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Chemical Blistering: Cellular and Macromolecular Components (U)

Annual Report

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M. J. Brabec, Subproject Director
R. C. Conolly, Subproject Director
R. H. Gray, Subproject Director
A. Kulkarni, Subproject Director
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19. ABSTRACT (Continue on reverse if necessary and identify by block number)

The objective of this project is to establish the molecular and cellular lesions responsible for tissue destruction as a result of exposure to toxic environmental chemical, bis-(β-chloroethyl) sulfide (BCES), are being studied in proliferating and differentiating cultures of epidermal keratinocytes obtained from neonatal rats and from human newborn foreskin.

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18. Subject Terms. (Continued)

Collagen Substratum, Nylon Membrane Substratum.

19. Abstract (Continued)

During the period of this report, techniques for growth of primary cultures of rat keratinocytes have been further refined to provide cultures similar in morphology to the epidermis in situ. Refinements include changes in the composition of the collagen substratum and the use of nylon membranes as substrata. Also, populations of basal and differentiated cells have been isolated and purified so that the effects of BCES on these two types of epidermal keratinocytes can be compared.

Studies of cross-linking and single-strand alkylation from BCES have been initiated. Initial exposures to BCES were at such high concentration that "nonspecific" cytotoxicity may have resulted. later experiments employed a lower dose at which cross-linking was still observed. Such low levels of BCES did not appear to affect protein synthesis, lactate production of glucose utilization significantly; these parameters were affected at higher exposures.

The biochemical lesion which is primarily responsible for the toxicity of BCES has not been defined, and it is not clear why the basal cell layer degenerates first when the tissue is exposed to BCES topically in vivo.

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I. SUMMARY

The objective of this project is to establish the molecular and cellular lesions that are responsible for the tissue necrosis which results from topical exposure of the human skin to the chemical vesicant bis-(}-chloroethyl) sulfide (BCES). The cell system studied is epidermal keratinocytes cultivated on a substratum at the air/liquid interface to form an epidermal-like tissue. Exposure to BCES is carried out in vitro.

Initial efforts to produce a tissue in vitro suitable for "topical" application of BCES were not successful. The culture destroyed the integrity of the collagen gel which was used as the substratum. As a result, the tissue did not have a smooth upper surface over the entire culture and had many holes. If a culture with this topography were topically exposed to BCES, the chemical could penetrate to the level of the basal layer without passing through the upper cell layers and therefore would not mimic the comparable situation in situ. This problem has been obviated during the second year of the project by altering the composition of the collagen gel or by using nylon membranes as substrata.

During the past year, techniques have been developed to obtain purified populations of basal and differentiated cells, and such populations are now available for comparison of their sensitivity to toxicity from BCES.

Techniques which were established for cultivation of murine keratinocytes have been successfully applied to keratinocytes from human newborn foreskin.

Preliminary data indicate that exposure of cultures to BCES leads to cross-linking of DNA, which later results in single-strand breakage. However, these experiments have to be repeated because use of too high a concentration of BCES produced non-specific toxicity. Use of 50 uM BCES should eliminate this general cytotoxicity. The rates of repair of BCES-induced lesions appear to be the same in basal cells and in differentiated cells.

Cultures exposed to BCES at 100 uM or lower exhibited a variable response in lactate production, while lactate production was stimulated at BCES levels between 100 and 300 uM. At 300 uM BCES, protein synthesis was decreased to 50% of normal and glucose utilization was increased 200-300 percent.

During the past year glutathione S-transferase and peroxidase have been isolated from rat skin and purified for use in the study of the epidermal metabolism of BCES.

During the third year of the project, planned work consists of ascertaining the degree of biochemical similarity between the culture and the tissue in situ, establishing a reproducible technique for topical application of BCES, comparing the degree of alkylation and cross-linking by BCES in basal vs differentiated cells, comparing the DNA repair capabilities of these two cell populations, comparing the effects of BCES on carbohydrate and protein synthesis in these cell types and examining the purified glutathione S-transferase and peroxidase for their ability to metabolize BCES and for their relative roles in the metabolism of BCES.

