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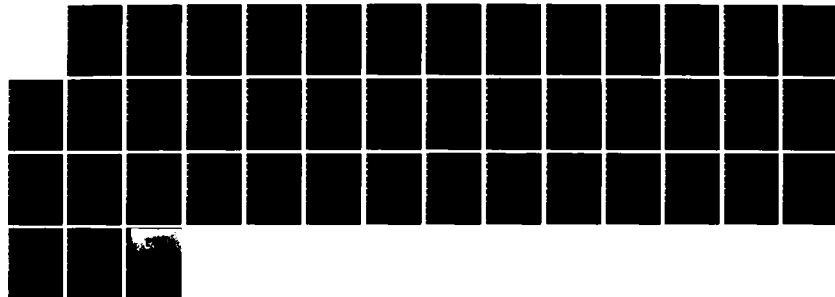
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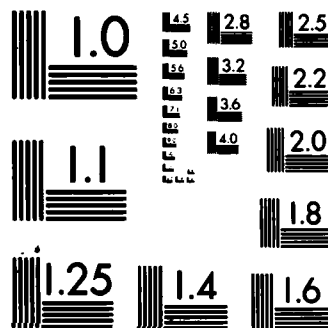
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These proceedings are of the first symposium on the mechanism of cyanide intoxication and its antagonism sponsored either by the Society of Toxicology or the American Society of Pharmacology and Experimental Therapeutics. It was held 15-19 August 1982 and was dedicated to Dr. K.K. Chen, one of the first scientists to utilize a molecular approach to the field of drug antagonism. His early scientific contributions provided much of the basic information for work discussed in the symposium. Papers included are the following:
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7. (continued)
John Wilson
Bryan Ballantyne

20. (continued)
"Mechanism of Cyanide Intoxication and Its Antagonism--Introduction"
James L. Way

"The High Resolution Three-Dimensional Structure of Bovine Liver
Rhodanese"
W.G.J. Hol, L.J. LIJK, and K.H. Kalk

"The Sulfurtransferases"
John Westley, Howard Adler, Laura Westley, and Craig Nishida

"Cyanide Antagonism"
James L. Way

"Treatment of Cyanide Poisoning with 4-Dimethylaminophenol (DMAP)--
Experimental and Clinical Overview"
Nick P. Weger

"Cyanide in Human Disease: A Review of Clinical and Laboratory
Evidence"
John Wilson

"Artifacts in the Definition of Toxicity by Cyanides and Cyanogens"
Bryan Ballantyne



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SYMPOSIUM

Mechanism of Cyanide Intoxication and its Antagonism^{1,2} INTRODUCTION

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This represents the first symposium on the mechanism of cyanide intoxication and its antagonism sponsored either by the Society of Toxicology or the American Society of Pharmacology and Experimental Therapeutics. It is being dedicated to Dr. K.K. Chen, one of the first scientists to utilize a molecular approach to the field of drug antagonism. His early scientific contributions provided much of the basic information for our discussion today (Chen *et al.*, 1933a).

Dr. K.K. Chen was born in China and matriculated at the University of Wisconsin and received a Ph.D. degree with Drs. Harold C. Bradley and Walter J. Meek. He returned to China to the Peking Union Medical College and it was there that he isolated ephedrine in crystalline form and described its pharmacologic properties. He returned to the United States and obtained his medical degree at Johns Hopkins School of Medicine and was associated with the Department of Pharmacology and Dr. John Jacob Abel. Subsequently, he became Director of Pharmacologic Research at Eli Lilly and Co. and it was during this period that he developed the nitrite-thiosulfate therapy for cyanide intoxication. This antidotal combination is still marketed by Eli Lilly and Co. for the treatment of cyanide intoxication. Dr. Chen was quite active in his professional society. He was elected Treasurer of the American Society of Pharmacology and Experimental Therapeutics in 1947, President in 1952, and President of FASEB in 1953.

He has received numerous recognitions for his scientific excellence and contributions. These awards include the China Foundation prize, and honorary Sc.D. degrees were conferred by the University of Wisconsin and Indiana-Purdue University. He also is the recipient of the Remington Honor Medal, Honorary membership in the Finnish Pharmacology Society and Honorary President of the International Union of Pharmacology.

Dr. K.K. Chen is best known for his discovery and development of the sympathomimetic amine, ephedrine. However, he also is one of the first toxicologists to employ a rational toxicologic approach in the development of the treatment of cyanide intoxication. It is difficult to conceive that it was fifty years ago when Dr. K.K. Chen reported on the use of the sodium nitrite-sodium thiosulfate antidotal combination in the treatment of

cyanide poisoning. More important, the conceptual basis for the toxicologic rationale for this antidotal combination was expounded. The nitrites were employed to oxidize hemoglobin to methemoglobin which would then bind the toxic cyanide anion by forming cyanmethemoglobin (Chen *et al.*, 1933b). The second cyanide antagonist was sodium thiosulfate, which served as a sulfur donor to the sulfurtransferase, rhodanese, so that the cyanide was metabolized to the less toxic thiocyanate (Chen *et al.*, 1934, 1952). This represented one of the first developments of drug antidotes which was based on a scientific toxicologic basis. More important, the concept was initiated that by combining two antidotes of different mechanism of action, an enhanced protective effect could be obtained. This foresight in the 1930's to employ a molecular approach for the development of drug antidotes has withstood the test of time, as the use of sodium thiosulfate-sodium nitrite still represents one of the most efficacious antidotal combinations for the treatment of cyanide intoxication, particularly when it is employed in combination with oxygen. This is truly a tribute to Dr. K.K. Chen that even today there are very few antidotal combinations which can protect as well as the nitrite-thiosulfate treatment. The present rapid expansion of research in this area owes a great deal of thanks to the pioneering effort of this dedicated individual.

It is generally not recognized that a widespread problem is chronic low level intoxication. This may occur from cigarette smoking, ingestion of cyanogenic staple food, *i.e.* cassava, in many of our Third World countries, and occupationally where cyanide is employed as a chemical intermediate. To emphasize the importance of the chronic toxicity from cyanide, we have initiated this symposium with the chronic toxic manifestations of cyanide in human diseases.

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The High Resolution Three-Dimensional Structure of Bovine Liver Rhodanese

W.G.J. HOL, L.J. LIJK and K.H. KALK

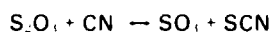
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ABSTRACT

The High Resolution Three-dimensional Structure of Bovine Liver Rhodanese. Hol, W.G.J., Lijk, L.J. and Kalk, K.H. (1983). *Fundam. Appl. Toxicol.* 3:370-376. The crystal structure of the sulfur complex of bovine liver rhodanese has been determined at a resolution of 2.1 Å. The three-dimensional structure of this sulfur-transfer enzyme reveals two domains of roughly equal size, with nearly identical conformations and very dissimilar amino acid sequences. The active site contains four elements which are carefully positioned with respect to each other in order to obtain efficient catalysis: (i) cysteine 247, (ii) a set of peptide nitrogen hydrogen bond donors, (iii) two positively charged residues, and (iv) a cluster of hydrophobic residues at the active site surface. The catalytic mechanism is described in some detail. The structures of the sulfur free enzyme and of the enzyme in complex with several metal cyanide inhibitors have been studied. These inhibitors appear to have one common binding site, blocking the entrance to the active site pocket and thus revealing the mode of inhibition by these, and probably other, anions.

INTRODUCTION

Rhodanese is an enzyme which catalyses the transfer of a sulfane sulfur atom from sulfur donors to sulfur acceptors. The best known reaction catalyzed is:



The enzyme has been observed in bacterial, fungal, plant, fish, amphibian, reptilian, avian and mammalian organisms (Westley, 1973; Sörbo, 1975; Dudek *et al.*, 1980). The presence of rhodanese has recently even been demonstrated in the large tube worm, *Riftia pachyptila*, which lives in deep-sea hydrothermal vents (Felbeck, 1981). In mammals, the enzyme is distributed

quite unevenly with respect to the various organs, the highest activities being found in liver and kidney. A study of Reinwein (1961) on the distribution of rhodanese in human tissues showed that the highest activity was found in liver, followed by kidney, suprarenals and thyroid gland. In mammalian liver, rhodanese is confined to the mitochondria (De Duve *et al.*, 1955; Dudek *et al.*, 1980).

The widespread occurrence suggests an important role of the enzyme. Although general agreement seems to exist that the enzyme is involved in the detoxification of cyanide, a large number of alternative, or additional, functions has been suggested (Westley, 1973; Sörbo, 1975). A possible "multi-functional" rhodanese could, for instance, also be important for

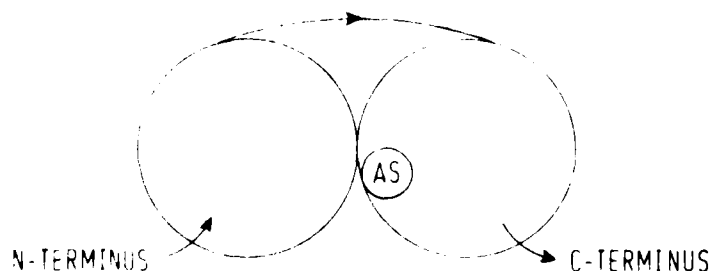
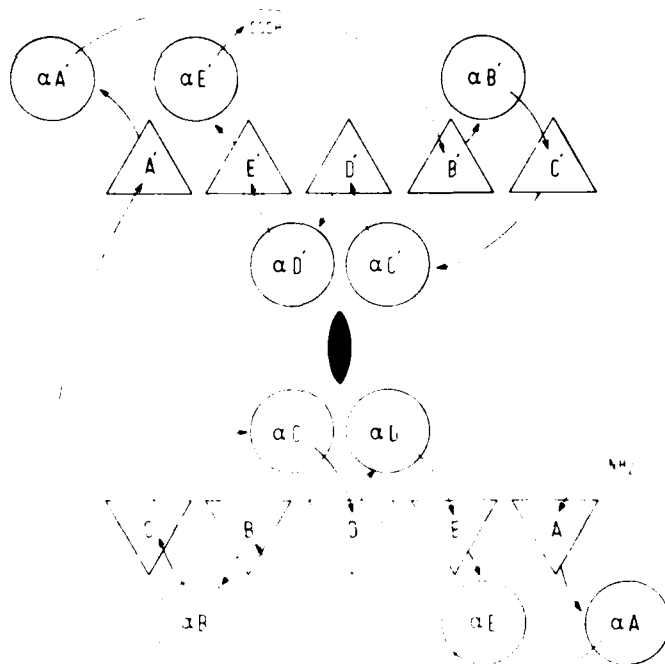


FIG. 1. Schematic representation of the rhodanese molecule showing the two domains of roughly equal size and the connecting loop at the surface of the molecule. The active site (AS) is close to the interface of the two domains.



RHD

FIG. 2. The secondary structure elements of bovine liver rhodanese. Triangles indicate β -strands and circles α -helices. Each domain contains a five-stranded parallel β -sheet labeled A to E in the first domain and A' to E' in the second domain. The β -sheets are covered on both sides by α -helices. The symbol in the center of the figure is the pseudo-two fold axis which relates the three-dimensional structures of the two domains.

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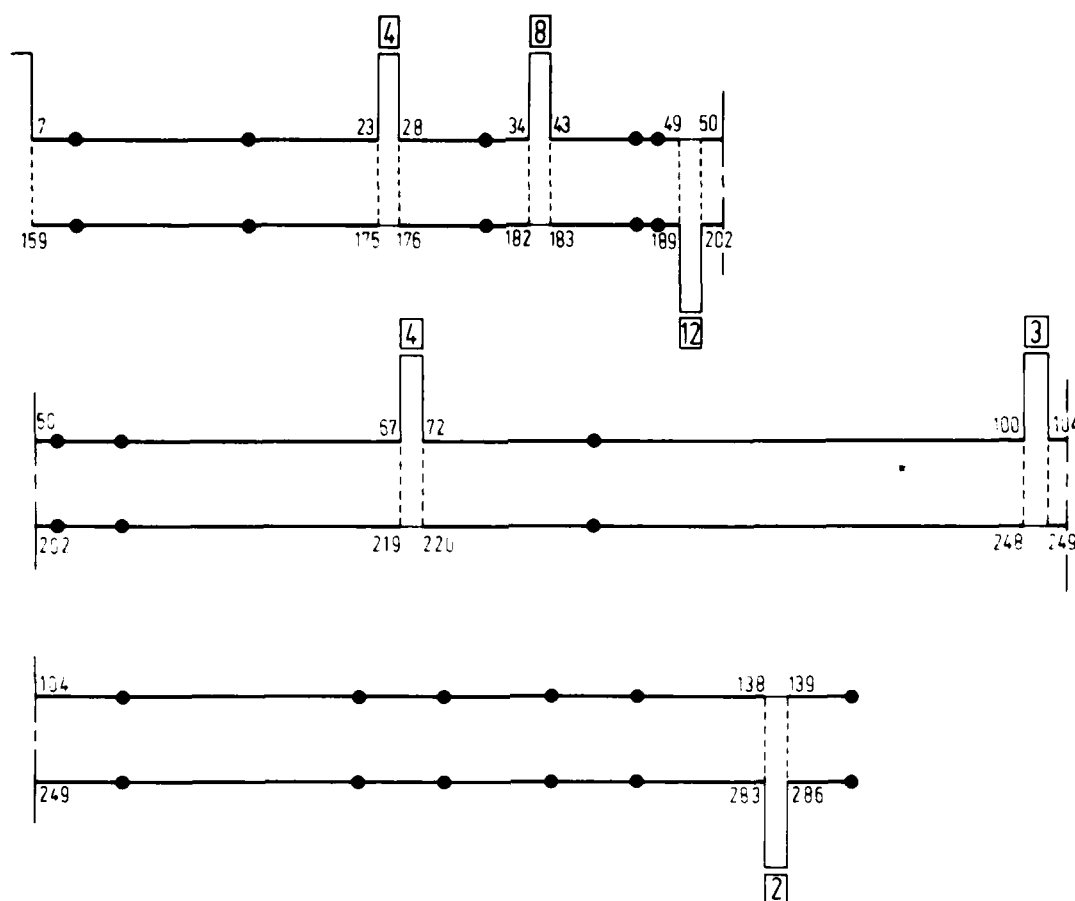


FIG. 3. Alignment of structurally equivalent residues in the two domains of rhodanese. The upper line represents the chain in the first domain, the lower line that of the second domain. The amino acid sequence numbers of the first and last residues of the equivalent chain segments are shown. The numbers in boxes give the lengths of the deletions required to bring the two sequences in register according to the three-dimensional structure. The block dots indicate the positions of the 14 equivalent amino acid residues which are identical in both domains.

- (i) the detoxification of inorganic sulfide;
- (ii) the incorporation of sulfur in iron-sulfur centra of redox proteins (Bonomi *et al.*, 1977a, 1977b); and
- (iii) the maintenance of the sulfane sulfur pool in organisms (Westley, 1977, 1980).

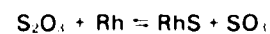
It seems hard to ascertain at present which of these additional possibilities are of real significance for the proper functioning of living organisms.

A protective role of rhodanese against low level cyanide poisoning is likely because of the high concentrations observed in mammalian liver mitochondria. There, it would be at the correct position for defending the sensitive cytochrome c oxidase system against cyanide which may be circulating in the blood as *e.g.* the result of the digestion of cyanide-containing food. This may be of great importance for human populations living in areas where cassava, which contains substantial amounts of cyanogenic compounds, is an important component of the diet.

In other areas, where the dependence on cyanogenic food-stuffs is virtually negligible, the importance of cyanide detoxification mechanisms is demonstrated by the occurrence of Leber's optic atrophy, a rare hereditary disease. Recently, it has been shown that rhodanese levels in the livers of patients

with this condition are substantially lower than in normal individuals (Cagianut *et al.*, 1981). This may explain the extreme sensitivity of these patients to low levels of cyanide as found in cigarette smoke (Wilson, 1965). In view of these lines of evidence, it seems warranted to focus in this contribution on the reaction between cyanide and thiosulfate, as catalyzed by rhodanese.

This sulfur-transfer reaction has been shown by Westley and collaborators to occur in two well-defined steps (Westley and Nakamoto, 1962):



where RhS is a "sulfur-rhodanese" intermediate, which is surprisingly stable in the absence of sulfur acceptors such as cyanide. From a series of mechanistic studies it became evident that bovine liver rhodanese contained a number of essential features in its active center (Westley, 1973, 1977)

- (i) an essential sulphhydryl group;
- (ii) a hydrophobic region;
- (iii) a cationic region containing two positive charges (Leininger & Westley, 1968).

TABLE 1
Ionic Interactions in Rhodanese

Salt Bridges in Domain I	Equivalent Residues in Domain II
Arg 41 - Glu 46	none and Arg 186
Lys 45 - Glu 49	Gly 185 and Gly 189
Arg 64 - Glu 61	Leu 216 and Met 213
Lys 66 - Glu 62	Glu 218 and Asp 214
Salt Bridges in Domain II	Equivalent Residues in Domain I
Arg 182 - Asp 180	Ser 34 and Asp 32
Arg 182 - Glu 193	Ser 34 and none
Arg 186 - Glu 193	Glu 46 and none
Lys 235 - Asp 214	Leu 87 and Glu 62
Lys 241 - Asp 238	Thr 93 and Ser 90
Arg 248 - Glu 193	Gly 100 and none
Arg 281 - Glu 165	Glu 136 and Lys 13 ^a
Arg 281 - Glu 277	Glu 136 and Asn 132
Interdomain Salt Bridges	Equivalent Residues in Other Domains
Arg 248 - Glu 71	Glu 100 and none
Arg 110 - Asp 272	His 255 and Asn 127

^aThe distance between the side chains of these residues is 10 Å which is much larger than the 3 to 4 Å required for a salt bridge.

The remainder of this paper will show that these inferences from chemical and kinetic studies were completely correct.

Bovine liver rhodanese contains a total of 293 residues in a single polypeptide chain with a molecular weight of approximately 32 000. The amino acid sequence has been elucidated by Heinrikson and co-workers (Ploegman *et al.*, 1978a; Russell *et al.*, 1978). In our laboratory, the three-dimensional structure has been determined by means of X-ray diffraction in a number of steps (Smit *et al.*, 1974; Bergsma *et al.*, 1975; Ploegman *et al.*, 1978b; Lijk *et al.*, in preparation) giving us a detailed picture of the architecture of this enzyme and a precise view of the nature and location of the atoms in its active center. This has led to the proposal of a mechanism for the sulfur transfer reaction (Ploegman *et al.*, 1979) and to an understanding of the mode of action of a number of inhibitors (Lijk, Kalk, Brandenburg and Hol, submitted for publication). In this contribution a number of the interesting properties of the bovine liver rhodanese molecule will be discussed.

DETERMINATION OF THE THREE-DIMENSIONAL STRUCTURE

The method used for isolating rhodanese from bovine liver was essentially that of Horowitz and De Toma (1970). Crystals suitable for X-ray diffraction studies were grown from a 2.0 M ammonium sulfate solution, pH 7.3, in the presence of 1 mM sodium thiosulfate. Shortly before mounting the crystal in a capillary for data collection, the thiosulfate was removed by soaking the crystal in a 2.0 M ammonium sulfate solution of pH 7.3, in order to reduce radiation damage by the X-ray beam. The form of rhodanese present in the crystal after this procedure is still the sulfur-rhodanese complex.

The multiple-isomorphous replacement method was used for solving the three-dimensional structure. For further details of this method the reader is referred to Blundell and Johnson

(1976). Hereto, six heavy atom derivatives were prepared of which the p-chloromercury benzene sulfonate and the sodium perrhenate derivatives were the most powerful. Intensities were collected on a 4-circle single-crystal diffractometer. From a 2.5 Å resolution electron density map, a Kendrew-Watson model of the molecule was constructed (Ploegman *et al.*, 1978a). This molecular model was subsequently refined with data to 2.1 Å resolution, using constrained and restrained refinement techniques. This has improved considerably the accuracy of the atomic positions and led to valuable information on the thermal motions of the atoms and positions of solvent molecules (Lijk, Van Nes, Kalk and Hol, in preparation).

THE ARCHITECTURE AND EVOLUTION OF THE RHODANESE MOLECULE

The crystallographic studies revealed a remarkable feature of bovine liver rhodanese. The polypeptide chain is folded into two so-called "domains", of virtually equal size (Figure 1). Domain I consists of residues 1 to 142 and domain II comprises residues 159 to 293. The residues 143 to 158 belong to an exposed loop which connects the two domains.

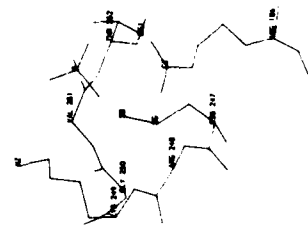
The folding of the polypeptide chain in the two domains is very similar. This is already apparent from Figure 2 where the secondary structure elements, α -helices and β -strands, of the molecule are indicated. Each domain consists of a five-stranded parallel β -sheet, flanked on both sides by α -helices. When precise superpositions of the C^α atoms of the two domains are carried out, it appears that 117 residues can be superimposed with a r.m.s. difference of 1.78 Å. This is a small number and one would expect that such a close similarity in folding pattern is due to a substantial amino acid sequence homology between the two domains. As Figure 3 illustrates, the number of residues with identical amino acids in equivalent positions is only 14, i.e. 13%. In addition, several insertions and deletions are required to obtain an optimal superposition of the two domains (Figure 3). This marginal similarity in sequence is in sharp contrast with the closely related conformations of the two domains. It remains a complete puzzle, but a great challenge to understand it at the same time, why two such dissimilar sequences give rise to such similar three-dimensional structures.

The superposition showed furthermore, that the two domains are related by a pseudo-twofold axis: the rotation deviates less than one degree from 180° and the translation parallel to the two-fold axis is less than one Ångström.

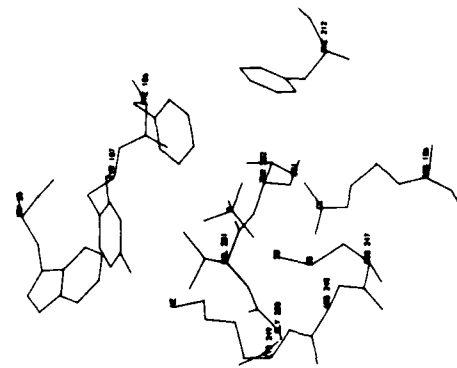
The architecture of the rhodanese molecule suggests that it may be a product of gene duplication and subsequent gene fusion in the course of evolution. After the duplication event, the two subunits probably formed a dimer, which later became a monomer of twice the size. After gene duplication, the amino acid sequences began to differ, while also deletions and insertions occurred which led eventually to the molecule we see at the present day. How little is preserved of the presumed original sequence identity, is illustrated in Figure 3, whereas Table 1 shows that none out of the 14 salt bridges occurring in the molecule has an equivalent counterpart in the other domain. More detailed analyses of the sequence and structure of bovine liver rhodanese are given by Ploegman *et al.* (1978b) and Keim *et al.* (1981).

THE CATALYTIC MECHANISM

The active site is a pocket which is situated close to the interface of the two domains, far away from the connecting



.....



