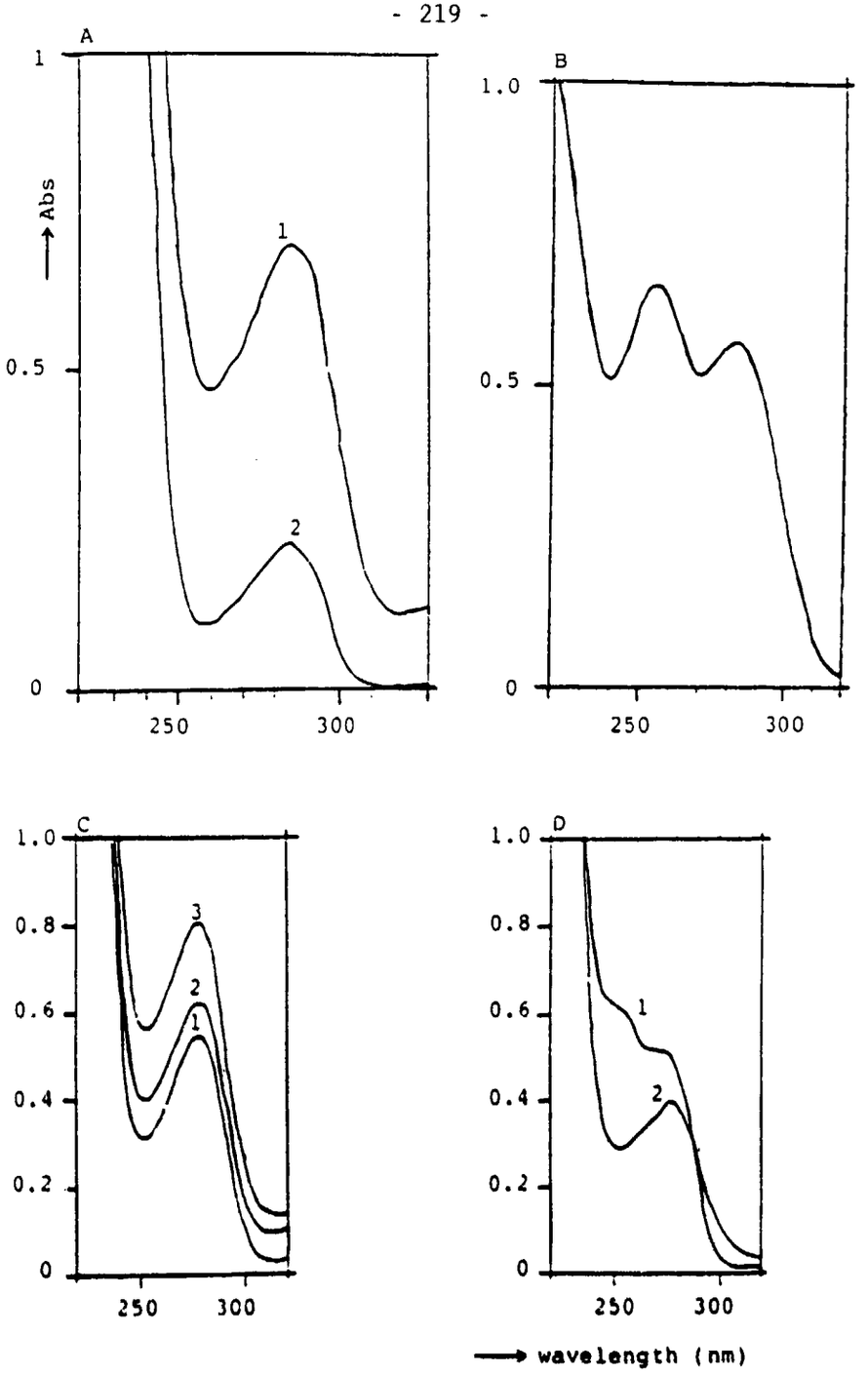

Figure 97. UV spectra of various compounds used for periodate coupling of guanosine-5'-phosphate (GMP) or N7-(2"-hydroxyethylthioethyl)-guanosine-5'-phosphate (GMP-7-HD) to Keyhole Limpet Hemocyanine (KLH) or Bovine Serum Albumin (BSA). Panel A: KLH (1) and BSA (2)in PBS. Panel B: GMP-7-HD at pH 7. Panel C: Coupling products of KLH with GMP-7-HD by periodate in PBS. The input ratio of adduct:protein was (w/w) 1:10 (1), 1:25 (2) and 1:100 (3). Panel D: Coupling products of BSA with GMP (1) and with GMP-7-HD (2) by periodate with an adduct:protein ratio (w/w) of 1:10 in PBS

EDC coupling

In this procedure, the phosphate group of the GMP is activated by 1-(3-dimethyl-aminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) in an imidazole buffer. This activated phosphate group can easily bind to the amino group of a lysine residue in the carrier protein. Only the coupling of GMP-7-HD to KLH was performed. Three different amounts of EDC (50, 100 or 250 mol EDC/mol GMP-7-HD) were used and UV spectra were taken after the coupling (Figure 98). The UV spectra show that GMP-7-HD was coupled to KLH. The coupling products obtained after reaction at a molar ratio of 50 and 100 mol EDC/mol GMP-7-HD showed the highest absorbance and both were used for the immunization of 8 mice to raise monoclonal antibodies.

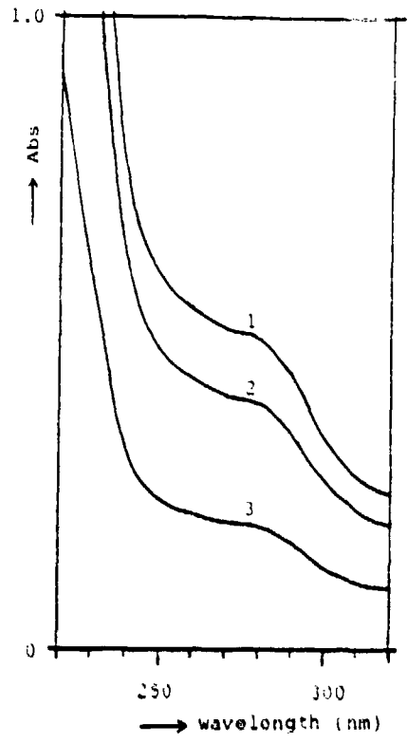

Figure 98. UV spectra of N7-(2"-hydroxyethylthioethyl)-guanosine-5'-phosphate (GMP-7-HD) coupled via 1-(3-dimethylamino-propyl)-3-ethylcarbodiimide hydrochloride (EDC) to Keyhole Limpet Hemocyanine (KLH). Three different input ratio's of EDC:GMP-7-HD were applied: 50 mol EDC/mol GMP-7-HD (1); 100 mol EDC/mol GMP-7-HD (2); 250 mol EDC/mol GMP-7-HD (3)

Immunizations and selection of antibody-producing clones

Four mice were immunized with the product of 50 mol EDC/mol GMP-7-HD (mouse 1, 2, 3 and 4) as immunogen and 4 mice with that of 100 mol EDC/mol GMP-7-HD (mouse 5, 6, 7 and 8). After 8 days blood samples of all mice were taken and the sera were tested for antibody activity

against ss-ct-DNA treated with 10 μM mustard gas in a direct ELISA. Figure 99 shows the results of the ELISA, at a serum dilution of 200.

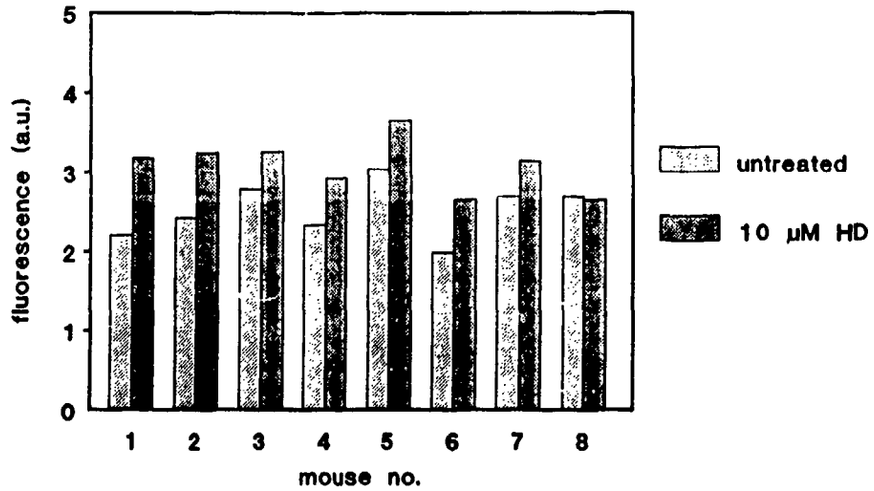

Figure 99. Antibody response of sera of eight mice at eight days after the first immunization, in a direct ELISA. Four mice (1-4) were immunized with the coupling product of N7-(2"-hydroxyethylthioethyl)-guanosine-5'-phosphate (GMP-7-HD) via EDC to Keyhole Limpet Hemocyanine (KLH) with a molar ratio of EDC:GMP-7-HD of 50:1 and a molar ratio of adduct:KLH of 100:1, and four mice (5-8) were immunized with the coupling product with a molar ratio of EDC:GMP-7-HD of 100:1 and a molar ratio of adduct:KLH of 100:1. The wells were coated with an excess of single-stranded calf-thymus DNA treated with 10 μM mustard gas or with untreated single-stranded calf-thymus DNA. The sera were diluted 200-fold

The mouse with the serum showing the best response against mustard gas-treated DNA was chosen for isolation of the cells to be used for fusion after a second immunization. The mice immunized with the coupling product of 50 mol EDC/mol GMP-7-HD appeared to be the best in this respect. Mouse 1 was chosen for the fusion experiment. After a second immunization the spleen and lymph node cells of this mouse were isolated; also blood was collected to check the antibody activity. Figure 100 shows the response of the serum of mouse 1 taken after the first and the second immunization against ss-ct-DNA treated with 10 μM mustard gas and untreated ss-ct-DNA, respectively. Evidently, the specific response against mustard gas-adducts had increased after the second immunization.

The lymph node and spleen cells of mouse 1 were fused with SP2/0 plasmacytoma cells and hybridomas were selected in HAT medium as described in II.13.7. In 100% of the wells (300 wells) containing "spleen"-hybridomas, clones were formed and, in first instance, supernatants of 62 wells showed a specific response against ct-DNA

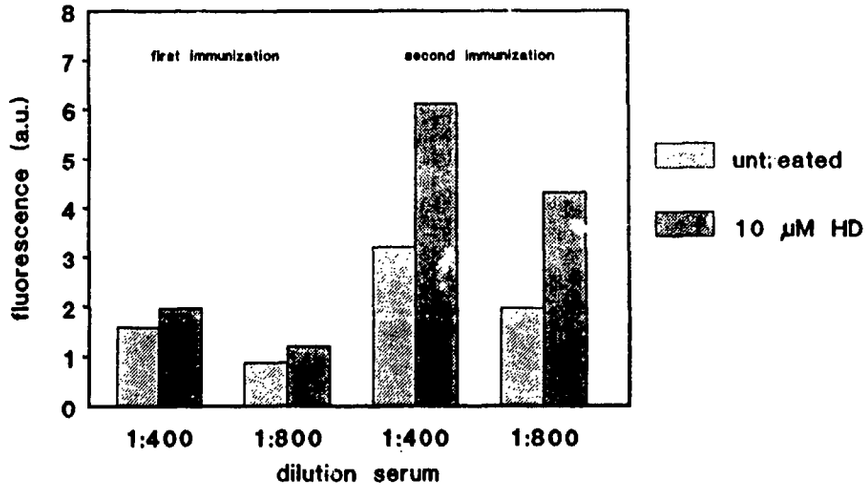

Figure 100. Antibody response of the serum of a mouse (1) eight days after the first immunization and four days after the second immunization, in a direct ELISA. The mouse was immunized with the coupling product of N7-(2"-hydroxy-ethylthioethyl)-guanosine-5'-phosphate (GMP-7-HD) via EDC to Keyhole Limpet Hemocyanine (KLH) with a molar ratio of EDC:GMP-7-HD of 50:1 and a molar ratio of adduct:KLH of 100:1. The wells were coated with an excess of single-stranded calf-thymus DNA treated with 10 µM mustard gas or with untreated single-stranded calf-thymus DNA. The sera were diluted 400- and 800-fold

treated with 10 µM mustard gas compared to untreated ct-DNA (specific response: ≥ 2x response of untreated DNA). Only in 67% of the wells (300 wells) containing "lymph node"-hybridomas, clones were formed and only nine wells showed positive response. The supernatants of the wells were screened in a direct ELISA, and when a positive response was observed, the supernatants were also tested in the cell-ELISA. However, as is often the case, many clones lost their antibody-producing capability with time. So finally cells from only four "spleen"-wells and one "lymph node"-well remained to be subcloned twice by limiting dilution, the procedure applied to make sure that monoclonal antibodies were obtained. For the second subculturing, only cells from a well in which one clone was grown were selected. The same procedure was followed for the selection of ten clones (two clones from each of the five wells).

The ten selected clones, producing antibodies with specific activity against ss-ct-DNA treated with mustard gas, were named 2C1, 2D4, 2F10, 1H4, 2F12, 2D3, 2A4, 2E3, 2F8 and 1H7. Two clones (2F10 and 1H4) were hybridomas from lymph node origin and the other eight came from spleen cells. The crude supernatants of these ten hybridomas were tested in a direct and a cell-ELISA. The results of the direct and cell-ELISA are shown in Figure 101. The supernatants were diluted tenfold and tested on ss-ct-DNA treated with 10 µM mustard gas (direct ELISA) and on WBC of whole blood treated with 1 mM mustard

gas. These results show that the last six clones in Figure 101 show a substantial response in the direct ELISA as well as in the cell-ELISA. Another important feature is the low response against untreated ss-ct-DNA and untreated WBC for all ten clones.Also the immunoglobuline-subclass was determined and all supernatants showed to contain specific antibodies of the IgG1-subclass.

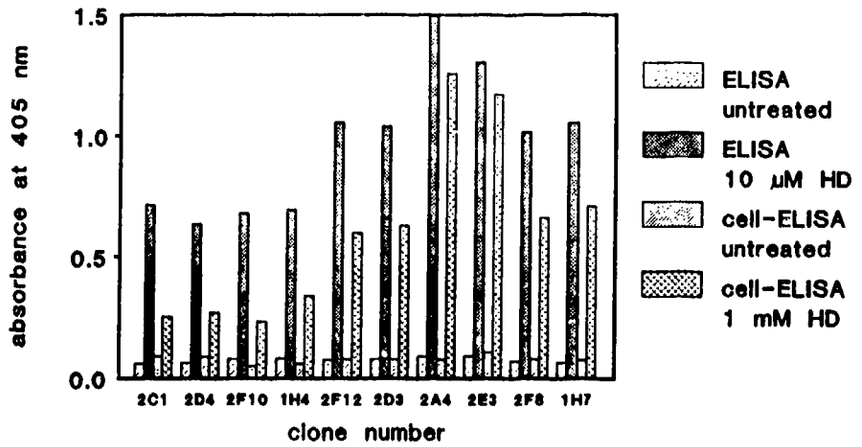

Figure 101. Antibody response of the supernatants of ten monoclonal cell cultures in a direct ELISA and in a cell-ELISA. The cell lines were isolated after fusion of the spleen- and lymph node cells of a mouse immunized with the coupling product of N7-(2"-hydroxyethylthioethyl)-guanosine-5'-phosphate (GMP-7-HD) via EDC to Keyhole Limpet Hemocyanine (KLH) (molar ratio of EDC:GMP-7-HD of 50:1) with SP2/0 plasmacytoma cells, and recloned twice by limiting dilution. The wells were coated with an excess of single-stranded calf-thymus DNA treated with 10 µM mustard gas or with untreated single-stranded calf-thymus DNA in the direct ELISA and with white blood cells (40,000/well) isolated from human blood treated with 1 mM mustard gas or from untreated blood in the cell-ELISA. The supernatants were diluted tenfold

Purification studies were performed on the monoclonal antibodies in saturated supernatants of each of the ten clones; ammonium sulfate precipitation followed by chromatography on a protein A column was applied. The protein content (Biorad assay) of the final, purified, monoclonal antibody preparations, originating from 100 ml of crude supernatants, is shown in Table 23.

Table 23. The amount of purified monoclonal antibodies isolated from the supernatant of ten hybridomas

Clone number	Protein content (μg/100 ml supernatant)
2C1	917
2D4	806
2F10	683
1H4	832
2F12	260
2D3	61
2A4	41
2E3	171
2F8	163
1H7	99

Characterization of the monoclonal antibodies

The ten purified MABs were screened in a direct ELISA and in a cell-ELISA (Table 24). Different amounts of the MABs (ng protein/well) were tested on immobilized ss-ct-DNA treated with 10 μM mustard gas and on WBC from whole blood treated with 1 mM mustard gas. After 1 h, the fluorescence was recorded and the amount of protein needed to reach a standard level of fluorescence (3,000 arbitrary units) was calculated. In the cell-ELISA the purified antibodies of the clone 2C1, 2D4 and 1H4 did not reach this level, so the fluorescence of the highest amount of incubated protein is shown. An important criterion for the selection of MABs is a high response to DNA treated with mustard gas and a low response to untreated DNA. Therefore, the corresponding response to untreated DNA/WBC is also shown in Table 24.

Table 24 shows that clones 2C1, 2D4, 2F10 and 1H4 respond very well to DNA treated with mustard gas, compared to untreated DNA; a relatively large amount of protein is needed, however, compared to the other clones. In the cell-ELISA they do not respond very well. The clones 2F12, 2E3 and 2F8 show a high background activity against untreated DNA, which is in contrast to the results shown in Figure 101 for the ELISA before purification of the same antibodies. The clones 2D3, 2A4 and 1H7 show the best results, in the direct ELISA as well as in the cell-ELISA. Less than 10 ng protein/well is needed to reach a fluorescence of 3000 units after 1 h of incubation, while the response to untreated DNA remains at an acceptable level.

The protein yield of the 6 clones 2F12, 2D3, 2A4, 2E3, 2F8 and 1H7 was not high; however, the specific activity (that is the amount of protein needed to reach a standard level of fluorescence of 3000 arbitrary units) was high. The low protein yield could be due to fluctuations in production capacity of the clones or loss of activity during the different purification steps (purification was done in the same order as the clones were numbered). In these experiments enough

Table 24. Direct ELISA and cell-ELISA on ten purified monoclonal antibodies (MABs)

Clone number	Direct ELISA			Cell-ELISA		
	fluorescence[a]		ng protein/ well	fluorescence[a]		ng protein/ well
	10 μM HD	0 μM HD[b]		1 mM HD	0 mM HD[c]	
2C1	3000	250	14	1925	425	40
2D4	3000	250	15	1875	425	40
2F10	3000	350	90	3000	875	600
1H4	3000	475	300	1850	850	1000
2F12	3000	1000	4.0	3000	800	7.5
2D3	3000	475	5.0	3000	450	8.5
2A4	3000	600	6.5	3000	600	10
2E3	3000	1175	2.5	3000	1125	4.0
2F8	3000	1275	3.0	3000	1075	6.0
1H7	3000	500	7.5	3000	500	10

[a] The fluorescence was recorded after 1 h.
[b] The fluorescence without MAB was 180.
[c] The fluorescence without MAB was 237.

MABs were isolated to perform screening tests to characterize the antibodies and to select one or two clones for the further productions of the antibodies. When new batches of antibody are needed in the future, more attention should be paid to the various purification steps in order to minimize loss of activity.

Competitive ELISA using monoclonal antibodies directed against N7-guanine monoadducts

The MABs were also tested in a competitive ELISA. The wells were coated with ss-ct-DNA treated with 10 μM mustard gas. Various amounts of competitor DNA (ss-ct-DNA treated with 10, 1 or 0.1 μM mustard gas) were incubated with a fixed amount of MAB. This amount was chosen such that a response of 3000 fluorescence units after 1 h of incubation was obtained, when no competitor DNA was added (100% point). To compare the activity of the various monoclonal antibodies with the rabbit antiserum W7/10, this serum was also tested with the same competitor DNA. Figure 102 shows the competition curves of MABs of clone 2D3 (5 ng protein/well) and of W7/10 (dilution: 1:40,000). The amount of competitor DNA required to reach the same level of inhibition was less for clone 2D3 than for W7/10. With ss-ct-DNA treated with 10 μM mustard gas as competitor, the 50% inhibition point was reached at 3 and 15.5 ng DNA/well with 2D3 and W7/10, respectively. As described before, it was shown that 1 out of 1,290 guanines is modified into a N7-G-HD monoadduct when ss-ct-DNA is treated with 10 μM mustard gas. Therefore, on the basis of these 50% inhibition points it can be calculated that, respectively, 1.8 and 9.1 fmol N7-HD-G monoadduct/well are present. Also some other clones were tested in a competitive ELISA. The results are summarized in Table 25.

The sensitivity of the competitive ELISA when antibodies of the six clones were compared showed little variation, only the antibodies produced by clone 2C1 appeared to be not very sensitive. It can be concluded that several hybridomas have been isolated that produce monoclonal antibodies with specificity for DNA damage due to exposure to mustard gas. The sensitivity of the competitive ELISA when performed with these antibodies is equal to that of the polyclonal antiserum W7/10, or somewhat better.

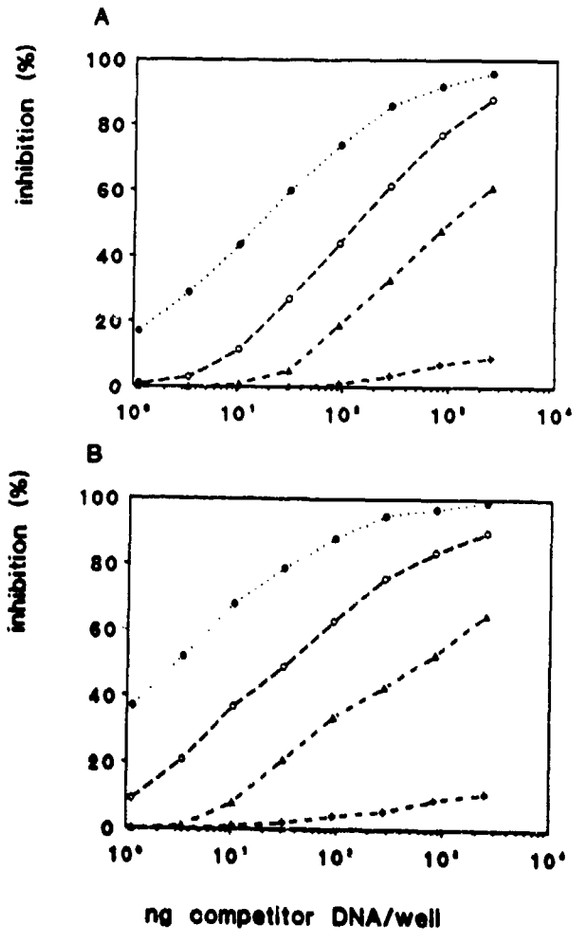

Figure 102. Competitive ELISA with rabbit serum W7/10 (panel A) and monoclonal antibodies produced by hybridoma 2D3 (panel B), and single-stranded calf-thymus DNA as competitor. The DNA was treated with 10 μM (●···●), 1 μM (o---o) or 0.1 μM (▲---▲) mustard gas or untreated (+---+). The wells were coated with an excess of single-stranded calf-thymus DNA treated with 10 μM mustard gas. The W7/10 serum was diluted 1:40,000 and monoclonal antibodies were added in 5 ng protein aliquots/well

Table 25. The amount of N7-(2'-hydroxyethylthioethyl)-guanine (N7-G-HD) monoadduct per well present in the competitor DNA at the 50% inhibition point in a competitive ELISA with various hybridomas

Serum	DNA at 50% inhibtion point (ng/well)	N7-G-HD-adduct at 50% inhibition point (fmol/well)
W7/10	15.5	9.1
2D3	3.0	1.8
2C1	103	60.4
2F12	2.3	1.3
2A4	5.1	3.0
2F8	2.3	1.3
1H7	2.6	1.5

Test for cross-reactivity

With two of these six clones, 2D3 and 2F8, the characterization was continued. Cross-reactivities towards different bases, nucleosides, nucleotides, unmodified and modified (alkylated by methyl, ethyl, hydroxyethyl, and hydroxyethylthioethyl group) were assessed in a competitive ELISA performed as described in II.13.2. The concentrations of the various compounds were measured spectrophotometrically on the basis of molar extinction coefficients taken from literature or determined in-house. Some compounds have a very low solubility. In such cases, the molar extinction coefficient of a structurally related compound was used. Table 26 shows the 50% inhibition point found for the various compounds in the competitive ELISA. The results show that both monoclonal antibodies 2D3 and 2F8 are primarily directed against the N7-GMP monoadduct of mustard gas. A low cross-reactivity can be seen with N7-Me-GMP, N7-Gua-HD and O6-Gua-HD as competitor. Especially the result with O6-Gua-HD is striking in this respect. No cross-reactivity can be detected with GMP. The monoclonal antibodies recognize only the adduct coupled to guanine with the intact imidazole ring. When this ring is opened, almost 5,000 times more competitor is required to reach the 50% inhibition point. It can be concluded that these monoclonal antibodies are specific for the ring-closed N7-GMP monoadduct of mustard gas.