II. FOREWORD

The source of animal tissue for primary cultures described in this report was neonatal rats derived from the CFN strain by random mating and reared in the School of Public Health's animal facility. This facility is under the supervision of the University Unit for Laboratory Animal Medicine. The University of Michigan is accredited by the American Accreditation Association for Laboratory Animal Care (AAALAC). In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

The source of human tissue was foreskin obtained at routine circumcisions done at Women's Hospital, The University of Michigan, and provided without any identification of the donor. The form utilized to obtain "informed consent" was the one in use by the hospital for routine circumcision. Signature on this form allows experimental use of tissues. The use of this tissue for the present project has been approved by a University Human Subjects Review Committee and, for the protection of human subjects, the investigators have adhered to applicable policies of Federal Law 45CFR46.

No biological material from human subjects was used in this investigation during the period of this report.

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

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III. NARRATIVE REPORT OF PROJECT PROGRESS

A. Statement of the Problem Under Study

Our knowledge of the basic biology of the epidermis has rapidly advanced over the past few years. The development of new techniques for cell culture, ultrastructural analysis and analysis of cellular components and metabolism now permits the investigation of the adaptive and pathological responses of the epidermis to environmental chemicals.

The technical objectives of this project are to develop appropriate culture systems of epidermal keratinocytes for use in the study of subcellular and macromolecular toxic manifestations of environmental chemicals, to establish the credibility of these systems for investigating the molecular mechanisms involved in chemical irritation and to determine whether these systems can be used to develop procedures by which toxic responses can be neutralized. Particularly, this project seeks to establish the molecular and cellular lesions responsible for the tissue destruction characterizing vesication produced by cutaneous exposure to a severe chemical irritant such as bis-(β)-chloroethyl) sulfide (BCES).

Application of BCES to human skin <u>in vivo</u> results in an initial erythema followed by blistering. In vesication, the accumulation of fluid appears to be secondary to fundamental damage to cellular structures (1). As noted by Warthin and Weller (2) and by Sinclair (3), the lesion initially involves destruction of the basal and lower spinous layers of the epidermis. The aim of the present project is to provide a molecular and cellular explanation for the toxicity of BCES and the preferential destruction of these lower layers when BCES is applied topically.

B. Background

1. Review of Appropriate Literature

Mustards are powerful alkylating agents for DNA, RNA and protein. Exposure to S and N mustards is accompanied by changes in cellular enzyme levels; inactivation of particular enzymes; and inhibition of respiration, glycolysis, transport and protein synthesis (4, 5). Alkylation of DNA could also lead to low-fidelity repair with consequent mitotic inhibition and cell death (6). Alkylation of RNA and protein could result in interference with the translation of genetic information and metabolic disruption. The subprojects of this investigation are designed to identify the biochemical and physiological parameters that are associated with exposure to BCES and to elucidate the roles of these parameters in the pathology which results.

2. Review of Annual Report No. 1

Annual Report No. 1 (7) described a methodology for growing primary cultures of rat keratinocytes on collagen gels for use in the various subprojects. However, techniques for raising the differentiated cultures were still being refined to increase morphological similarity between the epidermis in situ and the differentiated culture, and to obviate the problem of destabilization of the collagen used as the substratum for cell culture. When that report was submitted, promising results were being obtained with cells allowed to attach and grow on nylon membranes instead of collagen gels. Use of these membranes was viewed as a means of circumventing the "collagen destabilization" problem.