ACTIVE SITE NUMBER

373

loop, as sketched in Figure 1. At the bottom of the pocket cysteine 247 is situated. The electron density map clearly shows that the extra sulfur atom of sulfur rhodanese is attached as a persulfide to the sulfhydryl group of this essential cysteine residue (Ploegman *et al.*, 1979). This is in agreement with the interpretation of spectroscopic studies (Finazzi Agrò *et al.*, 1972). The persulfide is stabilized by a large number of hydrogen bonds with backbone NH groups and with the hydroxyl group of Thr 252 (Figure 4).

Close to the active site, several hydrophobic residues cluster together: Trp 35, Phe 106, Tyr 107, Trp 212 and Val 251 which are all in contact with the solvent region (Figure 4). Although the importance of this hydrophobic patch at one side of the active center is not clear for the reaction between cyanide and thiosulfate, it is obvious that it could play a role in binding other substrates like aromatic and aliphatic thiosulfonates. It also seems likely that this region is in contact with the aromatic moiety of inhibitors such as benzene sulfonate, when bound at the active site.

A further interesting arrangement near the active site entrance is a salt bridge network involving no less than six residues: Glu 71, Arg 248, Glu 193, Arg 182, Asp 180 and Arg

186 (Ploegman *et al.*, 1979). Of these residues, Arg 186 is situated at the entrance of the active site playing an important role in the electrostatic interactions which take place during the catalytic process. A second positive charge at the active site entrance is provided by the ϵ -amino group of Lys 249 (Figure 4). The presence of these two charges is in agreement with the conclusions drawn by Leininger and Westley (1968) on the basis of ionic strength dependent kinetic measurements.

The four elements at the active site of bovine liver rhodanese: (i) the sulfhydryl group of Cys 247; (ii) the "ring" of persulfide-stabilizing NH-groups; (iii) two positive charges at the entrance of the pocket, and (iv) a hydrophobic patch, are the essential ingredients for an efficient transfer of sulfur atoms from suitable donor molecules to acceptors. The role of the hydrophobic region has not been investigated by crystallographic studies and will therefore not receive further attention.

The catalytic mechanism as proposed in Figure 5 is based on the three-dimensional structure observed by X-ray investigations and on the establishment of the double displacement mechanism by the group of Westley. It is furthermore assumed that the three-dimensional structure of the "sulfur-free" enzyme is very similar to that of sulfur rhodanese as observed

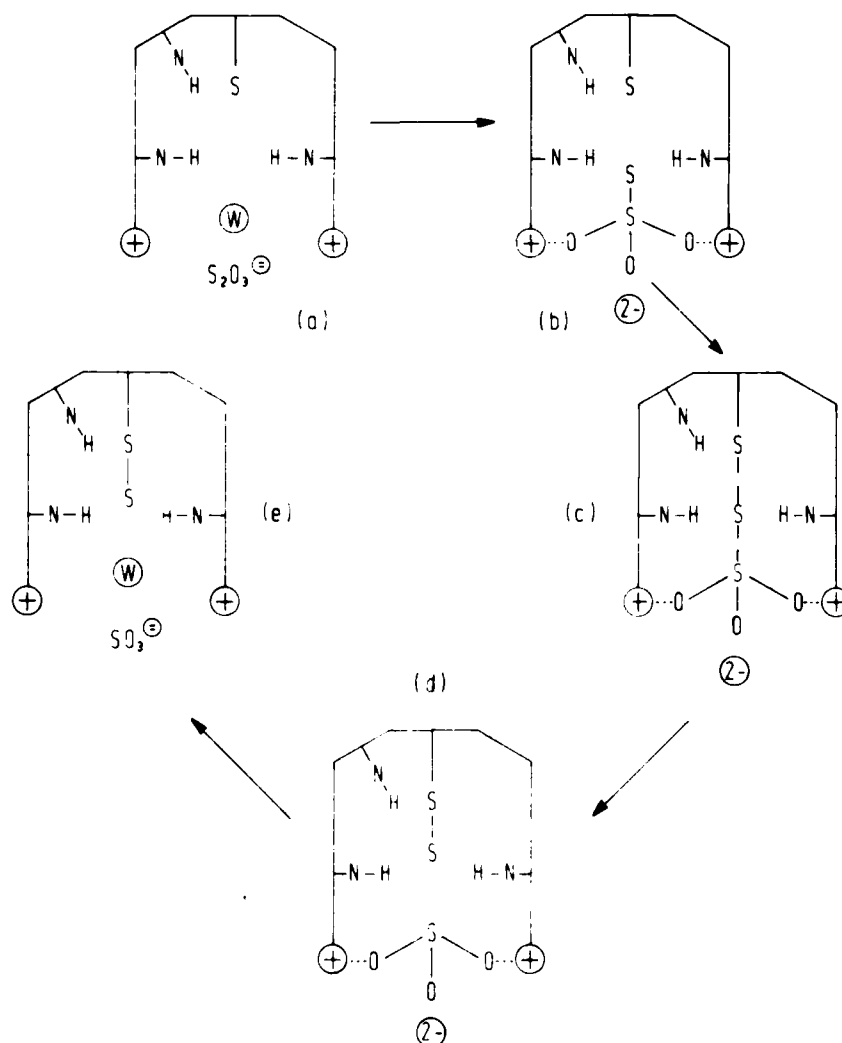


FIG. 5 Simplified diagram of the proposed steps in the reaction of rhodanese with thiosulfate (a), leading to the formation of sulfur rhodanese and sulfite (e). The transition state (c) is presumably stabilized by a number of NH $\cdots$ S hydrogen bonds

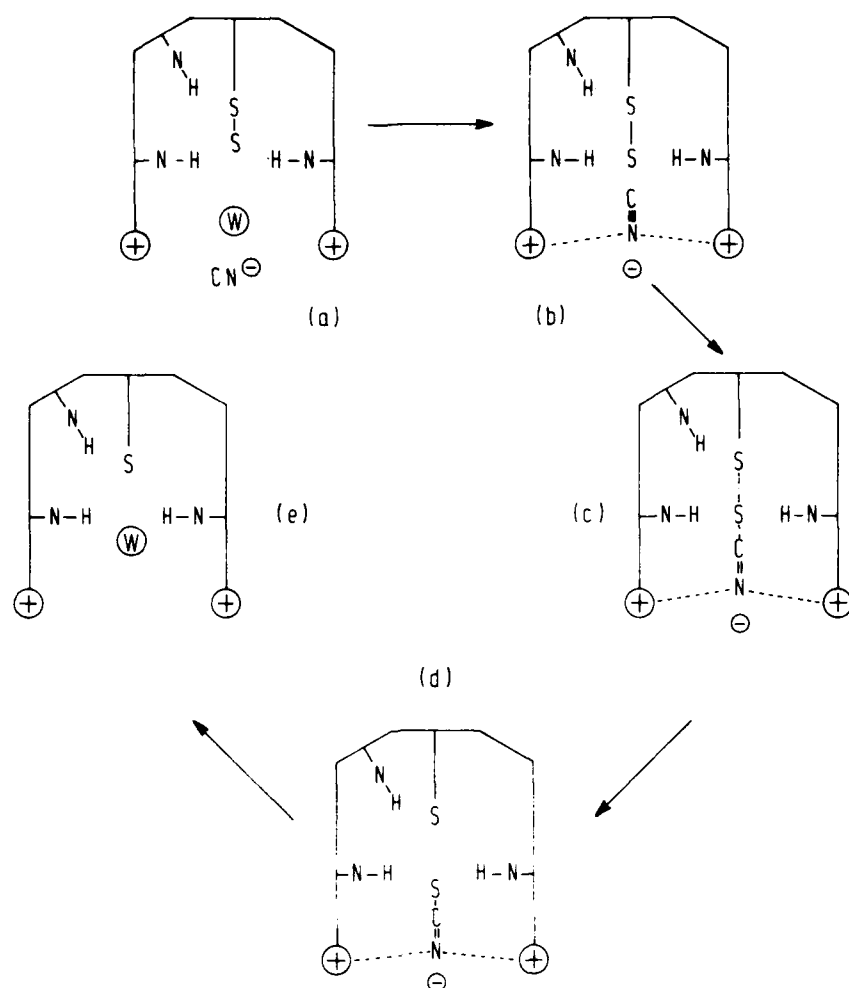


FIG. 6. Simplified diagram of the proposed steps in the reaction of sulfur rhodanese with cyanide (a) resulting in the formation of isothiocyanate and the regeneration of rhodanese (e).

in the crystalline state. Evidence for this comes from a number of studies in which the extra sulfur has been removed in the crystals by treatment with cyanide. The resultant structure differs only marginally from that of sulfur-rhodanese (Ploegman *et al.*, 1979; Lijk, Kalk, Brandenburg and Hol, submitted for publication). Other lines of evidence exist (Westley, 1977), which suggest that a significant change in conformation occurs upon binding of the first substrate, thiosulfate, which is only reversed upon release of the final product, thiocyanate. Even if this event would actually happen in solution, it would only require a minor modification of the mechanism shown in Figures 5 and 6, namely the addition of a conformational change at the beginning and at the very end of the catalytic cycle.

The reaction starts with the replacement of solvent molecules in the active site pocket by a thiosulfate ion (Figure 5). Electrostatic interactions with the positive charges of Arg 186 and Lys 249 side chains play an important role at this stage. Once bound at the active site entrance, the sulfhydryl group of Cys 247 performs a nucleophilic attack on the outer sulfur atom of thiosulfate. The resultant transition state intermediate (Figure 5c) is probably stabilized by hydrogen bonds from the "ring" of NH groups which, in the next step, also stabilizes the persulfide of sulfur rhodanese. The first product, sulfite, is

released from the enzyme and replaced by a solvent molecule. The sulfur rhodanese complex obtained is unusually stable in the absence of suitable sulfur acceptor molecules.

However, in the presence of cyanide a very fast, diffusion limited, reaction occurs which is essentially the reverse of the first reaction cycle (Figure 6): the sulfur atom is transferred from the persulfide to the cyanide ion and, in the final step, the product thiocyanate is replaced by a solvent molecule. Sulfur-free rhodanese is thus regenerated which may catalyse another sulfur transfer reaction.

INHIBITION BY METAL CYANIDES AND OTHER ANIONS

A variety of metal cyanides are reported to be inhibitors of bovine liver rhodanese (Volini *et al.*, 1978). This has led to an X-ray investigation of the mode of binding of metal cyanides to rhodanese. Platinum cyanide, $\text{Pt}(\text{CN})_4$, and gold cyanide, $\text{Au}(\text{CN})_2$, were studied as a complex with sulfur rhodanese, whereas nickel cyanide, $\text{Ni}(\text{CN})_4$, and zinc cyanide $\text{Zn}(\text{CN})_4$, were complexed with sulfur-free rhodanese in the crystalline state (Lijk, Kalk, Brandenburg and Hol, 1983). The conclusions from these studies are very clear. All these metal cyanides, irrespective of the nature of the metal and independent of the

presence of the extra sulfur atom, bind at one common position: at the entrance to the active site, in ionic interaction with the side chains of Arg 186 and Lys 249. Access to the essential Cys 247 residue is blocked in this manner which provides an elegant explanation for the inhibitory effect of these compounds.

From the high resolution studies and selenate replacement experiments (Lijk *et al.*, in preparation) it has been established that sulfate ions also bind at this position at the entrance to active site pocket (see also Figure 4). This explains the inhibition of the reaction by sulfate ions. From these observations, it is clear that this anion binding site is also a likely position for other inhibitors such as oxalate and α -ketoglutarate (Oi, 1975; Wang and Volini, 1973).

CONCLUSION

The crystallographic investigations described above have provided, in conjunction with the amino acid sequence determination and a wealth of kinetic results, a detailed picture of the bovine liver rhodanese molecule, in particular of the active site. A catalytic mechanism can be proposed in which three elements, a sulfhydryl group, a set of peptide nitrogen hydrogen bond donors and two positively charged side chains, are carefully positioned with respect to each other such that an efficient catalysis of the transfer of one single sulfur atom is ensured. The mode of inhibition of a wide variety of anions can be explained by binding to the two positive charges near the active site entrance thereby blocking access of the substrates to the essential sulfhydryl group.

A complete surprise of the crystal structure of rhodanese was the similarity of the conformation of the two domains, particularly in view of the large differences in amino acid sequence. Understanding this observation on the basis of physical chemical principles remains a challenge for theoretical biophysical chemistry.

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The Sulfurtransferases

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ABSTRACT

The Sulfurtransferases. Westley, J., Adler, H., Westley, L. and Nishida, C. (1983). *Fundam. Appl. Toxicol.* 3:377-382. The sulfurtransferases are a group of proteins that catalyze the formation, interconversion and reactions of compounds containing sulfane sulfur atoms. Serum albumin has properties that implicate it as a major potential sulfur carrier/transferase. The relevance of the sulfane pool system as a whole to cyanide detoxication appears clear. The mechanisms of action of the various components at the molecular level are still under investigation.

INTRODUCTION

From much that has appeared in the preceding papers, it must be clear that the major route of biological cyanide detoxication is by conversion to thiocyanate, which is relatively non-toxic. This conversion obviously requires a source of sulfane sulfur — that is, of divalent sulfur bonded only to other sulfur, since this is the form that can react with cyanide to produce thiocyanate. Sulfane sulfur atoms can occur either at the ends of poly- or per-sulfide chains, in which case they bear a negative charge and can accept a proton, or in the internal positions of polysulfide chains.

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Since a number of the topics dealt with in the present paper have been reviewed fairly recently, many of the references cited are review articles, from which detailed references to the original investigations may be obtained.

Such sulfane sulfur atoms occur in various compounds in biological systems (Westley, 1980).¹ As shown in Fig. 1, these include inorganic thiosulfate anion and both its oxidation products, the polythionates, and its organic analogs, the thiosulfonates. In addition, the persulfides ($R-S-S$) and polysulfides belong to this group, and so does the staggered eight-membered ring of sulfur atoms shown in the figure, which is elemental sulfur, containing nothing but sulfane sulfur atoms.

The dashed arrows in Fig. 1 are an artist's representation of the fact that these sulfane-containing materials undergo rapid equilibration *in vivo*. When an experimental animal is injected with thiosulfate containing ^{35}S in its sulfane position exclusively, all of these materials can be found labeled in the plasma as quickly as a sample can be obtained, although simply mixing such radioactive thiosulfate with plasma or whole blood *in vitro* achieves no such distribution (Schneider and Westley, 1969). These and other observations made in experiments with sulfane sulfur *in vivo* have indicated the occurrence of a physiological pool of cyanide-reactive sulfane materials, any or all of which might be prime candidates for the role of sulfur donor in cyanide detoxication (Westley, 1981).

A number of distinct enzymes, the sulfurtransferases, have been shown to catalyze reactions that either use or produce sulfane sulfur. These also must be considered as possible active participants in cyanide detoxication as well as in other processes that may compete with cyanide detoxication for sulfane sulfur. As indicated in Fig. 1, the sulfurtransferases

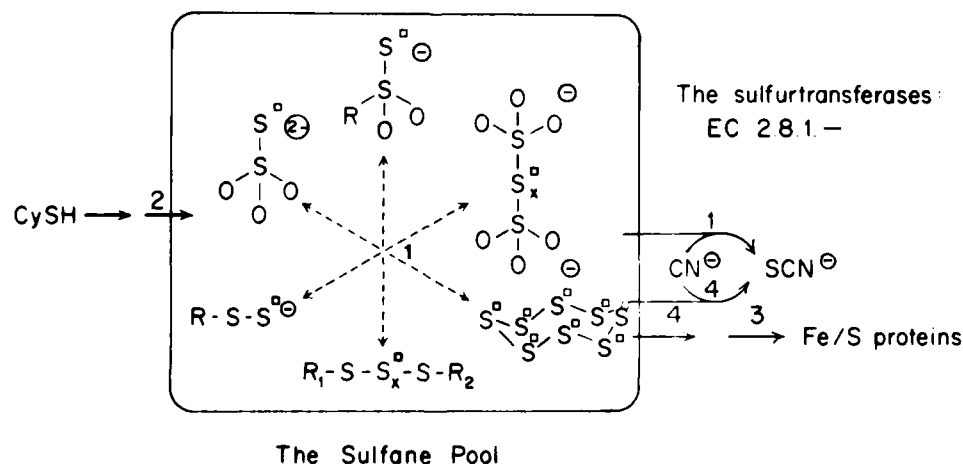


FIG. 1 Biological materials containing sulfane sulfur. Sulfane atoms are marked with the symbol $\square$. Numbers over reaction arrows indicate the participation of the following proteins: 1, rhodanese; 2, mercaptopyruvate sulfurtransferase; 3, thiosulfate reductase; 4, serum albumin.

TABLE 1
Distribution of Rhodanese Activity

Phylogenetic	Organ	Subcellular
Prokaryotes	Liver ^a	Nucleus ^c
Heterotrophic bacteria	Kidney ^b	Mitochondria
Actinomycetes	Heart ^c	Outer membrane ^c
Chemoautotrophic bacteria	Brain ^c	Intermembrane space ^c
Eukaryotes		Inner membrane ^c
Fungi	Intestine ^c	Matrix ^c
Plants	Testis ^b	Endoplasmic reticulum ^c
Animals	(Morris hepatoma) ^b	Cytosol ^c

^aThe richest sources

^bSources with 20-60% the specific activity of "a"

^cSources with ≤ 10% the specific activity of "a"

have Enzyme Commission registry numbers EC 2.8.1.-- and the probable positions of their participation in sulfane metabolism are indicated by the numbers over the reaction arrows. For example, the sulfurtransferase with the trivial name "rhodanese" is EC 2.8.1.1 and it is known mostly for its catalysis of the transfer of sulfane sulfur directly to cyanide. Less well known is the fact, noted in the figure, that rhodanese is also capable of catalyzing the rapid interconversions of all of the sulfane pool components.

EC 2.8.1.2 is 3-mercaptopyruvate sulfurtransferase, and its principal responsibility is thought to be the formation of sulfane sulfur *de novo* from the transamination product of cysteine. However, as is *not* noted in the figure, this enzyme can also transfer its mercaptopyruvate-derived sulfur to cyanide, and it may therefore be as much or as directly involved in cyanide detoxication as is rhodanese.

The sulfurtransferase known as "thiosulfate reductase" is not really EC 2.8.1.3, although it deserves to be, and very probably will be, when the Enzyme Commission considers the evidence. This enzyme (designated "3" in Fig. 1) is thought to be involved in the use of sulfane sulfur for the synthesis of iron/sulfur centers, which is the principal large-scale use of

inorganic sulfur (aside from sulfate) in mammals. It is noteworthy that thiosulfate reductase (at least the yeast enzyme, which is the only one thus far purified and studied) will *not* transfer sulfane sulfur to cyanide.

The "enzyme" labeled "4" in the diagram is perhaps something of a surprise — it is serum albumin! As Bo Sörbo showed more than 25 years ago (Sörbo, 1955), serum albumin can catalyze the cyanolysis of elemental sulfur. Moreover, the form in which elemental sulfur occurs in plasma is a complex with serum albumin (Schneider and Westley, 1969), which is thought to be a carrier for this form of sulfane sulfur from its site of formation in the liver to the peripheral tissues, where it can be used for the synthesis of iron/sulfur centers. Needless to say, such sulfur in transit may well be intercepted when cyanide is present.

The remainder of this presentation consists of a closer look at the three recognized sulfurtransferases — where they occur and what is known about how they work. Some previously unpublished experimental procedures and data on the sulfur loading capacity and cyanide reactivity (*i.e.*, the sulfur carrier/transferase properties) of serum albumin are also included.

THIOSULFATE: CYANIDE SULFURTRANSFERASE

Rhodanese is the longest-known, most studied and best understood sulfurtransferase (Sörbo, 1972, 1975; Westley, 1973, 1977). As shown in Table 1, this sulfurtransferase is practically ubiquitous biologically, at least in the sense that it occurs in all sorts of organisms. At a finer level of detail than the table presents, rhodanese is found in all phyla of the animal kingdom, including, quite notably, our own. The organ distribution differs somewhat from species to species, but what is shown in the table is a fair representation of the situation in the principal organs of laboratory rats (Koj *et al.*, 1977) and probably of people. Large quantities of rhodanese occur in liver, with all of it confined to the mitochondrial matrix (Koj *et al.*, 1975). The enzyme has been purified to homogeneity from various mammalian livers (including human liver) and from chicken livers and bovine kidneys. The bovine liver and kidney enzymes are evidently identical molecules

Bovine liver rhodanese has been the subject of extensive study as to both its mechanism of action (Schlesinger and Westley, 1974; Westley, 1977; Weng *et al.*, 1978) and its structure (Russell *et al.*, 1978; Ploegman *et al.*, 1978). What follows here is a brief description of the principal features of the mechanism. Readers interested in the structure are

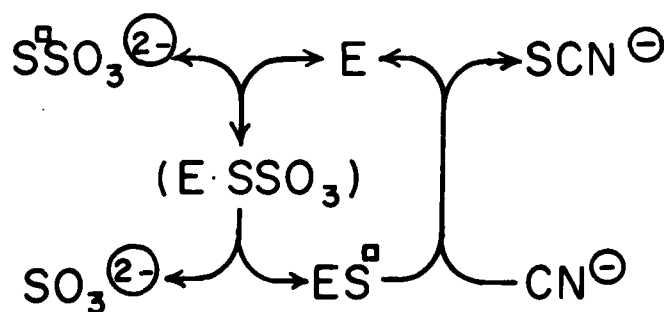


FIG. 2. Formal kinetic mechanism of rhodanese action. E, free enzyme; $(\text{E} \cdot \text{SSO}_3)$, enzyme-thiosulfate complex (noncovalent); $\text{ES}^\square$, sulfur-substituted enzyme.

TABLE 2
Distribution of 3-Mercaptopyruvate Sulfurtransferase Activity

Phylogenetic	Organ	Subcellular
Prokaryotes	Liver ^a	Nucleus ^c
Heterotrophic bacteria	Kidney ^a	Mitochondria
Eukaryotes	Heart ^a	Outer membrane ^c
Fungi	Brain ^a	Intermembrane space ^c
Animals	Intestine ^a	Inner membrane ^c
	Testis ^a	Matrix ^c
	Adrenal Cortex ^a	Endoplasmic reticulum ^c
	(Morris hepatoma) ^b	Cytosol ^b

^aThe richest sources

^bSources with 20-60% the specific activity of ""

^cSources with $\geq 10\%$ the specific activity of ""

referred to Dr. Hol's account of the elegant detailed structural studies of this enzyme by X-ray crystallographic techniques (Hol, 1983).