Table 26. Screening test of the supernatants of the monoclonals 2D3 and 2F8 for cross-reactivity in a competitive ELISA

Competitor[a]	Molar extinction coefficient ($l.mol^{-1}cm^{-1}$)	50% inhibition point (nmol/well)	
		2D3	2F8
Gua	10,700	> 0.45	> 0.45
2'deoxy-Guo	13,000	> 4.2	> 10
Guo	13,600	> 3.6	> 2.5
2'deoxy-GMP	13,700	> 10	> 10
GMP	13,700	> 4.4	> 10
Ade	13,400	> 3.6	> 7.5
2'deoxy-Ado	15,000	> 6.9	> 5.0
Ado	14,900	> 6.9	> 5.0
2'deoxy-AMP	15,300	> 6.1	> 4.8
AMP	15,400	> 5.7	> 4.8
N7-Gua-HD	7,200	0.18	0.67
N3-Ade-HD	12,000	> 6.5	> 10
N7-GMP-HD	10,000	0.00085	0.0014
N7-GMP-HD r.o.[b]	6,000	2.5	5.1
O6-dGuo-HD	9,120	> 4.6	> 10
O6-Gua-HD	8,500	0.53	0.50
N7-Me-GMP	9,800	0.082	0.102
N7-Guo-EtOH	9,250	4.5	1.9
N7-Guo-EtOH r.c.[b]	11,620	> 8.0	> 9.5
O6-Me-dGuo	8,490	> 2.0	> 4.0
O6-Me-Gua	7,180	> 1.2	> 2.5
O6-Et-dGuo	9,120	> 1.2	> 2.7
O6-Et-Gua	8,500	0.82	> 3.0

[a] Abbreviations used are: Gua, guanine; Guo, guanosine; GMP, guanosine-5'-monophosphate; Ade, adenine; Ado, adenosine; AMP, adenosine-5'-monophosphate; Me, methyl; Et, ethyl; EtOH, hydroxyethyl.

[b] The compound was treated with 5 N NaOH for 2 h in order to open the imidazole ring (r.o. = ring-opened).

"Sandwich" ELISA

An attempt was made to develop a more sensitive method for the detection of mustard gas-DNA adducts in calf-thymus DNA than the competitive ELISA. This so-called sandwich ELISA (115) is based on the following principles. Monoclonal antibodies directed against N7-guanine monoadduct are used to coat the walls of the wells of a microtiter plate (96-wells). Then, the test sample, i.e., single-stranded calf-thymus DNA treated with mustard gas, is added, which will become attached to the immobilized monoclonal antibodies when adducts are present. The method aims at the detection of exposure resulting in sparingly modified DNA, such that at most one adduct per DNA fragment is present. (During the handling of DNA, fragmentation

into pieces of ca. 10^4 nucleotides will occur by sonication.) Only the adduct-containing fragments will be bound. The amount of DNA attached to the wall is determined, which, consequently, is a measure of the number of mustard gas-DNA adducts present. At higher mustard gas concentrations, all DNA fragments will become labelled and the method loses its discrimination with regard to the adduct number. Therefore, the method is limited intentionally in its application to treatments at low mustard gas concentrations (< 10 μM). For the detection of bound DNA, a polyclonal rabbit serum (W6/39) is added which is directed against single-stranded DNA. This procedure is expected to result in a greatly enhanced detection signal when compared to the binding of antibody molecules to the adduct itself, since a long stretch of DNA is available for binding instead of one site. The quantitation of the anti-single-stranded DNA antibodies is performed via the binding of goat-anti-rabbit molecules to which alkaline phosphatase is attached. This enzyme converts a substrate into a fluorescing product. The fluorescence is a measure for the amount of DNA bound to the wells.

Some modifications were applied in alternative or control experiments. Instead of coating the walls with monoclonal antibodies, the polyclonal rabbit serum W7/10 (raised against single-stranded calf-thymus DNA treated with 1 mM mustard gas) or W6/39 (directed against single-stranded DNA) was used. After coating with W7/10, the monoclonal antibodies D1B (directed against single-stranded DNA) were applied in order to detect the DNA attached to the coating. In the case of W6/39, the monoclonal antibodies directed against the N7-guanine monoadducts were used. Goat-anti-mouse-Ig-alkaline phosphatase served as a third antibody.
Various conditions were tested:
(i) concentrations of the monoclonal antibodies and polyclonal antisera were varied,
(ii) washing steps between the incubations were introduced (PBS or PBS with 0.05% Tween 20),
(iii) blocking agents were applied (gelatin or FCS) in order to reduce the background signal,
(iv) various buffers for the monoclonal antibodies were used,
(v) varying amounts of DNA were added,
(vi) the molarities of the buffers, in which the DNA is diluted to stretch the DNA, were varied.

Disappointingly, the results of the experiments showed that the sensitivity of the sandwich-ELISA does not exceed the level reached with the competitive ELISA. In the competitive ELISA it was possible to discriminate betwen untreated DNA and DNA treated with 0.01 μM mustard gas, which was impossible in the sandwich-ELISA, even for DNA treated with 0.1 μM mustard gas. Also the background was high. The polyclonal serum W6/39 seemed to bind aspecifically to the coated monoclonal antibodies. A high aspecific binding of the third antibodies, goat-anti-mouse-Ig-alkaline phosphatase, was observed in the experiments with a coating of polyclonal sera.

In view of the difficulties encountered, it was decided not to pursue this approach but to optimalize the competitive ELISA (see III.14.2).

Preparation of large amounts of monoclonal antibodies

The clone 2F8 was selected for further investigations. First, a new batch of antibodies was produced. To this end the hybridoma culture was propagated in RPMI medium supplemented with 10% Fetal Calf Serum. The cells were subcloned. The supernatants of the wells containing only 1 clone/well were tested on specific activity against mustard gas-treated calf-thymus DNA. A few clones did not contain activity, indicating that some cells had lost their antibody-producing activity during storage at -180 °C. One subclone showing a high specific activity was cultured for the production of monoclonal antibodies. Cells (1.5×10^5/ml) were grown for 10 days without refreshing the medium. Each day samples of the cell culture were taken to count the living cell population, and to determine the specific antibody activity (dilution factor of the supernatant was 500 times).

Figure 103 shows the results of this experiment. After 3 days of culture a maximum number of living cells was reached. After 8 days the maximum antibody activity was reached. These results show that during this period proteinases destroying antibody activity were not released from the dead cells in significant amounts.

It was decided to culture cells for 7 days for the production of a large batch of monoclonal antibodies (1 liter supernatant). This crude supernatant was tested in the competitive ELISA to see whether the sensitivity of the assay with these monoclonal antibodies was still comparable with that of the W7/10 rabbit antiserum. On single- and double-stranded calf-thymus DNA treated with mustard gas and on DNA isolated from white blood cells of human blood exposed to mustard gas, similar sensitivities were obtained.

The proteins of the supernatant were precipitated overnight with a saturated ammonium sulfate solution (0 °C) as described in II.13.10. After centrifugation, the pellet was dissolved in PBS and dialyzed against PBS. The dialysate was loaded on a protein A column. Next, the monoclonal antibodies were eluted from the column with 0.1 M sodium citrate buffer, pH 4.7 (fraction 36-70 in Figure 104).

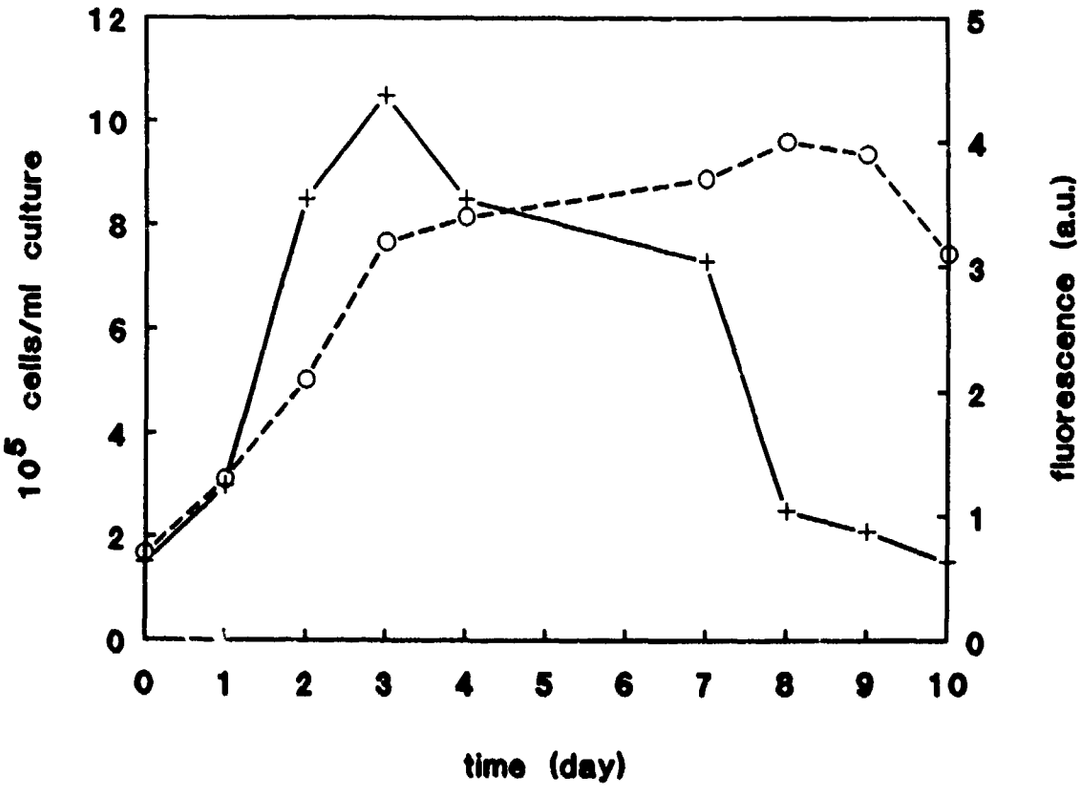

Figure 103. Growth curve of a 2F8 hybridoma culture and the production of specific antibodies. Viable cells (+) were counted, on the basis of trypan blue exclusion, in a counting chamber. Specific antibody activity (o) was detected in a direct ELISA. The wells were coated with an excess of single-stranded calf-thymus DNA treated with 10 μM mustard gas. The supernatants were diluted 500-fold

The fractions eluted with the sodium citrate buffer (fraction 42-67) contain both protein and specific antibody activity. From the area covered by the protein peak it was calculated that 8.3 mg of monoclonal antibodies were obtained from 1 liter of supernatant. The fractions 42-67 were pooled, concentrated with PEG 20,000 and dialyzed against PBS. The protein content of the resulting solution was measured spectrophotometrically; it amounted to only 4.4 mg, indicating that a substantial loss had occurred. This was probably due to protein adsorption on the dialysis tube.

The following samples were taken after the various steps in the purification procedure for testing in the direct ELISA in order to check loss of activity.

A: crude supernatant of the hybridomas,
B: supernatant after ammonium sulfate precipitation,
C: solution of the pellet of the ammonium sulfate precipitate after dialysis against PBS,
D: eluate of the protein A column during loading of the antibodies,

E: fractions of eluate during elution of the antibodies, and
F: final antibody solution after pooling of the eluate fractions, reduction of the volume by PEG 20,000, and dialysis.

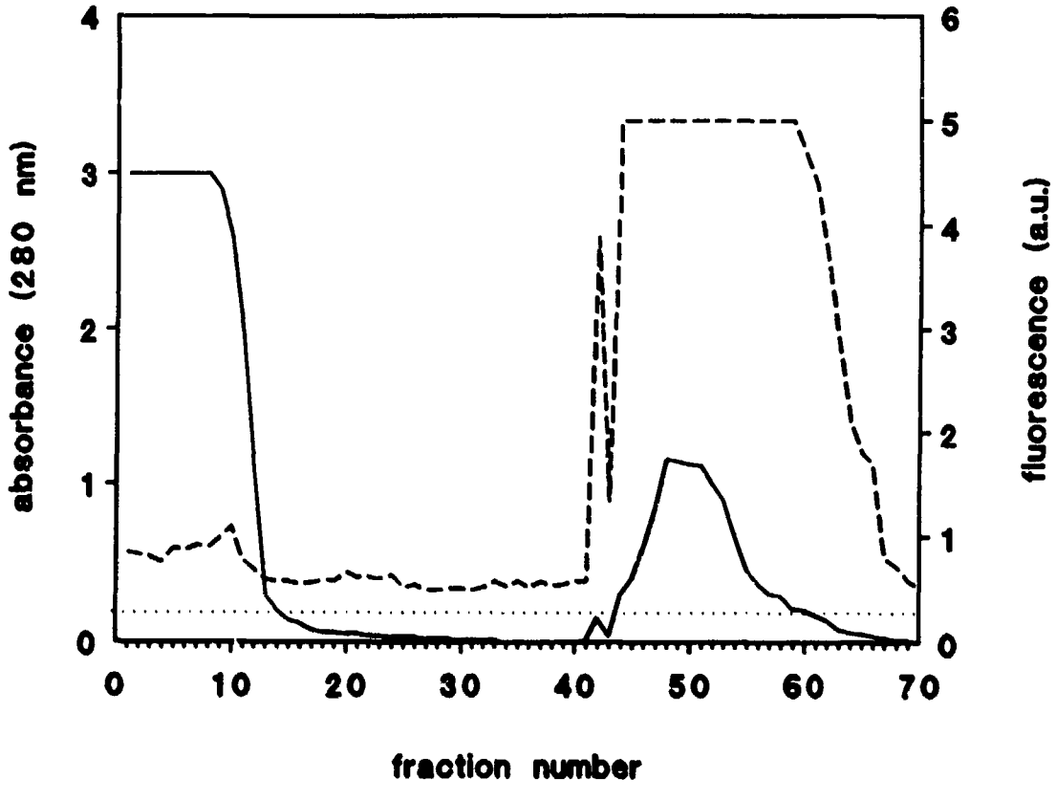

Figure 104. Purification of 2F8 monoclonal antibodies through a protein A column. The dialysate, obtained after ammonium sulfate precipitation, was loaded on a protein A column which was washed with binding buffer (1.5 M glycine, 3 M NaCl, pH 8.9) until the absorbance at 280 nm was less than 0.1. Fractions of 2 ml were collected (fraction 1-35). Next, the monoclonal antibodies were eluted from the column with 0.1 M sodium citrate buffer, pH 4.7 (fraction 36-70). The protein content (—, absorbance at 280 nm) and the specific antibody activity (- -) in a direct ELISA were measured of all samples. For the latter measurements, wells were coated with an excess of single-stranded calf-thymus DNA treated with 10 μM mustard gas and the samples taken from the fractions were diluted 1000-fold

During precipitation of the proteins in the crude supernatant, 18% of the original activity remained in the supernatant of the saturated ammonium sulfate solution (B). Accordingly, 82% was recovered in the dissolved pellet after dialysis (C). A small fraction (1%) was not retained during loading of the column with the monoclonal antibodies (D). Due to an experimental mishap, no data were obtained on the pooled column fractions. Only 33% of the activity was recovered in the final solution (F) containing 4.4 mg of protein. (The amount of

8.3 mg protein, calculated to be present in the antibody pool by summation of the protein content of the column, would have represented 62% activity.) Therefore, provided that the correction for the loss of protein is justified, a very satisfactory over-all recovery of pure antibody appears attainable. It was calculated that only 2 ng of protein per well from this purified 2F8 preparation was needed in order to obtain 3000 arbitrary fluorescence units after 2 h of incubation with substrate in our standard competitive ELISA (100% points).

In another 1-liter batch of the same subclone of 2F8 we obtained 5.1 mg of the purified monoclonal antibodies which contained 38% of the activity present in the crude supernatant.

III.14.5. Mustard gas-induced adducts to DNA in human skin

Recently, we have performed pilot experiments to detect local DNA damage in skin samples. Pieces of human skin obtained from cosmetic surgery were exposed to air saturated with mustard gas vapor for periods ranging from 2 to 10 min at 30 °C (1360 mg mustard gas/m^3). The pieces of skin were frozen to cut 5 μm slices with a microtome, which were fixed on glass slides. Proteins and RNA were degraded enzymatically on the slide, and DNA was unwound. Subsequently, the preparation was treated with the monoclonal antibody against the N7-guanine monoadduct. Next, the antibody molecules attached to the DNA damage were made detectable by binding to a goat-anti-mouse antibody that contains covalently a fluorescent group emitting green light to bind to them. The preparation was also treated with propidium iodide, which intercalates with DNA and emits red light when illuminated. The latter test serves to locate the cell nuclei in general. The slides were scanned for the green light emitted by the second antibody on the DNA damaged by mustard gas. under a laser-scan microscope. In a slice of skin exposed for 10 min to mustard gas vapor, it was observed that many of the nuclei of the epidermal cells had sustained damage, as evidenced clearly by the green fluorescence (Figure 105). At this preliminary stage of the investigations, the detection limit is at 1 min exposure, which corresponds with a Ct value of mustard gas (1360 $mg.min.m^{-3}$) that would not yet give blisters (1000-2000 $mg.min.m^{-3}$, ref. 116).

As a follow-up of the initial pilot experiments, we performed a preliminary study on the persistence of DNA damage induced by mustard gas in human skin. For this purpose, an area of human skin resulting from cosmetic surgery was exposed to mustard gas vapor-saturated air for 4 min at 30 °C and sections were kept in culture medium for 0, 2, 24 and 48 h. Next, cryostat sections were subjected to an immunostaining procedure as described in II.13.12 and were examined with fluorescence microscopy. The experiment has been carried out twice (Figure 106). The average FITC-fluorescence above the nuclei of the cells in the epidermis appeared to increase within the first 24 h after exposure to mustard gas whereas some decrease was observed within the next 24 h. At 48 h after mustard gas exposure, the FITC fluorescence equals the level observed directly after exposure, but

A

B

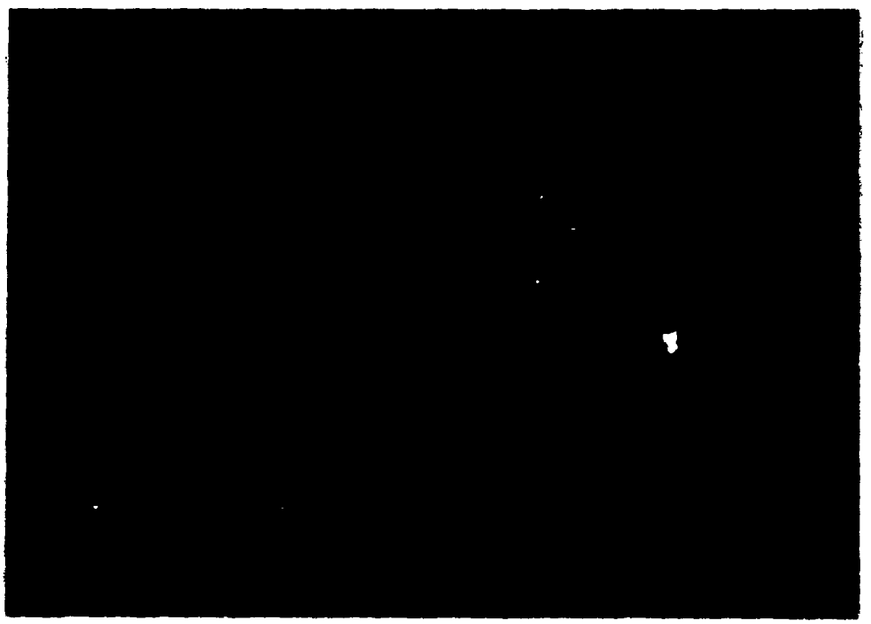

Figure 105A and B. Immunofluorescence microscopy of human skin exposed to mustard gas vapor-saturated air at 30 °C for 10 min (Ct = 13,600 mg.min.m^{-3}). A: FITC-fluorescence representing N7-guanine monoadducts; B: propidium iodide fluorescence representing DNA

C

D

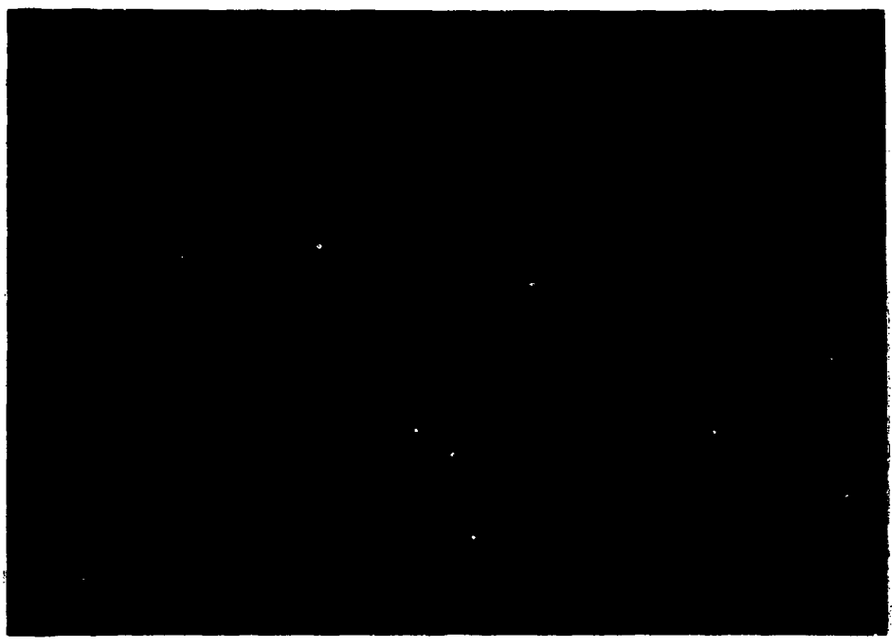

Figure 105C and D. Immunofluorescence microscopy of unexposed human skin. C: FITC-fluorescence; D: propidium iodide fluorescence (In comparison with A, C has been overexposed to show some fluorescence.)

is still above background. The skin could not be kept in culture medium for more than 48 h. Therefore, no conclusions can be drawn about the persistence of lesions beyond 48 h.

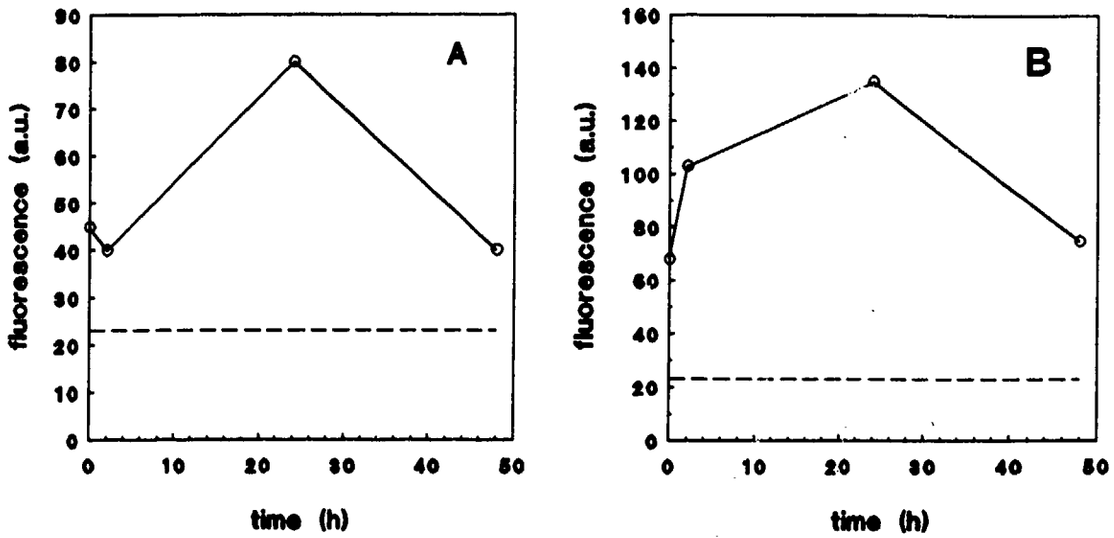

Figure 106. Persistence of mustard gas-adducts to N7-guanine in DNA of human skin exposed to mustard gas-vapor-saturated air for 4 min (Ct = 5440 mg.min.min^{-3}) and incubated for various times in culture medium at 37 °C. Cryostat sections were subjected to the immunostaining. The average fluorescence per nucleus was determined above the nuclei in the epidermis in two independent experiments (panels A and B). The horizontal dashed lines represent the background fluorescence of unexposed skin

III.15. Distribution of radioactivity between various blood components

Since adducts in DNA of WBC as well as those in hemoglobin of red blood cells are to be examined for their possible use in a dosimeter for mustard gas, the distribution of this agent between various blood components was studied after treatment of whole blood with 0.1 mM [^{35}S]mustard gas. After division of the blood into two equal parts (duplicate experiment), various blood fractions, including serum proteins, red and white blood cells, hemoglobin and DNA (as described in II.4) were isolated and the radioactivity was counted. The distribution of the radioactivity over the fractions was calculated. This experiment was done twice. The combined results of these experiments are shown in Figure 107.