Since BCES was not available during the first year of this project, alternative test chemicals were used to develop and validate the experimental techniques which were necessary for the project. Assays for DNA strand breakage, alkylation, depurination and repair were established. DNA strand breakage and repair were measured in a sample as small as the cells harvested from a single 60 mm plate of keratinocytes grown on a collagen gel. Procedures to measure respiratory activity and lactate production by cultures of keratinocytes were developed. Activities of several enzymes important in biotransformation were measured in preparations of whole skin. Substantial levels of peroxidase, lactate dehydrogenase and glutathione-S-transferase activities were found.

C. Subproject 1, Tissue Culture, and Subproject 2, Ultrastructural Characterization

Subproject Directors: Frizell L. Vaughan (Subproject 1) and Robert H. Gray (Subproject 2)

1. Progress Report

a. Objectives

The objectives of these two subprojects are to establish methods which can produce and maintain proliferating and differentiating cultures of cutaneous epidermal keratinocytes that exhibit structural and functional characteristics similar to those exhibited by the tissue in situ.

b. Background

During the first year of this project, a new procedure (8) for growing murine lingual epithelium on a substratum of collagen at the air/liquid interface was successfully adapted for the cultivation of epidermal keratinocytes from rat skin. This methodology produced a "tissue" in vitro which was very similar to, but not identical with, the epidermis in situ. However, a problem arose which would make these cultures unsuitable for this investigation. After 5-8 days of cultivation at the air/liquid interface, the culture began to distort the surface of the collagen and to dissolve the substratum itself, thereby producing holes in the collagen. Since this project will include an application of BCES to the surface of the culture, a piece of tissue with a relatively smooth and complete surface structure is necessary. A significant effort in the second year, therefore, was devoted to obviating this problem. The present report describes techniques by which the collagen substratum can be stabilized, thus providing relatively smooth culture surfaces.

Rat keratinocytes have also been successfully grown on several commercial synthetic membranes, with Puropor nylon providing the best substratum of the ones tested. Synthetic membranes offer several advantages over collagen as a substratum, including 1) better quality control of the substratum, 2) commercial availability in various sizes, 3) more stability for extended cultivation periods and 4) essentially labor-free substrata ready for immediate use. Biologically active attachment factors used to enhance the cultivation of mammalian cells did not significantly increase the efficiency of nylon membranes in promoting growth of rat keratinocytes. Thus, no pretreatment of nylon membranes is necessary in order to obtain a confluent monolayer that contains more cells per unit area than can be obtained with tissue culture plastics, as described in the previous report (7).

c. Methods and materials

1) Culture techniques

Epidermal basal cells are isolated from newborn rat epidermis (9) via trypsinization, purified on Ficoll gradients (Pharmacia) and suspended in complete growth medium [90% Eagle's minimum essential medium (MEM) (K.C. Biologicals); 10% fetal bovine serum (K.C. Biologicals); 10 ug/ml insulin and 10 ug/ml hydrocortisone (both from Sigma Chemical Company); 100 units/ml

penicillin and 100 fg/ml streptomycin (both from Pfizer Laboratories); and 0.05 fg/ml fungizone (GIBCO)] to a concentration of 10^5 cells per ml. The cells are then seeded onto a substratum and incubated submerged in growth medium until a monolayer is formed as a result of proliferation. The monolayer of keratinocytes is then lifted to the air/liquid interface using procedures appropriate to the substratum on which the cells have been grown (described below).

Keratinocytes from human foreskin are isolated and cultured essentially as reported (9) for the isolation of epidermal keratinocytes from newborn rat skin. The tissue is sterilized by soaking in 70% alcohol for 2 min, and the subcutaneous adipose tissue is removed by scraping with a dull scalpel. The tissue is then cooled to 4°C and incubated with 0.25% cooled trypsin (1:250 Difco) for 14 hr at 4°C. After rinsing with cold Eagles's Balanced Salt Solution (EBSS), the epidermis is separated from the dermis and basal cells are brushed from the underside of the epidermis using a camel's hair brush. The cell suspension is centrifuged at 60 x g for 5 min and plated on a collagen or plastic substratum.