As is indicated in Fig. 2, rhodanese is a double displacement enzyme. The sulfur-donor substrate enters a kinetically significant complex and is cleaved by the enzyme to form a covalently substituted sulfur-enzyme, with discharge of the first product. The sulfur-rhodanese is then attacked by the sulfur-acceptor substrate to produce the final product and regenerate the free enzyme. The occurrence of this formal mechanism is supported by the results of steady-state kinetic studies, which yield "ping-pong" initial velocity patterns, and by the isolation and characterization of the form ES. The isolated sulfur-enzyme reacts rapidly with sulfite to produce thiosulfate or with cyanide to produce thiocyanate. In terms of chemical mechanism, kinetic studies have shown that there is a cationic site on rhodanese for the anionic sulfur donor. This cationic site also serves as an electrophilic group polarizing the sulfur-sulfur bond of the substrate so that it is readily cleaved by an enzymic nucleophile, which has been shown to be an active-site sulfhydryl group. The sulfur-enzyme is thus a persulfide, and it reacts readily with cyanide. It is noteworthy that all of

this nice chemistry goes on in an essentially nonaqueous environment — the active site having been shown by experiments with reporter groups to be far less polar than the bulk solvent. This feature is also evident in the detailed X-ray crystallographic structure of the enzyme.

3-MERCAPTOPYRUVATE SULFURTRANSFERASE

Much less is known about the second of the sulfurtransferases, 3-mercaptopyruvate sulfurtransferase. However, as shown in Table 2, there is some information available concerning the biological distribution of this activity (Westley, 1980). Its occurrence in heterotrophic bacteria, fungi and various animal species is well established. In rats (and other mammals) its organ distribution is somewhat more even than that of rhodanese (Koj *et al.*, 1977). Not shown in the table is the fact that mammalian erythrocytes contain a substantial concentration of this activity. It does not appear to have been established clearly whether the enzymes from erythrocytes and from the several internal organs are identical. Within the liver cell, the activity is distributed bimodally, with some 20% appearing in the cytosol fraction of homogenates in which all

Mercaptopyruvate sulfurtransferase (EC 2.8.1.2)

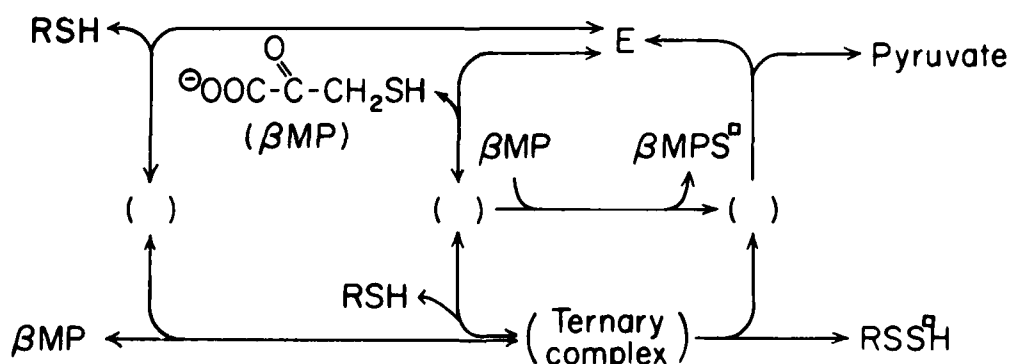


FIG. 3. Formal kinetic mechanism of mercaptopyruvate sulfurtransferase with a thiol (RSH) as sulfur-acceptor substrate. Parentheses indicate noncovalent enzyme-substrate complexes. RSSH, persulfide of the thiol substrate. $\beta\text{MPS}^\ominus$, mercaptopyruvate persulfide.

TABLE 3
Distribution of Thiosulfate Reductase Activity

Phylogenetic	Organ	Subcellular
Prokaryotes	Liver ^a	Nucleus ^c
Heterotrophic bacteria	Kidney ^a	Mitochondria
Chemoautotrophic bacteria	Heart ^b	Outer membrane ^c
Eukaryotes	Brain ^b	Intermembrane space ^c
Fungi	Intestine ^b	Inner membrane ^c
Animals	Testis ^b	Matrix ^c
	(Morris hepatoma) ^b	Endoplasmic reticulum ^c
		Cytosol ^b

^aThe richest sources

^bSources with 20-60% the specific activity of ""

^cSources with $\leq 10\%$ the specific activity of ""

of the rhodanese is confined to the mitochondrial matrix (Koj *et al.*, 1975). Again, it is not known whether the mitochondrial and cytosolic enzymes are the same molecule.

Mercaptopyruvate sulfurtransferase has been highly purified from rat liver, *E. coli*, erythrocytes, and bovine kidney. The enzyme prepared from acetone powders of rat liver was originally reported to contain essential copper ions, but more recent preparations of the rat liver enzyme do not; neither do the preparations from other sources.

The only full-scale bisubstrate kinetic studies reported for this enzyme were carried out with the bovine kidney mercaptopyruvate sulfurtransferase (Jarabak and Westley, 1978), and yielded the rather complex appearing conclusions shown in Fig. 3. However, this formal mechanism is in fact quite easily understood. The first thing to note is that it bears very little resemblance to the rhodanese formal mechanism. There is no sulfur-substituted enzyme intermediate, and this scheme does not yield a simple ping-pong initial velocity pattern of parallel double reciprocal plots. Instead, the necessity of forming a ternary complex of enzyme with both substrates, which can combine in either order, makes this a "sequential" mech-

anism, with an intersecting initial velocity pattern. The interesting "bridge" across the middle of this formal mechanism stems from the fact that the sulfur-donor substrate 3-mercaptopyruvate is itself a thiol and hence can serve also as sulfur-acceptor substrate. With either acceptor, the carbon-sulfur bond of the donor molecule is cleaved and the products formed are pyruvate and the persulfide of the acceptor. When cyanide serves as the sulfur acceptor in this reaction, only the inner portion of the formal mechanism given here is applicable; the other logically possible pathway (cyanide-first) does not function (Jarabak and Westley, 1980).

Essentially nothing is yet known about the chemical mechanism of this catalysis. The enzyme is unstable and rather difficult to purify highly from any source. Yet, it is important to find out whether the mechanism for cleaving the carbon-sulfur bond of mercaptopyruvate is essentially similar to that by which rhodanese cleaves the sulfur-sulfur bonds of its donor substrates. The difference in *formal* mechanisms may well hide a similarity of chemical mechanisms that includes electrophilic polarization of the target bond followed by nucleophilic cleavage (only in this case the nucleophile may be a

Thiosulfate reductase

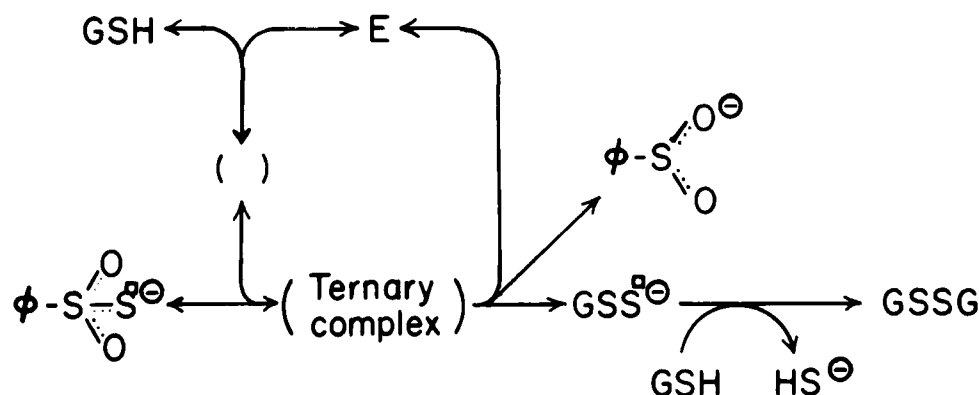


FIG. 4 Formal kinetic mechanism of thiosulfate reductase. Parentheses indicate noncovalent enzyme-substrate complexes; GSH, glutathione; GSSG, glutathione disulfide; GSS^- , glutathione persulfide; ϕ , benzene ring; $\cdots$, π bond.

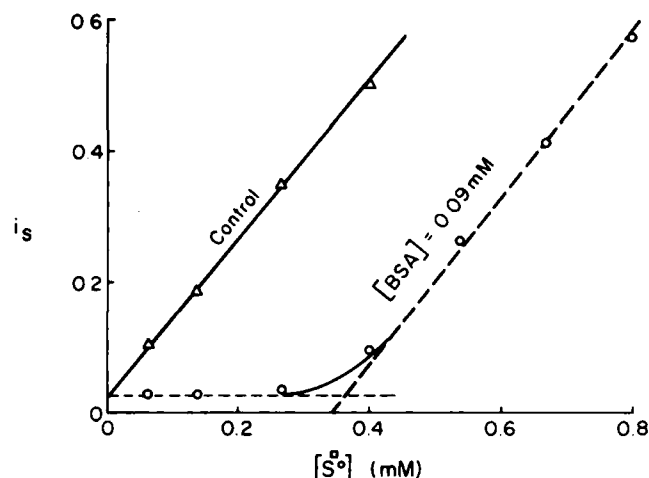


FIG. 5. Light scattering titration of bovine serum albumin for sulfur loading capacity. i_s , intensity of 546 nm light scattered at 90° viewing angle, arbitrary units; $[S^\circ]$, mM concentration of elemental sulfur, calculated as atomic sulfur. Experimental conditions: 2.0 mL of boric acid-NaOH buffer, pH 9.5, ionic strength 0.10, temperature 25°C . Sulfur was added with magnetic stirring as μL volumes of a saturated solution in pyridine ($0.4\text{ M } S^\circ$). The measuring instrument was a light scattering photometer from C.N. Wood.

sulfhydryl group of the acceptor substrate rather than of the enzyme itself) to form a persulfide.

THIOSULFATE REDUCTASE

The foregoing kind of mechanistic scenario has its origins in both the rhodanese mechanism and in recent work done with the third of the sulfurtransferases, thiosulfate reductase. This enzyme, which *in vivo* probably uses electrons from glutathione to reduce sulfane sulfur to the level of sulfide for use in the synthesis of iron/sulfur proteins, also has a broad biological distribution. As shown in Table 3, thiosulfate reductase activity distinct from the action of rhodanese has been demonstrated in a number of sources, although the activity in autotrophic bacteria is often directly dependent for its electrons on a hydrogenase system rather than on glutathione. In rat tissues, the liver and kidney are well-endowed with glutathione-dependent thiosulfate reductase activity, but all tissues contain a considerable amount, in accord with its presumed function. The subcellular distribution is like that of mercaptopyruvate sulfurtransferase.

Beyond the fact that it exists, however, our knowledge of the thiosulfate reductase of animal tissues extends to just one finding: It is extraordinarily unstable and, therefore, seemingly impossible to purify and study. For this reason all of our information about this enzyme comes from recent studies of thiosulfate reductase purified to homogeneity from yeast, which displays just enough stability to permit careful purification and kinetic observations. The catalyzed reactions of inorganic thiosulfate with glutathione and with cysteine have been studied in detail (Uhteg and Westley, 1979) and, more recently, the reaction of an organic thiosulfonate with glutathione has been examined (Chauncey and Westley, 1981). The formal mechanism established for this latter reaction is shown in Fig. 4.

This mechanism involves the ordered addition of the two substrates to form a ternary complex with the enzyme. The products discharged from the ternary complex in unspecified order (unspecified because we have not yet been able to prove what it is) are glutathione persulfide and, with this particular

donor, benzene sulfinate. The sulfane sulfur is then reduced to inorganic sulfide when the persulfide product reacts spontaneously with excess glutathione. Alternatively, when cyanide is present, much of the sulfane sulfur is converted to thiocyanate.

The overall reaction is the reductive dismutation of the thio-sulfonate by two molecules of glutathione, but the enzyme-catalyzed part of the process is simply a cleavage of the sulfur-sulfur bond of the sulfur-donor substrate with transfer of the sulfane sulfur to a sulfhydryl nucleophile. This is exactly like the rhodanese reaction except for the fact that the attacking sulfhydryl group in the case of the reductase belongs to the acceptor substrate rather than to the enzyme. Incidentally, thiosulfate reductase does contain one cysteine residue but, unlike the situation in rhodanese, this enzymic sulfhydryl group is not directly involved in the catalytic mechanism. Also unlike both rhodanese and mercaptopyruvate sulfurtransferase, thiosulfate reductase will not use cyanide as an acceptor substrate; that is, the presence of a thiol substrate is required for generation of a persulfide, which can only then transfer sulfur to cyanide, in a nonenzymic reaction.

SERUM ALBUMIN

In consideration of the sulfurtransferase facts outlined in the foregoing summaries, our working hypothesis for sulfane metabolism is as follows: Sulfane sulfur is formed largely in the liver and mostly by the action of mercaptopyruvate sulfurtransferase. The various sulfane forms are interconverted by rhodanese, still principally in the liver. Reduction of the sulfane sulfur to the sulfide level for incorporation into iron-sulfur centers is catalyzed by the thiosulfate reductase present in all tissues. When cyanide is present, it will react rapidly with any or all of the sulfane forms that it encounters. In this view, the question, "Which enzyme is primary in cyanide detoxication?" is simply not very helpful.

All that such a working hypothesis seems to lack is a sulfane carrier, to transport the sulfur from the liver, where it is formed, to the other organs. That carrier is serum albumin, which makes specific stoichiometric complexes with elemental sulfur in much the same way that it does with fatty acids, or bilirubin, or benzodiazepine drugs. It is easy to load serum albumin with sulfur both *in vivo* and *in vitro*. Fig. 5 presents a light-scattering titration curve from one such *in vitro* experi-

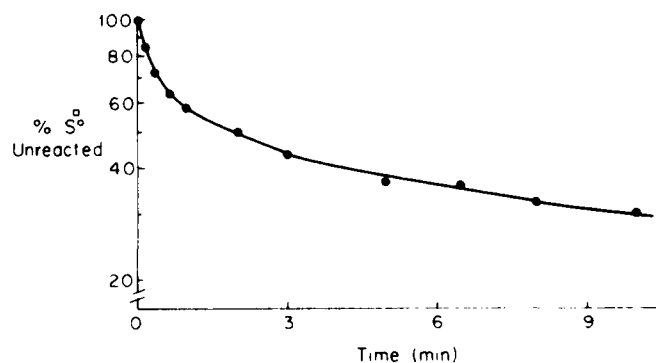


FIG. 6. Cyanolysis time course for sulfur-loaded serum albumin. Crystalline bovine serum albumin loaded with 4.0 S° atoms per molecule (Fig. 5) was incubated with 2 mM CN⁻ in boric acid-NaOH buffer, pH 9.5, ionic strength 0.10, at 25°C . At the indicated times 1-mL samples were analyzed for thiocyanate to determine the extent of reaction.

ment. Elemental sulfur is extremely insoluble in aqueous buffers. Addition of even a very small amount yields instant turbidity; hence, the light-scattering signal. However, in the presence of serum albumin (here at a level approximately one-tenth the concentration in plasma) the appearance of the scattering is delayed until all the sulfur sites on the albumin have been saturated. Very similar results are obtained when this experiment is carried out with whole plasma diluted ten-fold with borate buffer.

With a solution of crystalline bovine serum albumin, whether carefully delipidated or not, the titer represented by the intersection of the extrapolated linear portions of the plot corresponds to 4.0 ± 0.3 sulfur atoms per albumin molecule. When crystalline human serum albumin is used, the titer is 5.8 ± 0.3 sulfur atoms per albumin molecule.

At the present time, it is not entirely clear what these numbers mean, particularly since elemental sulfur is supposed to be largely S_8 . One thing that the numbers correctly suggest, however, is that this is by no means a simple system. This fact is seen also on other experimental approaches. The sulfur bound to albumin is quite reactive with cyanide, so that in fact serum albumin is a rather good catalyst for the cyanolysis of elemental sulfur to thiocyanate (Sörbo, 1955). One can thus study serum albumin by the conventional methods of steady-state enzyme kinetics, but the initial velocity patterns generated when such studies are extended to broad concentration ranges are very difficult to interpret. The reason for this becomes apparent when a simple time course for the cyanolysis of sulfur loaded albumin is determined (Fig. 6). This logarithmic plot would be linear if the sulfur-albumin consisted of a uniform collection of single sulfur atoms at identical binding sites. The fact is, however, that the best fit curve for the overall time course (which approaches 10% left unreacted at 100 min) requires equal contributions from no fewer than four different reactivities, very much as if each of the four sulfane atoms per albumin molecule were bound at a different kind of site.

Fig. 6 presents only the early time course of cyanolysis to stress the fact that at least two of the reactivity classes (sites?) represent reactions with cyanide that could be of toxicologic significance. Very similar cyanolysis time courses are obtained with sulfurated whole plasma diluted to the same albumin concentration in borate buffer. Considering the quantities of serum albumin *in vivo* and the rate constants for cyanolysis at the two most reactive "sites" (both with half times of < 1 min in 2 mM CN⁻ at 25°C) sulfur-albumin could well be the principal natural cyanide detoxication buffer in organisms having undepleted sulfane pools.

One final word about molecular mechanisms: Bovine serum albumin contains a sulfhydryl group that we expected to be involved in either the sulfur binding or the catalysis of the cyanide reaction. It is not. Both the light scattering titration of sulfur-loading capacity and the time course of cyanolysis are unchanged by alkylation of that sulfhydryl group.

ACKNOWLEDGEMENTS

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Cyanide Antagonism^{1,2}

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Development of antidotes to treat cyanide poisoning has been rather successful, as a series of highly efficacious agents are known which can antagonize the lethal effects of cyanide. Antidote(s) which can protect against one or two LD₅₀ doses of a toxicant normally would have important implications; however, this magnitude of protection would be relatively trivial with regard to cyanide because of the effective antagonists available. The history on the treatment of cyanide intoxication has been a paradox. Some of the older cyanide antagonists, *e.g.*, cobalt compounds, advocated almost 90 years ago (Antal, 1894), were discarded because of their toxicity, but now have generated renewed interest. Furthermore, some of the newer cyanide antidotes proposed are not nearly as efficacious as these older antagonists.

Part of the problem resides in the lack of standardization in methods to evaluate the efficacy of an antidote. Laboratories evaluating the efficacy of cyanide antagonists employed different experimental conditions. Some of these studies are conducted where the antidote(s) are administered prophylactically, whereas other studies administer the antidote(s) therapeutically. Various other conditions, such as using different animal species and room temperatures, are known to affect the results obtained. Also, in some instances, the analytical methods employed to determine the concentration of cyanide in biological fluids may be questioned, as the effect of various cyanide antagonists on these determinations were not investigated. Recent reports indicate that some of the procedures developed to measure cyanide in biological fluids in the presence of one or more of the cyanide antagonists were inaccurate (Morgan *et al.*, 1979; Morgan and Way, 1980; Sylvester *et al.*, 1982). This has important implications. A cyanide antagonist, presumed to be highly efficacious because a rapidly falling blood cyanide level was observed after its administration, may, in reality, have had no effect on cyanide disposition, but merely interfered with the method to analyze cyanide. Lastly, some basic classic concepts actually may have delayed some of the approaches to the development of cyanide antagonists. For example, it is well known that cyanide inhibits cytochrome oxidase and thereby inhibits the tissue utilization of oxygen. Therefore, the use of oxygen in cyanide antagonism should serve no useful purpose, as there is an abundance of oxygen already available in cyanide-poisoned animals. The paradox is that oxygen can be a very effective antagonist against the lethal effect of cyanide (Way *et al.*, 1972; Burrows *et al.*, 1973; Isom and Way, 1974).

Table 1 represents the generally reported mechanism(s) of cyanide intoxication and antagonism. Cytochrome oxidase is very sensitive to cyanide, and since this is the terminal oxidative enzyme on the mitochondrial respiratory chain, it occupies a critical biochemical location. The inhibition of this enzyme has been demonstrated *in vitro* and *in vivo* and most biochemical toxicity studies of cyanide have focused on this enzyme. Dr. K.K. Chen proposed the use of amyl and sodium nitrite to oxidize hemoglobin to methemoglobin, as cyanide has a low affinity for hemoglobin, but has a high affinity for methemoglobin (Chen *et al.*, 1933; Chen and Rose, 1952). Therefore, with methemoglobin formation, cyanide can combine with methemoglobin to form cyanmethemoglobin. The second mechanism of antagonizing cyanide is to metabolize cyanide to the less toxic thiocyanate. This is accomplished with sulfur donors in the presence of various sulfurtransferases. The combination of binding cyanide to methemoglobin and chemical conversion of cyanide to a less toxic product forms the basis to antagonize cyanide intoxication. With few exceptions, most of the compounds which presently are discussed either bind cyanide to a larger molecule or biotransform cyanide to a less toxic product.

The classification of cyanide antagonists which can protect against the lethal effects of cyanide are shown in Table 2. The first group are antidotes which are arbitrarily classified as those compounds which bind cyanide. The best known of this group are the methemoglobin formers such as the nitrites, amyl nitrite and sodium nitrite, and, more recently, dimethylaminophenol, DMAP. The other compounds which bind or complex cyanide are the cobalt-containing compounds. Most of the recent investigative efforts have focused on cobalt EDTA and hydroxocobalamin. The third class of compounds which binds cyanide is the carbonyl compounds. These compounds can form cyanohydrin; this is how various aldehydes and ketones exert their protective effects. One example of this type of compound is sodium pyruvate; however, it should be pointed out that there is a large series of compounds in this group. In addition to the compounds that bind cyanide, there are various compounds which metabolize cyanide to the less toxic adduct, thiocyanate. This would include compounds which serve as sulfur donors to rhodanese, *i.e.*, sodium thiosulfate and sodium thiosulfonate. There are various other sul-

TABLE 1
Mechanism of Cyanide Antagonism

CN + Cytochrome Oxidase +	• CN Cytochrome Oxidase
NO ₂ + Hemoglobin +	• Methemoglobin
CN + Methemoglobin +	• CNMethemoglobin
S ₂ O ₃ + CN	• SCN + SO

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TABLE 2
Classes of Cyanide Antagonist

I. Bind Cyanide	
A.	Methemoglobin formers -- nitrites, DMAP
B.	Cobalt compounds -- CoEDTA, hydroxocobalamin
C.	Carbonyl compounds -- pyruvate
II. Sulfur Donors	
A.	Thiosulfate
B.	Thiosulfonate
C.	Other sulfur sulfanes
III. Binding and Sulfur Donor	
A.	Mercaptopyruvate
IV. Unknown Mechanism	
A.	Oxygen
B.	Chlorpromazine

fur donors for other sulfurtransferases and the sulfanes will be discussed by Dr. Westley. There is another class of antidote which may have a combined effect of binding and metabolizing cyanide. These compounds, *i.e.*, mercaptopyruvate, serve as sulfur donors to sulfurtransferases other than rhodanese; this mercaptopyruvate sulfurtransferase is localized in the cytosol as well as the mitochondria. Whether mercaptopyruvate acts *in vivo* by binding cyanide to form the cyanohydrin or serves as a sulfur donor to form thiocyanate is being actively pursued at this time. Finally, we have a fourth category of cyanide antidotes which exert their effect by mechanisms which are still not clear. Compounds in this class would include compounds such as chlorpromazine and oxygen.