Almost all radioactivity appeared to be present in the serum (60%) and the erythrocytes (39%). Only 0.67% was associated with the WBC, with 0.02% bound to the DNA. Of the total amount of radioactivity, 31% was bound to globin, which is 1,500 times more than to DNA. Since the hemoglobin content in blood varies between 0.12 and 0.16 g/ml

with a mean value of 0.14 g/ml and the DNA-content is ca. 50 μg DNA/ml, the difference in available material amounts to a factor 2,800. When blood was treated with 0.1 mM [^{35}S]mustard gas, 1,500 times more radioactivity was bound to hemoglobin than to DNA; hence mustard gas binds 1.9 times more efficiently to DNA than to hemoglobin. However, the absolute number of mustard gas-adducts in hemoglobin in blood is much higher than in DNA because of the large amount of hemoglobin compared to DNA. It is expected that adducts in hemoglobin have a higher persistency due to the absence of repair for these types of lesions, in contrast to DNA adducts which -in general- can be repaired by cellular enzyme systems. Consequently, for a practical detection and the quantification of exposure to mustard gas, an immunochemical method based on antibodies that are specific for protein adducts might be attractive. However, on a weight basis more adducts are present in DNA and little DNA is needed for the assay. Furthermore, DNA adducts are more directly related to the adverse health effects of mustard gas.

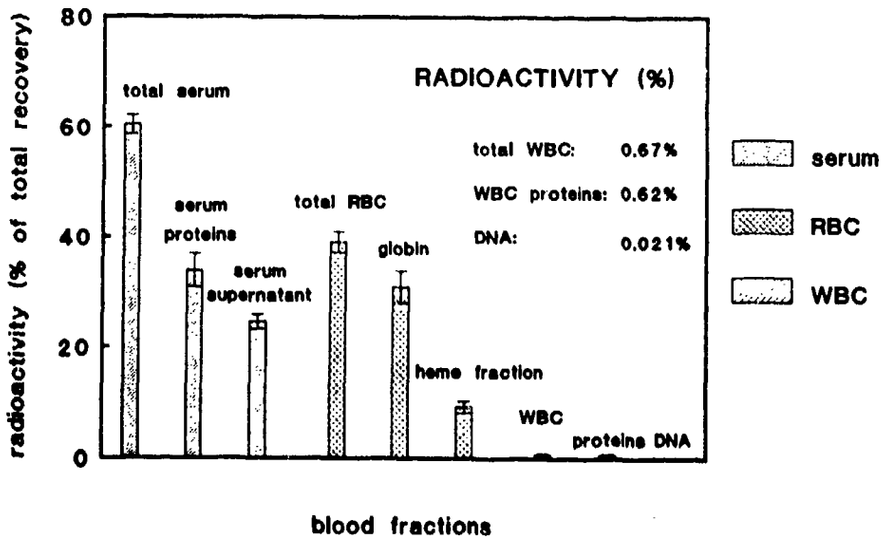

Figure 107. The distribution of radioactivity in human whole blood after treatment with 0.1 mM [^{35}S]mustard gas. The blood was separated in the serum fraction, the red blood cells (RBC) and the white blood cells (WBC). The serum was divided into the serum proteins and the serum supernatant containing the remaining components. The RBC were divided into the RBC proteins (globin) and the heme fraction. The WBC were divided into the DNA and the remaining components. The radioactivity is expressed as the percentage radioactivity in the fraction compared to the totally recovered radioactivity. The data are the mean values of four experiments (± SEM)

We repeated the above-mentioned experiment with a new batch of [^{35}S]mustard gas (see III.3). The data obtained with the new batch were in agreement with the earlier data. Approximately 50% of the radioactivity was found in the serum, ca. 50% was recovered bound to

hemoglobin whereas only ca. 0.01% was attached to DNA in the white blood cells.

III.16. Identification of reaction products of mustard gas in proteins of erythrocytes

III.16.1. Identification of the N-terminal heptaheptide of α-globin in hemoglobin digested with trypsin

Since it is known from literature (25) that the N-terminal amino acid valine of the α-chain of hemoglobin is a good target for alkylation, the detection of mustard gas-adducts to this amino acid has been chosen as a first approach. This valine is released from the α-globin chain by digestion with trypsin together with six other amino acid residues as the N-terminal heptapeptide val-leu-ser-pro-ala-asp-lys (HP).

An HPLC system has been developed to separate peptides and peptides alkylated by mustard gas. A C18 reversed phase column (25 cm x 4,6 mm) was used and the peptides were eluted with an acetonitrile gradient in 0.1% trifluoroacetic acid (TFA); the products were detected by their absorbance at 220 nm.

A method has been developed to isolate globin (Gb) chains from whole blood and from commercially available hemoglobin (Hb) as described in II.15.1 and II.15.2. In order to digest the proteins into peptides, Gb was hydrolyzed with trypsin at 37 °C for 2 h. Trypsin cleaves the protein specifically at the carboxyl-end of lysine and arginine residues. In this way, the heptapeptide could be released. Figure 108 shows the profile of Hb (Sigma) digested with trypsin and subsequently injected onto the HPLC column. Digestion and HPLC separation are very reproducible. Also Hb isolated from blood was digested and injected onto the HPLC column (Figure 109). The elution showed approximately the same profile as that obtained with the commercially available Hb (Figure 108). Figure 110 shows digested Gb, co-injected with the synthetic heptapeptide (see III.11.2) which was characterized by amino acid analysis. The heptapeptide was eluted after 18.5 min (peak 1). Peak 1 was collected from the sample as shown in Figure 108 and also analyzed by amino acid analysis. The amount of amino acids (nmol) was measured in the sample and expressed as the ratio of nmol amino acid:nmol ala. Ala served as a reference amino acid because it is very stable and will not be alkylated by mustard gas. Seven amino acids were detected with the molar ratio indicated between brackets: val(1.0), leu(1.0), ser(1.2), pro(1.3), ala(1.0), asp(0.9) and lys(1.1). The digestion method was considered suitable for releasing the HP from Hb and possibly also from hemoglobin reacted with mustard gas.

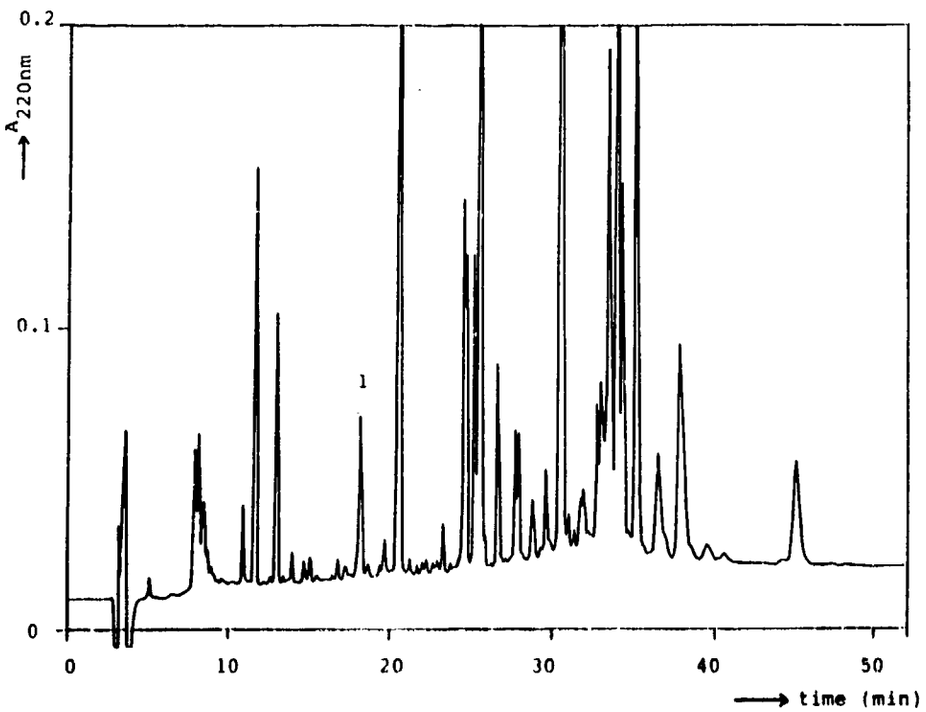

Figure 108. HPLC-chromatogram (ODS-Sephadex reversed-phase column) of hemoglobin (Sigma) hydrolyzed with trypsin. The absorbance was recorded at 220 nm. An acetonitrile gradient in 0.1% trifluoroacetic acid was used to elute the various peptides

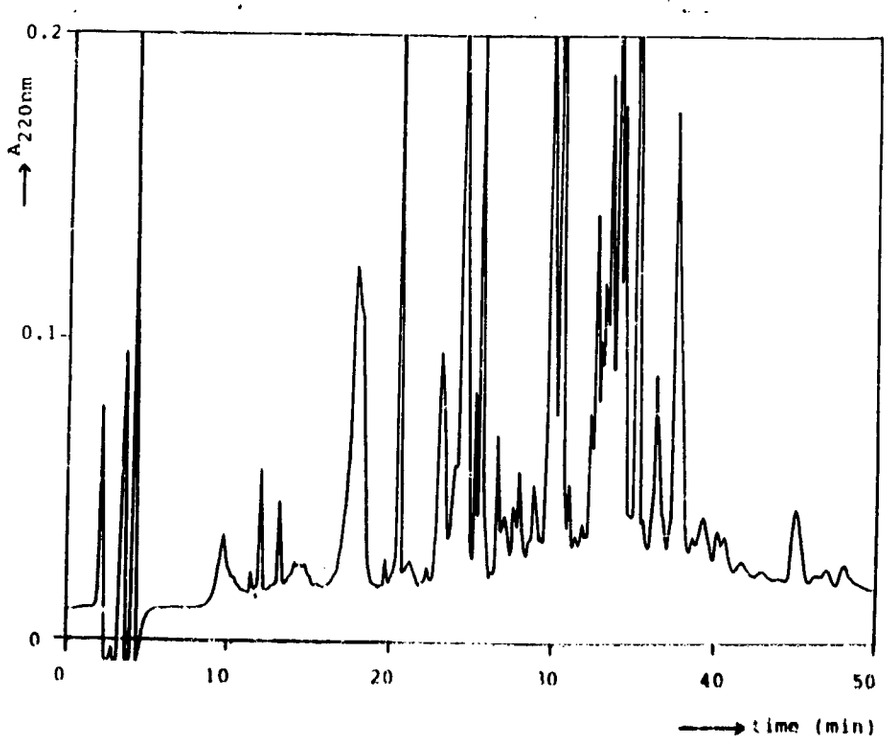

Figure 109. HPLC-chromatogram (ODS-Sephadex reversed-phase column) of trypsin-digested globin isolated from human blood. The absorbance was recorded at 220 nm. An acetonitrile gradient in 0.1% trifluoroacetic acid was used to elute the various peptides

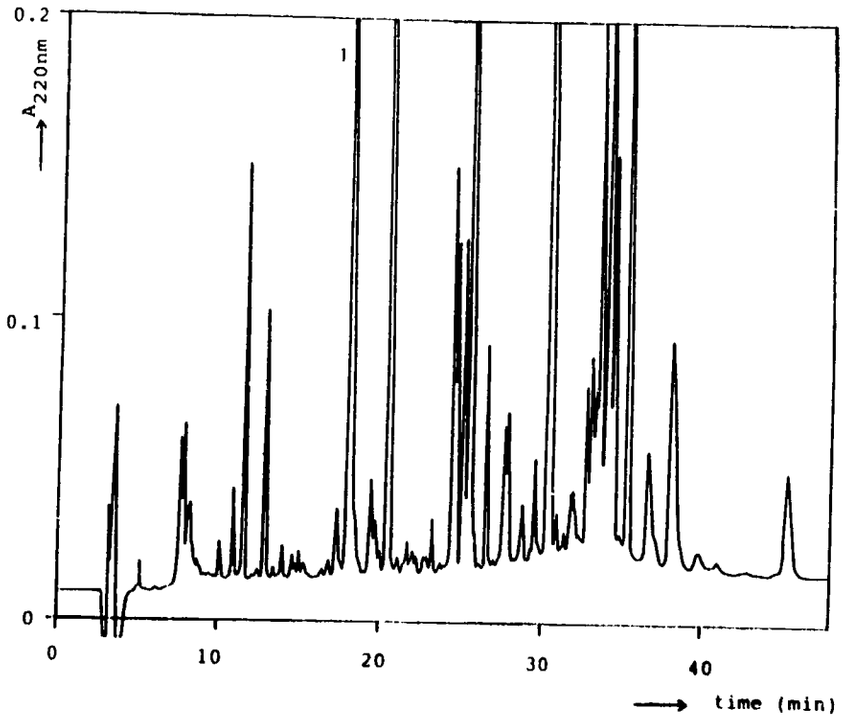

Figure 110. HPLC-chromatogram (ODS-Sephadex reversed-phase column) of hemoglobin (Sigma) hydrolyzed with trypsin and co-injected with the N-terminal heptapeptide of the α-chain of hemoglobin (peak 1). The absorbance was recorded at 220 nm. An acetonitrile gradient in 0.1% trifluoroacetic acid was used to elute the various peptides

III.16.2. <u>Identification of the alkylated peptide in hemoglobin treated with mustard gas</u>

Subsequent to the synthesis and the characterization of N-(2'-hydroxylethylthioethyl)-val-leu-ser-pro-ala-asp-lys, i.e., the heptapeptide alkylated with mustard gas at valine (see III.11.2), a larger amount of the heptapeptide (20 mg) was treated with mustard gas as described in III.11.2. The monoadduct of the heptapeptide was collected by HPLC on a semi-preparative column (ODS-Sephadex; 25 cm x 10 mm) and lyophilized. This product was used as a marker to identify the alkylated peptide in hemoglobin treated with mustard gas. Hemoglobin was treated with [^{35}S]mustard gas (1mM). Gb was isolated, digested with trypsin and the digest was injected onto the HPLC column. An aliquot of N-(2'-hydroxylethylthioethyl)-val-leu-ser-pro-ala-asp-lys was co-injected. Fractions eluted in 0.5-min time intervals were collected and the radioactivity was determined. Several radioactive peaks were observed (Figure 111), one of which was eluted with the reference material. Although no final proof was obtained that the material in the ^{35}S-peak is identical to the synthetic peptide, the results suggest that indeed the reaction of

mustard gas with the N-terminal valine of Hb could be a suitable indicator for exposure to this agent.

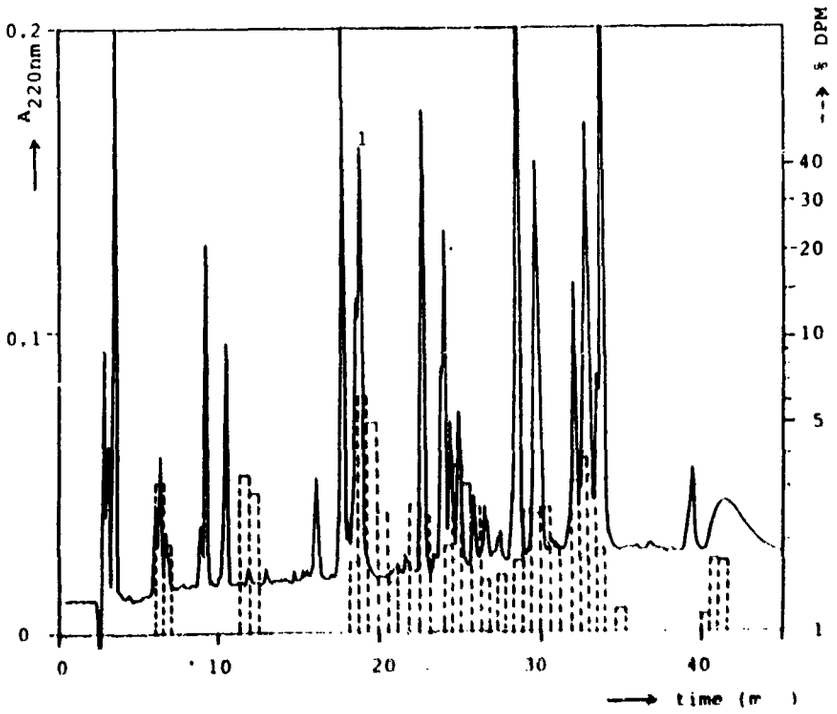

Figure 111. HPLC of trypsin-digested hemoglobin (Sigma) which had been treated with 1 mM [^{35}S]mustard gas. An aliquot of the N-terminal heptapeptide of the α-chain of hemoglobin alkylated with mustard gas at the valine residue [N-(2'-hydroxylethylthioethyl)-val-leu-ser-pro-ala-asp-lys; peak 1] was co-injected. The peptide mixture was analyzed on an ODS-Sephadex column; UV absorbance (220 nm) and radioactivity were monitored. The radioactivity of the fractions (collected over 0.5-min intervals) was counted for 10 min in a Mark III liquid scintillation counter (Packard, USA)

With a new batch of [^{35}S]mustard gas we repeated the identification of the alkylated peptides in hemoglobin treated with mustard. To adhere more closely to the practical situation for which the detection methods are intended, fresh human blood was incubated with the agent. The hemoglobin was isolated and digested, and the digest analyzed with HPLC. A broad spectrum of radioactive peaks was obtained. Among the ^{35}S-peaks that were eluted in the region of the large peptides (Figure 112), one was co-eluted with the reference, the alkylated N-terminal heptapeptide N-(2'-hydroxyethylthioethyl)-val-leu-ser-pro-ala-asp-lys, as also had been the case previously.

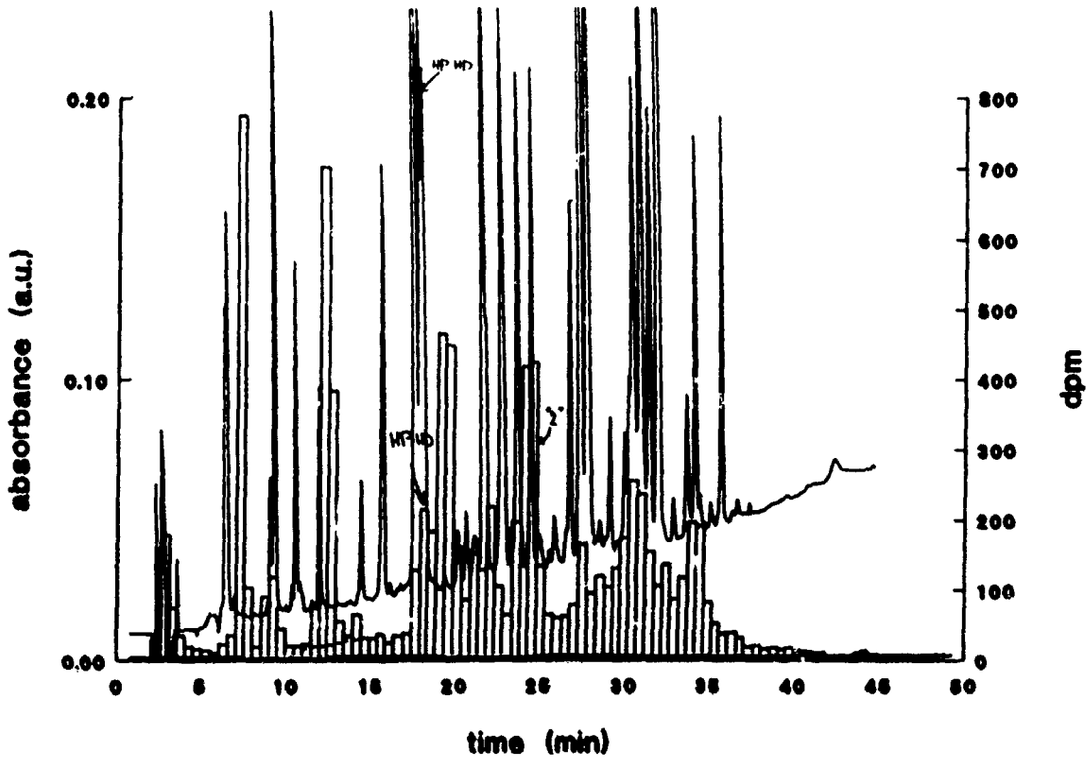

Figure 112. HPLC of trypsin-digested hemoglobin isolated from human blood which had been exposed to 1 mM [^{35}S]mustard gas (1 h at 37 ^{o}C). An aliquot of the N-terminal heptapeptide of α-hemoglobin alkylated with mustard gas at the valine residue [N-(2'-hydroxyethylthioethyl)-val-leu-ser-pro-ala-asp-lys], peak HP-HD, was co-injected. The peptide mixture was analysed on an ODS-Sephadex column; UV absorbance (220 nm) and radioactivity were monitored. The radioactivity of the fractions (collected over 0.5-min intervals) was counted for 10 min

In principle, mustard gas can react with numerous groups in hemoglobin. Many of the reaction products, however, may not be stable under acidic conditions. To see whether the radioactive material that was co-eluted with the synthetic peptide would be degraded by acid, a portion of the tryptic digest was incubated with 5 N aqueous HCl for 24h at 37 ^{o}C. Then it was neutralized and subjected to HPLC under identical conditions as before. Again, an aliquot of the synthetic alkylated heptapeptide was co-injected. Among the larger ^{35}S-peptides only two had remained (Figure 113), one was eluted at the same position as the marker peptide. Most other radioactivity was released from the peptides suggesting that less stable reaction products of mustard gas with carboxyl groups were involved. These results confirm the earlier preliminary conclusion that upon reaction of hemoglobin with mustard gas and subsequent tryptic digestion a substantial amount of alkylated heptapeptide can be isolated. The other (partially) acid-stable radioactive peak has not been identified yet.

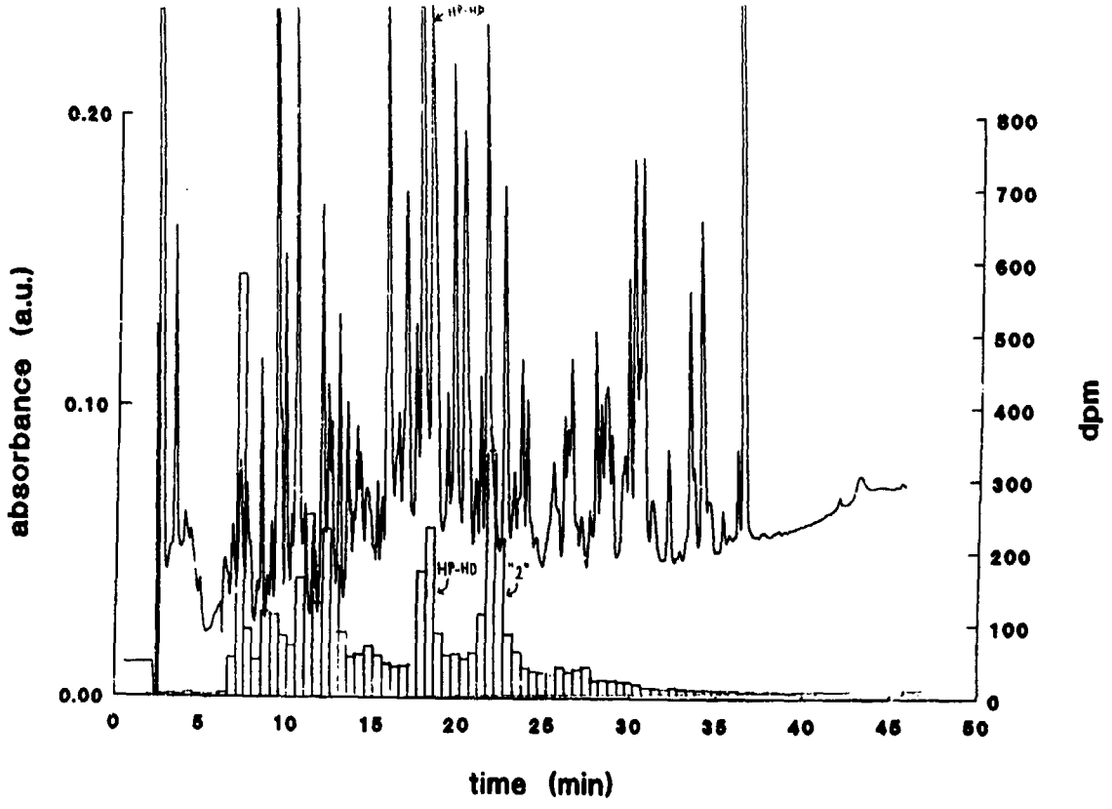

Figure 113. HPLC of trypsin-digested hemoglobin isolated from human blood which had been exposed to 1 mM [^{35}S]mustard gas (1 h at 37 °C). After trypsin digestion the sample was also treated with 5 M hydrochloric acid (24 h, 37 °C). An aliquot of the N-terminal heptapeptide of α-hemoglobin alkylated with mustard gas at the valine residue [N-(2'-hydroxyethylthioethyl)-val-leu-ser-pro-ala-asp-lys], peak HP-HD, was co-injected. The radioactive peak marked HP-HD was at the same position as the peak found for the tryptic digest not treated with acid (see Figure 112). The peptide mixture was analyzed on an ODS-Sephadex column; UV absorbance (220 nm) and radioactivity were monitored. The radioactivity of the fractions (collected over 0.5-min intervals) was counted for 10 min.