Keratinocytes grown on collagen gels are lifted by one of two methods: 1) cutting triangular segments of the collagen gel containing the monolayer of keratinocytes and placing the segments on organ culture grids suspended at the interface of the air/liquid medium and the atmosphere, or 2) growing the cells on collagen gels formed on nylon mesh and lifting them by removing the entire culture from the original dish and floating it in fresh medium.

Keratinocytes grown on synthetic membranes (Puropor nylon 0.2 u, Gelman) are lifted by one of two methods: 1) the organ culture method or 2) the glass fiber pad method, which consists of saturating a pre-filter pad with growth medium and placing the membrane on top of the pad so that the surface of the culture is exposed to the atmosphere and the bottom of the culture is in contact with the medium. These procedures are diagrammed in Figure 1.

Incubation is then continued with the cultures being fed by diffusion of nutrients through the collagen or nylon substratum.

2) Studies of substrata

The following substrata have been studied in an effort to encourage differentiation of keratinocytes and thus to produce cultures with more characteristics of the epidermis *in situ*. The first group of substrata studied consisted of collagen gels including Vitrogen 100 (Flow Laboratories, Inc.), rat tail collagen (Sigma) and rat tendon collagen prepared from rat tails in this laboratory. These collagens were studied alone or in combination for efficacy as substrata. The other type of substratum under investigation was the Puropor nylon membrane.

The attachment, proliferation and differentiation of keratinocytes seeded onto collagen substrata are monitored using phase contrast, light and transmission electron microscopy (TEM). Since membranes are not transparent and cultures on membranes cannot be monitored while growing, such cultures are fixed and sectioned prior to microscopic observation.

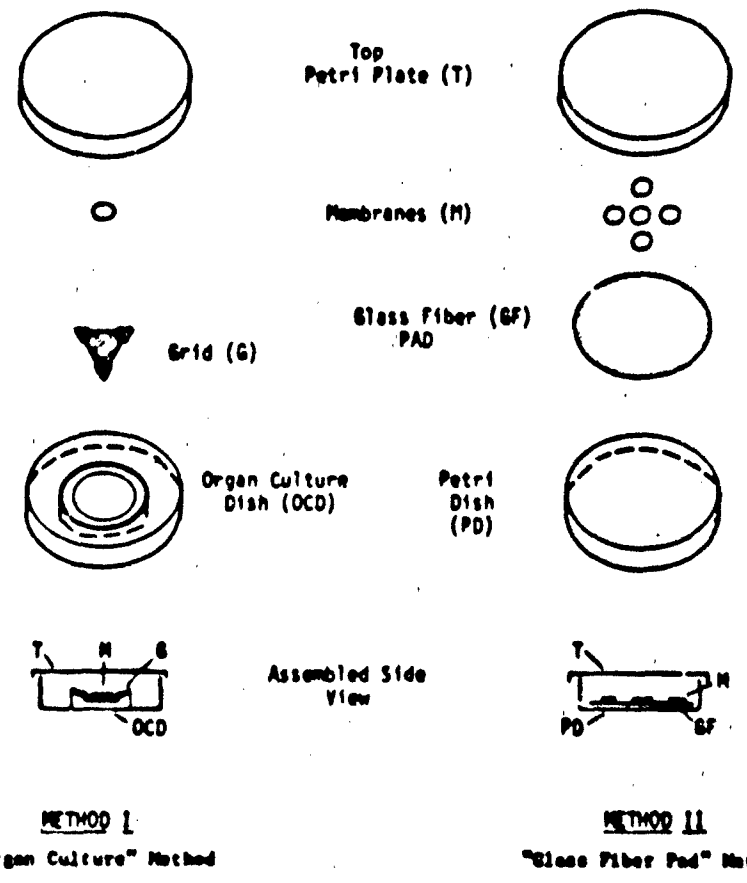


Figure 1. Schematic diagram illustrating two methods for culturing "raised" cutaneous epidermal cells at the air/liquid interface.