The methemoglobin formers, *i.e.*, sodium nitrite and DMAP, will not be discussed here, as Dr. Weger already has covered this topic in detail. The next class of compound which will bind cyanide is the cobalt-containing compounds. Depending on the experimental design employed, it is possible to demonstrate the superiority of one antidote(s) over another. In early studies employing cobalt EDTA, it was reported that cobalt EDTA was more efficacious in comparison to the classic nitrite-thiosulfate combination. These studies were conducted in dogs and the results were expressed in percent of survival rather than employing a dose-response designed experiment (Paulet, 1960). The rationale for the use of cobalt with EDTA is similar to the use of calcium EDTA in heavy metal poisoning. Cobalt EDTA would form a stable complex. Therefore cobalt toxicity should be minimized and, in the presence of cyanide, EDTA would be displaced and a stable cobalt-cyanide complex would be formed. There are some reports which have expressed some reservations with regard to the use of cobalt EDTA. Some emphasis also should be placed on species differences with regard to cobalt sensitivity. In studies conducted with mice and cobalt chloride (Table 3), cobalt alone is quite effective as a cyanide antagonist. When it is combined with sodium nitrite there is an additive effect. Moreover, when cobalt is administered in combination with sodium thiosulfate, a striking potentiation is noted; this combination is superior to the classic nitrite-thiosulfate antidotal combination. No further protection is observed if sodium nitrite is added to the cobalt-thiosulfate combination (Isom and Way, 1972). One would interpret from these data that cobalt and thiosulfate would be a superior combination to the classic nitrite-thiosulfate therapy. However, caution should be expressed in the interpretation of these results, as when similar studies were conducted in

sheep rather than mice the results were quite different. Sheep were unable to tolerate cobalt salts at the dosage regimen employed in mice. When the dose of cobalt chloride was reduced to a dose which would not be lethal to sheep, then cobalt alone was only minimally effective as a cyanide antagonist. Moreover, when cobalt was combined with sodium thiosulfate, it was not as effective as the classic nitrite-thiosulfate combination, and certainly inferior to the oxygen-nitrite-thiosulfate combination (Burrows and Way, 1977). Similarly, if the dose of cobalt chloride employed in mice now were reduced to the dose which is employed in sheep, then cobalt either alone or in various other combinations was not as effective as the nitrite-thiosulfate combination (Burrows and Way, 1979). It is of interest to note that with regard to cobalt as a cyanide antagonist, it was the first antidote proposed to complex cyanide; however, it was discarded because of the toxicity of cobalt (Antal, 1894). Now cobalt in a slightly different form is again proposed as an effective cyanide antagonist, but again there are some reports expressing some reservations with regard to its use because of its cardiac toxicity (Naughton, 1974; Nagler *et al.*, 1978).

Another class of compounds which form an adduct with cyanide to decrease its toxicity are various carbonyl organic compounds, *i.e.*, ketones and aldehydes. There is a series of these compounds which is quite efficacious against cyanide intoxication; however, only sodium pyruvate will be discussed as an example of this broad class (Schwartz *et al.*, 1979). Sodium pyruvate has been reported to be actively transported intracellularly and therefore would distribute to sites of cyanide localization (Halstrap, 1975). As would be expected, sodium pyruvate alone is quite effective as a cyanide antagonist (Table 3). However, sodium pyruvate does not enhance the protective effect of sodium nitrite and provides only a small enhancement to sodium thiosulfate. This latter combination is not nearly as efficacious as the sodium nitrite-sodium thiosulfate combination. However, if pyruvate is added to the nitrite-thiosulfate combination there is again further enhancement of the protective effect which is quite striking, as this antidotal combination is as effective as the nitrite-thiosulfate combination with oxygen. Studies are being continued with this class of compounds, as it provides the potential to manipulate the chemical structures to vary the electron density of the carbonyl group. Hopefully, this approach would enhance cyanohydrin formation and may lead to a more efficacious cyanide antagonist.

The next class of compounds exerts their effect to detoxify cyanide by the biotransformation of cyanide to the less toxic

TABLE 3
A Comparison of the Potency Ratios^a of Newer Cyanide Antagonists on KCN Intoxication

Antagonist	Nitrite			
	Alone	Nitrite	Thiosulfate	Thiosulfate
Control	1.0	1.0	1.0	1.0
Cobalt	2.5	1.5	3.0	1.8
Pyruvate	1.5	1.0	2.0	1.5
Mercaptopyruvate	2.8	2.3	1.2	1.0
Chlorpromazine	1.0	1.0	2.3	1.3
Oxygen	1.0	1.0	1.5	2.0

^aPotency ratio = LD of KCN with newer antagonists/LD₅₀ without newer antagonist

thiocyanate. The use of sodium thiosulfate as a substrate for the sulfurtransferase, rhodanese, was first reported about 50 years ago (Lang, 1933). Subsequently, Sorbo investigated the substrate specificity for this enzyme and various other substrates were developed. Some of the other rhodanese substrates developed were ethyl thiosulfonate and para toluene thiosulfonate (Sorbo, 1953). Both of these compounds serve as sulfur donors and convert cyanide to thiocyanate in the presence of rhodanese more rapidly than sodium thiosulfate. A considerable amount of attention was focused on rhodanese, as the tissue has a high content of this enzyme and this enzyme has a high turnover number. The effects of sodium nitrite and sodium thiosulfate either alone or in various combinations are summarized (Table 3). By combining two cyanide antagonists which exert their effect by different mechanisms of action, it is possible to obtain an efficacious antidotal combination to antagonize cyanide poisoning.

There are other sulfurtransferases besides rhodanese and they do require different substrates. Mercaptopyruvate is another antagonist which serves as a sulfur donor for the conversion of cyanide to thiocyanate. However, the enzyme involved is not rhodanese, which is localized in the mitochondria, but a mercaptopyruvate sulfurtransferase which is localized in the cytosol and mitochondria (Jarabak and Westley, 1980). With regard to mercaptopyruvate as a cyanide antagonist, it is quite effective alone in antagonizing cyanide poisoning. It does not enhance the effect of sodium thiosulfate as well as the nitrite-thiosulfate combination; however, nitrite, when administered in combination with mercaptopyruvate, is as effective as the classic nitrite-thiosulfate antidotal combination. Mercaptopyruvate has the potential of not only acting as a sulfur donor, but also as pyruvate to form a cyanohydrin. Although it appears to be acting primarily as a sulfur donor in exerting its protective effect, the mechanism of action of this antagonist is presently being actively pursued. The last class of cyanide antagonist is that which exerts its effect by mechanism(s) which have not been elucidated. The protective effect of chlorpromazine on cyanide toxicity has been reported (Guth and Spirtes, 1958; Levine and Kline, 1959). The mechanism for the protective effect of chlorpromazine was attributed to its hypothermic action. Under the conditions of our experiment (Way and Burrows, 1976), it was not possible to demonstrate any protective effect of chlorpromazine when used alone in antagonizing cyanide intoxication. Also, when chlorpromazine is combined with sodium nitrite, no apparent added protective effect from chlorpromazine was noted. However, when chlorpromazine is combined with sodium thiosulfate, a striking potentiation occurred, and again when chlorpromazine was administered with the nitrite-thiosulfate combination, the enhanced protective effect of chlorpromazine was quite apparent. Chlorpromazine is a compound with multiple pharmacologic properties and it is difficult to pinpoint the mechanism of this protective effect at this time. However, it is fairly apparent that chlorpromazine is probably not acting by its hypothermic properties. If the potency ratio of chlorpromazine as a cyanide antagonist is compared with the changes in basal body temperature, then chlorpromazine, when used either alone or in combination with sodium nitrite and/or sodium thiosulfate, shows an obvious discrepancy. Where the greatest changes in basal body temperature occurred, such as with chlorpromazine alone or chlorpromazine with sodium nitrite, no protective effect of chlorpromazine was noted. However, when chlorpromazine is employed with sodium thiosulfate either alone or in combination with sodium nitrite, the chlorpromazine induced

hypothermia is much less, but the protective effect against cyanide intoxication shows the greatest enhancement. Also, it should be noted that chlorpromazine does not form methemoglobin nor does it form an adduct with cyanide. Furthermore, chlorpromazine does not enhance the activity of rhodanese nor is it possible for this tranquilizer to serve as a sulfur donor.

The next cyanide antagonist whose mechanism of action has not been clearly elucidated is oxygen. Cyanide is believed to exert its predominant toxic effect by inhibiting cytochrome oxidase, thereby inhibiting the tissue utilization of oxygen. If this were true, then theoretically the administration of more oxygen should serve no useful purpose, as the oxygen which is present is not being utilized. Oxygen alone (Table 3) exerts only a minimal, if any, protective effect. Also, oxygen does not enhance the protective effect of sodium nitrite, but it does enhance the protective effect of sodium thiosulfate. This enhancement is most striking when oxygen is administered in combination with sodium nitrite-sodium thiosulfate (Way *et al.*, 1966; Sheehy and Way, 1968). This protective effect occurs both prophylactically and therapeutically. In the therapeutic-designed experiment, cyanide is administered orally; 60 seconds are allowed to elapse so that the toxic effect of cyanide is quite apparent. The sodium nitrite-sodium thiosulfate antidotal combination is then given intravenously and the animals are placed in an oxygen chamber. Since oxygen can markedly enhance the classic nitrite-thiosulfate combination and can be administered relatively safely, it is presently recommended that the classic sodium nitrite-sodium thiosulfate antidotal combination be given in combination with oxygen. Oxygen under these circumstances is not merely an adjunct therapy, but is an integral part of the antidotal combination to antagonize cyanide poisoning.

These studies conducted with various cyanide antagonists are summarized (Table 3) with respect to their effect alone and in combination with the classic combination of sodium nitrite and/or sodium thiosulfate. First, in experimental conditions where cobalt compounds or mercaptopyruvate are used *alone*, these probably represent some of the most effective newer cyanide antagonists that we have discussed. Under these conditions, sodium nitrite and sodium thiosulfate when used alone, or course, are quite effective. With regard to the various combinations of these antagonists, the most striking combination with sodium nitrite is mercaptopyruvate. Except for cobalt, none of the other newer agents that we have discussed today provided any additional protection with sodium nitrite. Cobalt salts or complexes provided a small, but significant enhanced protection in combination with sodium nitrite. When these newer antagonists are combined with sodium thiosulfate, cobalt salts and chlorpromazine produced the most striking potentiation. As we have indicated earlier, cobalt salts are efficacious with mice, since mice can tolerate a much higher dose of cobalt than other experimental animals. When cobalt salts are employed in a dose which can be tolerated by larger animals, *e.g.*, sheep, then the effectiveness of cobalt is greatly diminished. In fact, no enhancement of the nitrite-thiosulfate combination was noted when a reduced dose of cobalt salts was employed. The effect of cobalt salts in combination with sodium thiosulfate warrants additional consideration, as in proprietary use, cobalt EDTA is used alone. If it were combined with sodium thiosulfate, its ability to antagonize cyanide intoxication would be greatly enhanced. It should be pointed out again that under clinical conditions the use of cobalt EDTA in the treatment of cyanide intoxication can produce rather severe toxic effects on the heart. Last, the effect of these

newer antagonists, when they are administered in combination with sodium nitrite and sodium thiosulfate, are summarized (Table 3). Under these situations the most prominent enhancement observed was either with cobalt salts or oxygen and to a slightly lesser extent with sodium pyruvate. It should be noted that sodium pyruvate also prevented the convulsive seizures frequently observed with some cyanide poisoning.

In summary, there are a variety of effective cyanide antagonists. None of these antidotes are as effective alone as when they are employed in combination with other cyanide antagonists.

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Treatment of Cyanide Poisoning with 4-Dimethylaminophenol (DMAP) — Experimental and Clinical Overview

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INTRODUCTION

In August 1980, 303 pilgrims died in Riad, Saudi Arabia because one of them tried to prepare tea using a gas- or fuel-cooker inside of the airplane. The flames of the open fire contacted the plastic equipment nearby causing a smouldering combustion (almost all plastics contain nitrogen). As a result of the fire, the nitrogen within the plastic was set free as HCN.

A similar case occurred in Paris, in 1973, when, during an emergency landing, the crew of a Boeing 707 announced a fire on board. The reason for the fire was a burning cigarette left behind in the lavatory. First, the toilet began burning, then the plastic material next to it. The cyanide which was set free killed all 119 passengers. The crew in the cockpit, who had their own fresh air circulation was spared (Mohler, 1975).

In many cases, short circuits in electrical wires also led to combustion on ships, the plastic material of which was smouldering, producing cyanide and, consequently, causing cyanide poisonings (Levine *et al.*, 1978). In 1978, in an old-peoples'-home in Munich, many elderly people, as well as two firemen, died due to cyanide poisoning. Even though the firemen were wearing gas masks, no protection was afforded against cyanide (Daunderer, 1979). Various homicides were performed with cyanide and, in a few cases, cyanide became the background for the so called "perfect" murder. The murders of two Ukrainians, Mr. Rebet (1957) and Mr. Bandera (1959) in Munich would not have aroused the public had not the murderer Stachinskij, a Soviet agent, fled to the Federal Republic of Germany, where he reported having used a gas pistol containing cyanide to kill them (Anders, 1963).

In 1978, 912 members of a religious sect in Guyana died as a result of cyanide mixed into their orange juice. In this case of mass cyanide poisoning, theoretically only a very rapidly acting antidote could have helped to save their lives.

In September 1982, seven people in Chicago died as a result of potassium cyanide which was filled into capsules with Tylenol, a pain reliever. Cyanide has also been suggested as a possible chemical agent in chemical warfare. Due to its rapid vaporization at about 25°C, its use is considered for a surprise attack. It permits the attacker to move quickly, with only minor precautions, across the contaminated area. The aggressor is impeded by cyanide for a maximum period of only about 30 min (Weger, 1981).

REACTION MECHANISM OF CYANIDE POISONING

One of the first symptoms of cyanide poisoning is a deep and rapid hyperventilation caused by stimulation of the carotid sinus (Heymans *et al.*, 1931). Cyanide reacts with the enzyme cytochrome oxidase and thereby inhibits the biological respira-

tion of the cell, especially the cells of the central nervous system. At first, low concentrations of cyanide cause headache, nausea, and vomiting, then, depending on the dose, cramps with opisthotonus, and finally respiratory arrest. Four or 5 minutes later the heart beat ceases. The time of contamination with cyanide and respiratory arrest depends on the dose of cyanide. After inhalation of very high concentrations of cyanide, respiratory arrest occurs in a few seconds to a few minutes, while in lower doses of cyanide, respiratory arrest may not occur until several hours have elapsed. After inhalation, oral or percutaneous absorption of cyanide, the cyanide ion reacts with the Fe²⁺ of the cytochrome oxidase inhibiting the biological oxidation in the cell (Warburg, 1924) (Fig. 1). Therefore, the oxygen cannot react with the hydrogen to form H₂O in the cell and remains in the venous blood. This is why people poisoned with cyanide exhibit red faces and red skin. The concentration of hydrogen emerging from the metabolic pathway causes an acidosis in the cells. This acidosis depends, of course, on the time lapse and the blocking of the cytochrome oxidase by cyanide. This reaction mechanism of blocking the cytochrome oxidase by cyanide follows an S-curve. Therefore,

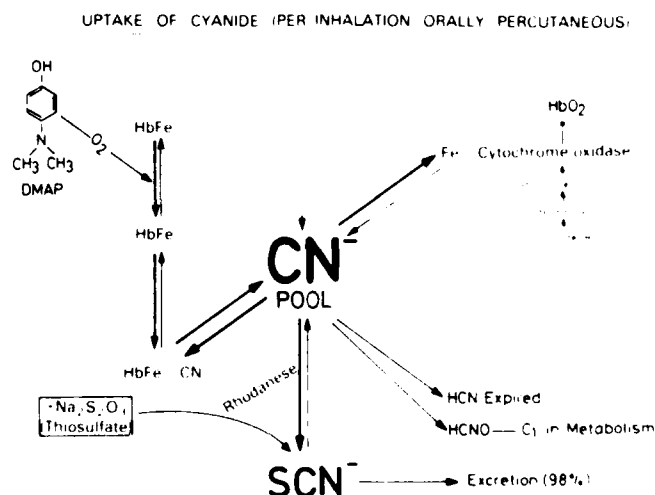


FIG. 1 Overview of the effects in cyanide poisoning and therapy. The Warburg flow is inhibited by CN⁻ due to its reaction with Fe²⁺ of the cytochrome oxidase. Ferrihemoglobin, which is produced in combination with 4 dimethylaminophenol (4 DMAP) under the influence of oxygen by partial oxidation to hemoglobin has a higher affinity to CN⁻ by forming a HbFe²⁺-CN complex in the blood. Rhodanese catalyzes the formation of thiocyanate (SCN⁻). Thiosulfate is reactivating this reaction (98% of the cyanide uptake was excreted as thiocyanate salts. Only a small portion is eliminated by excretion and metabolized by the C₁ metabolism process.

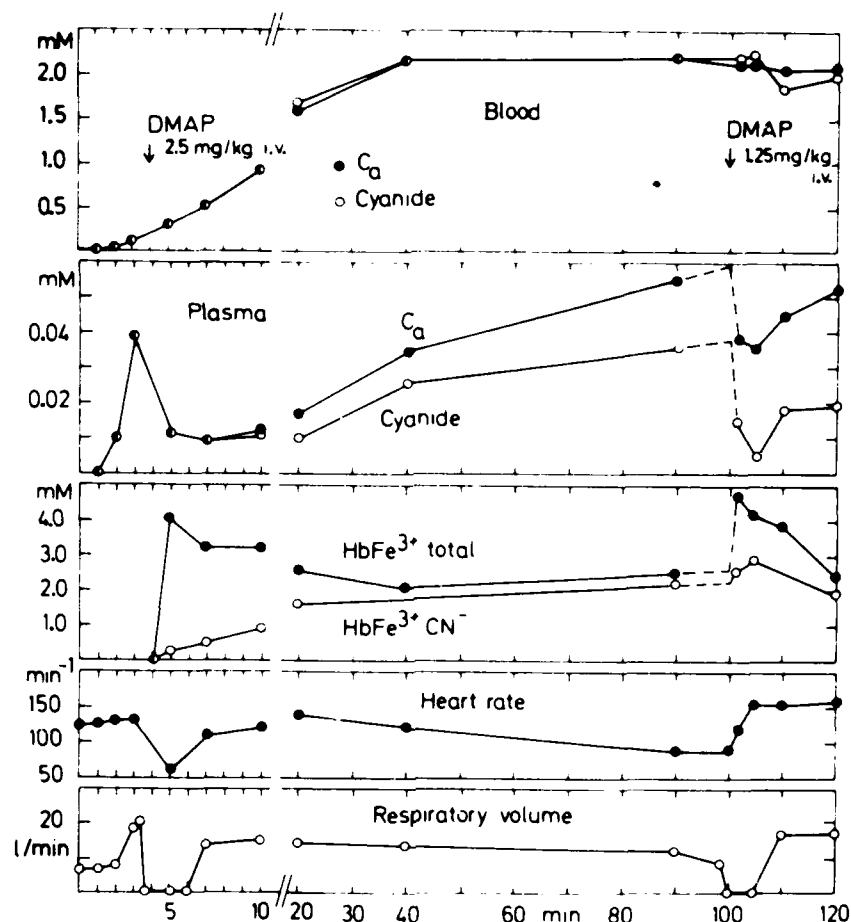


FIG. 2. Cyanide and cyanide metabolites in the blood of a dog and the effect of 4-dimethylaminophenol. Dog 10.5 kg received 3.87 mMoles KCN (0.37 mMoles or 24 mg/kg) through stomach tube after 2 mMoles HCl. Initial hemoglobin content of the blood 9.0 mM iron. C_a is the molarity of all radioactive compounds calculated on the assumption that one mole of cyanide yields one mole of metabolite.

the cytochrome oxidase in the brain shows a relatively long-term activity. Thus, in experiments of cyanide poisoning in humans, no symptom of damage in the central nervous system was noted, provided the treatment was started before the heart stopped.

In the study of pharmacokinetics of cyanide poisoning with C^{11} labeled cyanide, the concentration of cyanide in the blood increased relatively slowly compared to the plasma, where the concentration of cyanide increased relatively rapidly. Within 3 minutes the concentration of cyanide in plasma reached 40 μ M/mL (Fig. 2). Upon reaching this concentration, respiration ceases (Fig. 2). In this experiment on dogs, DMAP 2.5 mg/kg was given intravenously one minute after respiratory arrest (Fig. 2). The quick formation of ferrihemoglobin by i.v. injection of DMAP, after plasma cyanide had risen to or above 40 μ M, decreased the cyanide concentration in plasma and restored respiration while cyanide was accumulated in red cells by formation of ferrihemoglobin cyanide. These experiments in dogs show that respiration is arrested by a concentration of 40 μ M cyanide/mL in the plasma. If the concentration falls below 40 μ M cyanide/mL, respiration occurs spontaneously without any artificial respiration. In most cases, if the concentration of cyanide in the plasma was nearly twice as much (70 μ M/mL) the heart rate ceased about 5 min after respiratory arrest (Christel *et al.*, 1977).

In order to determine more accurately the concentration of cyanide in plasma which causes respiratory arrest, cyanide was intravenously infused at rates which caused a slow increase in cyanide in plasma. Figure 3 presents the results of such an experiment. Intravenous infusion of KCN, 0.07 mg/kg/min, caused a slow increase in cyanide in plasma and an increase in respiration rate and respiratory volume. Forty minutes after the start of the infusion, when the cyanide concentration in the plasma had risen beyond 30 μ M, respiration decreased and, with 40 μ M cyanide in the plasma, respiration suddenly ceased. In this experiment an intravenous dose of sodium thiosulfate, 500 mg/kg, sufficed to bring plasma cyanide down below 30 μ M in 3 min with prompt return of respiration, although the slow infusion of cyanide was continued. The heart rate decreased during respiratory arrest. The EKG did not indicate any effect of the lethal cyanide concentration on cardiac function.

The effect of $Na_2S_2O_4$ on the biotransformation of cyanide was also investigated in these experiments. Figure 4 shows that a 13-fold increase in the rate of biotransformation by $Na_2S_2O_4$ was calculated.