The radioactive material that was acid-stable and was co-eluted with N-(2'-hydroxyethylthioethyl)-val-leu-ser-pro-ala-asp-lys amounted to ca. 6% of the total ^{35}S bound to hemoglobin. Taking into account the distribution of the radioactivity over the various blood components, as described above, it can be calculated that in human blood mustard gas binds about 7 times more efficiently to the N7 of guanine per weight unit of DNA than to the N-terminal heptapeptide per weight unit of hemoglobin. However, on a molecular basis, mustard gas reacts 3.7 times more efficiently with the N-terminal valine in the α-chain of hemoglobin in the erythrocytes of human blood than with N7-guanine

in DNA of the WBC. It can be concluded that the reaction of mustard gas with the N-terminal valine is potentially a suitable indicator for exposure to this agent.

III.16.3. Stability of mustard gas-adducts in hemoglobin

In addition to the experiments described in III.16.2, experiments were carried out to determine the amount of alkali- and acid-labile adducts in mustard gas-treated Hb. Adducts to carboxylic acid groups are described in the literature to be alkali- and acid-labile, whereas adducts to amino groups, and those to Cys or His (when formed) are supposed to be stable. Hb was treated with 1 mM [^{35}S]mustard gas at pH 8. A part of the Hb was precipitated and Gb was isolated (see II.15.2). Hb and Gb were treated for 0-120 h at 37 ^{o}C with 5 N aqueous solutions of NaOH, or methanesulfonic acid (MSA), or HCl. Treatment with the 5 N solutions led to precipitation of Hb, so this protein was treated with 1 N solutions of these reagents. The treatment was stopped by neutralization of the mixtures with HCl or NaOH, followed by precipitation of the proteins with ice-cold 10 mM HCl in 99% acetone. The radioactivity was determined in the supernatant (containing the heme group -if present- and the components derived from alkali- or acid-labile adducts) and in the precipitated "globin fraction" after it had been dissolved in distilled water. The percentages of alkali- and acid-labile adducts were calculated. In the same way, Hb treated with 1 mM [^{35}S]mustard gas was incubated at pH 7, 37 ^{o}C, in order to study its stability under these conditions. After precipitation of the protein, the radioactivity was determined in the supernatant and in the precipitate.

When Gb, isolated from Hb which had been treated with 1 mM [^{35}S]mustard gas, was treated for various periods of time with 5 N NaOH, HCl or MSA, it appeared that 30% of the initial amount of radioactivity bound to Gb was still bound to precipitable protein (acid- and alkali-stable adducts) after 24 h, whereas 70% was present in the supernatant (originating from acid- and alkali-labile adducts), as can be derived from Figure 114. The release of acid- or alkali-labile adducts (30% of total) is complete within 4 h. The effects of the three treatments, with either acid or alkali, were similar. Probably, the acid- and alkali-labile adducts are mustard gas residues bound to carboxylic acid groups of amino acids.

When 1 mM Hb treated with 1 mM [^{35}S]mustard gas was incubated with 1 N of MSA or NaOH, 50% of the adducts bound to the globin within Hb were alkali- and acid-stable and were still present after 48 h (Figure 115), while in Gb treated with [^{35}S]mustard gas only 30% remained bound after 4 h. These differences could be due to the molarities of the acid and alkali treatments (5 versus 1 N) and may also be ascribed to the configuration of free Gb and Gb: the globin structure in Hb may be more compact and may thus protect the adducts against hydrolysis. When Hb treated with 1 mM [^{35}S]mustard gas was incubated at neutral pH, 37 ^{o}C, in order to study the stability of the adducts over several days, 75% of the radioactivity initially

present in Gb was still bound to protein after 24 days (Figure 116). Hb started to precipitate after 24 days, so the experiment was stopped.

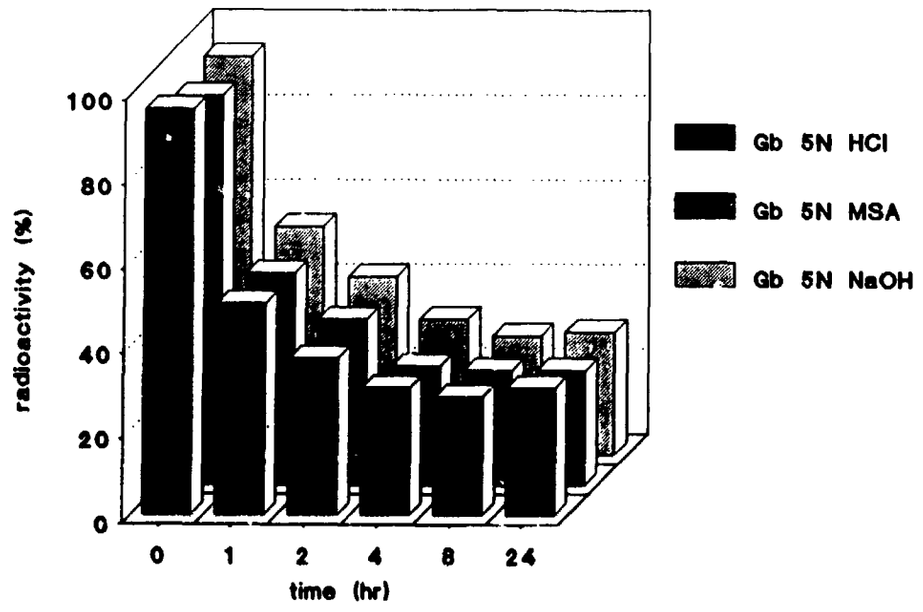

Figure 114. Stability of mustard gas-adducts to globin in acidic and in alkaline solution. Human blood was treated with 1 mM [^{35}S]mustard gas and the hemoglobin was isolated. Globin was isolated from the hemoglobin and treated with 5 N HCl, 5 N methanesulfonic acid or 5 N NaOH at room temperature for several hours. After acid-acetone precipitation (-20 ^{o}C) of the protein, the radioactivity in the precipitate was counted and compared to total radio-activity, to determine the percentage of adducts of mustard gas still attached to globin

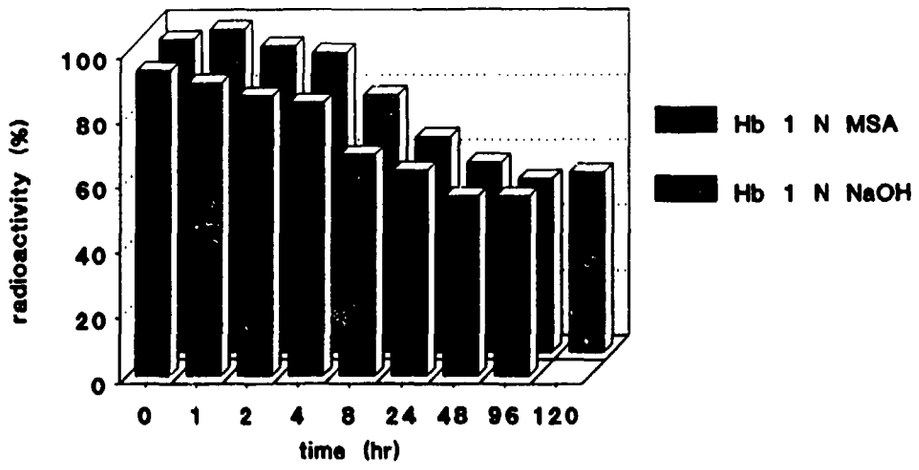

Figure 115. Stability of mustard gas-adducts to hemoglobin in acidic and alkaline solution. Human blood was treated with 1 mM [^{35}S]mustard gas and hemoglobin was isolated and treated with 1 N methanesulfonic acid or 1 N NaOH at room temperature for several hours. After acid-acetone precipitation (-20 ^{o}C) of the protein, the radioactivity in the precipitate was counted and compared to total radioactivity, to determine the percentage of adducts of mustard gas still attached to hemoglobin

The results indicate that Hb exposed to mustard gas contains a high proportion of mustard gas-adducts that is relatively stable (75% of the initial amount is still present after 24 days of incubation under physiological conditions; 30% of the adducts resist acid or alkali treatment). This might indicate that a substantial fraction of the mustard gas-adducts to Hb formed in vivo (at 37 ^{o}C) remains in the body for a prolonged period of time, perhaps long enough after exposure to permit retrospective detection after several days or even weeks.

However, the identification of the various mustard gas adducts to protein is hampered by the acid-instability of a large proportion of the reaction products. To establish to which amino acid the mustard gas residue is attached, the protein has to be digested to yield the free amino acids. Among these, certain amino acids modified by mustard gas can subsequently be characterized. The usual procedure for complete hydrolysis of proteins involves heat treatment in the presence of 6 N HCl, during which treatment at least 70% of the adducts will decompose. Therefore, a different procedure has to be developed for identification of the acid-labile adducts.

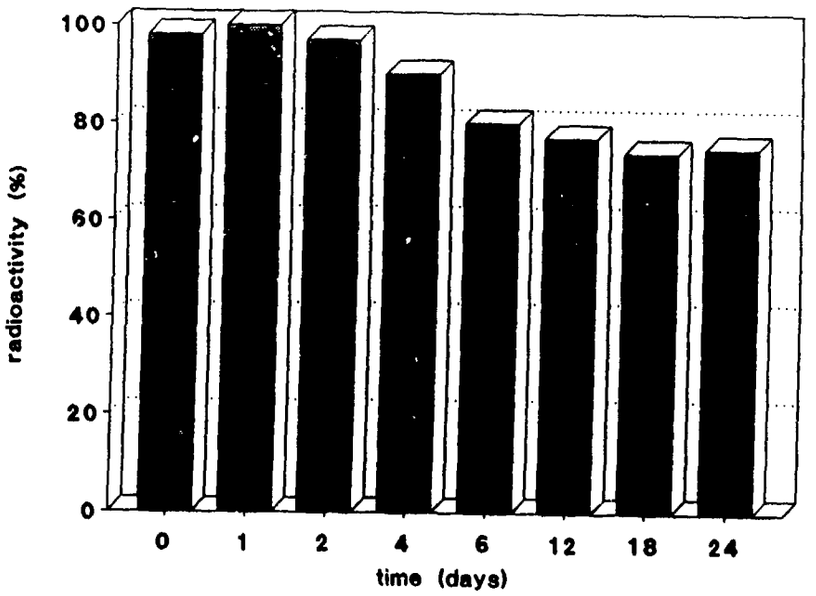

Figure 116. Stability of mustard gas-adducts to hemoglobin at neutral pH. Human blood was treated with 1 mM [^{35}S]mustard gas and hemoglobin was isolated and incubated at 37 ^{o}C for several days. After acid-acetone precipitation (-20 ^{o}C) of the protein, the radioactivity in the precipitate was counted and compared to total radioactivity, to determine stability of adducts during that time. The radioactivity in the globin fraction immediately after treatment with [^{35}S]mustard gas was set at 100%

III.17. Immunochemical methods for the detection of mustard gas adducts in proteins of erythrocytes

III.17.1 Polyclonal antiserum raised against mustard gas adducts to hemoglobin

For the immunochemical detection of mustard gas adducts in (blood) proteins, two approaches are being followed up to the present. One aims at the detection of the mustard gas-modified N-terminal valine in Hb, the other is a less specific approach, involving antibodies raised against mustard gas-protein adducts in general. In first instance, it was attempted to obtain polyclonal antisera suitable for the devolopment of detection and selection procedures. Two different rabbit antisera were raised, for which purpose Hb treated with 1 mM mustard gas and with N-(2'-hydroxyethylthioethyl)-D,L-valine coupled to Keyhole-Limpet Hemocyanine (KLH) via EDC, respectively, were used as immunogen. Immunizations were performed at 4 week intervals. Two weeks after the third immunization the rabbits were bled and the antisera collected.

The antiserum raised against Hb treated with 1 mM mustard gas was tested in a direct ELISA with Gb, Gb treated with 1 mM mustard gas, human serum albumin (HSA), and HSA treated with 1 mM mustard gas, used as antigens to coat the wells of the microtiter plates (1 μg/ml). No difference in signal between the mustard gas-treated and untreated proteins was detected (Figure 117). As expected, the response against Gb was much higher than that against HSA. There may be several reasons for the negative result of these preliminary experiments: (i) the adducts in the coating proteins are not accessible to the antibodies, (ii) treatment of the proteins with 1 mM mustard gas resulted in too few adducts that can be recognized by the antibodies, (iii) no antibodies are formed, because the adducts are too weakly immunogenic, and (iv) only one rabbit was used for immunization.

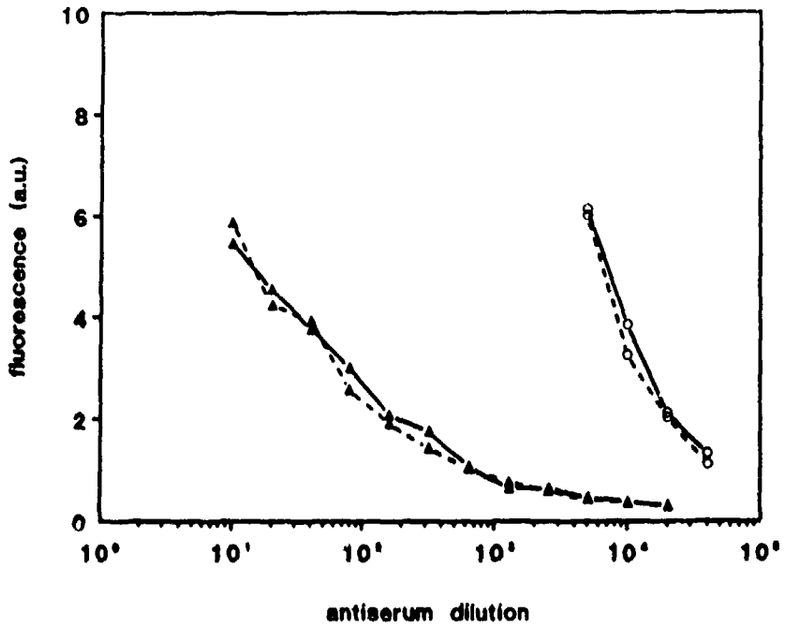

Figure 117. Antibody response of the serum of a rabbit immunized with hemoglobin treated with 1 mM mustard gas, in a direct ELISA. The wells were coated with 50 ng of untreated globin (Gb) (o---o), globin treated with 1 mM mustard gas (o——o), untreated human serum albumin (HSA) (▲---▲), and HSA treated with 1 mM mustard gas (▲——▲)

The antiserum of the rabbit immunized with N-(2'-hydroxyethylthioethyl)-D,L-valine coupled to KLH was tested in a direct ELISA with HSA, HSA treated with 1 mM mustard gas, N-(2'-hydroxyethylthioethyl)-D,L-valine coupled to HSA (HSA-Val-HD), HP, HP treated with 5 mM mustard gas, Hb, and Hb treated with 1 mM mustard gas as coating antigens in the wells of the microtiter plates (1 μg/ml). Various serum dilutions were tested (Figures 118 and 119). Figure 118 shows an equally low response of the serum against HSA and HSA treated with

1 mM mustard gas and a high response against HSA-Val-HD. Also the responses against HP, HP treated with 5 mM mustard gas, Hb and Hb treated with 1 mM mustard gas were low (Figure 119). It can be concluded that antibodies specific for mustard gas adducts are not present in the serum. The high response to HSA-Val-HD should probably be attributed to artifical determinants present in immunogen and testing antigen that are induced by the coupling with EDC. Such misleading EDC-determinants are encountered frequently in studies like these. Therefore, at this moment, an antiserum against protein adducts of mustard gas is not available for the development of methods to screen hybridoma supernatants or detection procedures.

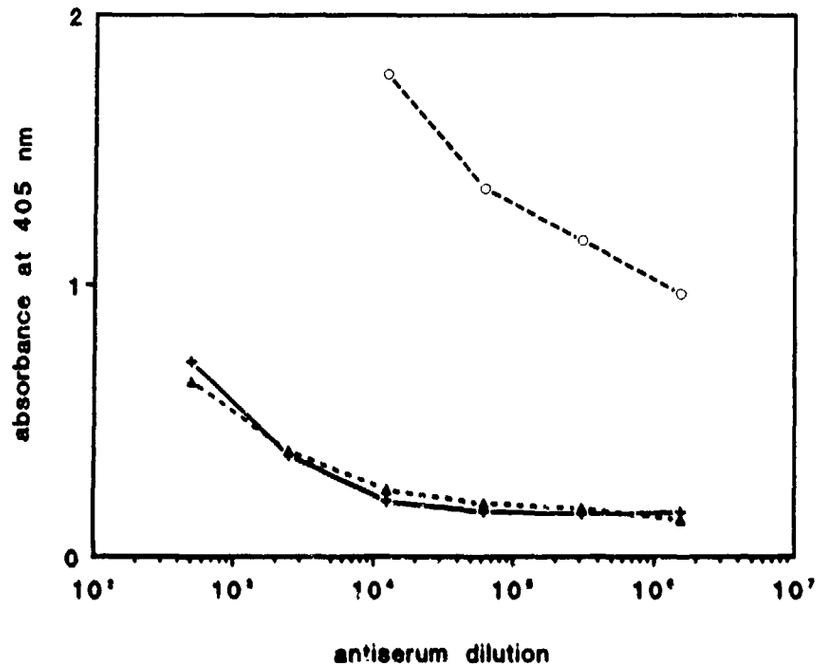

Figure 118. Antibody response of the serum of a rabbit immunized with N-(2'-hydroxyethylthioethyl)-D,L-valine coupled to Keyhole Limpet Hemocyanine (KLH) via EDC, in a direct ELISA. The wells were coated with 50 ng of untreated human serum albumin (HSA) (+——+), HSA treated with 1 mM mustard gas (▲---▲), or N-(2'-hydroxyethylthioethyl)-D,L-valine coupled to HSA via EDC (o---o)

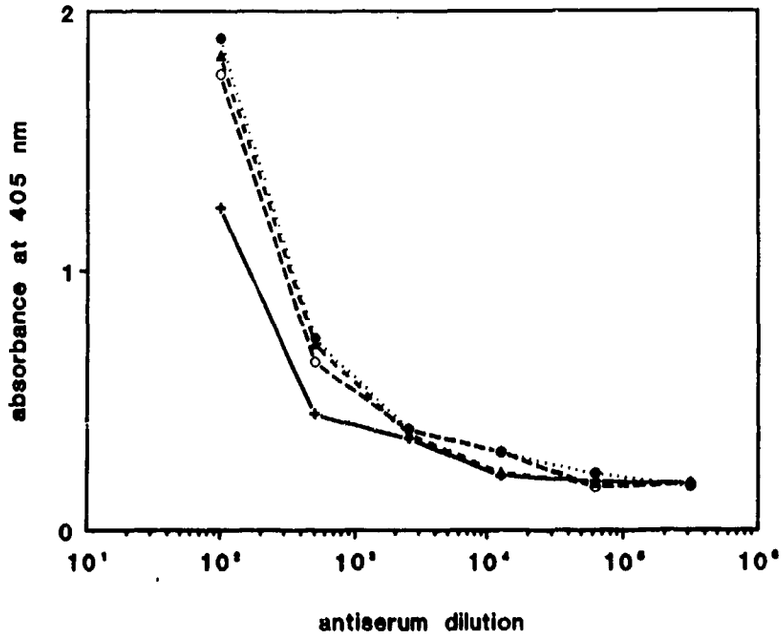

Figure 119. Antibody response of the serum of a rabbit immunized with N-(2'-hydroxyethylthioethyl)-D,L-valine coupled to KLH via EDC, in a direct ELISA. The wells were coated with 50 ng of the N-terminal heptapeptide of the α-chain of hemoglobin (HP) (+——+), HP treated with 5 mM mustard gas (---), hemoglobin (Hb) (o---o), or Hb treated with 1 mM mustard gas (o···o)

III.17.2. Monoclonal antibodies for hemoglobin-mustard gas adducts

The N-terminal heptapeptide of hemoglobin alkylated by mustard gas and coupled to KLH via EDC, was used in attempts to obtain monoclonal antibodies. Four mice were immunized with this material (see II.16.3) and the sera sampled after the first and the second immunization were tested in a direct ELISA. For the screening of the sera we used a commercially available pentapeptide, val-leu-ser-glu-gly, the first three residues of which are identical to the N-terminal sequence of the heptapeptide. This peptide was alkylated with mustard gas at the amino group of valine, and attached via EDC coupling to BSA. As a control, also the non-alkylated pentapeptide was coupled to BSA. Both substances were used as coating material in the ELISA. The sera of the four mice were diluted 100, 200 and 400 times. Disappointingly, no significant specific antibody activity was detected. It was nevertheless decided to continue this approach and to carry out fusion experiments. After the third immunization, cells of the spleen of one mouse were fused with mice SP2/0 cells as described in

II.13.7. The supernatants of the resulting hybridomas were screened on mustard gas-alkylated hemoglobin and on native hemoglobin. The supernatants of six wells showed a positive response in the direct ELISA against mustard gas-alkylated hemoglobin, one of which also showed a positive response against the native hemoglobin.

These hybridomas were subcloned by limiting dilution which yielded three monoclonals. All three appeared to be of the IgM subclass. Attempts to set up a competitive assay with these monoclonal antibodies so far did not yield satisfactory results. Furthermore, a direct ELISA with hemoglobin treated with $\leq$ 1 mM mustard gas, instead of 5 mM, did not result in a positive response on the mustard gas-treated hemoglobin.

A new fusion experiment was performed with the spleen cells of mice immunized with the heptapeptide treated with mustard gas in our attempt to obtain antibodies of the IgG type. After screening on mustard gas-treated hemoglobin, a number of positive hybridomas remained. These were tested on the production of IgG antibodies but so far, no clones producing such antibodies were found.

In a more generalized approach to obtain antibodies directed against protein adducts of mustard gas, a mouse was immunized with chicken gammaglobulin that had been treated with mustard gas (see II.16.3). After the standard boostering procedure, the spleen cells of the mouse were used for a fusion experiment. This yielded 14 monoclonals producing antibodies with a positive response against hemoglobin treated with 5 mM mustard gas. Unfortunately, these also were all of the IgM type.

III.17.3 The hapten gly-gly-gly-glu-5-(2'-hydroxyethylthioethyl) ester-1-amide

The alkylated tetrapeptide gly-gly-gly-glu-5-(2'-hydroxyethyl-thioethyl) ester-1-amide was synthesized to serve as a hapten for generation of monoclonal antibodies recognizing protein adducts in skin biopsis. This antigen was already coupled to a carrier protein (see II.16.3), but immunizations have not been carried out so far.