In method I, the seeded cells are grown in submerged cultures on the membranes for 5-7 days. A metal grid (G) is used to support the membrane on which the cells are growing. The cultures are then maintained in organ culture dishes (OCD) at the air/liquid interface for periods up to 2-3 weeks.

In method II, cells are seeded on membranes and grown in submerged cultures for 5-7 days. The membranes are transferred to a Petri dish (PD) containing a glass fiber filter in fresh medium where the culture on the membrane continues to differentiate at the liquid/air interface.

The bottom diagram for each method shows a longitudinal section through both of the culture systems

Specimens selected for TEM are fixed for a minimum of 16 hr in Karnovsky fixative, followed by three rinses in a 0.2M sucrose-0.1M sodium cacodytate buffer. Specimens are then post-stained for 2 hr in 2% aqueous osmium tetroxide, rinsed, stained for 1 hr in 2% uranyl acetate, rinsed again, dehydrated through a series of ethanol washes (70-100%), placed in a 1:1 ethanol:propylene oxide (PO) solution, then in absolute PO and infiltrated with a 1:1 mixture of PO and epoxy resin for 3-4 hr. Specimens are placed in Epon overnight, rinsed, transferred to fresh Epon and polymerized at 65% for 2 days. Embedded tissues are trimmed and sections are cut at 1 fm and stained with toluidine blue for light microscopic evaluation and for selection of regions to be cut for TEM thin sections. Thin sections, 70-100 nm, are cut with a diamond knife, post-stained with lead citrate and uranyl acetate and examined on an AEI Corinth 275 TEM at 60 Kv.

3) Studies of solvent effects

Because of the ease of preparing nylon membranes for keratinocyte cultivation, this membrane has been used to study the effect of various solvents on culture viability. A non-toxic solvent for BCES that will result in uniform distribution of the agent over the surface of the culture is being sought. The solvents under investigation include acetone, ethanol, hexane and methylene chloride (nonaqueous) and dioxane and dimethylsulfoxide (aqueous).

d. Results

1) Cultivation of keratinocytes (from the rat) on collagen gels.

As noted above, growth and differentiation of keratinocytes on Vitrogen was unsatisfactory because the collagen gel became unstable after 5-8 days of lifted cultivation. Attempts to alter the gel in an effort to overcome this problem were not successful. As a result, studies were initiated using collagens from other sources as substrata to support the cultures. As described below, combining Vitrogen 100 with other collagens provided an effective substratum for cultivation of keratinocytes from the cutaneous epidermis of the newborn rat.

Rat tail and rat tendon collagens supported keratinocyte growth equally well. By 24 hr following seeding on these collagens, numerous areas of confluent growth resulted so that almost 3/4 of the area of the culture vessel was covered. Confluence resulted after 4 days of incubation as compared to 6-7 days required for cultures grown on plastic. However, because of the low viscosity of collagen from both rat tail and rat tendon, cutting and lifting these cultures to the air/liquid interface was very difficult.

Mixing Vitrogen 100 with rat tail collagen in ratios of 1:1, 1:4, 3:1 and 4:1 (w/w) alleviated the problem of low viscosity with the latter collagen and provided the advantage of using collagen as a substratum. These mixtures have the optical qualities necessary for phase contrast microscopic observation of growing cultures and provide a solid substratum for convenient lifting. Cells attached and spread evenly on these substrata and confluence was reached as rapidly as it was with cells seeded on rat tail or rat tendon collagen per se. However, stratification proceeded more uniformly throughout the culture seeded on the collagen mixtures as compared with the softer collagens. The 1:1

mixture was used to study the stratification and differentiation of keratinocytes after incubation at the air/liquid interface. Figure 2 is a cross-sectional TEM micrograph of a culture grown on the 1:1 collagen mixture at the air/liquid interface for 7 days. As can be seen, the cells nearest the collagen gel had a cuboidal shape with a single nucleus, one or two large nucleoli, desmosomes, tonofilaments, numerous free ribosomes, secondary lysosomes and numerous microvilli extending from the cell surfaces. A few hemidesmosomes (not shown in Figure 2) have been found where basal cell membranes attached to the collagen gels. Large electron-dense inclusions resembling keratohyalin granules also have been observed. Up to 20 layers of flat, cornified cells resembling the stratum corneum have been found on the top of lifted cultures that had been incubated for 14 days at the air/liquid interface.