REACTION MECHANISM OF TREATMENT

Efforts to find a treatment for persons suffering from cyanide poisoning date back many years. Various methods have been

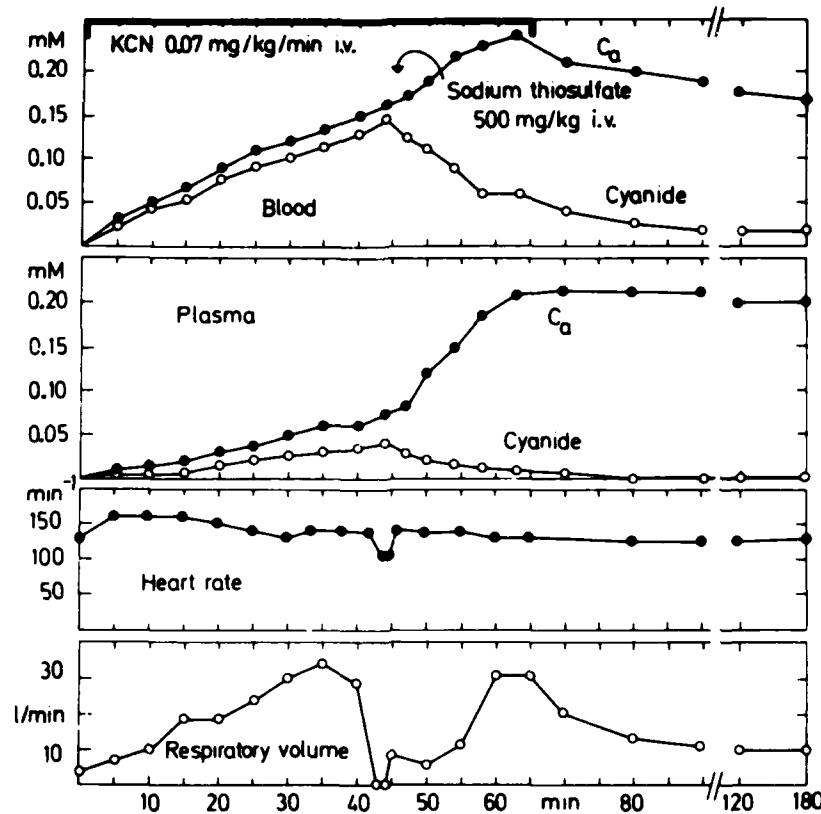


FIG. 3. Cyanide and cyanide metabolites in blood of a dog during intravenous infusion of cyanide, $1.08 \mu\text{M}$ or 0.07 mg/kg/min . Dog 16.5 kg. For C_a see Fig. 2.

considered based on the knowledge in chemistry and biochemistry of cyanide poisoning. The following detoxification mechanisms were tested *in vivo*:

Injections of thiosulfate to accelerate the transformation of HCN to HSCN: S. Lang (1895); Chen *et al.* (1933b).

Thiosulfate with Rhodanese: Clemenson *et al.* (1954).

Formation of ferrihemoglobin in combination with methylene blue, sodium nitrite: Hug (1932, 1933a, 1933b).

Sodium nitrite, amyl nitrite: Chen *et al.* (1933a, 1933b); Hug (1932, 1933a, 1933b).

p-Aminopropionphenone: Jandorf and Bodansky (1946).

Aminophenols: Kiese *et al.* (1966); Kiese and Weger (1965a, 1965b, 1966).

4-Dimethylaminophenol-HCl (DMAP): Weger (1968).

Injection of cobalt compounds in combination with HCN.

Hydroxocobalamin: Mushett *et al.* (1952).

Cobalt EDTA: Paulet (1957, 1958).

Cobalt histidine.

Albaum *et al.* (1946), as well as Schubert and Brill (1968), have shown that cytochrome oxidase *in vitro* inhibited by hydrocyanic acid could be reactivated with ferrihemoglobin. The team of Paulet (Paulet, 1957, 1958) and Lendle (Friedberg *et al.*, 1965) has shown in their publications that in cases of cyanide poisoning treated with nitrite, the velocity of ferrihemoglobin formation was too slow. In poisonings where high doses of cyanide are taken up rapidly, a rapid removal of cyanide from cytochrome oxidase is the major goal for this treatment. In investigations of the ferrihemoglobin forming

properties of various aminophenols it was found that several of them can rapidly form limited amounts of ferrihemoglobin.

The o-aminophenol was studied in various animals because of its exceptionally rapid reaction with hemoglobin and its low toxicity. In the treatment of cyanide poisoning in mice and dogs,

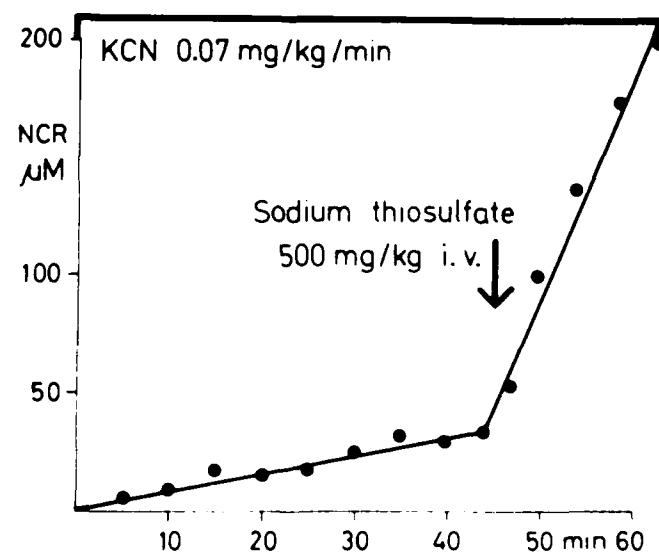


FIG. 4. Effect of thiosulfate on the rate of metabolite formation from ^{14}C -labeled cyanide indicated by the concentration of non-cyanide radioactivity in blood plasma. Dog 16.5 kg. Intravenous infusion of KCN, 0.07 mg/kg/min . NCR = molarity of non-cyanide radioactive compounds calculated on the assumption that one mole of cyanide yields one mole of metabolite.

o-aminophenol was superior to sodium nitrite compared with different doses of cyanide (Kiese and Weger, 1966, 1969).

In order to test the effects of various HCN antidotes on the circulatory system, the blood flow and blood pressure in various vessels, and the respiration were measured on cats in light anesthesia (Kiese *et al.*, 1968; Kiese and Weger, 1969).

The i.v. injection of 4 mg KCN/kg proved in all experiments without antidotes to be immediately fatal. The breathing stopped about 4 min after HCN. Blood pressure and pulse frequency dropped rapidly with respiratory arrest. The heart rate stopped at the latest after 11 min. The effects of NaNO_2 and DMAP were compared after poisoning with 8 mg KCN/kg. The antidotes were injected immediately after the cyanide injection. The doses were chosen to produce 30% of ferrihemoglobin.

Figure 5 shows that nitrite was not capable of stopping the total cessation of breathing. Blood flow, blood pressure and pulse dropped to zero after breathing had stopped. After injection

of DMAP the frequency of respiration increased very rapidly. The blood flow in the carotid artery rose to 200% of the original value (OV). In the femoral artery, the blood pressure and pulse remained at first slightly reduced and, after 10 min, returned to normal.

Experiments with several aminophenols showed that 4-dimethylaminophenol and 4-methylaminophenol both rapidly produced limited amounts of ferrihemoglobin in the blood of various species of animals *in vitro* and *in vivo*. The rapid reaction of 4-DMAP was also observed in humans after i.v. injection. Doses which oxidize 30-40% of hemoglobin produce the half maximum of ferrihemoglobin concentration in 1 minute without an immediate effect on the cardiovascular system. Sodium nitrite in doses of 4 mg/kg, recommended for the treatment of cyanide poisoning, was found to oxidize slowly about 7% of the hemoglobin producing half the maximum amount of ferrihemoglobin within 10 min, i.e. 3.5% of the total

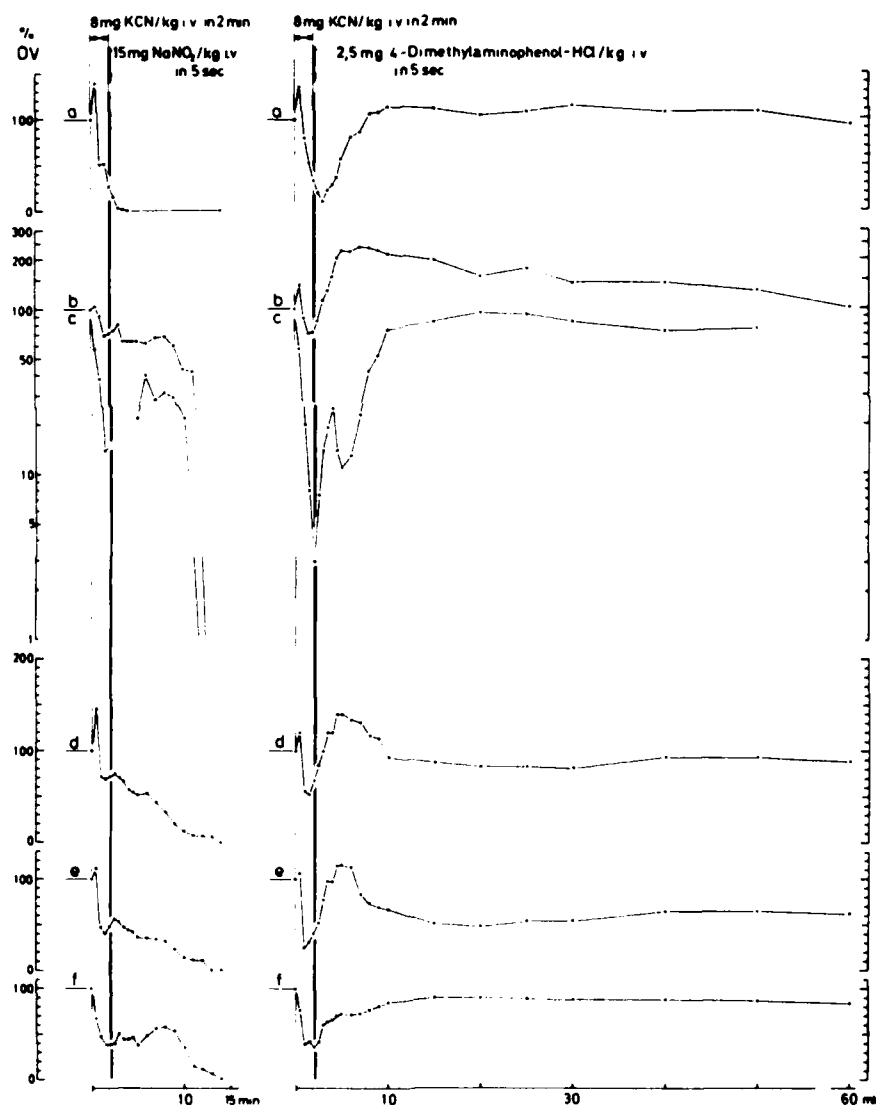


FIG. 5. Effects of 15 mg NaNO_2 /kg and 2.5 mg DMAP/kg on cats infused with 8 mg KCN/kg over a period of 2 min. The antidote was injected immediately afterwards, within 5 sec. The changes in the individual curves are shown in percentages of the original value (OV). Because of the great changes, logarithmic ordinates were used for the blood flow. These are the results of 8 experiments: a) respiratory frequency; b) blood flow in the carotid and c) femoral artery; d) systolic and e) diastolic blood pressure; f) pulse frequency.

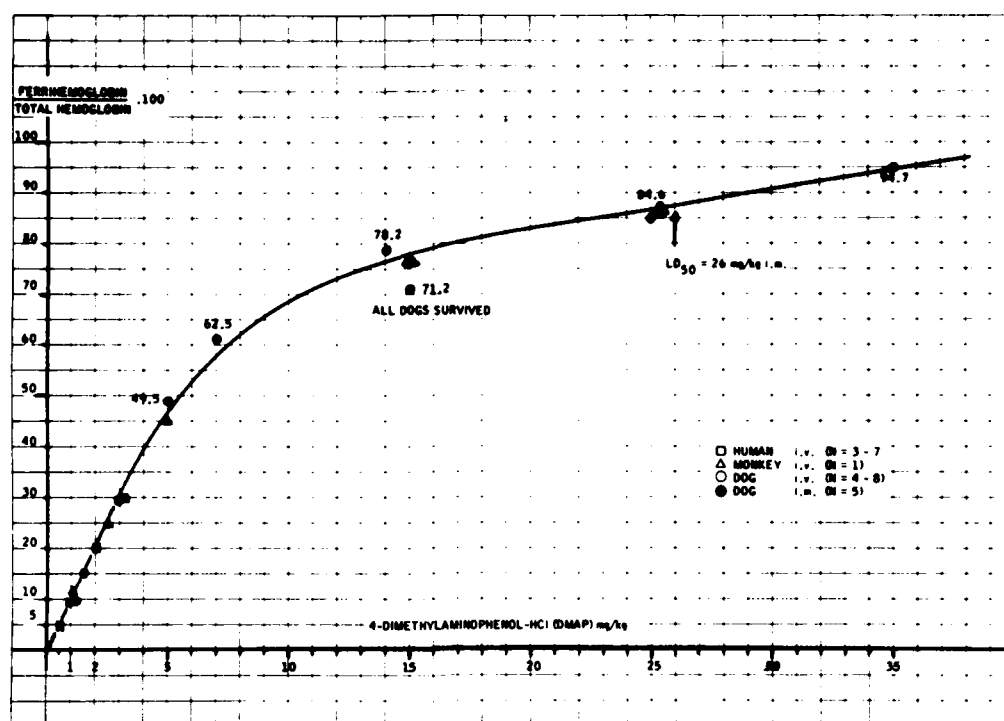


FIG. 6. Maximal ferrihemoglobin concentration in human ($\square$), monkey (Δ), and beagle dogs ($\circ$) after i.v. and i.m. injection of various doses of 4-DMAP. The LD_{50} in dogs was found at 26 mg 4-DMAP/kg, a dose which oxidizes 85% of hemoglobin to ferrihemoglobin.

hemoglobin. Nitrite caused a decrease in arterial blood pressure in all experiments and the orthostatic collapse of several test persons (Kiese and Weger, 1969).

Cyanide is bound to ferrihemoglobin in a complex form and, because of the higher affinity to ferrihemoglobin, cyanide is set free from the cytochrome oxidase.

REACTIONS OF 4-DIMETHYLAMINOPHENOL WITH HEMOGLOBIN, AND AUTOXIDATION OF DMAP

The reactions between 4-dimethylaminophenol and hemoglobin were studied with DMAP ^{14}C -labeled either in the methyl groups or in the ring (Eyer *et al.*, 1974). In the absence of oxygen, DMAP was stable in red cell suspensions or hemoglobin solutions. In the presence of oxygen, oxyhemoglobin rapidly oxidized 4-dimethylaminophenol. The following reaction products were found in incubates of 4-dimethylaminophenol with red cells or hemoglobin: ferrihemoglobin, formaldehyde, dimethylamine, and hemoglobin with derivatives of DMAP covalently bound to its protein moiety.

DMAP catalytically transferred electrons from ferrohemoglobin to oxygen. It was oxidized by oxyhemoglobin, and oxidized DMAP was reduced to DMAP by ferrohemoglobin with formation of ferrihemoglobin. Hydrolysis of oxidized DMAP, N,N-dimethylquinonimine, and its covalent binding to globin limited the catalytic ferrihemoglobin formation by DMAP to an average between 50 and 100 electron transfers per molecule of DMAP, when DMAP concentration was low and hemoglobin concentration was high. Since DMAP reduced ferrihemoglobin to ferrohemoglobin, though more slowly than the catalytic cycle produced it, the increase in ferrihemoglobin content did not indicate the amount of ferrihemoglobin produced.

In red cell suspensions at 37°C DMAP, 0.58 mmol/L disappeared in 10 min, but dimethylamine continued to be formed, obviously from protein-bound derivative(s) of DMAP.

The rate of autooxidation of DMAP was found to be much lower than the oxidation of DMAP by oxyhemoglobin. After autooxidation of DMAP several products were isolated and identified namely hydroquinone, 4-methylaminophenol, 4-aminophenol, 2-dimethylamino-1,4-benzoquinone, and a purple and a yellow dye identified as an epoxide of 2-(N-methyl-N-(p-hydroxyphenyl)-amino-benzoquinone).

BIOTRANSFORMATION OF DMAP: REACTION WITH GLUTATHIONE, AND SOME PROPERTIES OF THE REACTION PRODUCTS

DMAP forms ferrihemoglobin by catalytic transfer of electrons from ferrohemoglobin to oxygen. In solutions of purified human hemoglobin, quick binding of oxidized DMAP to the globin moiety of hemoglobin terminates this reaction (Eyer and Kiese, 1976). Reduced glutathione in high concentrations, as in the red cell, substantially diminished binding of oxidized DMAP to hemoglobin by formation of S,S,S-(2-dimethylamino-5-hydroxy-1,3,4-phenylene)-tris-glutathione (tris-(GS)-DMAP), which does not form ferrihemoglobin. In the presence of reduced glutathione, DMAP disappeared more rapidly from hemoglobin solutions than in its absence. The formation of tris(GS)-DMAP in red cells was found to be of importance for the termination of catalytic ferrihemoglobin formation by DMAP *in vivo*.

With low concentrations of GSH, DMAP in hemoglobin solutions formed another conjugate, bis(GS)-DMAP, S,S,-(2-dimethylamino-5-hydroxy-1,3-phenylene)-bis-glutathione. Similar to DMAP, bis(GS)-DMAP catalyzed the formation of ferrihemoglobin. As the oxidized bis(GS)-DMAP was bound to hemoglobin more slowly and to a lesser extent, it produced more ferrihemoglobin than DMAP. In contrast to the reactions of DMAP with hemoglobin, hydrogen peroxide and superoxide radicals are involved in the ferrihemoglobin formation by

TABLE 1
An Overview of the Medical Therapy of 15 Cases of Cyanide Poisoning

Case No.	Poison	Way of Poison	Min. 1st Sympt.	Min. DMAP Ther.	Min. Uncont.	DMAP Given by	Surv./Death	Sex	Age
1	KCN	Oral	1	15	30	Tox Center	Surv.	Male	27
2	KCN	Oral	20	300	20	Tox Center	Surv.	Male	43
6	KCN	Oral	1	87	-	Helicopter crew	Surv.	Female	26
8	KCN	Oral	3	80	3	Tox Center	Death	Male	25
10	KCN	Oral	1	15	-	Tox Center	Surv.	Male	27
11	> 35 Bitter almonds	Oral	1	500	-	Helicopter crew	Surv.	Female	30
12	KCN	Oral	1	60	20	Tox Center	Surv.	Male	66
13	> 35 Bitter almonds	Oral	60	120	-	Tox Center	Surv.	Female	38
14	KCN	Oral	1	90	15	Hospital	Surv.	Male	28
15	KCN	Oral	1	15	5-10	Hospital	Surv.	Male	19
3	Galy. bath	Cutan.	10	12	-	Emergency phys.	Surv.	Male	29
5	Galy. bath	Cutan.	1	120	-	Tox Center	Surv.	Male	19
7	Galy. bath	Cutan. + oral	60	120	60	Emergency phys.	Surv.	Male	19
4	HCN-gas	Inhal.	1	55	-	Tox Center	Surv.	Female	20
9	Benzyl-Cyanide	Inhal.	60	315	-	Tox Center	Surv.	Female	50

bis(GS)-DMAP. The radicals accelerate the oxidation of bis(GS)-DMAP and thereby ferrihemoglobin formation.

CIRCULATION, RESPIRATION, AND BLOOD HOMEOSTASIS IN CYANIDE-POISONED DOGS AFTER TREATMENT WITH 4-DIMETHYLAMINOPHENOL OR COBALT COMPOUNDS

The effects of intravenously injected 4-dimethylaminophenol-HCl (DMAP), Co₂EDTA, and Co(histidine)₂ on the survival rate and several physiological parameters were studied on dogs after acute intravenous poisoning with a double lethal dose of potassium cyanide (Klimmek *et al.*, 1979).

All dogs survived when the antidotes were administered 1 min after poisoning. When the therapy began 4 min after poisoning more dogs were rescued in the DMAP group than in the cobalt groups. DMAP, Co₂EDTA, and Co(histidine)₂ restored circulation and respiration of the surviving animals in a similar manner. The increase in the plasma concentrations of glucose and lactate was much higher in the Co₂EDTA group than in the DMAP group. The injection of Co₂EDTA produced a sharp rise in the lactate/pyruvate ratio. The lactate/pyruvate ratio stayed unchanged 15 min after injection of DMAP before rising. The total dose of KCN (4 mg/kg) was bound to the ferrihemoglobin formed by DMAP. The arterial pO₂ increase, caused by liberation of oxygen from oxyhemoglobin during the formation of ferrihemoglobin, was less when cyanide was given before DMAP.

Also the effects of DMAP and 100% oxygen on cerebral blood flow (CBF), peripheral circulation, arterial and venous blood gases, and other parameters have been investigated in dogs in the course of slow cyanide infusion (Klimmek *et al.*, 1982).

The i.v. infusion of KCN increased the respiratory minute volume, accompanied by a rise in arterial pO₂, pH and a decrease in arterial pCO₂, while the venous lactate concentra-

tion increased by about 500% and the hemoglobin content and hematocrit by about 30%. Heart rate and carotid artery blood flow decreased. Local CBF in the cingulum as measured with thermocouples rose steadily, and the brain and esophagus temperature were lowered. The breathing of 100% oxygen raised the local CBF, the temperature, and the arterial pCO₂.

During the infusion of KCN into the femoral artery of artificially ventilated dogs the femoral venous pO₂ increased continuously by some 40 mm Hg, attended with a decrease in pCO₂ of 15 mm Hg. The femoral blood flow, however, rose sharply within 3 min. 100% oxygen induced a rise in pCO₂ and a diminution of pH in the femoral vein and in the sinus sagittalis, and the femoral flow rose rapidly. After DMAP i.v. the values of most of the parameters returned to normal or finally stabilized below or above the initial level. The rise in the hemoglobin content, hematocrit, and lactate concentration was stopped, but the arterial and venous pH remained or were lowered. DMAP elicited a rapid, strong decrease in the pO₂ of the femoral vein and the sinus sagittalis with a concomitant marked increase in pCO₂.

BIOTRANSFORMATION OF DMAP IN THE DOG

After an i.v. injection, DMAP quickly forms ferrihemoglobin by catalytic transfer of electrons from ferrohemoglobin to oxygen. This reaction is rapidly terminated by covalent binding of oxidized DMAP to the reactive SH-groups of hemoglobin and to reduced glutathione within the red cells, and by conjugation with glucuronic or sulfuric acid presumably in the liver. Fifteen min after i.v. injection of DMAP, 3.25 mg/kg, ¹⁴C-labeled in the ring, no intact DMAP was detected in the blood. The concentrations of metabolites in the blood were as follows: 33 μM DMAP covalently bound to hemoglobin, 30 μM S.S.S-(2-dimethylamino-5-hydroxy-1,3,4-phenylene)-Tris-glutathione (Tris-(GS)-DMAP) 90% of it located within the red cells, 5 μM DMAP-glucuronide, and 22 μM DMAP-sulfate. Within 3 days,

90% of the radioactivity was excreted in the urine, 4% in the feces. In the 24 hr urine, 25% of the DMAP injected was excreted as DMAP-sulfate, 15% as DMAP-glucuronide, and 23% as DMAP-thioethers, mainly as S,S,S-(dimethylamino-5-hydroxy-1,3,4-phenylene)-Tris-cysteine. When DMAP, ^{14}C -labeled in the methyl groups, was administered, 11% of the radioactivity was excreted in the urine as dimethylamine. It is concluded that most of the thioethers found in the urine derived from Tris-(GS)-DMAP which had been produced within the red cells indicating an important role of the red cells on biotransformation of DMAP (Eyer and Gaber, 1978).

TOXICITY OF DMAP

The effects of i.v.-injected DMAP on cerebral blood flow, brain temperature, blood gases, and lactate concentration in the sinus sagittalis blood were measured in male beagle dogs anesthetized with chloralose (Klimmek *et al.*, 1981).