IV. DISCUSSION

Identification of mustard gas-adducts to calf thymus DNA and DNA of human white blood cells

To the best of our knowledge, the products arising from alkylation of DNA due to in vivo exposure to mustard gas have not been investigated. In vitro alkylation of DNA and RNA by mustard gas has been studied by Lawley et al. (30-35) in the early sixties. They suggested that foremostly the N7 nitrogen of guanine is alkylated, leading to N7-(2'-hydroxyethylthioethyl)-guanine, as well as to the corresponding intrastrand and interstrand (36) di-adduct di-(2-guanin-7'-yl-ethyl) sulfide. The authors also report that the N3 nitrogen of adenine in DNA is alkylated to give N3-(2'-hydroxy-ethylthioethyl)-adenine. In RNA the N1 adduct of adenine is also formed, presumably because this position is not hydrogen-bonded, as it is in double-stranded DNA. Similar DNA adducts have been reported by Kirchner and Brendel (117) upon exposure of yeast cells to mustard gas. Until now, all these products were characterized only on the basis of similarity of their UV spectra and chromatographic behavior with that of analogous alkyl-substituted purines. In most cases, except for the N7 guanine monoadduct, it was unclear whether enough of the adducts had been isolated to allow further analysis and characterization.

We have (re)synthesized and characterized several adducts of mustard gas with guanine and adenine for use as markers in the identification of adducts formed upon exposure of calf-thymus DNA or human WBC to mustard gas. Using the early, but highly reproducible, work of Brookes and Lawley as a starting point, we have now developed methods of synthesis and purification for the N7 mono- and di-adducts of guanine, as well as the N3 adduct of adenine, which yield the pure adducts on a 10-100 mg scale. This allowed full characterization of these adducts based upon thermospray and electron impact mass spectrometry, as well as on ^{1}H- and ^{13}C-NMR spectroscopy.

In more recent investigations, Ludlum et al. have studied the reaction of monofunctional sulfur mustard (chloroethyl ethyl sulfide; CEES) with 2'-deoxyguanosine (98) and with calf-thymus DNA (37). In both investigations, the alkylation product of CEES at the O6-position of 2'-deoxyguanosine was found to be a minor product (0.1% of the total alkylation). Attempts to depurinate this product to O6-(ethylthioethyl)-guanine failed, presumably due to rapid dealkylation at O6 in acidic aqueous solution. It has not become clear whether the authors obtained the corresponding O6-adduct of mustard gas with 2'-deoxy-guanosine (97). We have used a very recent method of synthesis for O6-adducts of 2'-deoxyguanosine to obtain O6-(2'-hydroxyethyl-thioethyl)-2'-deoxyguanosine. The key step in this method is the replacement of a 1-methylpyrrolidinium group at the 6-position of 2'-deoxyguanosine by an appropriate alcohol, in this case 2-acetoxy-ethyl 2'-hydroxyethyl sulfide or 2-t-butyldimethyl-silyloxyethyl 2'-hydroxyethyl sulfide. The desired adduct was obtained in 12% overall yield. In contrast with the results of Ludlum et al. with the

corresponding CEES derivative, we obtained O6-(2'-hydroxyethylthioethyl)-guanine via acid-catalyzed depurination of the 2'-deoxyguanosine derivative, without overriding dealkylation at O6. In our route of synthesis of the O6-derivative we used for the first time new acyl and trialkylsilyl derivatives of thiodiglycol and semi-mustard gas which we designed in order to circumvent problems due to the bifunctionality of thiodiglycol and semi-mustard gas. At the end of the synthesis route, the hydroxyl group of the 2'-hydroxyethylthioethyl moiety is deprotected, in alkaline medium for the acyl derivatives and in acidic medium in case of the trialkylsilyl derivatives.

In other studies on the alkylation of 2'-deoxyguanosine with CEES in aqueous solution (pH 6.0) Sack et al. (118) have found evidence for the formation of a small amount of N2-(ethylthioethyl)-2'-deoxyguanosine (Figure 120a), in addition to the expected large amount of the N7 adduct. In our studies on the alkylation of 2'-deoxyguanosine with mustard gas in aqueous solution (pH 7.5), we have not found an N2 adduct, possibly due to the fact that we have not attempted to isolate and characterize all minor reaction products. However, we have isolated N1-(2'-hydroxyethyl-thioethyl)-2'-deoxyguanosine (Figure 120b), i.e., a hitherto unreported reaction product of mustard gas with nucleosides, as a minor but significant product from the reaction mixture. This product was fully characterized by means of ^{1}H- and ^{13}C-NMR (HETCOR) spectroscopy, thermospray mass spectrometry and UV spectra. The formation of this product is rather surprising because the N1-position in 2'-deoxyguanosine is known to be highly unreactive in aqueous solution. So far, N1-adducts have only been obtained by alkylation in basic, nonaqueous media in which the 2'-deoxyguanosine anion is prevalent (119).

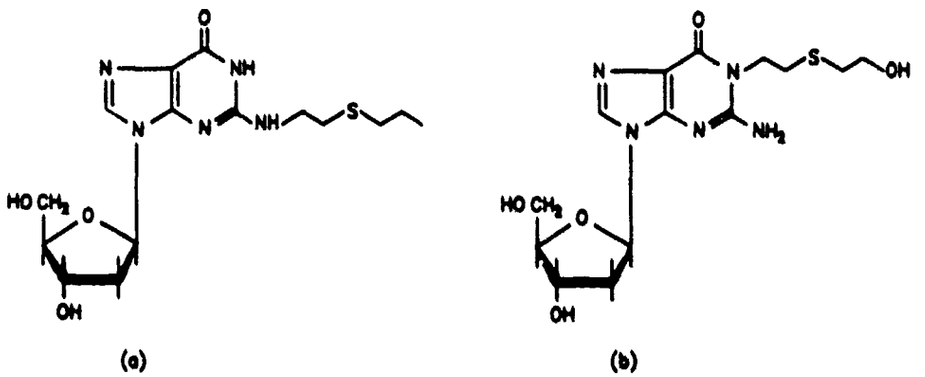

Figure 120. Chemical structures of N2-(ethylthioethyl)-2'-deoxyguanosine (a) and of N1-(2"-hydroxyethylthioethyl)-2'-deoxyguanosine (b)

In the present study, prevalence of the adduct and its stability and persistence in the living cell are important aspects in selection of the reaction product between DNA and mustard gas for use in the immunization of mice and subsequent isolation of hybridomas that

produce specific antibodies against DNA damage due to mustard gas. In experiments with double-stranded calf-thymus DNA and white blood cells exposed to [^{35}S]mustard gas, N7-(2'-hydroxyethylthioethyl)-guanine was shown to be the major adduct. Approximately 60% of the radioactivity reacted at the N7-position of guanine resulting in this monoadduct. The N7 di-adduct and the N3-adenine monoadduct were formed to a lesser extent.

With the techniques available, it was impossible to detect O6-(2'-hydroxyethylthioethyl)-guanine. If this adduct is formed, it is present only as a trace adduct (less than 0.5% of total detected radioactivity). A very low degree of alkylation at O6 of the guanine moiety by mustard gas should be expected on the basis of the high s-value (0.95) of the episulfonium ion in the Swain-Scott equation (120,121). Furthermore, we found that the O6-(2'-hydroxyethyl-thioethyl)-guanine is rather unstable due to relatively rapid dealkylation. For double-stranded DNA, the three detectable adduct peaks represented ca. 90% of all radioactivity. Upon alkylation of single-stranded DNA, the corresponding peaks represented only 66% of all radioactivity. Two additional large peaks were detected in the hydrolysis products, containing ca. 11 and 16% of total radioactivity. These two adducts are not identified yet. One of the unidentified peaks may correspond with N1-(2'-hydroxyethyl-thioethyl)-adenine, which is also formed in substantial amounts upon alkylation with mustard gas of RNA, in which N1 of adenine is not hydrogen-bonded (32,35). Further studies will have to show whether the N1-adduct of guanine is formed upon alkylation of double- or single-stranded DNA. In this study we have focused on the adduct pattern induced in double-stranded DNA, representing the prevalence of alkylation by mustard gas in biological samples.

Our results show that mustard gas is a very effective alkylating agent. Even in blood, in which numerous other reactive constituents are present, 1 out of 124 guanine bases was alkylated to form the N7 monoadduct upon exposure to 1 mM mustard gas. When double-stranded DNA was treated, even 1 out of 10 guanines was alkylated by 1 mM mustard gas. Comparable experiments with diethyl sulfate showed an alkylation degree of only one N7-ethyl-guanine adduct per 400 unmodified guanines upon exposure of white blood cells to 100 mM diethyl sulfate, whereas an alkylation level of one N7-guanine adduct per 45 unmodified guanines was observed for double-stranded DNA after exposure to 77 mM diethyl sulfate (122). The concentrations of mustard gas mentioned above are very high in comparison with the so-called biologically relevant doses which are used, for instance, in studies to determine cell survival as expressed by means of colony-forming ability. When Chinese hamster ovary cells are exposed to 1.4 µM mustard gas, 37% of the cells in the population survive and will form colonies after exposure.

These studies suggest that the biologically relevant doses are probably in the micromolar range. For cells in blood, the critical concentration might be somewhat higher than for the hamster cells in culture medium, but a comparison of the induction of DNA interstrand

crosslinks under the two conditions (Figures 73 and 75) suggests that the difference will not be more than a factor of 2-3. This implies that calibration of the methods for adduct assay should be performed preferably on DNA that is alkylated by mustard gas to the same extent as the DNA of cells in blood exposed in the micromolar range (0.1 to 10 μM). The calibration method applied was based on radioactively labelled mustard gas. However, in order to establish the degree of alkylation in DNA exposed to such low doses of mustard gas, extrapolation of the ratio of alkylated bases versus non-modified bases in DNA from blood cells exposed to much higher doses (0.1 - 1 mM) had to be applied. This was a consequence of the relatively low specific activity of the batches of [^{35}S]mustard gas available for the experiments (850 and 286 MBq/mmol), which did not permit derivation of sufficiently accurate data for the degree of alkylation of DNA from cells exposed in blood to concentrations of mustard gas below 0.1 mM. At this concentration, an alkylation level of about one adduct per 4000 non-modified nucleotides was observed for the predominant N7-guanine monoadduct. The values at lower concentrations were estimated by linear extrapolation. For calibration purposes, also isolated DNA was treated with [^{35}S]mustard gas, which permitted the use of concentrations down to 1 μM. These DNA preparations were used to calibrate the alkylation degree of identical preparations exposed in parallel to "cold" mustard gas. The latter preparations were used to calibrate the quantitative ELISA procedure.

In future experiments, these extrapolations as well as the use of radioactive material can be avoided if an alternative method of analysis for alkylated bases in digested DNA can be developed based upon HPLC with electrochemical detection. In this way, Park et al. (123) were able to detect 100 fmol N7-ethylguanine. In our laboratory, the detection limit amounted to 0.45 N7-ethylguanine/10^6 nucleotides in liver cells from rats that were exposed to hydrazine (122; for comparison, the highest sensitivity ever reached with our ^{35}S procedure after exposure of purified DNA amounted to about 50 adducts/10^6 nucleotides). Possibly, this HPLC procedure can be used for the calibration of immunochemical assays.

The development of immunochemical detection methods: ELISA

The immunization of rabbits with calf-thymus DNA that had been exposed to mustard gas resulted in the polyclonal antiserum W7/10 with a high specificity for adducts of DNA with mustard gas. With this antiserum a screening method for specific antibody activity of hybridoma-supernatants could be developed and optimized.

Next to a screening method for supernatants of hybridomas, a competitive ELISA was developed with single-stranded calf-thymus DNA treated with mustard gas as competitor. This competitive ELISA appears suitable to measure the level of mustard gas adducts in biological samples such as human blood exposed to mustard gas. It was shown that untreated DNA does not give any inhibition, not even at high amounts. This is a criterion for the selectivity of the antiserum.

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The N7-guanine monoadduct is the most abundant modification in DNA that is converted into the ring-opened derivative during treatment with alkali. Since DNA exposed to mustard gas after treatment with alkali is no longer recognized by the antiserum, it is likely that this antiserum is directed mainly against the N7-guanine monoadduct. The sensitivity of the competitive ELISA at the 50% inhibition point is a few femtomoles per well of this adduct. When the 20% inhibition point instead of the 50% inhibition point was chosen as a criterion, 0.4 fmol N7-guanine monoadduct/well is detectable in single-stranded calf-thymus DNA. Very low levels of alkylation can be detected because of the high sensitivity of the method, together with the low cross-reactivity of the antibodies with untreated DNA. With single-stranded calf-thymus DNA treated with 0.1 μM mustard gas, the detection of one N7-guanine monoadduct amongst 1.3×10^5 unmodified guanines was shown to be possible. A tenfold lower level could be reached with the DNA treated with 0.01 μM mustard gas, provided that the 20% inhibition point was used, thus allowing the detection of one adduct amongst 5.2×10^6 unmodified nucleotides.

In order to reach a sensitivity of 0.4 fmol adduct/well (20% inhibition level) using competitor DNA with a very low alkylation degree ($\leq$ 0.01 μM mustard gas) it is necessary to add more competitor DNA and/or to decrease the concentration of the N7-guanine monoadduct-specific antibodies with a simultaneous increase of the incubation period. Until now the highest amount of added competitor DNA was 2.5 μg/well. To obtain the required amount of DNA from biological specimens (DNA from white blood cells), large samples are needed. Sometimes it is impossible to reach the level of 20% inhibition by adding more DNA. Therefore, a better method should be developed to detect low levels of damage (< 0.1 μM mustard gas). Possibly, one such method is the amplification of the detection signal by use of the avidin-biotin complex (124).

For the initial development and optimization of the competitive ELISA to detect damage due to mustard gas, purified single-stranded calf-thymus DNA was used. In this type of DNA, the antigens are presented in an optimal way for antibody recognition. However, the immunochemical methods for this project are aimed at the detection of mustard gas-damage in biological samples, i.e., human blood or skin biopsies. After exposure to the same concentration of mustard gas, much more double-stranded DNA is required for effective competition than single-stranded DNA treated with mustard gas, probably due to shielding of the damage by the complementary DNA strand, as well as to interstrand crosslinks of mustard gas which prevent an optimal presentation of antigen. A 13-fold decrease in sensitivity relative to single-stranded DNA was measured. Therefore, DNA in biological samples should be converted into the single-stranded form which is accessible for the antibodies.

In the biological samples, the DNA is present in the cell nucleus, as double-stranded material, in tight interaction with the nucleus proteins, the histones. The isolation of this DNA and the preparation for the ELISA require adequate methods to disrupt the cell wall and the nuclear membrane, to digest the proteins, to remove RNA, and to release the DNA in single-stranded form without changing or destroying the adducts with mustard gas. The usual method for disrupting the cell wall and releasing the DNA in the single-stranded form is treatment of the WBC with alkali (93). However, this method is not suitable, because of opening of the imidazolium ring* in the N7-guanine adducts which destroys the binding of the antibodies to the mustard gas adducts. Also, extensive heating at 100 °C or lowering the pH in combination with denaturating agents cannot be used, because of the release of the N7-modified guanines from the DNA backbone.

Formamide is another agent for the induction of single-strandedness. In preliminary experiments with single-stranded DNA treated with 70% formamide at 56 °C, we found that formamide did not modify or destroy the adducts and did not interfere with the ELISA when solutions with high DNA concentrations (1 mg/ml) were treated with 70% formamide, provided that the solution was diluted 25-fold before being used in the ELISA. However, formamide did induce single-strandedness, although slightly less effectively than alkali did (100% with alkali versus 90% with formamide). When the dilution step was omitted, the high concentrations of formamide caused substantial background signals in the ELISA. Since only a small amount of DNA can be isolated from biological samples ($\leq$ 25 μg/ml blood), extensive dilutions cannot be applied to avoid these high background signals. Therefore, formamide should be removed by, e.g., dialysis or precipitation of the DNA. Howerver, our attempts to remove formamide did not yield satisfactory results.

Another problem was encountered with formamide when high concentrations of this compound were used to disrupt cell walls of white blood cells and to release the DNA. Similar competition curves were obtained when DNA from untreated white blood cells or from white blood cells treated with various concentrations of mustard gas were used, which indicated that the mustard gas adducts were not recognized. Possibly, the formamide treatment does not lead to complete degradation of the cells and complete removal of the histones from the DNA for optimal presentation of the antigens. In view of the various difficulties encountered with formamide, its use at high concentrations was discontinued and other ways were explored for cell disruption and DNA isolation and for the conversion of DNA into a single-stranded form.

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* Kinetic measurements showed that the rate of ring-opening of N7-(2"-hydroxyethylthioethyl)-guanosine-5'-phosphate at pH 11.2 is virtually the same as that of the corresponding N7-methyl derivative (see III.7).

competition was obtained with double-stranded DNA isolated from white blood cells exposed to 0.1 mM mustard gas. In the mean time a suitable method for the unwinding of double-stranded DNA was developed, viz., mild heating (25 min at 52 °C) of the DNA at low ionic strength in the presence of low concentrations of formamide (4%) and formaldehyde (0.2%). By applying this treatment, N7-guanine monoadducts induced in double-stranded DNA could be detected with the same efficiency as those induced in single-stranded DNA. When this method was applied to DNA isolated with the sodium dodecyl sulfate procedure, adducts could be detected in DNA from white blood cells that had been exposed to 10 or 2 μM mustard gas. However, ca. 20 fmol of N7-guanine monoadduct were required to obtain 50% inhibition in the assay with DNA from blood treated with 10 μM mustard gas, whereas only a few fmol of the monoadduct were needed after exposure of dissolved single-stranded DNA. Probably, the antigen recognition in DNA from blood treated with mustard gas was still not optimal for reasons which are not yet understood.

As mentioned before, it should be possible under optimal conditions to detect with this competitive ELISA 1 modified guanine amongst 5.2×10^6 unmodified nucleotides in single-stranded DNA exposed to 0.01 μM mustard gas. Theoretically, it should also be possible to detect 1 modified guanine amongst 5.2×10^6 unmodified nucleotides when DNA is isolated from WBC exposed to mustard gas and when complete single-strandedness is induced. This corresponds with an exposure of the white blood cells to ca. 0.1 μM mustard gas, as derived by linear extrapolation from exposure to higher concentrations. In order to optimize detection conditions, the presentation of the antigens in DNA of white blood cells exposed to mustard gas should be improved by using conditions at which the adducts are not destroyed.

Methods to achieve a high sensitivity in the competitive ELISA can be developed by a combination of various procedures to release the DNA from the cells or tissues, to digest the proteins and to induce single-strandedness in the DNA. The procedure now available for the disruption of the cell walls and membranes, i.e., the use of high concentrations of sodium chloride or sodium dodecyl sulfate followed by protein digestion appears satisfactory. The influence of extraction of DNA with phenol and precipitation with ethanol upon the stability of the adducts should be studied to see whether these steps should be omitted or replaced. The induction of single-strandedness in DNA that has been treated by mustard gas in solution can now be achieved in a satisfying manner. Further studies are needed to establish the effectiveness of the procedure for DNA isolated from mustard gas-exposed cells. For the induction of single-strandedness, alternative methods are still available, such as digestion by exo- and endonucleases in combination with an unwinding treatment and sonication to prevent renaturation.

In the competitive ELISA for analysis of DNA treated with an unknown amount of mustard gas, it is necessary to measure the concentration of DNA. A method should be developed to determine small quantities of DNA from blood (10-50 ng/sample) for use in the competitive ELISA.

For the quantitation of DNA adducts in experimental samples, a standard batch of mustard gas-treated DNA with an exactly known degree of alkylation should be available for calibration purposes. A large batch of this material has been prepared, which will remain available.

Monoclonal antibodies

Upon treatment of DNA with mustard gas, the N7-guanine monoadduct was shown to be the major adduct in all circumstances. For that reason it was decided to synthesize a hapten based on this adduct for use in the immunization of mice and the subsequent isolation of hybridomas which produce specific antibodies against damage in DNA induced by mustard gas.

In our first attempt to obtain a hapten we have tried to alkylate the N7-position of 2'-deoxyguanosine-5'-phosphate with mustard gas at pH 7.5, and to use the phosphate moiety for subsequent coupling of the hapten to a carrier protein. However, the alkylation proceeded almost exclusively at the phosphate moiety (125) instead of the N7-position. Attempts to shift the regiospecificity of the alkylation reaction to N7 by means of protonation of the least acidic function at phosphate at pH 4.5 (59) led to some alkylation at N7, but also to complete depurination. Even alkylation at N7 of 2'-deoxyguanosine with mustard gas at pH 7.5, to be followed by enzymatic phosphorylation at the 5'-position (100), led to depurination. In view of these results we decided to attempt alkylation at N7 of guanosine-5'-phosphate, since (i) guanosine derivatives depurinate several orders of magnitude less rapidly than the corresponding 2'-deoxyguanosine analogs (126) and (ii) other investigators had learned that the presence of an extra hydroxyl group at the 2'-position of the sugar moiety in the hapten does not decrease the binding activity of antibodies towards adducts in DNA (101). Treatment of guanosine-5'-phosphate with mustard gas in aqueous solution at pH 4.5 gave a reaction mixture from which both the desired N7-(2"-hydroxyethylthioethyl)-guanosine-5'-monophosphate and the corresponding di-adduct could be isolated on a 10-100 mg scale by means of various chromatographic techniques. The products were characterized with ^{1}H- and ^{13}C-NMR spectroscopy, whereas the molecular weight of the monoadduct was confirmed with FAB-ionized mass spectrometry. Coupling of the N7-guanine monoadduct to carrier protein with 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide via an in situ prepared imidazolium derivative at the phosphate moiety proceeded smoothly.

After immunization of mice with N7-(2"-hydroxyethylthioethyl)-guanosine-5'-phosphate (N7-GMP monoadduct) coupled to a carrier protein, fusion experiments yielded 10 hybridoma clones producing monoclonal antibodies that recognize adducts of mustard gas. The clones were selected on single-stranded DNA treated with 10 μM mustard gas, i.e., not on the immunogen itself coupled to a carrier. In competition experiments it was found that the most promising monoclonal antibodies, 2D3 and 2F8, are primarily directed against the N7-GMP monoadduct of mustard gas. A low cross-reactivity was seen

when N7-Me-GMP, N7- and O6-guanine monoadducts of mustard gas were used as competitor. Especially the low cross-reactivity with O6-guanine monoadduct is striking. No cross-reactivity has been detected with GMP. The monoclonal antibodies recognize the adduct only when present as alkylated guanine with an intact imidazole ring. When this ring is opened, almost 5,000 times more competitor is required to reach the 50% inhibition point. It can be concluded that these monoclonal antibodies are specific for the ring-closed N7-GMP monoadduct of mustard gas.

It appeared in competitive ELISA's that the monoclonal antibodies recognize DNA exposed to mustard gas better than the free nucleotide or guanine alkylated with mustard gas: the antibodies were selected on single-strand DNA and part of the DNA-backbone might be involved in the antigen presentation. This aspect is of importance with regard to the preparation of DNA samples from human origin for a detection method to be used for the verification of exposure to mustard gas in casualties. Since mustard gas adducts in DNA are better recognized than those in the alkylated nucleotides, it appears more advisable to use well purified DNA than to aim at the detection of the adducts in nucleotides or bases released from the DNA.

Starting with the crude supernatants obtained after culturing of the selected clones, the monoclonal antibodies were purified via ammonium sulfate precipitation and affinity chromatography on a protein A column. In the initial experiments, low recoveries were obtained. The possibility that the antibodies were degraded by proteinases released from dead cells could be excluded: no significant decrease in antibody activity in the supernatants occurred over a period of 7 days. Further studies showed that both the precipitation step and the chromatography caused significant losses of antibody activity, but nevertheless, a satisfactory over-all recovery of pure antibody appeared attainable. A 1-liter culture yielded on the average 4.7 mg of purified monoclonal antibodies. These antibodies can be stored in PBS at -20 °C for months.

The antibodies produced by six clones were tested in the competitive ELISA. For five clones, viz., 2D3, 2F12, 2A4, 2G8 and 1H7, the sensitivity was comparable to that of the assays performed with the polyclonal antiserum W7/10. These antibodies recognize the N7-guanine monoadducts of mustard gas containing an intact imidazolium ring, since after exposure of the alkylated DNA with ring-disrupting agents such as alkali, inhibition no longer occurred. Consequently, alkali should also not be used in a method for the sensitive detection of mustard gas exposure with the monoclonal antibodies.