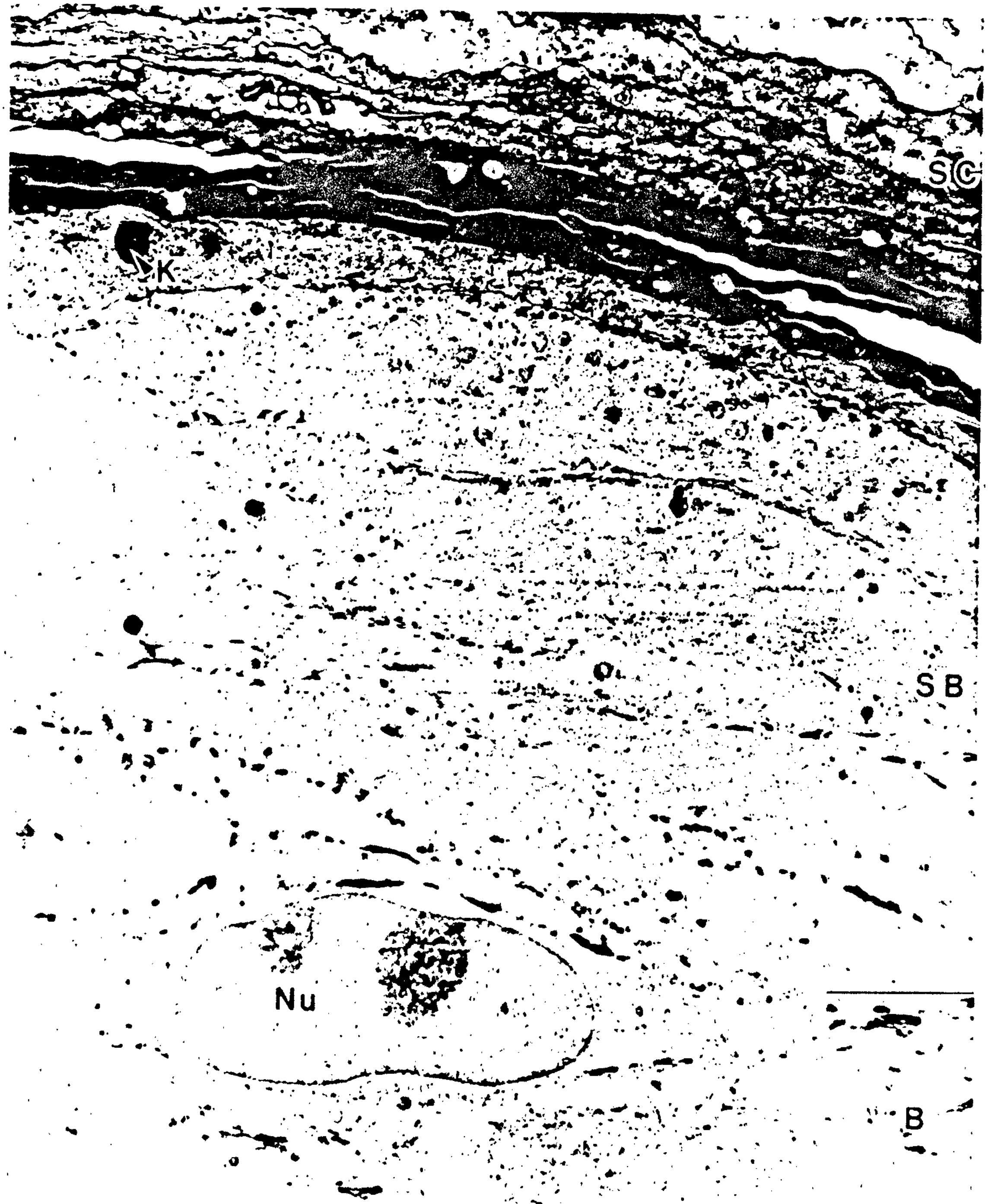
2) Cultivation of keratinocytes (from the rat) on Puropor nylon membranes.