An increase in cerebral blood flow became measurable when 5% or more of the hemoglobin was oxidized to ferrihemoglobin. The local cerebral blood flow of the cingulum region and the flow in the sinus sagittalis increased, while the sinus pO_2 decreased. An increase in the ferrihemoglobin content of some 20% of the total hemoglobin at a constant arterial pO_2 and pCO_2 was attended with a decrease in the sinus pO_2 of about 10 mm Hg when less than 40% of the heme iron was oxidized. The sinus pO_2 approached a threshold value of some 8 mm Hg when the ferrihemoglobin content was increased above 40%. The lactate concentration began to rise very rapidly when 40-50% of the hemoglobin was oxidized. At the same time pCO_2 increased and pH decreased in the sinus blood, the brain temperature remained unchanged. The behavior of conscious dogs with a ferrihemoglobin content of 40% of the total hemoglobin showed no abnormalities in muscular coordination or general behavior.

In cooperation with the U.S. Biomedical Laboratory in Edgewood, Maryland, it could be shown that the dose-lethality curve of DMAP corresponded well with the concentration-

lethality curve of ferrihemoglobin in man, dog, and monkey (Fig. 6). The LD_{50} of DMAP in beagle dogs was found to be 26 mg/kg, which oxidized 85% of hemoglobin. The dogs died if more than 85% of ferrihemoglobin was formed. Below 80% ferrihemoglobin, all dogs survived. Therefore, the LD_{50} of DMAP was due to methemoglobinemia. This data corresponds to the results of Bodansky (1951). Restlessness, ataxia, hyperventilation, prone to defecate, and urination occurred in dogs when more than 60% of hemoglobin was oxidized. The same was also observed in humans treated with twice the therapeutic dose of DMAP. With 6.5 mg/kg about 60% of ferrihemoglobin was measured. Dogs and humans recovered completely.

In order to investigate the preliminary toxic effects of DMAP, different studies *in vivo* and *in vitro* were performed. In rats, a dose of DMAP (20 mg/kg i.v.) oxidized as much as 50% of the hemoglobin to ferrihemoglobin, but did not cause kidney lesions (Kiese *et al.*, 1975). In highly toxic doses only, *e.g.* twice the LD_{50} , DMAP produced tubular necrosis. These results led us to more detailed studies on the mechanism of tubular necrosis produced by DMAP.

Isolated rat kidneys were perfused (single pass) with 4 to 40 μM solution of 4-dimethylamino-(^{14}C)phenol (DMAP) (Elbers *et al.*, 1980a). Nephrotoxicity was not detected at concentrations of ^{14}C -DMAP up to 18 μM ; higher concentrations resulted in irreversible loss of physiological functions with simultaneous five-fold increase in covalent bound ^{14}C .

At all concentrations, 85% of the post-renal ^{14}C was due to unchanged DMAP, while 15% corresponded to DMAP conjugates. These conjugates were identified as DMAP-glucuronide (90% total) and DMAP-thioethers. All DMAP conjugates were concentrated in the urine with urine perfusate concentration ratios in the range 5-7 for the glucuronide, 50-100 for the sulphate, and 10-20 for the thioethers.

Also, isolated rat livers were perfused with various concentrations of 4-dimethylaminophenol-(^{14}C) (DMAP). During single-pass perfusion with modified protein-free Krebs-Henseleit solution up to 70 μM prehepatic DMAP were metabolized by the liver (Eyer and Kampffmeyer, 1978). The main route of biotransformation was conjugation. Thus, at low substrate concentrations, the sulfate conjugation exceeded glucuronidation, whereas at high substrate concentration the ratio of conjugates was reversed. In contrast to DMAP-sulfate, some DMAP-glucuronide was stored by the liver and was released with a half-life of about 15 min which showed positive correlation with the dose of DMAP during the washout period. The results obtained from the isolated metabolic system resemble the hepatic part of the overall metabolism under *in vivo* conditions.

Addition of 4-dimethylaminophenol (DMAP) to suspensions of isolated rat kidney tubules increased extracellular lactate dehydrogenase (LDH) at concentrations which did not markedly affect gluconeogenesis. ATP content was also decreased by DMAP but this did not occur until the membrane became permeable to LDH. There was no similar leakage of the mitochondrial enzyme glutamate dehydrogenase (Sznicz *et al.*, 1979; Sznicz and Weger, 1980). After i.v. injection of DMAP to rats in doses which did not inhibit gluconeogenesis, kidney glutathione was decreased and the urinary LDH was increased. DMAP was irreversibly bound to tissue in rat, with the highest binding in the kidney. The highest binding occurs in those tissues in which DMAP causes necrosis. In isolated rat hepatocytes, DMAP caused toxic effects which were similar but less extensive than occur on addition of DMAP to kidney tubules. The formation of acid-soluble metabolites was higher in iso-

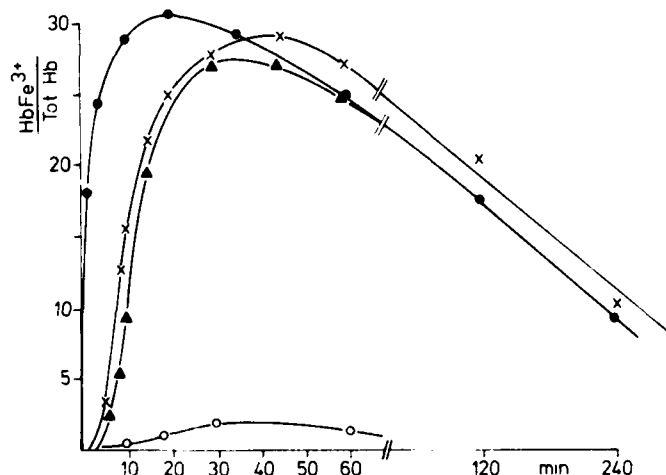


FIG. 7. Comparison of the kinetics of ferrihemoglobin formation in man at DMAP dosage which oxidizes 30% hemoglobin after (●) i.v., (×) i.m., and (▲) oral application. After i.v. injection of 3.25 mg DMAP/kg a half maximal ferrihemoglobin concentration was reached within about 1 min; after i.m. injection within about 7 min; after oral application within about 10 min. 10 ampules of amylnitrite (0.33 mL each) broken inside the protection mask produced 2% of ferrihemoglobin after 30 min.

lated hepatocytes (20 nmol/mg protein) than in rat kidney tubules (4 nmol/mg protein). DMAP-glucuronide and DMAP-sulphate comprised the major acid-soluble metabolites in both preparations; conjugates of DMAP with glutathione or cysteine were also found.

To evaluate the influence of DMAP on cellular intermediary metabolism, isolated rat livers were single-pass perfused with subtoxic (0.3 mM) and toxic (1 mM) concentrations of DMAP. The rate of glycolysis and oxygen consumption both increased with biphasical kinetics immediately after the onset of DMAP infusion (Elbers *et al.*, 1980). After a transient reduction, the cytosol NAD system was oxidized by DMAP; the mitochondrial NAD system, except for a brief initial oxidation, remained almost unaffected. DMAP caused an intracellular alkalinization. At 0.3 mM, this alkalinization was confined to the cytosol; at 1 mM the mitochondria were alkalinized. The cellular content of CoA was unchanged at 0.3 mM but diminished by 65% at 1 mM, in accordance with the unchanged rate of ketogenesis at 0.3 mM and inhibition at 1 mM.

The chronic toxicity of DMAP was studied on beagle dogs in a 16-week period (4 male, 4 female, and 2 controls). Three milligrams of DMAP/kg were injected twice a week oxidizing 35% of hemoglobin but causing neither gross nor microscopical changes on lung, liver, kidney, heart, spleen, pancreas, salivary gland, testicles, ovaries as well as on blood.

Clinical cases

More than 20 cyanide poisonings were successfully treated with DMAP and thiosulfate in the Toxicological Dept. of the II. Medical Clinic Center, Technical University Munich.

Table 1 shows an overview of 15 cases of cyanide poisoning that were treated during a one-year period in Munich. The time course between contact of poisoning and DMAP therapy is in contrast to the general opinion that cyanide is fatal within a few seconds. Therapy with DMAP and thiosulfate was successful, if the poisoned person was still breathing, or at the latest 5 min after respiratory arrest, that is, prior to cardiac standstill, and also in some cases several hours after contact with the poison (Daunderer, 1981).

The following describes the course of a typical case of CN-poisoning which was treated with DMAP for the first time; however CoEDTA was also used in this treatment (Daunderer *et al.*, 1974). A 26-year-old female patient was brought into the hospital 15 min after oral intake of about 10 g of potassium cyanide. According to the criminal investigation, the amount of poison vomited was minor. The patient was brought in unconscious, she was still breathing spontaneously, however unregularly. Her body was pale red, lips and extremities were partly cyanotic. Until arrival of the antidotes, a stomach cleansing was carried out, whereby a heavy meal, smelling intensely of bitter almonds, was discharged. The patient was oxygenated. Only 45 min after oral intake of the poison, it is possible to apply the antidotes 4-dimethylaminophenol-HCl (250 mg) and 1 amp. of Kelocyanor® (300 mg CoEDTA). In the 70th minute after intake of the poison and about 25 min after i.v. application of 4-DMAP and Kelocyanor, sodium-thiosulfate was injected intravenously. The patient awoke immediately and fully regained consciousness.

Seven hours later, the patient, now in good general condition, was moved from intensive care to the general ward for further treatment. The following day, an increase of the generalized edema was noticed. Eye lids, backs of hands, and feet

TABLE 2
Therapy of HCN-Poisoning

I. Self Aid	
HCN - inhalation no vomiting	3 DMAP-tbl afterwards: 1 DMAP-tbl/h
II. First Aid	
By medical personnel for unconscious persons and/or vomiting patients	1 amp DMAP i.m. afterwards: 1 DMAP-tbl/h
III. Medical Therapy	
After Pretreatment	1 amp i.v. sodium thiosulfate
IV. Medical Therapy	
	1 amp DMAP i.v. afterwards: through the same canula 1 amp sodium thiosulfate

were heavily swollen. These appearances vanished slowly by themselves without therapy within the next 5 days. At no other time lapse were such a heavy decrease of blood pressure or edema observed. Experiments with CoEDTA in animals revealed that these effects were due to cobalt.

Colleagues treating the patient should take into account the fact that 30% ferrihemoglobin turns skin and mucus membranes cyanotic. Thus, one day I received a call from a colleague who was treating a woman under the oxygen tent because she still showed a blue face — although she was fully conscious and her blood pressure normal.

A further case emphasizes our experiments in animals (Fig. 2). A colleague called angrily, because he had spent a full day injecting DMAP every hour and a half into the patient who showed recurring symptoms of poisoning, but which disappeared at once after half of the doses were administered. I explained to him that he should immediately infuse thiosulfate (100 mg/kg).

Here, a dramatic case of cyanide poisoning of a child: a child was taken to a hospital "after having eaten a few bitter almonds". The doctor treating the child applied a 5-fold DMAP-doses (as a result of a calculation error). The child at once had turned extremely cyanotic, unconscious, and fell into a coma. Happily, the doctor realized what was happening, and she injected toluidine blue (2 mg/kg i.v.), whereby ferrihemoglobin of over 80% spontaneously decreased under the 10% level (Kiese *et al.*, 1972).

DMAP is also very effective in H₂S and mercaptan poisonings, because these poisons react with cytochrome oxidase in the same manner as does HCN. In several cases of poisoning with H₂S-CH₃ and H₂S, DMAP was successfully engaged. Because of the strong irritability of these irritants on the alveoli, dexamethasone must be inhaled at the same time (5 strokes every 10 min). Application of thiosulfate in this case of poisoning is useless. If after poisoning with H₂S or H₂S-CH₃ ferrihemoglobin is measured "fatal concentrations of ferrihemoglobin" may be found, while the patient is still feeling very well. Sulphur reacts with hemoglobin by formation of sulfhemoglobin.

Both after poisonings with cyanide and H₂S, as well as with mercaptans, a consequent edema must be taken into account, which ought to be treated with Dextrane (10% 4000).

If exactly dosed, about 3 mg DMAP/kg, no side effects of DMAP were to be seen. If, in case of poisoning, 2 amp. of DMAP were given, that is about 6-7 mg DMAP/kg, more than 60% methemoglobin would be measured. This increases methemoglobin concentration in the erythrocytes causing damage to the red blood cell, and about 24 h later partly destroys the erythrocyte, which results in hemolysis. This hemolysis is stronger and more critical to the patient, if not to say dangerous, should 3 or more amp. of DMAP be applied, and the critical methemoglobin concentration of about 80% be reached.

Therapy under field conditions

For treatment of cyanide poisonings under field conditions and under conditions of mass casualties of poisonings an intravenous application of DMAP by the physician would keep relatively few people alive. An i.m. therapy of cyanide poisoning by the medical personnel, or a self- and fellow aid by oral therapy with DMAP would be most welcome. Considering the oral application of DMAP, the 3-fold doses of i.v. or i.m. application form, is required to form about 30% methemoglobin. After oral application, a half maximal ferrihemoglobin concentration is reached after about 10 min, whereas after i.m. injection of DMAP the half maximal concentration is attained in about 7 min. However, in both cases, relatively quick minor methemoglobin concentrations are shown (Fig. 7).

Oxidation of hemoglobin to ferrihemoglobin occurs parallel with the oxidation of DMAP-metabolites. For this coupled reaction of oxidation the presence of oxygen is necessary. In nitrogen atmosphere one may unite hemoglobin and DMAP for an unlimited period without the occurrence of any methemoglobin. DMAP and hemoglobin are able to react only in the presence of oxygen, whereby methemoglobin and DMAP metabolites arise. DMAP itself can no longer be found in man after a few minutes. If it does not react in a few minutes with oxygen and hemoglobin and thus be transformed into metabolites, it will form glucuronide and sulfate by passing through the liver and more than 50% will be excreted in the urine (Jancso *et al.*, 1981).

If DMAP is applied intravenously, there should be sufficient oxygen available in the blood. If DMAP is applied intramuscularly, ferromyoglobin and the equivalent amount of ferromyoglobin are oxidized to ferrimyoglobin. This oxidation reaction is extracting oxygen from the muscle tissue at the injection site. Therefore, it leads to a local deficiency of oxygen in the tissue, and consequently to a delayed destruction of the tissue or to a local sterile infection by an invasion of leukocytes, and discharge of the damaged tissue. CPK concomitantly increases reaching a maximum at about 24 h, and simultaneously heavy pain is felt locally. After i.m. application of DMAP, due to local anesthesia no pain is felt for the time being. The pain starts at about 12 h after DMAP injection, and reaches its maximum at about 24 h parallel to the maximal CPK-concentration. After another 24 h the pain has disappeared, again a parallel decrease of CPK-concentration. The danger of an abscess at the injection site is of minor importance, since DMAP, like other phenols, has a disinfecting effect.

The degree of pain due to the i.m. application of DMAP is dependent on the concentration of DMAP at the injection site, i.e. the higher the amount of DMAP and the smaller the volume of the applied DMAP-solution, the greater pain is felt and the higher is the CPK-concentration. For this reason, we are trying to make applicable as first aid a sufficient DMAP dosage of 120 mg in an amount of 6 mL solution to be injected

intramuscularly through a double injector at two injection sites. These experiments are not yet concluded. The double injector, however, is already available.

For therapy of cyanide poisoning, Table 2 roughly indicates some guidelines.

Self- and fellow aid

If there are grounds for suspicion of cyanide poisoning because of the smell of bitter almonds when nausea and/or vomiting has not yet started, then immediately take 3 DMAP tablets or ampoules.

First aid measures

By medical personnel: unconscious persons or patients who were vomiting should be given 1 amp. DMAP i.m.

In both cases, the patient should be taken to the hospital for treatment. 1-2 h after oral or i.m. application form of DMAP, 1 amp. of sodium thiosulfate (100 mg/kg) must be infused.

Optimal effect, however, in the medical treatment of cyanide poisoning results in 1 amp. DMAP i.v. and thereafter through the same cannula 1 amp. sodium thiosulfate.

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Cyanide in Human Disease: A Review of Clinical and Laboratory Evidence

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ABSTRACT

Cyanide in Human Disease: A Review of Clinical and Laboratory Evidence. Wilson, J. (1983). *Fundam. Appl. Toxicol.* 3:397-399. Experimental cyanide exposure in animals causes demyelination and circumstantial clinical and laboratory evidence suggest that there are human parallels. In Leber's hereditary optic atrophy there appears to be a defect in the conversion of cyanide to thiocyanate because of deficient rhodanese activity. For transmitters of the disease smoking carries the risk of blindness and in the most severely affected patients, there is diffuse neurological disease. It is possible that other hereditary optic atrophies (dominant and recessive) may also reflect inborn errors of cyanide metabolism. In the retrobulbar neuritis and optic atrophy of vitamin B₁₂ deficiency there may be a conditional abnormality of cyanide metabolism in smokers, and likewise in so-called tobacco-alcohol amblyopia in which there are more complex nutritional deficiencies. Epidemiological evidence (differing sex ratios, excess of smokers) indicates that defective cyanide metabolism may contribute to the development of sub-acute combined degeneration of the cord in vitamin B₁₂ deficiency. In protein-malnourished populations consuming large amounts of cyanide or cyanogens, viz. in tropical Africa where the staple diet includes cassava containing large amounts of linamarin, similar maladies occur as acquired disorders. There may be a similar explanation for lathyrism. The known pathways of human cyanide metabolism are reviewed and evidence supporting the clinical data is presented.

INTRODUCTION

Although cyanide is not among the most toxic of agents, its acute effects are universally known and make the experimental study of more chronic effects so difficult. There is, however, good epidemiological, therapeutic and experimental evidence suggesting that there are important effects — mostly neurological — of chronic exposure to cyanide and cyanogens. It is this evidence which I would like briefly to review.

Metabolism and detoxification

There are traces of cyanide in body fluids and in exhaled air in humans (Boxer and Rickards, 1952a). Although it is possible that this is derived from the metabolism of gut bacteria, viz. *E. coli* and *Ps. Pyocyaneus* it may also be generated endogenously from thiocyanate (Goldstein and Rieders, 1953; Vesey and Wilson, 1978).

Endogenous metabolism of cyanide is probably regulated by hydroxocobalamin with which it easily combines to form cyanocobalamin — its presumed entrée to the 1-C metabolic pool

(Boxer and Rickards, 1952b). Although this is probably not important from the standpoint of detoxification for stoichiometric reasons, it is potentially important in toxicity (Wokes, 1958; Smith, 1961).

The main detoxification route is by the enzymic conversion to thiocyanate for which thiosulphate and/or mercaptopyruvate are substrates (Figure 1). These substrates are derived from cysteine (Smith and Malcolm, 1930; Lang, 1933; Fielder and Wood, 1956).

It has been shown that large amounts of cyanide given to rats will combine directly with cystine to yield 2-amino-4-thiazolidocarboxylic acid (Figure 2) but it is uncertain if this reaction is of importance in humans (Wood and Cooley, 1956). I have been unable to identify the compound in urine of heavy smokers. Likewise the metabolic significance of a reaction between cyanide and ascorbic or dehydroascorbic acid to form cyanohydrin is not known (Sprince *et al.*, 1982).

Clinical abnormalities

In humans, clinical abnormalities apparently occur when cyanide exposure is high or when there is an abnormality of detoxification or when there is a combination of both factors. Abnormalities of detoxification may arise when there is either an inborn metabolic error or a paucity of substrate secondary to malnutrition.

Excessive cyanide exposure

Tobacco amblyopia

In tobacco amblyopia there is usually, but not invariably, in addition to a history of heavy smoking (especially pipe-smoking) evidence of vitamin B₁₂ depletion (Wokes, 1958). It has been inferred that interrelated abnormalities of cyanide and vitamin B₁₂ metabolism are responsible for both this condition and for retrobulbar neuritis complicating pernicious anaemia because hydroxocobalamin is more effective therapeutically than cyanocobalamin (Smith, 1961; Chisholm *et al.*, 1967). Cyanocobalamin levels are elevated in plasma (Wilson *et al.*, 1971).

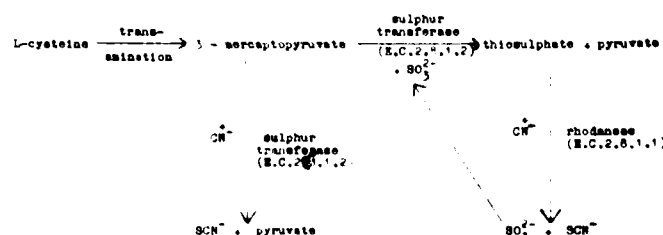


FIG. 1 The main route of cyanide detoxification

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linamarin and lotaustralin are present in the integument of the cassava tuber and it is likely that the glycoside itself as well as free cyanide is ingested and contributes to the neuropathy. In an area where animal protein intake tends to be poor, the substrate for detoxification is also an important determining factor. Plasma levels of sulphur-containing amino acids were also very low in untreated patients with the disease (Osuntokun *et al.*, 1968).

As in many other situations where there is both undernourishment and malnourishment the interrelation of contributory dietary factors is likely to be complex, but in endemic areas, there is no evidence that sickling is a factor.

Although it has been claimed that it is the different levels/concentration of glycoside in different varieties of cassava (bitter versus sweet) which relates to the varying prevalence in different areas, it is more likely that differing culinary practices as well as the general nutritional state of the population are more important determinants of prevalence. It is not, therefore, surprising that ataxic neuropathy appears to be recognized most frequently in areas where cassava is a relatively recent cultivation.

Lathyrism

So far there has been no convincing laboratory evidence linking neurolathyrism to cyanide toxicity although clearly nitriles generally are potential sources of cyanogenesis *in vivo*.

It has been suggested that swayback in lambs reared on copper-deficient pasture may represent the superadded neurotoxic effects in vulnerable animals of nitriles derived from clover, since outbreaks of swayback coincide with cultural conditions favoring rich growth of clover.

Teratogenic effects of cyanide

Whereas the low birth weight of infants of smoking mothers might be due to unfavorable competition for sulphur-containing amino acids between fetus and cyanide detoxification, or even the direct histotoxic effect of cyanide itself, there is no evidence of an increased rate of malformation among such offspring. Nevertheless, it has been claimed that there is an increased rate of teratogenesis in rats fed on cassava (Singh, 1981) but this study needs to be repeated using other species, because the rat is notoriously resistant to cyanide.

Cassava as goitrogen

In an interesting contrast to the nutritional conditions favoring the occurrence of ataxic neuropathy in the tropics, goiter has been reported elsewhere in the tropics in cassava-eating populations with relatively low iodine intake (Ermans *et al.*, 1980).

CONCLUSIONS

Although there is little or no evidence of overtly chronic toxic effects of cyanide or cyanogens in otherwise well-nourished and metabolically normal subjects, adverse effects are seen in circumstances where cyanide detoxification is abnormal *viz.* substrate deficiency due to inadequate diet, inadequate absorption, inadequate mobilization or substrate competition or enzymic deficiency, *viz.* inborn, maturational inadequacy or nutritional. While circumstantial evidence supporting this view is very strong, more direct investigation of this potent and ubiquitous histotoxic agent is needed.