Recently, we have performed experiments to detect local DNA damage in skin samples. Pieces of human skin obtained from cosmetic surgery were exposed to air saturated with mustard gas vapor at 30 °C (1360 $mg.m^{-3}$) for periods ranging from 1 to 10 min. By using the monoclonal antibodies we were able to detect N7-guanine monoadducts of mustard gas in the skin samples by means of immunofluorescence microscopy. In skin exposed for 10 min the DNA in many epidermal cells was clearly

$mg.m^{-3}$) for periods ranging from 1 to 10 min. By using the monoclonal antibodies we were able to detect N7-guanine monoadducts of mustard gas in the skin samples by means of immunofluorescence microscopy. In skin exposed for 10 min the DNA in many epidermal cells was clearly shown to be substantially alkylated. In this pilot experiment the detection limit was at 1 min exposure, which corresponds with a Ct value of mustard gas (1360 $mg.min.m^{-3}$) that would not yet give blisters (116). An additional pilot experiment was performed to study the persistence of this type of lesion in human skin. For this purpose, human skin resulting from cosmetic surgery was exposed to mustard gas vapor-saturated air for 4 min. Surprisingly, the nuclear fluorescence in the cells of the epidermis appeared to increase during the first 24 h after exposure; thereafter a decrease was observed. It is not clear yet whether indeed more adducts are formed within the initial 24 h or that structural changes in the nuclear DNA take place which facilitate the procedures applied to make the adducts accessible to the antibodies. Formation of more adducts during the initial 24 h appears rather unlikely in view of the limited stability of mustard gas in the culture medium, although storage of small amounts of intact mustard gas in the skin after cutaneous exposure cannot be excluded (116).

Adducts to proteins and model peptides

It is expected that adducts of mustard gas to proteins, for instance hemoglobin or serum albumin, have a higher persistency due to the absence of repair systems for these types of lesions. This stands in contrast with DNA adducts, which are often repaired by cellular enzyme systems. For the detection and the quantification of exposure to mustard gas in practical situations, it might be attractive to develop an immunochemical method of analysis with antibodies that are specific for protein adducts. Moreover, sufficient amounts of proteins are readily obtained from relatively small blood samples. For example, the mean value for the hemoglobin content in blood is 0.14 g/ml as compared to 50 µg DNA/ml blood. It was shown that mustard gas binds only 1.9 times less efficiently to protein than to DNA. Therefore, immunochemical detection of mustard gas damage in proteins for dosimetry of exposure to mustard gas might be advantageous, provided that the protein adducts can be concentrated by way of purification.

Little is known about the reactivity of mustard gas towards individual amino acids in proteins or the structure and stability of the adducts (22-24). For this reason we have investigated the alkylation of simple model peptides, i.e., N-acetyl-amino acid-methylamides [$CH_3C(O)NH\text{-}CH(Y)\text{-}C(O)NHCH_3$], in which Y represents a group that can be alkylated by mustard gas. We studied the reactions with mustard gas in aqueous solution (pH 7.5) at room temperature of such model peptides derived from aspartic acid, glutamic acid, histidine, cysteine, and methionine. In order to elucidate the reaction products of mustard gas with terminal amino groups, we have also studied the alkylation of valine-methylamide, $NH_2CH(iPr)C(O)NHCH_3$ (see Figure 36). All model peptides were readily

alkylated. In the model peptides derived from aspartic and glutamic acid the free carboxylic groups were alkylated leading to the formation of (2'-hydroxyethylthioethyl) esters. The ester derived from the aspartic acid model peptide underwent a rapid secondary reaction with expulsion of the alcohol moiety by nitrogen of the peptide bond and formation of a succinimide derivative. Evidently, this secondary reaction spoils the use of aspartic acid derivatives for verification purposes. Both ring nitrogens of the histidine peptide were alkylated at approximately the same rate, whereas a small amount of product also was formed in which both nitrogens had reacted. The methionine peptide is alkylated at sulfur, leading to a ternary (and chiral) sulfonium derivative, which decomposes in acidic or alkaline medium. As expected, the primary reaction product of valine-methylamide with mustard gas is the N-(2'-hydroxyethyl-thioethyl) derivative. The model peptide derived from cysteine reacts rapidly with mustard gas. The peptide is alkylated at the thiol moiety of the side chain.

The alkylation products formed in aqueous solution were all identified by means of thermospray-LC-MS. These products were also synthesized by means of independent routes and their identity with the products formed by alkylation of the model peptides in aqueous solution was confirmed with HPLC.

The alkylation products were used as references for competition experiments, in which the relative order of reactivity of the various peptides vis-a-vis mustard gas was studied. The qualitative results obtained clearly showed that cysteine is by far the most reactive amino acid towards mustard gas.

Identification of mustard gas-adducts to hemoglobin

It is known that human erythrocytes have a life-span of ca. 120 days, i.e., a potential target protein such as hemoglobin (Hb) will stay in the circulation for a long time. Hemoglobin is easily isolated after separation of serum and cell fractions, followed by lysis of the erythrocytes. Another protein present in blood in large quantities, serum albumin, is more difficult to isolate because of the presence of many other proteins in the serum fraction from which albumin should be separated by chromatography. Studies on the stability of mustard gas adducts to hemoglobin showed that 70% of the adducts bound to globin are acid- and alkali-labile (treatment with 5 N alkali or acid, during 4 h at room temperature). These data indicate that identification of mustard gas adducts to hemoglobin based upon amino acid analysis after the complete degradation of hemoglobin into amino acids by means of the standard procedure involving treatment with 6 N HCl at 110 °C for 24 h is not feasible. Alternatively, the proteins can be degraded into amino acids or small peptides via digestion with specific enzymes.

Under physiological conditions, however, the mustard gas adducts of proteins are rather stable. Not less than 75% of the initial amount of $[^{35}S]$mustard gas was still bound to hemoglobin after 24 days of

Analogous to the work of Wraith et al. (25) we investigated the alkylation with mustard gas of the N-terminal valine of the α-chain of hemoglobin which was shown to be a good target for alkylation by ethylene oxide. The N-terminal heptapeptide val-leu-ser-pro-ala-asp-lys is conveniently isolated by means of tryptic digestion of the protein, followed by HPLC.

For use as a marker, the heptapeptide was synthesized and mono-alkylated with mustard gas specifically at the amino group of terminal valine. The reaction product was purified by means of HPLC and was characterized by means of amino acid analysis, thermospray mass spectrometry and ^{1}H-NMR spectrometry. With [^{35}S]mustard gas we obtained evidence that exposure of hemoglobin to mustard gas indeed results in alkylation at the N-terminal valine. A broad spectrum of radioactive peaks was obtained after exposure of human blood, isolation of the hemoglobin, digestion with trypsin and chromatographic separation. One of the peaks coincided with the marker, the alkylated synthetic heptapeptide. In contrast to all but one other ^{35}S-peak, this one also was found, unaltered, when the digest had been mildly treated with hydrochloric acid prior to HPLC, in agreement with the acid-stable character of alkylations at the amino group. Upon treatment with acid, most ^{35}S was released, which suggests that also in blood mustard gas mainly forms the acid-labile reaction products with free carboxyl groups in hemoglobin. The ^{35}S-peak that was co-eluted with the alkylated heptapeptide represented about 6% of the total radioactivity bound to hemoglobin. From this figure and the distribution of reacted radioactivity over the various blood constituents, it was derived that mustard gas reacts about 3.7 times as efficiently with the amino group of the N-terminal valine in hemoglobin in human blood than with the N7 of guanine of DNA in WBC.

Together, the results demonstrate that the alkylation of the N-terminal valine of hemoglobin could be a suitable indicator for exposures to mustard gas. Further research will be required to develop a practical detection method based on the formation of this reaction product. The most direct approach would be the isolation of antibodies that recognize specifically this adduct in intact hemoglobin, but the facile isolation of the heptapeptide offers the possibility to enrich the structure to be detected, if desired. As an alternative, the second acid-stable alkylated tryptic peptide might be considered. However, this product will have to be identified first.

The attempts to obtain antibodies directed to protein adducts of mustard gas initially were aimed at a polyclonal rabbit antiserum that could be used to set up detection methods and a system for the screening of hybridomas for the production of adduct-specific antibodies. Analogous to the "DNA-approach," rabbits were immunized with hemoglobin treated with mustard gas and with N-(2'-hydroxyethylthioethyl)-D,L-valine coupled to a carrier protein. Unfortunately, serum with specific activity against mustard gas-damage was not obtained, possibly because of poor immunogenicity of the alkylated proteins. In view of time limitations, no new rabbit

immunizations were performed. Instead, mice were immunized. As immunogen, the alkylated heptapeptide coupled to a carrier protein was used. However, also in these experiments, no specific antibody activity could be detected in the sera, not even after repeated immunizations. Nevertheless, fusions were performed, resulting in a few hybridomas that produced antibodies discriminating between mustard gas-alkylated hemoglobin and the native protein in a direct ELISA. The antibodies of these clones appeared of limited practical use, however, since attempts to set up a competitive ELISA with these monoclonals remained without success. Furthermore, they failed to discriminate between native and alkylated hemoglobin when exposure was lowered from 5 to 1 mM mustard gas. Finally, the antibodies were all of the less suitable IgM type. New fusion experiments aiming at the isolation of more satisfactory monoclonals, preferably of the IgG type, have as yet not been successful.

In another approach it was attempted to raise antibodies to less strictly defined protein adducts of mustard gas by immunizations of mice with chicken gammaglobulin that had been alkylated with mustard gas. Fusion experiments yielded a number of hybridomas producing antibodies, all of the IgM type, with specificity for highly alkylated (5 mM) hemoglobin. It is unknown which determinant(s) they recognize or whether these antibodies might be more suitable for practical applications.

Haptens based on amino acids with high reactivity towards mustard gas

In the absence of an analysis of the most abundant adducts formed by alkylation of proteins, and in addition to the above-mentioned approach involving the terminal heptapeptide in the α-chain of hemoglobin, we are developing haptens based on amino acids which show a high reactivity towards mustard gas. Our competition experiments show that cysteine is highly reactive. Therefore, we intend to synthesize the adduct of cys-gly-gly-gly with mustard gas as a hapten. Attempts to synthesize the tetrapeptide, however, did not succeed. We did obtain the hapten gly-gly-gly-glutamic acid-5-(2'-hydroxyethylthioethyl) ester-1-amide. Such adducts can be used to characterize the monoclonal antibodies that can be obtained after immunization with, e.g., chicken gammaglobulin that has been exposed to mustard gas. Another possibility is the use of these antigens, after coupling to a carrier protein, to generate antibodies. Both approaches might yield antibodies which are especially useful to detect mustard gas damage to proteins in skin biopsies. Obviously, antibodies which are specific for mustard gas adducts of hemoglobin will be very useful to detect damage in blood, but not in skin biopsies. Investigations published shortly after the second world war (38), indicate that the alkali-labile adducts of mustard gas, which we interpret as ester adducts to, e.g., glutamic acid, are stable for weeks or even months in the skin of human volunteers.

V. CONCLUSIONS

1. Routes for the synthesis and purification of the mono- and di-adducts of mustard gas at the N7-position of guanine, for the monoadduct at the O6-position of guanine and at N3 in adenine, were developed, leading to full characterization of these adducts.

2. The hitherto unreported monoadduct of mustard gas at the N1 position of guanine has been identified as an alkylation product of 2'-deoxyguanosine with mustard gas in aqueous solution at neutral pH.

3. O-Acetylated and O-trimethylsilylated derivatives of thiodiglycol and semi-mustard gas are highly useful synthons to obtain monoadducts of mustard gas with DNA and proteins.

4. The monoadduct of mustard gas at the N7-position of guanine was shown to be the major DNA adduct upon exposure of double-stranded calf-thymus DNA and human white blood cells to mustard gas. The corresponding di-adduct and the monoadduct at N3 of adenine were formed to a lesser extent, whereas the monoadduct at O6 of guanine could not be detected with the available techniques.

5. For double-stranded calf-thymus DNA, the three identified reaction products with mustard gas represent ca. 90% of all adducts.

6. Mustard gas is a very effective alkylating agent: even in human blood treated with 1 mM mustard gas, 1 out of 124 guanine bases is converted into the monoadduct at N7.

7. The biologically effective concentration of mustard gas is ca. 1 μM, as evidenced by survival experiments with Chinese hamster ovary cells.

8. Cross-linking of DNA by mustard gas in Chinese hamster ovary cells disappears within a time period of 4 h.

9. Immunization of rabbits with calf-thymus DNA exposed to mustard gas resulted in a polyclonal antiserum W7/10 with a high specificity for adducts of DNA with mustard gas.

10. With the polyclonal antiserum against mustard gas adducts of DNA immunochemical detection methods were developed, such as a competitive ELISA and an ELISA used in the screening of hybridoma cells for production of adduct-specific antibodies, based upon the use of single-stranded calf-thymus DNA exposed to mustard gas as a coating material for the wells.

11. A competitive ELISA was developed in which mustard gas adducts to DNA could be detected with a minimum detectable amount of a few femtomoles of adducts per well and a selectivity which allows detection of one monoadduct at N7 of guanine amongst 1.3×10^6 unmodified guanines.

12. A suitable procedure was developed for conversion of double-stranded DNA into single-stranded without disrupting the N7-guanine monoadduct, which allows the detection of adducts in double-stranded DNA with the same efficiency as in single-stranded DNA.

13. The minimum detectable amount in the ELISA of adduct with DNA in human white blood cells exposed to mustard gas is ca. two orders of magnitude higher than for single-stranded DNA, probably due to shielding by the complementary strand and proteins as well as to interstrand cross-linking, all preventing optimal presentation of antigen.

14. Methods for the synthesis, purification and full characterization of the mono- and di-adducts of mustard gas at the N7-position of guanosine-5'-phosphate were developed, for use as haptens to generate monoclonal antibodies against such adducts in DNA.

15. Hybridomas were isolated which produce monoclonal antibodies that recognize specifically the N7-guanine adducts with an intact imidazolium ring structure. These hybridomas were obtained after immunization of mice with the N7-substituted monoadduct of mustard gas of guanosine-5'-phosphate, coupled to a carrier protein via the phosphate moiety.

16. The sensitivity of the competitive ELISA with the monoclonal antibodies is similar to that observed in the assays performed with the polyclonal antiserum W7/10.

17. A single-cell assay has been developed with the antibodies, which allows the detection of adducts in DNA of human skin due to exposure to non-blistering Ct values of mustard gas vapor. Adducts are still detectable up to 48 h after exposure.

18. Since mustard gas binds almost as effectively to proteins as to DNA, immunochemical detection of mustard gas-damage for dosimetry of exposure to mustard gas based on protein adducts might be advantageous in view of the abundant availability of proteins, provided that the adducts can be concentrated by way of purification.

19. Our studies on the reaction products resulting from alkylation of model peptides with mustard gas provide valuable information on the nature, abundance and stability of the amino acid adducts which are possibly formed upon exposure of proteins to this agent.

20. Cysteine is by far the most reactive amino acid towards alkylation by mustard gas.

21. Since approximately 70% of the adducts of mustard gas to globin is acid- and/or alkali-labile, enzymatic degradation to amino acids and peptides might be a more viable approach to identification of the adducts than the standard approach involving complete degradation into amino acids by means of acid hydrolysis.

22. The N-terminal heptapeptide from the α-chain of hemoglobin, i.e., val-leu-ser-pro-ala-asp-lys, was synthesized and mono-substituted with mustard gas specifically at the terminal amino group of valine, for use as a hapten to generate monoclonal antibodies against adducts of mustard gas with hemoglobin.

23. Alkylation at the N-terminal amino group in the α-chain of hemoglobin takes place upon treatment of human blood with [^{35}]mustard gas, amounting to ca. 6% of the total radioactivity bound to hemoglobin.

24. Immunization of mice with the N-terminal heptapeptide from the α-chain of hemoglobin, mono-substituted with mustard gas specifically at the terminal amino group of valine and coupled to a carrier protein yielded hybridomas that produce monoclonal antibodies of the IgM type. The antibodies recognize hemoglobin alkylated with mustard gas but are not sufficiently selective for practical detection purposes.

25. Immunization of mice with mustard gas-alkylated chicken gammaglobulin yielded hybridomas that produce monoclonal antibodies of the IgM type, which recognize mustard gas-treated hemoglobin.

26. Since mustard gas appears to be especially reactive towards cysteine and possibly also to glutamic acid in proteins, mustard gas adducts to these amino acids bound to gly-gly-gly are synthesized, specifically for use as haptens to generate monoclonal antibodies for immunochemical detection of mustard gas exposure in skin biopsies.

LITERATURE CITED

1. Report of the specialists appointed by the Secretary-General to investigate allegations by the Islamic Republic of Iran concerning the use of chemical weapons. UN document S/16, 433 of 26 Mar 1984.

2. New Compounds in Biological and Chemical Warfare: Toxicological Evaluation, Ghent, May 21-23, 1984, (Chairman: Prof. A. Heyndrickx). Abstract Book.

3. EMBER, L.R. (1984). "Yellow Rain". Chem. Eng. News 62 (2), 8-34.

4. HEYNDRICKX, A. (1986). Mission to South Africa and Angola from 25/08/1986 until 01/09/1986. September, 8, 1986.

5. HEYNDRICKX, A. (1988). Toxicological report on the second mission, February 15-20, 1988, in Angola (Unita-Mr. Savimbi). March 8, 1988.

6. WILLEMS, J.L., DE BLEECKER, J. AND LISON, D. (1989). Organophosphorus induced neuromuscular paralysis. Proceedings of the November 1989 meeting of RSG.3 on Prophylaxis and Therapy against Chemical Agents, AC/243(Panel 8)/1, Vol. 1, The Hague, 13-17 November, pp. 18/1-18/12.

7. The Problem of Chemical and Biological Warfare, Volume 1: The rise of CB weapons (SIPRI). Almqvist and Wiksell, Stockholm, 1971, pp. 159-161.

8. EMBER, L.R. (1988). Fashioning a global chemical weapons treaty. Chem.Eng. News 66(13), 7-17.

9. BENSCHOP, H.P. AND DE JONG, L.P.A. (1988). Toxicokinetic investigations of C(±)P(±)-soman in the rat, guinea pig and marmoset at low dosages-Quantification of elimination pathways. Midterm Report for Grant No. DAMD17-87-C-7015, Prins Maurits Laboratory TNO, Rijswijk, The Netherlands.

10. DE JONG, L.P.A. AND VAN DIJK, C. (1984). Formation of soman (1,2,2-tri-methylpropyl methylphosphonofluoridate) via fluoride-induced reactivation of soman-inhibited aliesterase in rat plasma. Biochem. Pharmacol. 33, 663-669.

11. DE BISSCHOP, H.C.J.V., AUWERX, E. VANSTEENKISTE, S. AND MARTEAU, L. (1989). Fate of reactivatible soman-binding sites in guinea pig tissue. Proceedings of the November 1989 meeting of RSG.3 on Prophylaxis and Therapy against Chemical Agents, AC/243(Panel 8)/1, Vol. 1, The Hague, 13-17 November, pp. 4/1-4/8.

12. DRASCH, G., KRETSCHMER, E., KAUERT, G. AND VON MEYER, L. (1987). Concentrations of mustard gas [bis(2-chloroethyl)sulfide] in the

tissues of a victim of a vesicant exposure. J. Forensic Sci. 32, 1788 - 1793.

13. VYCUDILIK, W. (1985). Detection of mustard gas [bis(2-chloroethyl)-sulfide] in urine. Forensic Sci. Int. 28, 131-136.

14. VYCUDILIK, W. (1987). Detection of bis(2-chloroethyl)-sulfide (yperite) in urine by high resolution gas chromatography-mass spectrometry. Forensic Sci. Int. 35, 67-71.

15. STADE, K. (1964). Pharmakologie und Klinik Synthetischer Gifte, Deutscher Militarverlag, Berlin, p. 137.

16. BLACK, R.M. AND READ, R.W. (1988). Detection of trace levels of thiodiglycol in blood, plasma and urine using gas chromatography-electron-capture-negative-ion chemical ionisation mass spectrometry. J. Chromatogr. 449, 261-270 (1988).

17. WILS, E.R.J., HULST, A.G., DE JONG, A.L., VERWEIJ, A. AND BOTER, H.L. (1985). Analysis of thiodiglycol in urine of victims of an alleged attack with mustard gas. J. Anal. Toxicol. 9, 254 -257.

18. WILS, E.R.J., HULST, A.G. AND VAN LAAR, J. (1988). Analysis of thiodiglycol in urine of victims of an alleged attack with mustard gas, Part II. J. Anal. Toxicol. 12, 15-19.

19. DAVISON, C., ROZMAN, R.S. AND SMITH, P.K. (1961). Metabolism of bis-ß-chloroethyl sulfide (sulfur mustard gas). Biochem. Pharmacol. 7, 65-74.

20. ROBERTS, J.J. AND WARWICK, G.P. (1963). The metabolism of bis-2-chloro-ethylsulphide (mustard gas) and related compounds. Biochem. Pharmacol. 12, 1329-1334.

21. Methods for detecting DNA damaging agents in humans: applications in cancer epidemiology and prevention. Proceedings of a symposium held in Espoo, Finland 2-4 September 1987. (Eds. BARTSCH, H., HEMMINKI, K. and O'NEILL, I.K.), IARC Scientific Publication No. 89, Lyon, 198?

22. NEUMANN, H.-G.(1984). Analysis of hemoglobin as a dose monitor for alkylating and arylating agents. Arch. Toxicol. 56, 1-6.

23. WOGAN, G.N. AND GORELICK, N.J. (1985). Chemical and biochemical dosimetry of exposure to genotoxic chemicals. Environm. Health Perspect. 62, 5-18.

24. FARMER, P.B., NEUMANN, H.-G. AND HENSCHLER, D. (1987). Estimation of exposure of man to suostances reacting covalently with macromolecules. Arch. Toxicol. 60, 251-260.

25. WRAITH, M.J., WATSON, W.P., EADSFORTH, C.V., VAN SITTERT, N.J., TORNQVIST, M. AND WRIGHT, A.S. (1988). An immunoassay for

monitoring human exposure to ethylene oxide. In: Methods for detecting DNA damaging agents in humans: applications in cancer epidemiology and prevention. Proceedings of a symposium held in Espoo, Finland 2-4 September 1987. (Eds. BARTSCH, H., HEMMINKI, K. and O'NEILL, I.K.), IARC Scientific Publication No. 89, Lyon, pp. 271-274.

26. SANTELLA, R.M., LIN, C.D. AND DHARMAJAMA, N.(1986). Monoclonal antibodies to a benzo(a)pyrene diolepoxide modified protein. Carcinogenesis 7, 441-444.

27. ASHBY, J.(1988). Comparison of techniques for monitoring human exposure to genotoxic chemicals. Mutat. Res. 204, 543-551.

28. STADE, K. (1964). Pharmakologie und Klinik Synthetischer Gifte, Deutscher Militarverlag, Berlin, pp. 135-204.

29. BOURSNELL, J.C., FRANCIS, G.E., AND WORMALL, A. (1946). Studies on mustard gas (ßß'-dichlorodiethyl sulphide) and some related compounds. 7. The immunological properties of protein treated with mustard gas and some related compounds. Biochem. J. 768-774.

30. BROOKES, P. AND LAWLEY, P.D. (1960). The reaction of mustard gas with nucleic acids in vitro and in vivo. Biochem. J. 77, 478-484.

31. BROOKES, P. AND LAWLEY, P.D. (1961). The reaction of mono- and di-functional alkylating agents with nucleic acids. Biochem. J. 80, 496-503.

32. BROOKES, P. AND LAWLEY, P.D. (1963). Effects of alkylating agents on T2 and T4 bacteriophages. Biochem.J. 89, 138-144.

33. LAWLEY, P.D. AND BROOKES, P. (1963). Further studies on the alkylation of nucleic acids and their constituent nucleotides. Biochem. J. 89, 127-138.

34. LAWLEY, P.D. AND BROOKES, P. (1968). Cytotoxicity of alkylating agents towards sensitive and resistant strains of Escherichia coli in relation to extent and mode of alkylation of cellular macromolecules and repair of alkylation lesions in deoxyribonucleic acids. Biochem. J. 109, 433-447.

35. SHOOTER, K.V., EDWARDS, P.A. AND LAWLEY, P.D. (1971). The action of mono- and di-functional sulphur mustards on the ribonucleic acid-containing bacteriophage μ2. Biochem. J. 125, 829-840.

36. EDWARDS, P.A. AND SHOOTER, K.V. (1971). Sedimentation characteristics of DNA multiply crosslinked by a difunctional alkylating agent, mustard gas. Biopolymers 10, 2079-2082.

37. LUDLUM, D.B., KENT, S. AND MEHTA, J.R. (1986). Formation of O6-ethylthioethylguanine in DNA by reaction with the sulfur mustard, chloroethyl sulfide, and its apparent lack of repair by O6-alkylguanine-alkyltransferase. Carcinogenesis 7, 1203-1206.

38. RENSHAW, B. (1946). Mechanisms in production of cutaneous injuries by sulfur and nitrogen mustards. In "Chemical Warfare Agents and Related Chemical Problems", Parts III-IV. Summary Technical Report of Division 9, NDRC. Volume 1, Office of Scientific Research and Development, National Defense Research Committee, Washington, D.C., chapter 23.