Rat keratinocytes were seeded onto nylon membranes (13 mm in diameter), placed in multiwell plastic culture vessels and incubated until a monolayer developed (approximately 6 days). The growth of the cells was monitored by fixing samples taken at various times and preparing them for histological examination. After the monolayer was formed, the culture growing on the membrane was raised to the air/liquid interface by one of the two methods described above and incubated for 4-23 days, after which it was fixed for TEM observation.

TEM micrographs from specimens taken at selected intervals between 4 and 23 days showed that after incubating the lifted cultures for 4 days (Figure 3), 4-5 cell layers had developed, with the topmost cell layer containing degenerated nuclei. By day 7 (Figure 4), additional layers were formed and more flattened cornified cells appeared at the surface. Characteristic desmosomal attachment plaques were seen scattered throughout the cell layers. In older lifted cultures, i.e., those incubated at the air/liquid interface for 12 or 17 days (Figures 5-7), 6-7 nucleated cell layers and approximately 10 layers resembling the cornified cells of the normal epidermis had formed. Several epidermal morphological markers were observed via TEM, e.g., desmosomes, keratohyalin-like granules, tonofilaments and layers of squamous cells on the outer surface that form the stratum corneum of the epidermis in situ. The total number of cell layers appeared to have increased with time of incubation at the air/liquid interface and to have reached a maximum at approximately 14-23 days. The surfaces of the differentiated keratinocyte cultures on nylon were more uniform than those on collagen gels and the attachment of the cells to the substratum was more even on nylon as compared with collagen.

3) Cultivation of human newborn keratinocytes on collagen gels.

Attachment of cells was satisfactory on either a collagen or plastic substratum. Small groups of round cells were seen by 2-3 days after seeding. Clumps were connected by long cellular bridges several cells wide. Faster growth was observed on a substratum of Vitrogen 100 than on a plastic surface. Figures 8a and b show the relative sizes of a particular culture at 2 and 5 days on Vitrogen 100. By 21 days, cells on Vitrogen covered about 1/3 of a



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[illegible] epidermal cells on collagen [illegible] gel, [illegible] days sub- [illegible] keratohyalin (K), basal cells (B), suprabasal cells [illegible] stratum corneum [illegible]