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Artifacts in the Definition of Toxicity by Cyanides and Cyanogens

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ABSTRACT

Artifacts in the Definition of Toxicity by Cyanides and Cyanogens. Ballantyne, B. (1983). *Fundam. Appl. Toxicol.* 3:400-408. Misleading conclusions may be drawn in defining toxicity from administered cyanides or cyanogens if meticulous attention to detail is not given in the design, conduct and interpretation of experimental and analytical procedures. Problems may occur if specimens are not appropriately stored or if interfering factors, such as antidotal agents, are present. Measurement of whole blood cyanide concentrations is valuable for diagnostic purposes, but plasma concentrations may give a better functional index of blood cyanide providing that samples are immediately analyzed. The most appropriate tissues for cyanide and cytochrome oxidase determinations are brain and ventricular myocardium. Analyses should be carried out immediately on freshly sampled tissue. In addition to the use of biochemical techniques for determination of cytochrome oxidase activity, dynamic quantitative histochemical methods are useful for assessing effects of cyanide on regional parenchymal enzyme activity. In determining cyanide-related cyanogen toxicity, the signs are useful, but comparison of molar lethal toxicity data requires caution. Confirmatory antidotal studies should be carefully designed with respect to both the nature and timing of antidotal procedures. *In vitro* studies assist in confirming cyanide liberation and are of value for investigating mechanisms of cyanogenesis. Variations in toxicity between cyanides and cyanogens are due to both the influence of inherent toxicity of the cyanogen molecule and differences in the rate of accumulation of biologically active cyanide.

INTRODUCTION

Acute cyanide poisoning may result directly from exposure to simple cyanides such as hydrogen cyanide and its alkali salts, or from the biotransformation of more chemically complex cyanogens. There are many occasions when it may be necessary to determine or confirm toxicity, including lethal toxicity, from cyanides or cyanogens. These include studies on the experimental toxicology of cyanides or cyanogens, and where cyanide poisoning is suspected in clinical or forensic circumstances.

This paper discusses investigational approaches and technical problems in defining cyanide toxicity, including cyanide-related toxicity of cyanogens. It is convenient to discuss the confirmation of cyanide poisoning initially, since many of the principles also apply to cyanogen intoxication where the considerations are more extensive. Thus, the nature, magnitude and

onset of effects produced by acutely administering a cyanogen may be different from those of giving a single dose of a free cyanide by the same route. This is due to both the possibility for intrinsic toxicity from the cyanogen, and the fact that the rate of accumulation of toxicologically significant cyanide concentrations is slower for cyanogens than results from acute administration of free cyanide. The decreased rate of accumulation is due to the requirement for biotransformation and the proportionately greater detoxification of cyanogen-liberated cyanide.

CYANIDE TOXICITY

Four major areas often considered in relation to the diagnosis or confirmation of acute cyanide poisoning are signs of intoxication, autopsy features, measurement of body fluid and tissue cyanide concentrations, and assessment of the degree of inhibition of the biologically vital target tissue enzyme cytochrome oxidase.

Signs of toxicity

A list of signs typical of acute cyanide poisoning, and common to both animals and humans, is given in Table 1. All these signs are characteristic of acute cyanide intoxication, but not diagnostic on their own. As a consequence of the cytotoxic hypoxia in acute cyanide poisoning, there is a shift from aerobic to anaerobic metabolism and, hence, the development of a lactate acidosis (Graham *et al.*, 1977; Yamamoto and Yamamoto, 1977). A combination of tachypnoea, convulsions and lactate acidosis is strongly suggestive of acute cyanide poisoning.

Autopsy findings

Postmortem findings are few and nonspecific in acute cyanide poisoning (Ballantyne, 1973; Sunshine and Finkle, 1964). Tracheal congestion and hemorrhages, with subpleural and confluent alveolar hemorrhages, cerebral and pulmonary edema, and petechiae of the brain, meninges and pericardium are common by most routes of exposure (Ballantyne, 1970, 1973). Also, in peroral poisoning it is common to find gastric erosions.

TABLE 1
Signs of Toxicity in Acute Cyanide Poisoning

Tachypnoea
Incoordination of movements
Cardiac irregularities
Convulsions
Coma
Respiratory failure
Death

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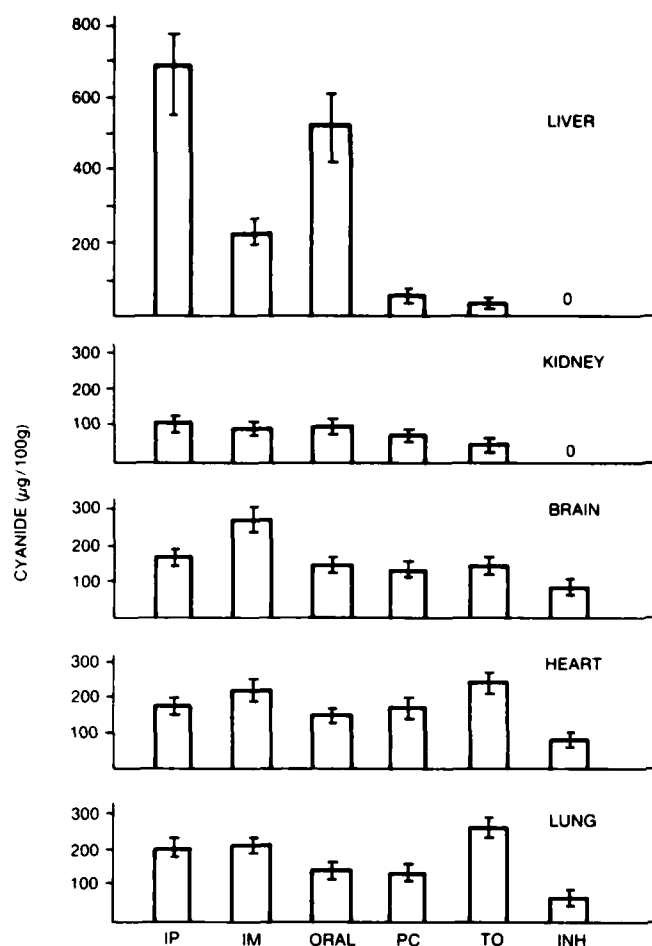


FIG. 1. Concentrations of cyanide in various tissues taken from rabbits following lethal poisoning from hydrogen cyanide administered by various routes of exposure. For each route the values represent the mean $\pm$ standard error for six animals. IP - intraperitoneal; IM - intramuscular; PC - percutaneous; TO - transocular; INH - inhalation.

In spite of the cytotoxic hypoxia, resulting in a significantly reduced utilization of arterial oxygen and, thus, a narrowing of the arteriovenous oxygen tension difference, the coloration of venous blood is variable, being bright red in only about half the cases, and is not a reliable guide as to cause of death (Ballantyne, 1970; Pryce and Ross, 1963). In clinical situations, however, and in the absence of a readily available means for rapid blood cyanide analysis, the measurement of a normal arterial pO_2 with reduced A-V pO_2 difference is useful supportive evidence for a diagnosis of acute cyanide poisoning.

Although the threshold level for olfactory detection of atmospheric hydrogen cyanide is about 1 ppm (Guatelli, 1964), its odor may be missed for various reasons. These include the presence of other odors, and the fact that 20% to 40% of the population is unable to detect a cyanide odor (Brown and Robinette, 1967; Gwilt, 1961).

Measurement of cyanide concentrations

The measurement of cyanide concentrations in biological fluids and tissues is a major consideration in defining lethal toxicity from cyanide. However, considerable caution is necessary with respect to the sampling of tissues, their storage and

analysis, and the interpretation of the possible adverse biological significance of the results. These factors are considered in detail below.

Choice of tissues

The selection of tissues for analysis, in both experimental acute cyanide poisoning and in fatal human cases, depends, in part, on the route of exposure. Figure 1, for example, shows the concentration of cyanide in various tissues following lethal poisoning from hydrogen cyanide by various routes of exposure. The concentration of cyanide in liver varies markedly with route and, as expected, is high following intraperitoneal and peroral poisoning, and low by the percutaneous and inhalation routes. Indeed, in this particular experimental situation cyanide could not be detected in specimens of liver removed from animals killed by high inhalation dosages of hydrogen cyanide vapor (Ct of 2500 mg min/ m^3 for a 5-minute exposure). Comparison of similar tissues for the different routes of exposure indicates that the lowest tissue cyanide concentrations, overall, are to be found in animals receiving lethal doses of hydrogen cyanide by inhalation. This is probably due to the rapid and continuous uptake of cyanide from alveolar air into the pulmonary circulation, the absence of first-pass detoxification in the liver, and, thus, the rapid attainment of toxic tissue concentrations.

In general, and with respect to differences in the routes of exposure, least variability in cyanide concentrations occurs in brain and ventricular myocardium, which contain concentrations of diagnostic significance. The concentrations in brain are similar in grey and white matter (Ballantyne, 1975).

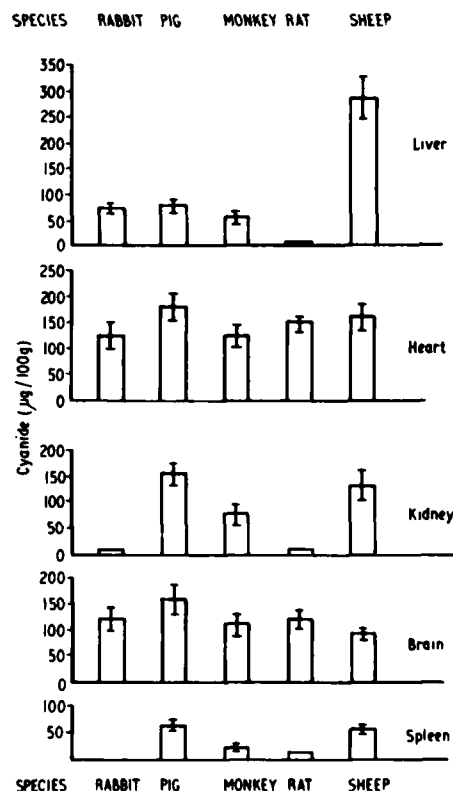


FIG. 2. Concentrations of cyanide in various tissues taken from several species following lethal poisoning by potassium cyanide given as an intramuscular injection. For each species the values represent the mean $\pm$ standard error for six animals.

The influence of species on the differential concentrations of cyanide in various tissues is shown in Figure 2. There is marked variability in kidney and spleen cyanide concentrations. This species difference in spleen concentrations contrasts with the situation in human fatal cases of acute cyanide poisoning, where the splenic cyanide concentrations may be several times those measured in blood (Ansell and Lewis, 1970; Sunshine and Finkle, 1964). The differences may reflect variations in the proportionate volume of blood perfusing the spleen in different species. The concentrations of cyanide in brain and ventricular myocardium are similar for the different species, and generally greater than $100 \mu\text{g}/100 \text{ g}$ wet tissue. This finding, coupled with the demonstration that brain and myocardial cyanide concentrations are consistently higher for different routes of exposure, indicate the value of these two tissues for the reliable diagnosis or confirmation of acute lethal cyanide poisoning. However, and particularly for diagnostic purposes, it is preferable that multiple tissue samples be taken (Finck, 1969).

Blood cyanide concentrations may be measured on samples taken at autopsy, or which have been removed sequentially during the course of poisoning. Typical whole blood cyanide concentrations for rabbits receiving hydrogen cyanide by different routes of exposure are shown in Figure 3. These are high concentrations which are of clear diagnostic significance. Figure 4 shows the similarity in blood cyanide concentrations for various species given lethal intramuscular potassium cyanide, and the fact that they are all high and of diagnostic significance. Similar lethal blood concentrations have been reported by Egekeze and Oehme (1979a).

Serum concentrations are also similar between species, but significantly lower than whole blood values (Fig. 4). Plasma values are similar to serum concentrations (Ballantyne, 1975). Depending on the species, plasma and serum concentrations are usually one-third to one-half those of whole blood. For the diagnosis or confirmation of acute cyanide poisoning, blood is the fluid of choice (Goenechea, 1982). However, with respect to the experimental toxicology of cyanide, it is often more meaningful to measure plasma concentrations of cyanide. A major reason is that plasma cyanide is a prime determinant of tissue fluid and parenchymal cyanide concentrations, and, hence, may give a reasonable index of likely functional tissue

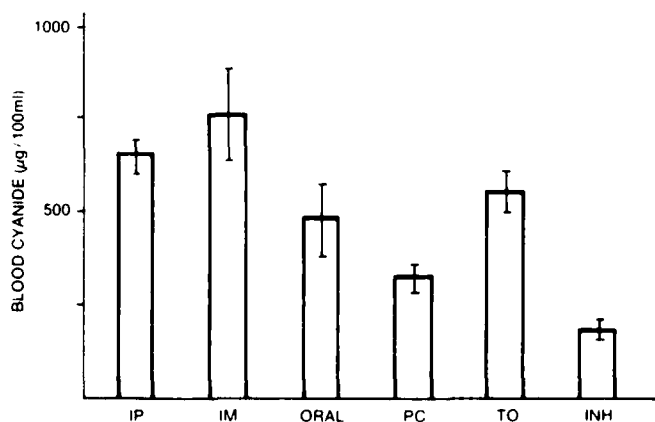


FIG. 3. Cyanide concentrations in whole blood taken from rabbits immediately after death from hydrogen cyanide given by different routes of exposure. For each route the values represent mean $\pm$ standard error for six rabbits. IP - intraperitoneal; IM - intramuscular; PC - percutaneous; TO - transocular; INH - inhalation.

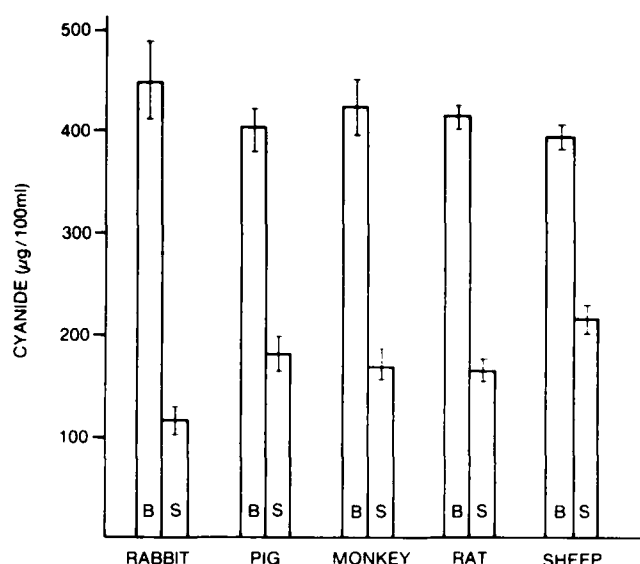


FIG. 4. Cyanide concentrations in whole blood (B) and serum (S) for various species given a lethal intramuscular injection of potassium cyanide. For each species the values represent the mean $\pm$ standard error for six animals.

dose (Ballantyne, 1975). Several studies have demonstrated that blood cyanide concentrates in erythrocytes, and that this sequestration may imply a protective role for erythrocytes in cyanide poisoning (McMillan and Svoboda, 1982; Vesey and Wilson, 1978). The apparent inability of intact erythrocytes to oxidize cyanide avoids the compromising of oxygen transport functions by carbamylation, and cyanide probably binds to unaltered hemoglobin (McMillan and Svoboda, 1982).

Factors influencing measured cyanide concentrations

Several factors may affect the measured concentration of cyanide in biological fluids and tissues, and can markedly influence the interpretation of results. These include postmortem formation of cyanide, postmortem transformation of cyanide, and changes in normal blood occurring after its collection.

Several studies have apparently demonstrated the formation of cyanide in tissues kept under various storage conditions (Curry *et al.*, 1967). However, it has not been our experience that cyanide production occurs postmortem in otherwise normal tissues. For example, when rabbits killed by cervical fracture were kept for up to 3 weeks at room temperature, it was not possible to detect any cyanide in liver, kidney, lung, brain, spinal cord and myocardium using a quantitative colorimetric method (Epstein, 1947) able to measure down to $1 \mu\text{g}/100 \text{ g}$ tissue. Additionally, we have not detected cyanide in liver, kidney, myocardium, intestine, brain, spinal cord and lung, kept at room ($c. 20^\circ\text{C}$) or refrigerator (4°C) temperature for up to 6 weeks following their removal from normal rats or rabbits.

Measured concentrations of cyanide in normal human blood are shown in Table 2. Cyanide concentrations are significantly higher in smokers than in nonsmokers, and this is also reflected in the higher plasma thiocyanate concentrations in smokers. When whole blood samples from normal individuals are collected and stored over several weeks, little change is noted at refrigerator temperature, a gradual decrease in cyanide concentration occurs at room temperature, and at deep-freeze temperature (-20°C) there is significant increase in measured cyanide concentration up to 15 to $20 \mu\text{g}/\text{dL}$ (Fig. 5 Ballantyne, 1977a). Thus, while this increase in cyanide con

TABLE 2
Blood Cyanide and Plasma Thiocyanate
Concentrations in a Group of Cigarette
Smokers (n = 10) and a Group
of Nonsmokers (n = 14)^a

Measurement	Concentration ($\mu\text{g/dL}$) as Mean $\pm$ S.E.		p^b
	Nonsmokers	Smokers	
Blood cyanide	1.6 $\pm$ 0.2	4.1 $\pm$ 0.4	> 0.0005
Plasma thiocyanate	47 $\pm$ 6	154 $\pm$ 14	> 0.0005

^aBlood analyzed immediately after collection.

^bSignificance of difference between smokers and nonsmokers.

centration at deep-freeze temperature is not to levels of lethal significance, the effect may represent a serious artifact in studies on cyanide concentrations in smokers, those having diets high in cyanogens, or in patients receiving cyanogenic drugs. This increase in cyanide concentration occurs if blood is kept below its freezing point (Ballantyne, 1977a) and is probably due to the conversion of thiocyanate to cyanide, a reaction which is catalyzed by free hemoglobin liberated as a consequence of mechanical hemolysis due to the freeze-thaw process. Although intact erythrocytes are apparently not capable of converting thiocyanate to cyanide (McMillan and Svoboda, 1982), this has been shown to occur with free hemoglobin (Chung and Wood, 1971) at an optimum around pH 4.5 (Vesey and Wilson, 1978). Another probable artifact with normal blood cyanide measurements has been described by Vesey and Wilson (1978) who found that the amount of cyanide released on acidifying whole blood is greater than the total determined by separate analyses of erythrocytes and plasma. They noted, as did we (Ballantyne, 1977a), that the measured whole blood cyanide concentration varies directly with plasma thiocyanate concentrations. Thus, cyanide concentrations, measured using techniques involving liberation by acid treatment, may be artifactually high. Here is a further reason why it may be more appropriate to measure plasma rather than whole blood cyanide. Vesey and Wilson (1978) recommend the separate assay of plasma- and saline-washed erythrocytes.

Transformation of cyanide in body fluids and tissues may cause significant decreases in cyanide concentration after death, and this could result in false negative diagnoses. Figure 6, for example, shows the effects of delaying the removal of tissues from cyanide-poisoned animals, and also the effect of delaying analysis of tissues removed immediately after death from cyanide-killed animals. If removal of tissues from poisoned animals was delayed for several days, then it was not possible to detect cyanide in kidney, liver, brain and lung. After 3 weeks, cyanide could not be detected in the contents removed from the cardiac ventricles. If tissues were removed immediately after death, but analyses delayed and tissues kept at room or refrigerator temperature, then again it would not be possible to detect cyanide in tissues within a few days of their removal. However, in contrast with the results involving delay to autopsy and removal of tissues, the blood cyanide concentrations did not decrease as rapidly as did those of the intraventricular contents, and toxicologically significant concentrations of cyanide could still be measured at 3 weeks in blood samples (Ballantyne *et al.*, 1974). The effects of storage conditions on high blood cyanide concentrations are dis-

cussed in more detail below. Postmortem transformation of cyanide has been investigated in sheep by Terblanche *et al.* (1964), who found that measurable cyanide disappeared from liver and skeletal muscle, with respective disappearance times of 12 and 28 hours. The postmortem transformation of cyanide may be a result of various mechanisms, including conversion to thiocyanate, hydrolysis to ammonium formate, and reaction with aldehydes and polysulphides in postmortem tissues (Ballantyne, 1973; Guatelli, 1964; Ioanid and Bors, 1961). These pathways are readily available in the intact dead animal and also in most tissues removed from animals, but not so readily available in samples of blood removed from animals immediately after death. This may well account for the fact that postmortem transformation of cyanide is not so rapid in samples of blood removed within a short time of death.

The rate of transformation of toxicologically significant concentrations of cyanide in serum and whole blood has been investigated in detail (Ballantyne, 1973, 1976). When blood containing cyanide at concentrations of lethal significance is stored at room (20°C), refrigerator (4°C) or deep-freeze (-20°C) temperature, least change is seen over a 3-month storage period in the samples stored in the deep-freeze cabinet. When initial cyanide concentrations were of borderline significance in respect of lethal toxicity then storage at room temperature, but not deep-freeze or refrigerator temperature, resulted in measured concentrations of cyanide decreasing to levels not normally considered to represent lethal blood concentrations. In general, for the lower toxicologically significant cyanide concentrations, decreases in measured levels were less at refrigerator than at room temperature, which was an observation also made by Egekeze and Oehme (1979b).

When there is a delay to the analysis of samples of serum or plasma containing added cyanide, there is a rapid decrease in measurable cyanide concentrations. In one series of experiments, for example, by 1 hour after the addition of a known amount of cyanide the recovery from serum was 31 to 34%, with the most rapid loss occurring during the first 20 minutes (Ballantyne *et al.*, 1973). Hence, the value of whole blood measurements for the diagnosis or confirmation of acute cyanide poisoning

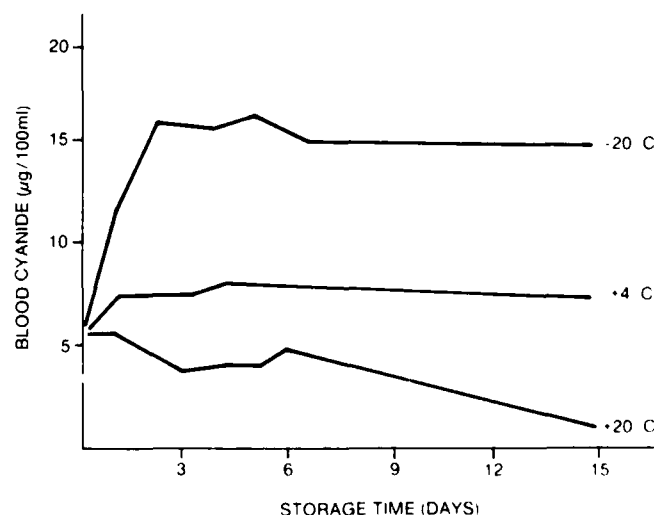


FIG. 5. Changes in measured normal blood cyanide concentrations for various storage times and temperatures, blood from smokers.

The above observations indicate the care necessary in sampling biological fluids and tissues for measurement of cyanide concentrations. When possible, they should be removed immediately after death and promptly analyzed. Brain and myocardium are the best tissues for analysis. Whole blood concentrations, which are consistently high for all routes of exposure in several species, are useful for diagnostic or confirmatory purposes, but plasma concentrations may give a better index of functional levels in experimental situations. However, it is particularly important to analyze plasma and serum at the earliest time possible after their separation because of the significantly greater rate of transformation compared with whole blood (Ballantyne *et al.*, 1973). When it is necessary to store blood, the anticipated concentration may dictate storage and conditions; when high concentrations are envisaged then storage at deep-freeze temperature is preferable, but when interest is in low concentrations then refrigerator conditions are more appropriate.