39. SMITH, P.K., NADKARNI, M.V., TRAMS, E.G. AND DAVISON, C. (1958). Distribution and fate of alkylating agents. Ann. N.Y. Acad. Sci. 68, 834-849.

40. BOURSNELL, J.C. (1948). Some reactions of mustard gas (ßß'-dichlorodiethyl sulphide) with proteins. In: "The biochemical reactions of chemical warfare agents," Biochemical Society Symposium No. 2, (Ed.: R.T. WILLIAMS), Cambridge University Press, pp. 8-15.

41 PHILIPS, F.S. (1950). Nitrogen mustards and sulfur mustards. In: Pharmacological Reviews, Vol. 2 (Ed.: GOODMAN, L.S.), Baltimore, pp. 291-292.

42. ROSS, W.C.J. (1962). Biological Alkylating Agents, Butterworths, London, chapter 3.

43. STADE, K. (1964). Pharmakologie und Klinik Synthetischer Gifte, Deutscher Militarverlag, Berlin, pp.169-171.

44. FUNG, L.W.M., HO, E.F., ROTH, AND NAGEL, R.L. (1975). The alkylation of hemoglobin S by nitrogen mustard. J. Biol. Chem. 250, 4786-4789.

45. ORMSBEE, R.A., HENRIQUES, F.C., AND BALL, E.G. (1949). The reaction of mustard gas with skin proteins. Arch. Biochem. 21, 301-312.

46. DAVIS, E.B. AND ROSS, W.F. (1947). The reaction of mustard gas with proteins. J. Am. Chem. Soc. 69, 1177-1181.

47. PIRIE, A. (1947). The action of mustard gas on ox cornea collagen. Biochem. J. 41, 185-190.

48. HERRIOTT, R.M., ANSON, M.L. AND NORTHROP, J.H. (1946). Reactions of enzymes and proteins with mustard gas (bis ß-chloroethyl sulphide). J. Gen. Physiol. 30, 185-210.

49. (a) MOORE, S., STEIN, W.H. AND FRUTTON, J.S. (1946). Chemical reactions of mustard gas and related compounds. II. The reactions of mustard gas with carboxyl groups and with the amino

groups of amino acids and peptides. J. Org. Chem. 11, 675-680; (b) STEIN, W.H. AND MOORE, S. (1946). Chemical reactions of mustard gas and related compounds. III. The reaction of mustard gas with methionine. J. Org. Chem. 11, 681-685.

50. BOURSNELL, J.C., FRANCIS, G.E. AND WORMALL, A. (1946). Studies on mustard gas and some related compounds. III. The preparation and use of mustard gas containing a) radioactive sulfur and b) deuterium. Biochem J. 40, 743-745.

51. DE BORST, C AND MOES, G.W.H. (1970). The synthesis of ^{35}S-labelled mustard gas on a micro scale. PML Report 1970-14.

52. LEE, C.R., ESNAUD, H. AND POLLITT, R.J. (1987). Simple and highly sensitive technigue for radio-gas chromatography. J. Chromatogr. 387, 505-508.

53. STANFORD, F.G.(1967). Separation of Mustard Gas and Hydroxy Analogues by Thin-Layer Chromatography, The Analyst 92, 64.

54. SELIGMAN, A.M., RUTENBERG, A.M., PERSKY, L. AND FRIEDMAN, O.M. (1952). Effect of 2-chloro-2'-hydroxydiethyl sulfide (hemi sulfur mustard) on carcinomatosis with ascites. Cancer 5, 354-63.

55. GAFFNEY, B.L., MARKY, L.A. AND JONES, R.A. (1984). Synthesis of a set of four dodecadeoxyribonucleoside undecaphosphates containing O6-methyl guanine opposite adenine, cytosine, guanine and thymine. Biochemistry 23, 5688-5691.

56. GAFFNEY, B.L. AND JONES, R.A. (1982). Synthesis of O-6 alkylated deoxyguanosine nucleosides. Tetrahedron 23, 2253-2260.

57. VAN BOOM, J.H. (1990). Leiden University, The Netherlands, unpublished results.

58. SINGER, B AND GRUNBERGER,D. (1983). Molecular Biology of Mutagens and Carcinogens, Plenum Publishing Corp., New York, Appendix B.

59. GRIFFIN, B.E. AND REESE, C.B. (1963). The synthesis of N1-and N6-Methyl-adenosine 5'-pyrophosphates. Possible substrates for polynucleotide phosphorylase. Biochim. Biophys. Acta 68, 183-192.

60. SEELA,F. AND WALDEK,S.(1975). Agarose linked adenosine and guanosine-5'-monophosphate;a new general method for the coupling of ribonucleotides to polymers through their cis-diols. Nucleic Acid Res. 2, 2343-2354.

61. CHETSANGA, C.J. BEARIE, B., AND MAKAROFF, C. (1982). Alkaline opening of imidazole ring of 7-methylguanosine. 1. Analysis of

the resulting pyrimidine derivatives. Chem. Biol. Interact. 41, 217-233.

62. CHETSANGA, C.J. AND MAKAROFF, C.(1982). Alkaline opening of imidazole ring of 7-methylguanosine. 2. Further studies on reaction mechanisms and products. Chem. Biol. Interact. 41, 235-249.

63. KALLAMA, S AND HEMMINKI, K.(1986). Stabilities of 7-alkylguanosines and 7-deoxyguanosines formed by phosphoramide mustard and nitrogen mustard. Chem. Biol. Interact. 41, 85-96.

64. MULLER, N. AND EISENBRAND, G.(1985). The influence of N7 substituents on the stability of N7-alkylated guanosines. Chem. Biol. Interact. 53, 173-181.

65. POIRIER, V AND CALLEMAN, C.J. (1983). The nucleophilic reactivity of valine methylamide. A model of N-terminal valine residues of hemoglobin. Acta Chem. Scand. B37, 817-822.

66. SHALATIN,Y. AND BERNARD, S.A.(1966). Cooperative Effects of Functional Groups in Peptides. II. Elimination reactions in aspartyl-(O-acyl)-serine derivatives. J. Am. Chem. Soc. 88, 4711-4721.

67. BODANSKY, M. AND BODANSKY A.(1984). The Practice of Peptide Synthesis, Springer Verlag, Berlin, New York, pp. 107-108.

68. BODANSKY, M. AND BODANSKY A.(1984). The Practice of Peptide Synthesis, Springer Verlag, Berlin, New York, pp. 153-156.

69. ROGOSKIN, V.A, KRYLOV, A.J. AND KHLEBNIKOVA, N.S.(1987). Gas chromatographic determinations of 1- and 3-methylhistidine in biological fluids. J. Chromatogr. 423, 33-40.

70. CALLEMAN, C.J. AND WACHTMEISTER, C.A.(1979). Synthesis of Nπ- and Nτ-(2-hydroxyethyl)-L-histidine. Acta Chem. Scand. B37, 277-280.

71. ZAHN, H. AND STROKA, W. (1967). N-Acetyl-L-cystein-methylamid und einige thiolsubstituierte Derivate. Monatshefte Chem. 98, 745-754.

72. GRANT, W.M AND KINSEY, V.E. (1946). Synthetic preparation of 2-chloro-2'-hydroxydiethylsulfide, reaction with cysteine and valine and measurement of reaction rate in aqueous media. J. Am. Chem. Soc. 68, 2075-2076.

73. BODANSKY, M. (1984). Principles of Peptide Synthesis. Springer Verlag, Berlin, New York, pp. 175, 178.

74. BARBER, J.R. AND CLARKE, S.(1985). Demethylation of protein carboxyl methyl esters: a nonenzymatic process in human erythrocytes? Biochemistry 24, 4867-4871.

75. NEISES,B. AND STEGLICH, W. (1978). Einfaches Verfahren zur Veresterung von Carbonsauren. Angew. Chem. 90, 556-557.

76. CAMPBELL, J.B. (1983). The synthesis of N(τ)-(2-hydroxypropyl)histidine, N(π)-(2-hydroxyethyl)histidine, and their deuterated analogues, J. Chem. Soc. Perkin Trans. 1, 1213-1217.

77. VAN DENDEREN, J., HERMANS, A., MEEUWSEN, T., TROELSTRA, T., ZEGERS, N.D., BOERSMA, W.,GROSVELD, G. AND VAN EWIJK, W. (1989). Antibody recognition of the tumor-specific bcr-abl joining region in chronic myeloid leukemia. J. Exp. Med. 169, 87-98.

78. MERRIFIELD, R.B.(1963). Solid phase peptide synthesis. I. The synthesis of a tetrapeptide. J. Am. Chem. Soc. 85, 2149-2154.

79. JANSSEN, P.S.L., VAN NISPEN, J.W., MELGERS, P.A.T.A., VAN DEN BOGAART, H.W.M., HAMELINCK, R.L.A.E. AND GOVERDE, B.C. (1986). HPLC analysis of phenylthiocarbamyl (PTC) amino acids. Evaluation and optimization of the method. Chromatografia 22, 345-350.

80. JANSSEN, P.S.L., VAN NISPEN, J.W., MELGERS, P.A.T.A., VAN DEN BOGAART, H.W.M., HAMELINCK, R.L.A.E. AND GOVERDE, B.C. (1986). HPLC analysis of phenylthiocarbamyl (PTC) amino acids. II. Application in the analysis of (poly)peptides. Chromatografia 22, 351-357.

81. PECK, R.M. O'CONNELL, A.P. AND CREECH, H.J. (1966). Heterocyclic derivatives of 2-chloroethyl sulfide with antitumor activity. J. Med. Chem. 9, 217-221.

82. MURTHY, M.S.S., CALLEMAN, C.J., OSTERMAN-GOLKAR, S., SEGERBACK, D. AND SVENSSON, K. (1984). Relationship between ethylation of hemoglobin, ethylation of DNA and administered amount of ethyl methanesulfonate in the mouse. Mutat. Res. 127, 1-8.

83. TE KOPPELE, J.M., ESAJAS, S.W., BRUSSEE, J., VAN DER GEN, A. AND MULDER, G.J. (1988). Stereoselective glutathione conjugation of the separate α-bromoisovalerylurea and α-bromoisovaleric acid enantiomers in the rat in vivo and in rat liver subcellular fractions. Biochem. Pharmacol. 37, 29-35.

84. PETERS, J.A., VIJVERBERG, C.A.M., KIEBOOM, A.P.G. AND VAN BEKKUM, H. (1983). Lanthanide(III) salts of (S)-carboxymethyloxysuccinic acid: chiral lanthanide shift reagents for aqueous solution. Tetrahedron Lett. 24, 3141-3143.

85. BODANSKY, M. AND BODANSKY A.(1984). The Practice of Peptide Synthesis, Springer Verlag, Berlin, New York, pp.143-144.

86. BODANSKY, M. AND BODANSKY A.(1984). The Practice of Peptide Synthesis, Springer Verlag, Berlin, New York, pp.170-171.

87. BODANSKY, M. AND BODANSKY A.(1984). The Practice of Peptide Synthesis, Springer Verlag, Berlin, New York, p.113.

88. SHILOH, Y., VAN DER SCHANS, G.P., LOHMAN, P.H.M. AND BECKER, Y.(1983). Induction and repair of DNA damage in normal and ataxiatelangiectasia skin fibroblasts treated with neocarzinostatin. Carcinogenesis 4, 917-921.

89. VAN DER SCHANS, G.P., CENTEN, H.B. AND LOHMAN, P.H.M.(1982). DNA lesions induced by ionizing radiation. Prog. Mutat. Res. 4, 285-299.

90. PLOOY, A.C.M., VAN DIJK, M. AND LOHMAN, P.H.M.(1984). Induction and repair of DNA cross-links in Chinese Hamster Ovary cells treated with various platinum coordination compounds in relation to platinum binding to DNA, cytotoxicity, mutagenicity and antitumor activity. Cancer Res. 44, 2043-2051.

91. VAN DER SCHANS, G.P., PLOOY, A.C.M. AND BERENDS, F. (1984). Distribution of molecular weights in alkali-denatured DNA samples with randomly induced single-strand breaks and interstrand crosslinks. Cancer Res. 44, 2049-2050.

92. SCHUTTE, H.H., VAN DER SCHANS, G.P. AND LOHMAN, P.H.M. (1988). Comparison of induction and repair of adducts and of alkali-labile sites in human lymphocytes and granulocytes after exposure to ethylating agents. Mutat. Res. 194, 23-37.

93. VAN DER SCHANS, G.P., VAN LOON, A.A.W.W., GROENENDIJK, R.H. AND BAAN, R. (1989). Detection of DNA damage in cells exposed to ionizing radiation by use of anti-single-stranded A monoclonal antibody. Int. J. Radiat. Biol. 55, 747-760.

94. OTT, D.G., REISFELD, M.J. AND WHALEY, T.W. (1986). Synthesis of isotopically labeled threat agents. In: "Synthesis and applications of isotopically labeled compounds 1985": Proceedings of the Second International Symposium, Kansas City, MO, U.S.A., 3-6 September 1985 (MUCCINO, R.R., Ed.). Elsevier, Amsterdam, pp. 409-414.

95. WIELAND, T. AND KRANTZ, H. (1958). Notiz zur einfachen Darstellung von ^{35}S-Schwefelwasserstoff. Chem. Ber. 91, 681-682.

96. BROOKES, P. AND LAWLEY, P.D. (1961). The alkylation of guanosine and guanylic acid. J. Chem. Soc. 3923-3928.

97. LUDLUM, D.B.(1983). Protection against the acute and delayed toxicity of mustards and mustard-like compounds. Annual summary report, contract no. DAMD17-82-C-2203, U.S. Army Medical Research and Development Command; AD-A146 943.

98. LUDLUM, D.B., TONG, W.P., MEHTA, J.R., KIRK, M.C. AND PAPIRMEISTER, B. (1984). Formation of O6-ethylthioethyl deoxyguanosine from the reaction of chloroethyl ethyl sulfide with deoxyguanosine. Cancer Res. 44, 5698-5701.

99. HAINES, J.A., REESE, C.B. AND LORD TODD. (1962). The methylation of guanosine and related compounds with diazomethane. J. Chem. Soc., 5281-5288.

100. BILLICH, A., STOCKHOWE, U. AND WITZEL, H. (1986). Nucleoside Phosphotransferase from Malt Sprouts. I. Isolation, Characterization and Specificity of the Enzymes. Biol. Chem. Hoppe-Seyler, 367, 267-278.

101. MULLER, R. AND RAJEWSKY, M.F. (1981). Antibodies specific for DNA components structurally modified by chemical carcinogens. J. Cancer Res. Clin. Oncol. 102, 99-113.

102. DARZYNKIEWICZ, E., ANTOSIEWICZ, J., EKIEL, I., MORGAN, M.A., TAHARA, S. AND SHATKIN, A.J. (1981). Methyl esterification of m7G5'p reversibly blocks its activity as an analog of eukaryotic mRNA 5'-caps. J. Mol. Biol. 153, 451-458.

103. RUPPRECHT, K.M., SONENBERG, N., SHATKIN, A.J. AND HECHT, S.M. (1981). Design and preparation of affinity columns for the purification of eukaryotic messenger ribonucleic acid cap binding protein. Biochemistry 20, 6570-6577.

104. BLAIR, A.H. AND GHOSE, T.I.(1983). Linkage of cytotoxic agents to immunoglobulins. J. Immunol. Methods 59, 129-143.

105. CALLEMAN, C.J. AND POIRIER, V.(1983). The nucleophilic reactivity and tautomerism of the imidazole nitrogens of N2-acetyl-histidine methylamide and N2-acetyl-histidine. Acta Chem. Scand. B37, 809-815.

106. GUNDLACH, H.G, MOORE, S. AND STEIN, W.H.(1959). The reaction of iodoacetate with methionine. J. Biol. Chem. 234, 1761-1764.

107. RAMIREZ, F., FINNAN, J.L. AND CARLSON, M. (1973). In vitro decomposition of S-methylmethioninesulfonium salts. J. Org. Chem. 38, 2957-2960.

108. CAPPS, P.A. (1974). Sulphonium salt formation from the reaction of methionine with some aziridinium alkylating agents. J. Chem. Soc., Chem. Commun., 321-322.

109. BODANSKY, M.(1984). Principles of Peptide Synthesis, Springer Verlag, Berlin, p.37.

110. WUTHRICH, K. (1986). NMR of Proteins and Nucleic Acids, Wiley, New York.

111. FICHTINGER-SCHEPMAN, A.M.J., VAN DER VEER, J.L., DEN HARTOG, J.H.J., AND LOHMAN, P.H.M. (1985). Adducts of the antitumor drug cis-diamminedichloroplatinum(II) with DNA: Formation, identification, and quantitation. Biochemistry 24, 707-713.

112. BERANEK, D.T., WELS, C.C., AND SWENSON, D.H. (1980). A comprehensive quantitative analysis of methylated and ethylated DNA using high pressure liquid chromatography. Carcinogenesis 1, 595-606.

113. SCHUTTE, H.H., VAN DER SCHANS, G.P., AND LOHMAN, P.H.M. (1988). Comparison of induction and repair of adducts and of alkali-labile sites in human lymphocytes and granulocytes after exposure to ethylating agents. Mutat. Res. 194, 23-37.

114. FICHTINGER-SCHEPMAN, A.M.J., VAN OOSTEROM, A.T., LOHMAN, P.H.M., AND BERENDS, F. (1987). cis-Diamminedichloroplatinum(II)-induced DNA adducts in peripheral leukocytes from seven cancer patients: quantitative immunochemical detection of the adduct induction and removal after a single dose of cis-diamminechloro-platinum(II). Cancer Res. 47, 3000-3004.

115. CAMPELL, A.M. (1984). Monoclonal antibody technology. Laboratory techniques in biochemistry and molecular biology (Eds. BURDON, R.H. and VAN KNIPPENBERG, P.H.), Elsevier, Amsterdam, pp. 33-65.

116. PAPIRMEISTER, B., FEISTER, A.J., ROBINSON, S.I. AND FORD, R.D. (1991). Medical defense against mustard gas: toxic mechanisms and pharmacological implications, CRC Press, Boca Raton.

117. KIRCHNER, M. AND BRENDEL, M. (1983). DNA alkylation by mustard gas in yeast strains of different repair capacity. Chem.-Biol. Interact. 44, 27-39.

118. SACK, G.H., FENSELAU, C., KAN, M.-N.N., AND KAN, L.S. (1978). Characterization of the products of alkylation of 2'-deoxyadenosine and 2'-deoxyguanosine by chloroethyl ethyl sulfide. J. Org. Chem. 20, 3932-3936.

119. SINGER, B.(1985). In vivo formation and persistence of modified nucleosides resulting from alkylating agents. Environ. Health Perspect. 62, 41-48.

120. SWAIN, C.G AND SCOTT, C.B. (1953). Quantitative correlation of relative rates. Comparison of hydroxide ion with other nucleophilic agents toward alkyl halides, esters, epoxides and acyl halides. J. Am. Chem. Soc. 75, 141-147.

121. LAWLEY, P.D.(1974). Alkylation of nucleic acids and mutagenesis. In: PRAKASH, L., SHERMAN, F., MILLER, M.W., LAWRENCE, C.W. AND TABER H.W. (Eds.). Molecular and Environmental Aspects of Mutagenesis, C.C.Thomas, Springfield, Ill., pp. 17-31.

122. VAN DELFT, J.H.M., TNO-Medical Biological Laboratory, Rijswijk, The Netherlands, unpublished results.

123. PARK, J.-W., CUNDY, K.C., AND AMES, B.N. (1989). Detection of DNA adducts by high-performance liquid chromatography with electrochemical detection. Carcinogenesis 10, 827-832.

124. WILCHEK, M. AND BAYER, E.A. (1988). Review; The avidin-biotin complex in bioanalytical applications. Anal. Biochem. 171, 1-32.

125. ABELL, C.W., ROSINI, L.A. AND RAMSEUR, M.R (1965). Alkylation of polyribonucleotides: the biological, physical, and chemical properties of alkylated polyuridylic acid. Biochemistry 54, 608-615.

126. SINGER, B AND GRUNBERGER, D. (1983). Molecular Biology of Mutagens and Carcinogens, Plenum Publishing Corp., New York, p.19.

APPENDIX A

Molecular weight distribution of fragments in alkali-denatured DNA samples with randomly induced single-strand breaks and interstrand crosslinks

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In the appendix by Van der Schans et al. (A1), added to the paper of Plooy et al. (A2), curves have been derived and presented for the calculation of the average number of interstrand crosslinks related to the fraction of DNA eluted. The fraction of eluted DNA of cells pretreated with a crosslinking agent and subsequently irradiated was assessed at the 50% elution point of DNA of cells which have been only exposed to radiation. However, in the case of irradiated DNA, the fraction of DNA eluted at the 50% point cannot be determined accurately because the amount of DNA in the first fraction of the elution is not exactly known (due to the presence of a small but varying amount of fluorescing material not related to DNA). The effect of this uncertainty on the calculation of the number of interstrand crosslinks can be reduced when the calculation is carried out for later fractions in the elution, i.e., at the 80% point.

For non-crosslinked DNA, the molecular weight of single-stranded DNA fragments that are passing through the filter at the point in the elution curve where 80.1% of the material has been eluted, is just equal to 3 times the number-averaged molecular weight of all fragments (m_n), when a random distribution of the breaks over the molecule is assumed. Since $m_n = M/p$, Eq. 4 of Van der Schans et al. (A1) can be written as:

$$X_m = \int_0^m m_n^{-2} \cdot e^{-m/m_n} \cdot (1 - e^{(x/p)(m/m_n)})dm \qquad \text{(Eq. 6)}$$

where X_m is the weight fraction of molecules with a molecular weight between 0 and m containing at least one link.

The value p is the average number of breaks and x is the average number of links, both per single-stranded DNA molecule with molecular weight M. Solution of this equation yields:

$$X_m = 1 - (m/m_n + 1)e^{-m/m_n} \cdot (1 + x/p)^{-2} + (1 + x/p)^{-2} \cdot$$
$$(m/m_n + (m/m_n).(x/p) + 1)e^{-(m/m_n)(1+x/p)} \qquad \text{(Eq. 7)}$$

In the experimental determination of crosslinks, we use the percentage of total DNA already eluted at the point where non-crosslinked single-stranded material with $m = 3m_n$ is expected to appear in the eluate (the 80% point). It is relevant, therefore, to ask what

will be the elution behavior of the material in fraction X_{3mn}. Which part will acquire a molecular weight $>3m_n$ by the crosslinking? Analogous to Van der Schans et al. (A1), this question can be easily answered for two extreme situations:

1. All crosslinked fragments have acquired a total molecular weight exceeding $3m_n$ and are no longer eluted in the category $m<3m_n$. In this case, Eq.7 directly gives the weight fraction that disappears from this category. Hence, the original 80.1% eluted with $m<3m_n$ will be reduced to $0.801-X_{3mn}$.
2. Only the fragments crosslinked to pieces with $m>3m_n$ disappear from the category with $m<3m_n$. As the crosslinks have a random distribution and since - by definition - 0.199 of the material has a molecular weight $>3m_n$, in this case only 19.9% of the crosslinks will be effective in shifting the fragments to the category with $m>3m_n$. This implies that in Eq. 7, x has to be substituted by the number of effective crosslinks, i.e., by 0.199x, in order to obtain the weight fraction of material disappearing from the population with $m<3m_n$.

In Figure A1, the weight fraction expected to be collected in the eluate at the point when 80.1% would have been eluted in the absence of crosslinks, has been plotted as a function of x/p, for both cases. It will be clear that the real curve will lie somewhere between these two extremes.

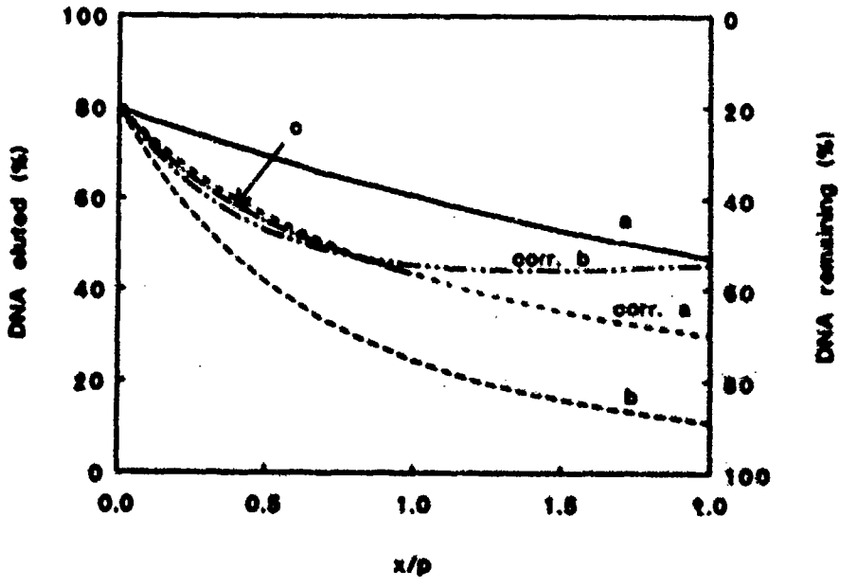

Figure A1. The fraction of irradiated DNA treated with mustard gas eluted at the 80.1% elution point of irradiated untreated DNA as a function of x/p. Curve a: 0.199 of the links in molecules with molecular weight $m<3m_n$ result in a retention beyond the 80.1% elution point; curve b: all links in molecules with $m<3m_n$ result in retention up to or beyond the 80.1% elution point; curve c: best estimate of the real curve on the basis of the subdivision of molecules with $m<3m_n$ into four weight categories.

For a better approximation of this real curve, it has to be considered which fraction of the molecules with $m<3m_n$ that are crosslinked to fragments of the same category will become larger than $3m_n$ because of this combination. As a first approach, this group of fragments can be subdivided into the following classes of molecules:

a1: with m between 0 and $0.75m_n$
a2: with m between $0.75m_n$ and $1.5m_n$
a3: with m between $1.5m_n$ and $2.25m_n$, and
a4: with m between $2.25m_n$ and $3m_n$.

Integration of Eq. 1 of Van der Schans et al. (A1) over these intervals, with the use of Eq. 3 from that paper, shows that 17.3% falls into category a1, 26.9% into category a2, 21.5% into category a3 and 14.4% into category a4. As the number of links is proportional to the amount of DNA, the links will show the same distribution. In either class, 0.199 of the links will be connected to fragments with $m>3m_n$ (according to the above situation 2).

Combination of a fragment of category a1 with a fragment of either category a1, a2, or a3 will certainly result in material with $m<3m_n$. Combination of a fragment of category a2 with a fragment of category a4 will result in material with $m>3m_n$, whereas m will remain $<3m_n$ when two molecules of class a2 are crosslinked or if a fragment of category a2 is linked to a fragment of category a1 and so on. In the Table A1 the possible combinations are summarized. With the use of Eq.7 we can calculate the weight fraction of fragments containing a link in the categories a1, a2, a3 and a4, as a function of x/p.

Table A1. Possible combinations of linked fragments with m larger or smaller than $3m_n$

Class	$m>3m_n$		$m<3m_n$	
a1: (17.3%)			a1+a1	
			a1+a2	
		(0.199x)	a1+a3	(0.657x)
a2: (26.9%)	a2+a4		a2+a1	
		(0.344x)	a2+a2	(0.442x)
a3: (21.5%)	a3+a3		a3+a1	
	a3+a4	(0.559x)		(0.173x)
a4: (14.4%)	a4+a2			
	a4+a3			
	a4+a4	(0.827x)		

As the breaks are random, it can be considered a matter of chance which two links (in different fragments) are connected together. This implies that only 19.9% of the links in material of class a1 will

result in material with $m>3m_n$. Consequently, in the formula used for the calculation of X_{a1}, x should be replaced by 0.199x in order to arrive at the fraction that with certainty is no longer eluted before the 80.1% point. For the calculation of X_{a2}, x should be replaced by 0.344x; for X_{a3}, 0.559x and for X_{a4} by 0.827x.

Calculation of the sum of X_{a1}, X_{a2}, X_{a3} and X_{a4} with respectively 0.199x, 0.344x, 0.559x and 0.827x, for the various values of x/p between 0 and 2 yields a corrected version of curve a in Figure A1. In an analogous manner curve b can be corrected upwards by adding X_{a1} (0.657x), X_{a2} (0.442x) and X_{a3} (0.173x). In this way the distance between the two graphs can be narrowed from 14.7% at x/p=0.2 to 3.2%, whereas at x/p=0.86 the curves even cross each other. The latter has to be ascribed to an overcorrection of curve b, since it has not been taken into account that at high x/p values more than two different fragments can be linked together, resulting in molecules with $m>3m_n$. So, the corrected curve a in Figure A1 is a fair approximation of the real curve in the range x/p 0.86-2, whereas for x/p 0-0.86 an average of the corrected curves of a an b (curve c) is a fair one.

More refined calculations are without meaning, because, as mentioned above, in this approach the possibility is ignored that more than 2 fragments are linked together when multiple links per fragment are present, which will lead to inaccuracies in particular at higher values of x/p.

LITERATURE CITED:

A1. VAN DER SCHANS, G.P., PLOOY, A.C.M. AND BERENDS,F. (1984). Distribution of molecular weights in alkali-denatured DNA samples with randomly induced single-strand breaks and interstrand crosslinks. Cancer Res. 44, 2049-2050.

A2. PLOOY, A.C.M., VAN DIJK, M. AND LOHMAN, P.H.M. (1984). Induction and repair of DNA cross-links in Chinese Hamster Ovary cells treated with various platinum coordination compounds in relation to platinum binding to DNA, cytotoxicity, mutagenicity and antitumor activity. Cancer Res. 44, 2043-2051.

APPENDIX B

Detection with a diode array detector of adduct peaks from calf-thymus DNA exposed to 1 mM mustard gas

HPLC conditions:

Pump	: Waters Associates
Flow (ml/min)	: 1.0
Eluent	: 0.025 M ammonium bicarbonate in 20% aqueous methanol
Column	: Beckman; 250 x 5 mm; ultrasphere ODS; 5 μm
Detector	: Waters Associates, Diode Array, 190-350 nm
Sample	: calf-thymus DNA, treated with 1 mM mustard gas (45 min, 37 ^{o}C), degraded enzymatically into nucleosides, heat treated (10 min, 100 ^{o}C).

Figure B1 shows the HPLC chromatogram of the sample described above. Of the six main components the UV spectra are shown which agree with the spectra of commercially available markers and the synthesized and characterized adduct markers (dC: 2'-deoxycytidine; dmC: 2'-deoxy-methylcytidine; dG: 2'-deoxyguanosine; T: thymidine; dA: 2'-deoxy-adenosine; N7-G-HD: N7-(2'-hydroxyethylthioethyl)-guanine. Figure B2 shows an enlargement of the HPLC chromatogram from 8-25 min, which reveals the presence of, N3-(2'-hydroxyethylthioethyl)-adenine (N3-A-HD) and di-(2-guanin-7'-yl-ethyl) sulfide (N7-G-HD-G). The UV spectra of these two adducts are shown in Figure B3.

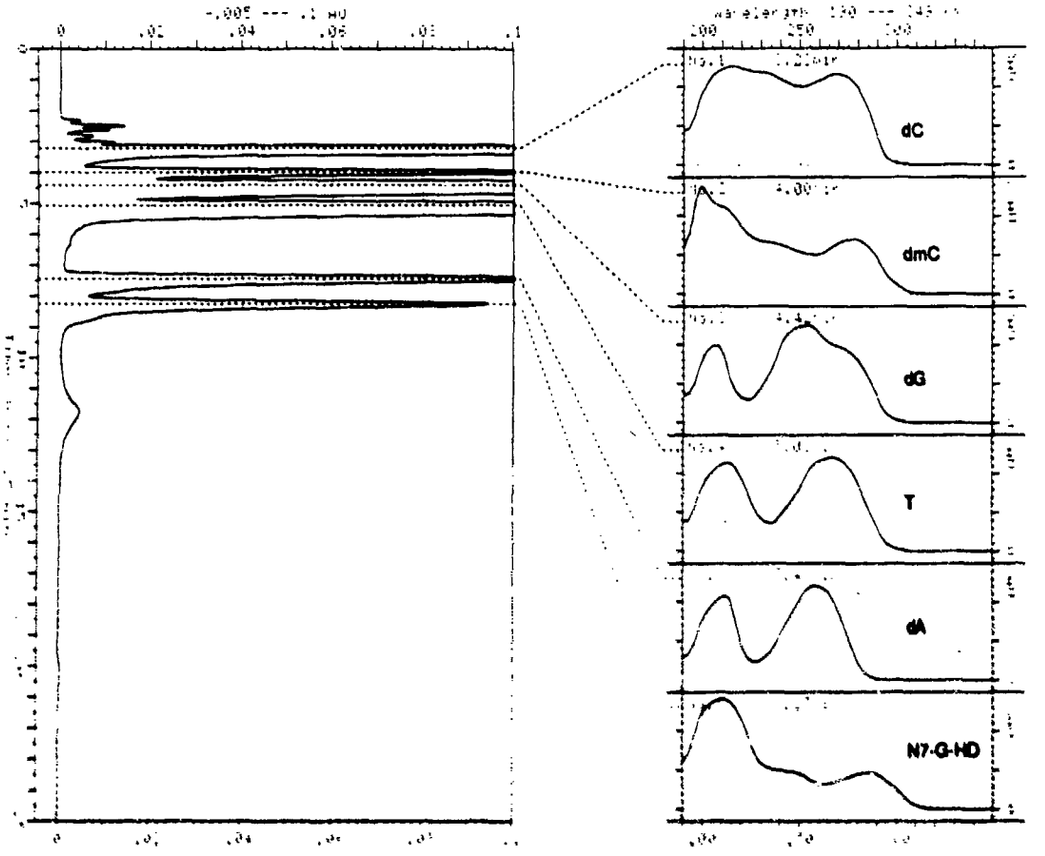

Figure B1. HPLC-chromatogram and UV spectra of calf-thymus DNA after treatment with 1 mM mustard gas for 45 min at 37 °C, and enzymatic degradation into nucleosides. The absorbance at 285 nm was recorded with a diode array detector (Waters Associates; 190-350 nm). dC: 2'-deoxycytidine; dmC: 2'-deoxy-methylcytidine; dG: 2'- deoxyguanosine; T: thymidine; dA: 2'-deoxyadenosine; N7-G-HD: N7-(2'-hydroxy-ethylthioethyl)-guanine.

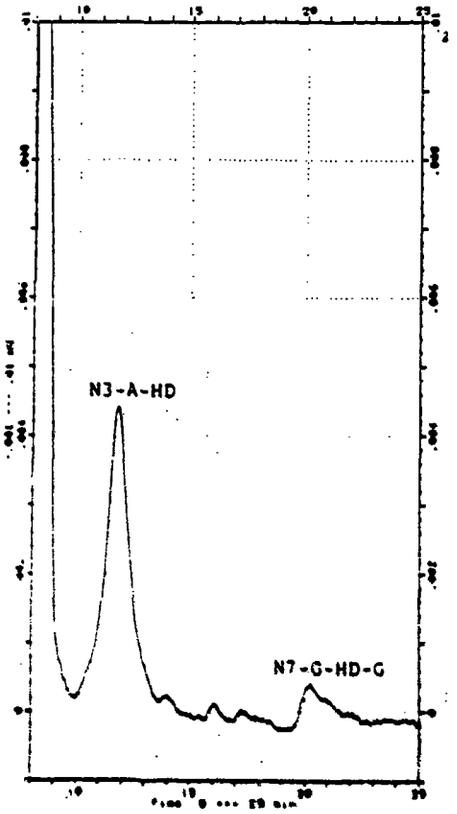

Figure B2. Enlargement of the HPLC chromatogram shown in Figure B1 for retention times in between 8 and 25 min. N3-A-HD: N3-(2'-hydroxyethylthioethyl)-adenine; N7-G-HD-G: di-(2-guanin-7'-yl-ethyl) sulfide.

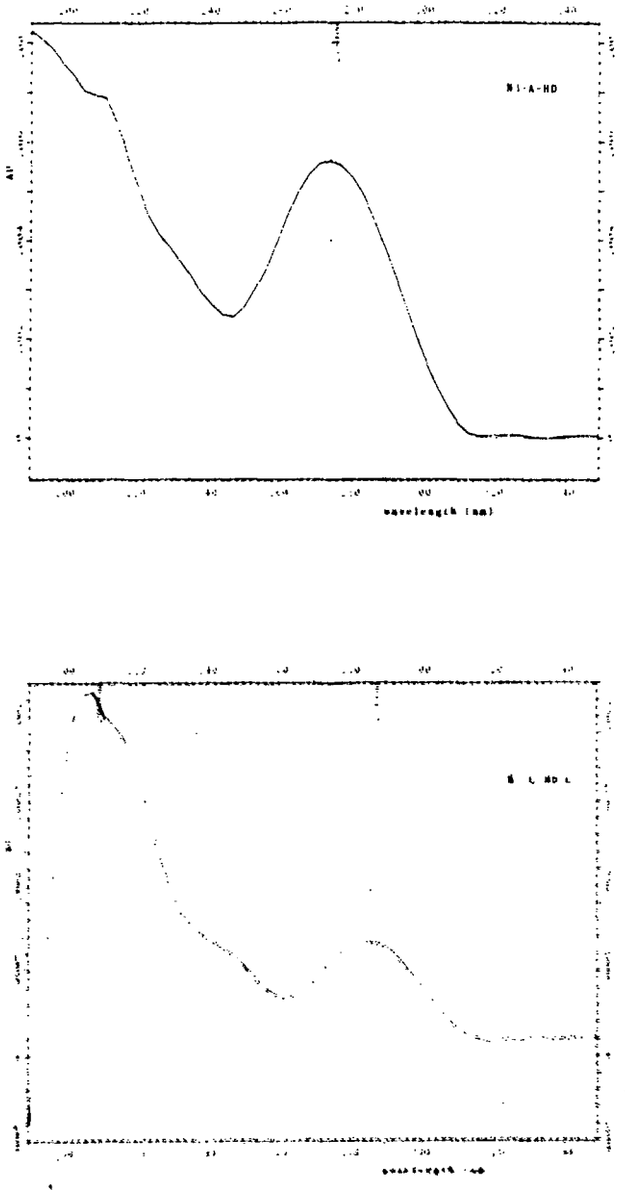

Figure B3. UV spectra of N3-(2'-hydroxyethylthioethyl)-adenine (N3-A-HD) and di-(2-guanin-7'-yl-ethyl) sulfide (N7-G-HD-G) recorded with a diode array detector (Waters Associates; 190-350 nm). Both compounds were detected in calf-thymus DNA after treatment with 1 mM mustard gas for 45 min at 37 °C and enzymatic degradation into nucleosides.

APPENDIX C

Detection of adduct peaks in fragmented calf thymus DNA treated with 1 mM mustard gas by means of thermospray-LC-MS

LC-parameters:

Column type	: Lichrosorb RP-18 (AR270)
Particle size (μm)	: 7
Length (cm)	: 25
Diameter (mm)	: 5
Eluent	: gradient: eluent A: 50 mM ammonium acetate eluent B: 50 mM ammonium acetate: methanol (6/4) In 20 min linear gradient from 100% A to 100% B
Flow (ml/min)	: 1.5
pH	: 7
Sample	: calf-thymus DNA, treated with 1mM mustard gas, enzymatically degraded into nucleosides, heat treated (10 min, 100 °C)

Thermospray-MS parameters:

Tip temperature (°C)	: 260-250
Source temperature (°C)	: 230
Discharge (on/off)	: off
Mode (pos/neg ions)	: pos
Repeller (volt)	: 150
Multiplier (volt)	: 500
Ion energy (volt)	: -10
Scan conditions	: m/z= 100-450 in 1 sec.

Figure C1 shows the mass chromatograms at single ion monitoring of the molecular ions (MH^+) of the various peaks (dC: 2'-deoxycytidine; dmC: 2'-deoxy-methylcytidine; dG: 2'-deoxyguanosine; T: thymidine; dA: 2'-deoxyadenosine; N7-G-HD: N7-(2'-hydroxyethylthioethyl)-guanine; N3-A-HD: N3-(2'-hydroxyethylthioethyl)-adenine; N7-G-HD-G: di-(2-guanin-7'-yl-ethyl) sulfide; TDG: thiodiglycol). Figure C2 shows the complete mass spectra of the various peaks.

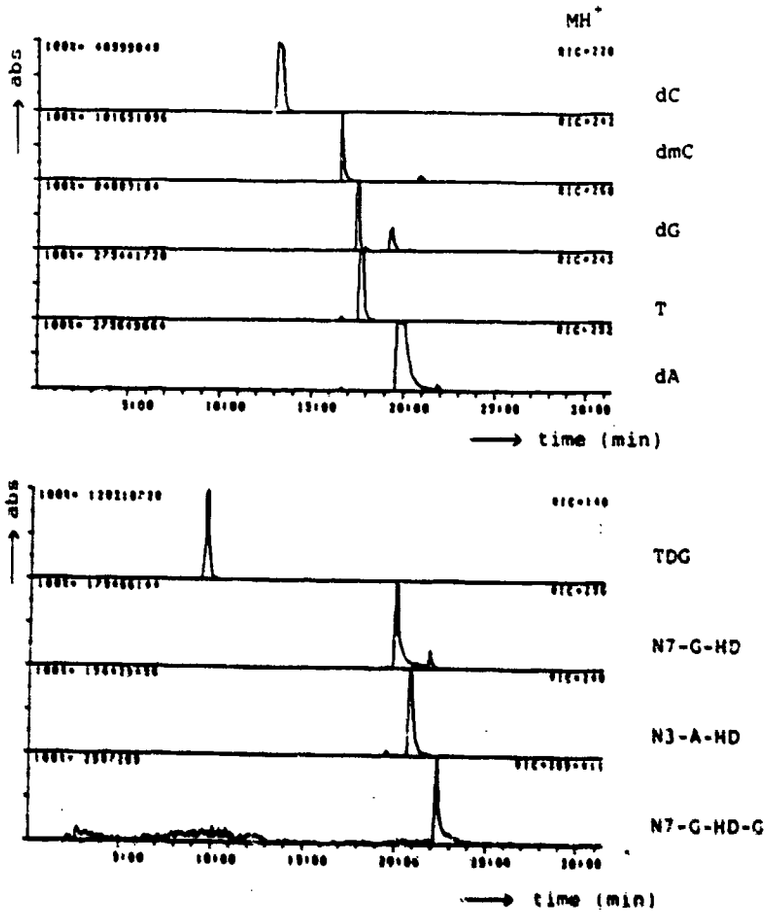

Figure C1. Mass chromatogram (thermospray MS) of calf-thymus DNA after treatment with 1 mM mustard gas for 45 min at 37 °C, and enzymatic degradation into nucleosides. dC: 2'-deoxycytidine; dmC: 2'-deoxy-methylcytidine; dG: 2'-deoxyguanosine; T: thymidine; dA: 2'-deoxyadenosine; N7-G-HD: N7-(2'-hydroxyethylthioethyl)-guanine; N3-A-HD: N3-(2'-hydroxyethylthioethyl)-adenine; N7-G-HD-G: di-(2-guanin-7'-yl-ethyl) sulfide; TDG: thiodiglycol.

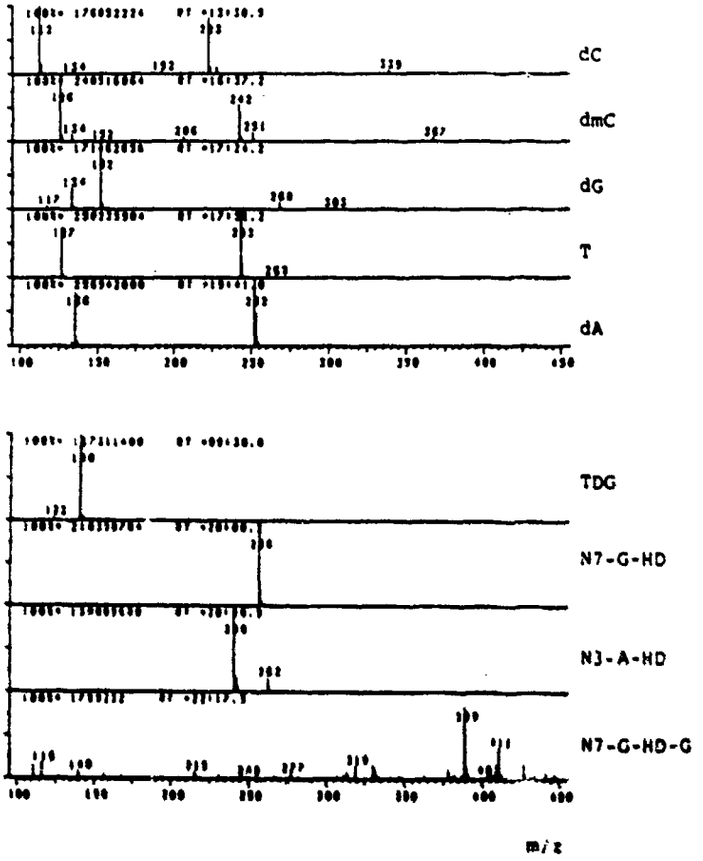

Figure C2. Thermospray mass spectra of calf-thymus DNA after treatment with 1 mM mustard gas for 45 min at 37 °C, and enzymatic degradation into nucleosides. dC: 2'-deoxycytidine; dmC: 2'-deoxy-methylcytidine; dG: 2'-deoxyguanosine; T: thymidine; dA: 2'-deoxyadenosine; N7-G-HD: N7-(2'-hydroxyethylthioethyl)-guanine; N3-A-HD: N3-(2'-hydroxyethylthioethyl)-adenine; N7-G-HD-G: di-(2-guanin-7'-yl-ethyl) sulfide; TDG: thiodiglycol.

BIBLIOGRAPHY OF PUBLICATIONS AND MEETING ABSTRACTS

BENSCHOP, H.P., MOES, G.W.H., FIDDER, A., SCHEFFER, A.G., AND VAN DER SCHANS, G.P. (1989). Immunochemical detection of mustard gas adducts with DNA: identification of the adducts, Proceedings of the 1989 Medical Defense Bioscience Review, U.S. Army Medical Research and Development Command, August 1989, pp. 1-8.

SCHEFFER, A.G., VAN DER SCHANS, G.P., AND BENSCHOP, H.P. (1989). Dosimetry of HD-DNA adducts by immunochemical methods after exposure of mammalian cells to sulfur mustard, Proceedings of the 1989 Medical Defense Bioscience Review, U.S. Army Medical Research and Development Command, August 1989, pp. 67-70.

BENSCHOP, H.P., MOES, G.W.H., FIDDER, A., VAN DEN BERG, G.R., VAN DER HOLST, J.P.J., BRENKMAN, T., SCHEFFER, A.G., AND VAN DER SCHANS, G.P. (1990). Immunochemical detection of mustard gas adducts with DNA and proteins: identification of the adducts, Proceedings of the Meeting of NATO AC/243 Panel 8 RSG 3, November 1989, The Hague, pp. 21.1-21.19.

SCHEFFER, A.G., VAN DER SCHANS, G.P., AND BENSCHOP, H.P. (1990). Dosimetry of HD-DNA adducts by immunochemical methods after exposure of mammalian cells to sulfur mustard, Proceedings of the Meeting of NATO AC/243 Panel 8 RSG 3, November 1989, The Hague, pp. 22.1-21.26.

BENSCHOP, H.P., SCHEFFER, A.G., VAN DER SCHANS, G.P., AND BAAN, R.A. (1991). Verification of exposure to chemical agents: immunochemical detection of exposure to mustard gas, Proceedings of the Meeting of NATO AC/243 Panel 8 RSG 3, April 1991, La Tronche/Grenoble, pp. 148-166.

BENSCHOP, H.P., SCHEFFER, A.G., VAN DER SCHANS, G.P., AND BAAN, R.A. (1991). Immunochemical diagnosis and dosimetry of exposure to sulfur mustard, Proceedings of the 1991 Medical Defense Bioscience Review, U.S. Army Medical Research and Development Command, August 1991, pp. 67-74.

VAN DER SCHANS, G.P., AND BENSCHOP, H.P. (1991). Immunochemical detection of adducts to DNA after exposure of mammalian cells to sulfur mustard, Proceedings of the 1991 EUROTOX Congress, p. 275.

VAN DER SCHANS, G.P., SCHEFFER, A.G., MARS-GROENENDIJK, R., BAAN, R.A., AND BENSCHOP, H.P. (1991). Verification of exposure to chemical agents: immunochemical detection of exposure to mustard gas, Proceedings of the Fourth German-Netherlands Symposium on "Protection against Highly Toxic Compounds", September 1991, Rijswijk, pp. 283-288.

BENSCHOP, H.P., DE JONG, L.P.A., VAN DER SCHANS, G.P., AND BAAN, R.A. (1992). Diagnosis and dosimetry of exposure to sulfur mustard, Proceedings of the Program Review: Biotechnology, U.S. Army Medical Research Institute of Chemical Defense, May 1992.

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