Measurement of cytochrome oxidase activity

Cyanide inhibits a variety of enzymes, of which the most functionally important is cytochrome oxidase. This results in a

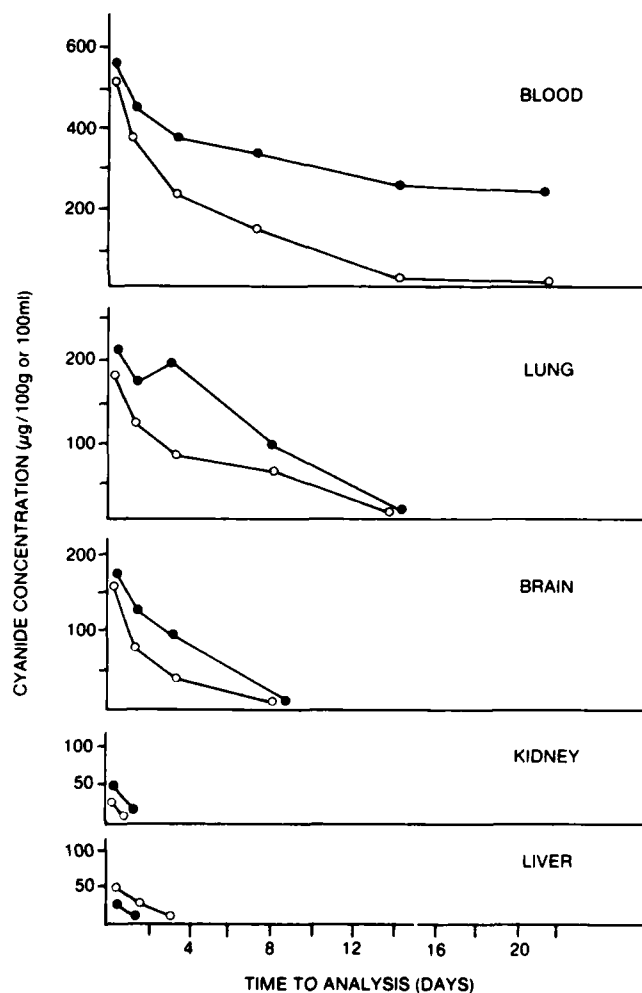


FIG. 6. Changes in cyanide concentrations in blood and various tissues following death as a result of giving potassium cyanide by intramuscular injection (8 mg CN/kg body weight). ●—● values for tissues stored for various times after removal from animals shortly after death; ○—○ values for tissues removed from animals at various times after death.

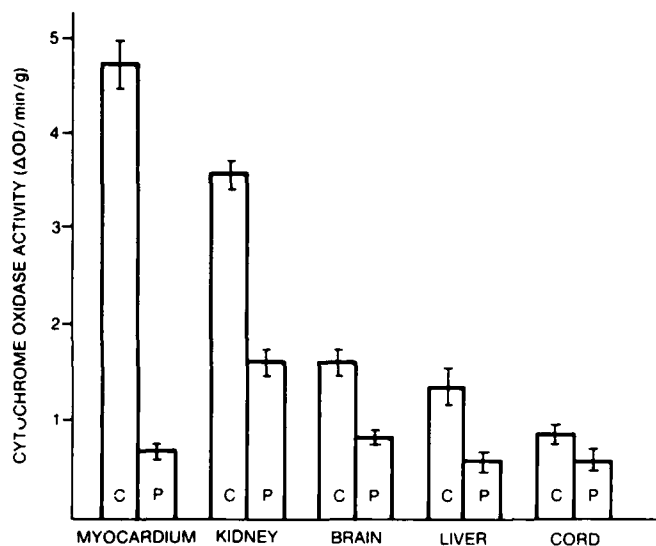


FIG. 7. Cytochrome oxidase activities in tissues from normal rabbits (C) and those killed by a lethal intramuscular injection of potassium cyanide (P).

cytotoxic hypoxia. Biochemical measurements generally show a decrease in cytochrome oxidase activity which is proportional to the cyanide content of the tissue (Fig. 7; Schubert and Brill, 1968). Myocardium and brain are usually the best tissues for the confirmation of poisoning by assessment of enzyme inhibition; decreases in cytochrome oxidase activity are marked and there is little variation with route and species. The significant inhibition of myocardial cytochrome oxidase activity in lethal poisoning accords with the low I_{50} of 2.74 μ M measured *in vitro* for the tissue (Ballantyne, 1977b). Also, Camerino and King (1966) found that heart muscle preparations treated with cyanide exhibited only 10% of the activity of untreated controls.

As with measurement of cyanide concentrations in tissues, the determination of enzyme activity should be made on freshly sampled tissue. Delay in removing tissues will result in measured cytochrome oxidase activities which are similar to those found in control tissues (Fig. 8; Ballantyne, 1977b).

Biochemical measurement of enzyme activity gives useful information about inhibition of cytochrome oxidase activity as determined in tissue homogenates. However, since such homogenates also contain cyanide liberated from blood vessels in the tissue, the degree of inhibition in tissue homogenates may not be an accurate reflection of the situation *in vivo*. Determination of regional parenchymal enzyme activity is possible by the use of quantitative histochemical methods, in which the effect of cyanide released from blood vessels may be obviated. A commonly employed histochemical method for the demonstration of sites of cytochrome oxidase activity is to incubate tissue sections in a medium containing soluble amine substrates, which are converted to insoluble azine dye and precipitated at the sites of enzyme activity (Burstone, 1960). If sections, prepared from tissues removed from normal animals, are incubated in histochemical media to which have been added differing concentrations of cyanide, then a clear relationship between inhibition of cytochrome oxidase activity and cyanide concentration may be demonstrated. However, if sections of tissues removed from animals killed by acute cyanide poisoning are incubated in the usual normal histochemical medium for cytochrome oxidase, then apparently normal tissue enzyme activity exists. This is due to the fact that during the

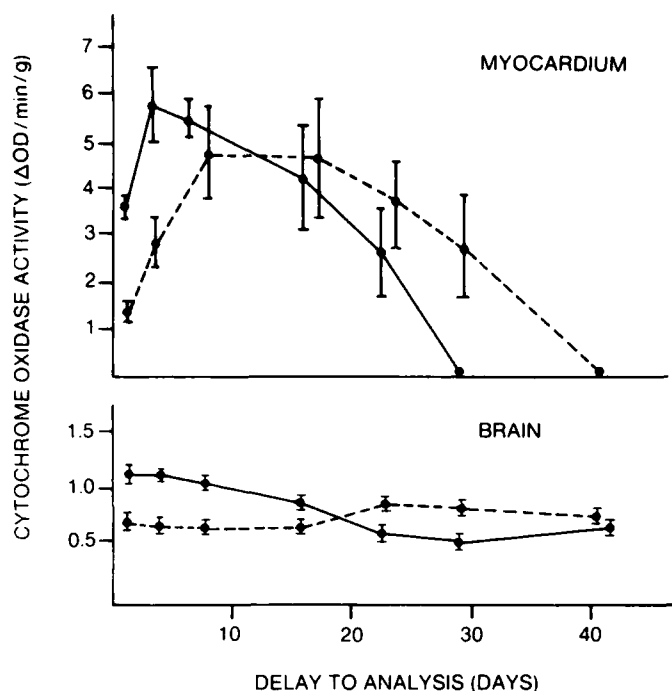


FIG. 8. Changes in cytochrome oxidase activity in myocardium and brain removed from rabbits immediately after being killed by cervical fracture (●—●) or an intramuscular injection of potassium cyanide (○---○).

incubation time normally required for this histochemical technique the comparatively large volume of incubating fluid favors the dissociation of the enzyme-inhibitor complex, and reactivation of cytochrome oxidase activity occurs (Ballantyne, 1977b).

In order to reduce the likelihood for reactivation of inhibited enzyme, it is necessary to obtain conditions which are not favorable to the dissociation of enzyme-inhibitor complex formed *in vivo*, and to make measurements of residual enzyme activity before any significant reactivation occurs. This we have found possible by the use of a technique involving laying tissue sections on gel films containing amine substrates (Ballantyne and Bright, 1979). Azine dye production begins immediately, and its rate of appearance is proportional to cytochrome oxidase activity in the tissue sections over the time of measurement. The rate of azine dye can be measured microdensitometrically by recording changes in optical density of the sections at 550 nm. The initial measurements give a reading of tissue cytochrome oxidase activity before any enzyme reactivation has occurred, and this represents a reasonable estimate of *in vivo* parenchymal enzyme activity in the presence of inhibitor. Correlation with biochemical measurements is good, although the results from kinetic microdensitometric procedures may give values indicating a lesser degree of enzyme inhibition. However, quantitative histochemical measurements may be a more reliable index of *in vivo* enzyme inhibition, since only cellular components are examined. In contrast, homogenates used for biochemical estimates contain fragments of metabolically nonrelevant cells as well as cyanide released from blood in tissue vessels.

CYANOGEN TOXICITY

Cyanogens, which may be synthetic or naturally occurring, are materials which contain biologically available cyanide but

with individual variations in nitrile group lability. Synthetic nitriles, used for a variety of industrial, domestic and therapeutic purposes, include acetonitrile, acrylonitrile, fumaronitrile, malononitrile, propionitrile, sodium nitroprusside and succinonitrile (NIOSH, 1978; Hartung, 1982). A major source of naturally occurring cyanogens is in plants, of which the cyanogenic precursor has been identified as a cyanogenic glycoside in about 200 species (Vennesland *et al.*, 1982). Reviews of naturally occurring cyanogens are to be found in Montgomery (1969), Nartey (1980), Nestel and MacIntyre (1973), and Towill *et al.* (1978).

Currently used approaches to assess the relative contribution of released cyanide in the toxicity of cyanogens are as follows:

- Signs of toxicity
- Comparison of molar LD_{50} values
- Studies on the effects of cyanide antidotes
- Measurement of cyanide concentrations
- *In vitro* studies
- Assessment of cytochrome oxidase activity inhibition

No one method is totally satisfactory, but a combination of several approaches provides rewarding information.

Signs of toxicity

Although the signs of acute cyanide poisoning are not diagnostic, as a group they are characteristic and therefore useful in preliminary assessments of cyanogenic potential. Sometimes a clear chemical determinant may be seen in the expression of signs of toxicity. For example, Ahmed and Farooqui (1982) found it possible to differentiate saturated nitrites, causing characteristic central nervous system signs of acute cyanide poisoning, from unsaturated nitriles which produced moderate to severe cholinomimetic signs.

Comparison of LD_{50} data

Comparison of route specific molar LD_{50} values for cyanogens and cyanides might appear a reasonable approach for assessing

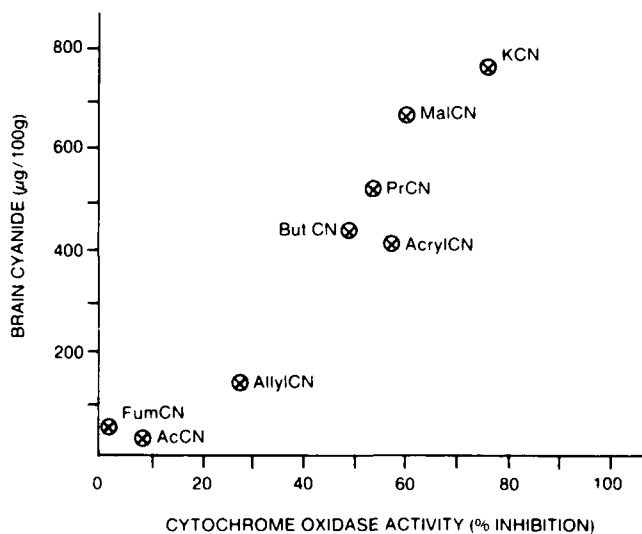


FIG. 9. Relationship between brain cyanide concentrations and inhibition of brain cytochrome oxidase activity following lethal doses of various aliphatic nitriles; KCN - potassium cyanide; MalCN - malononitrile; PrCN - propionitrile; ButCN - butyronitrile; AcrylCN - acrylonitrile; AllylCN - allyl cyanide; FumCN - fumaronitrile; AcCN - acetonitrile. Plotted from data given by Ahmed and Farooqui (1982).

the relative contribution of cyanide in the lethal toxicity of cyanogens. However, for two major reasons, considerable caution is necessary in making such comparisons. First, any free cyanide which is administered to an animal is immediately available as a bolus dose to exert its toxicity, but with cyanogens there is a need for the cyanide radical to be liberated from the parent molecule by an appropriate biotransformation mechanism, and this occurs at variable rates according to the structure of the discrete cyanogens; thus, cyanide liberated from cyanogen is made available to the organism at a slower rate than with bolus administered cyanide. Second, both administered free cyanide and cyanogen-liberated cyanide will be detoxified, mainly by sulfurtransferase mechanisms (Sorbo, 1975). However, since the rate of presentation of cyanide to the organism is slower with cyanogens, the proportionate detoxification of cyanogen-liberated cyanide will be greater than with administered free cyanide. Taking both of these factors into account, it will be apparent that the rate of accumulation of biologically active cyanide will be significantly slower with cyanogens. Thus, compared with simple free cyanides, the cyanogens may show a prolonged latent period before toxicologically significant cyanide concentrations are attained, and this will clearly influence the time to onset of signs of toxicity and also the magnitude of cyanogen dose required to produce lethality due to cyanide release. Thus, differences in LD_{50} values may be due to the influence of nitrile group lability as well as the contribution of the intrinsic toxicity of the cyanogen. For this reason, lethality data need to be carefully examined along with all other relevant toxicological information including, particularly, signs of toxicity and their times to onset.

Experiments with cyanogens should be structured to allow contributing toxicity, other than cyanide induced effects, to be detected and its relevance determined with respect to the overall toxicity of the cyanogen under investigation. For example, in addition to cyanide release, the following may be important in the acute toxicity of specific cyanogens. Nephrotoxicity with benzyl cyanide (Guest *et al.*, 1982); interaction with cellular sulphhydryls and other nucleophiles for acrylonitrile (Szabo *et al.*, 1977)

Use of cyanide antidotes

The modifying effect of administered cyanide antidotes on cyanogen toxicity might reasonably be expected to yield information of value in determinations on the relative contribution of cyanide release in the toxicity of cyanogens. However, published studies are often unhelpful in this respect, and for a variety of reasons to which Willhite and Smith (1981) have drawn attention. They found, for example, that with a single dose of thiosulphate the plasma levels of this antidote may be significantly reduced by the time that toxic levels of cyanogen-liberated cyanide are accumulating, and thus having minimal protective effects. This may be corrected by the later administration of thiosulphate, as determined by the clinical picture, or by giving repeated injections of thiosulphate spaced at appropriate intervals. Additionally, when nitrite is administered, the liberated cyanide may be transiently captured as cyanomethemoglobin, but as the complex dissociates it will add to the accumulating toxicologically available cyanide which continues to be liberated from the cyanogen. Thus, the observation that a single dose of antidotal agent fails to protect against nitrile poisoning cannot necessarily be taken as evidence against a cyanogenic component in the toxicity of the material tested. Studies should be designed to take into account the

continual titration of cyanide from cyanogens, and the slow accumulation of biologically active cyanide. In appropriately conducted studies, protection by several antidotes having different mechanisms of action is good evidence for a major involvement of cyanide intoxication by cyanogens.

A further problem that may be encountered in studies on the effects of antidotes in cyanogen toxicity is the possibility for certain antidotes to interfere with methods used for the quantitative determination of cyanide concentrations. For example, Morgan *et al.* (1979) demonstrated that sodium thiosulphate may interfere with the measurement of cyanide by colorimetric procedures which utilize microdiffusion separation, this being due to the conversion of thiosulphate to sulphite. They were able to modify the analytical procedure by taking advantage of differing pK_a values of hydrogen cyanide and polythionic acids, formed on acidification of the thiosulphate ion. By using pH 5.2 buffered solution rather than sulphuric acid, they were able to measure cyanide in the presence of thiosulphate. Also, thiosulphate has been shown to interfere with the potentiometric determination of cyanide, particularly in the presence of blood, and produce falsely elevated cyanide concentrations which are believed to be due to the presence of the sulphide anion (Sylvester *et al.*, 1982). The interference can be eliminated by oxidation of sulphide with hydrogen peroxide. In this context, measurement of cyanide concentrations in antidotal studies can be valuable for confirming decreased cyanide concentrations accompanying reduction in both mortalities and signs of toxicity in cyanogen-treated animals.

Measurement of cyanide concentrations

Measurement of the concentration of cyanide in biological fluids and tissues is of importance in assessing cyanogen toxicity, particularly if measurements are made on the rate of increase in blood or plasma cyanide and related to the appearance and development of signs of toxicity. It has been our experience, as with acute free cyanide poisoning, that concentrations of cyanide in whole blood, plasma, brain and myocardium are convenient and reliable indicators of cyanide related cyanogen toxicity, providing that specimens are removed soon after death and immediately analyzed. Concentrations of cyanide in biological fluids and tissues following acute cyanogen poisoning are generally lower than is the case with lethal free cyanide poisoning. This is in part related to the slower accumulation of biologically active cyanide with cyanogens. Materials producing typical cyanide effects generally have high cyanide concentrations, with significantly lower concentrations in those cases where the evidence indicates that cyanogenesis is not a major factor in their toxicity.

With any specific cyanogen, the range of lethal brain concentrations is often relatively narrow. However, as anticipated from the differing metabolism and pharmacokinetics, the mean and range of brain cyanide concentrations vary between cyanogens of differing nitrile group lability (Ahmed and Faraogha, 1982; Willhite and Smith, 1981). As discussed previously, reduction in measured cyanide concentrations in appropriately conducted antidotal studies can be valuable confirmatory evidence of the relative role of cyanide liberation in the mechanism of toxicity of cyanogens.

In vitro studies on cyanogenesis

The finding of reduced *in vivo* lethal toxicity of many aliphatic nitriles after pretreatment of animals with an acutely hepatotoxic agent, such as carbon tetrachloride, confirms the

need for normal hepatic function in the biotransformation processes for cyanogens (Willhite, 1981; Willhite and Smith, 1981). The information from *in vivo* biochemical and metabolic studies can often be supplemented by well controlled *in vitro* investigations. The latter are useful for both confirming cyanogenic potential and studying mechanisms of cyanogenesis. Some illustrative examples are given below.

Using a variety of aliphatic nitriles, Willhite and Smith (1981) found that cyanide was liberated from n-butyronitrile and succinonitrile when incubated with mouse liver slices or NADPH-fortified hepatic microsomal preparations. Such liberation of cyanide did not occur with liver from carbon tetrachloride pretreated animals or when SKF-525A was added to the *in vitro* normal liver slice preparation.

In acute acrylonitrile poisoning there may be signs which resemble those of acute cyanide poisoning, and high blood and tissue cyanide concentrations with reduced cytochrome oxidase activity are measured after lethal doses of acrylonitrile (Ahmed and Farooqui, 1982). *In vitro* studies (Abreu and Ahmed, 1980) have demonstrated that hepatic enzymes involved in the liberation of cyanide from acrylonitrile are located in the microsomal fraction, and require NADPH, oxygen and magnesium chloride for maximum activation. These findings, coupled with the demonstration of decreased cyanide liberation in the presence of SKF 525A or carbon monoxide, strongly indicate that acrylonitrile is metabolized to cyanide via a cytochrome P-450-dependent mixed function oxidase system. However, in considering the relative contribution of cyanogenesis in the acute toxicity of acrylonitrile the fact that the total urinary excretion of thiocyanate only accounts for a small proportion of an administered dose of acrylonitrile must be taken into account (Farooqui and Ahmed, 1982). Additionally, both *in vivo* and *in vitro* studies indicate that cytoplasmic and membrane protein binding of acrylonitrile may be equally important as cyanide liberation in the acute toxicity of acrylonitrile (Appel *et al.*, 1981; Farooqui and Ahmed, 1982; Gut *et al.*, 1975).

The above examples clearly indicate that *in vivo* and *in vitro* studies should be carried out as complementary investigations.

Measurement of cytochrome oxidase activity

As with acute free cyanide poisoning, following acute cyanogen poisoning there is usually a direct relationship between the cyanide concentration in tissues and their degrees of cytochrome oxidase inhibition. Also, the proportionate reduction in cytochrome oxidase activity appears to relate well with cyanide dependent toxicity of the cyanogen. Brain and myocardium are the most convenient and reliable indicators of reduced cytochrome oxidase activity due to cyanide released from cyanogens. Ahmed and Farooqui (1982), for example, showed a positive correlation between cyanide concentration in the brain and the proportionate reduction in cytochrome oxidase activity (Fig. 9). However, as stressed by Willhite and Smith (1981) in such studies it is essential to determine, by appropriate *in vitro* investigations, if the parent cyanogen is itself an enzyme inhibitor.

Dynamic quantitative histochemical methods for measurement of parenchymal cytochrome oxidase activity, using a substrate-gel technique (Ballantyne and Bright, 1979), show good agreement with biochemical estimates for cytochrome oxidase inhibition by administered cyanogens.

CONCLUSIONS

Studies which are carried out to define acute toxicity from administered cyanides and cyanogens should receive meticulous attention to detail in respect of their design, conduct and interpretation. Problems may occur if specimens are not appropriately collected and stored, or if interfering factors such as antidotal agents are present. Determination of the concentration of cyanide in whole blood is valuable for the diagnosis or confirmation of acute cyanide poisoning, but plasma concentrations may give a better functional index of blood cyanide providing that samples are analyzed immediately after their separation. The most appropriate tissues for cyanide and cytochrome oxidase determinations are brain and ventricular myocardium. Tissues should be removed immediately after death, and promptly analyzed. Dynamic quantitative histochemical methods are useful for assessing inhibition of regional parenchymal enzyme activity, and can supplement the information derived by standard biochemical procedures. In assessments of cyanide-related cyanogen toxicity the signs are useful, but a comparison of lethal toxicity with that due to free cyanide requires considerable caution. Confirmatory antidotal studies need careful design with respect to both the nature and the timing of the antidotal procedures. *In vitro* studies are useful both for confirming and investigating the mechanism of cyanogenesis, but require to be reviewed in the light of other available *in vivo* toxicity information.

The overall effects of cyanide liberated by the biotransformation of various nitrile-containing materials may be different from the effects produced by bolus administration of free cyanide. In view of the necessity for biotransformation of cyanogen, and since there is a proportionately greater detoxification of cyanide so released, the rate of accumulation of toxicologically significant cyanide will be slower for cyanogens than with molar equivalents of acutely administered free cyanide. This rate will also vary between cyanogens, depending on the pharmacokinetics of the individual material and its rate of biotransformation. Such differences in the rate of accumulation of biologically active cyanide partly account for differences in time to onset, magnitude, duration and nature of the toxic effects of cyanogens. Additional variation may be due to the expression of the intrinsic toxicity of the cyanogen molecule.

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