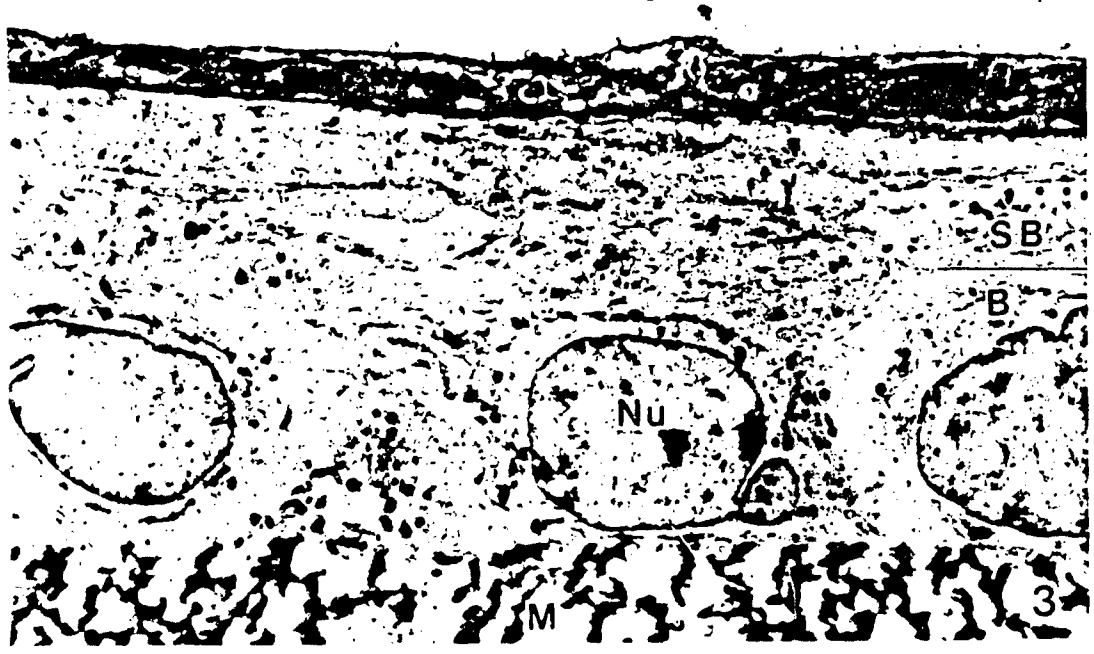


Figure 3. Cultured cutaneous epidermal cells on nylon membrane (M) for 4 days. Note cuboidal shape of basal (B) cell layers and nuclei (Nu) still retained in uppermost suprabasal (SB) layers. x5500

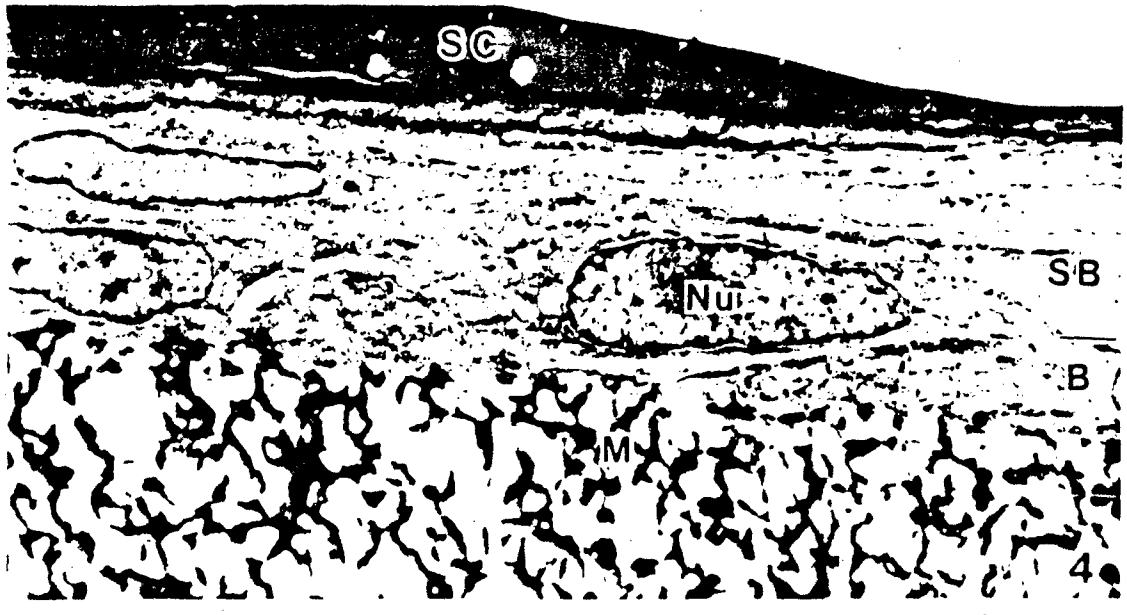
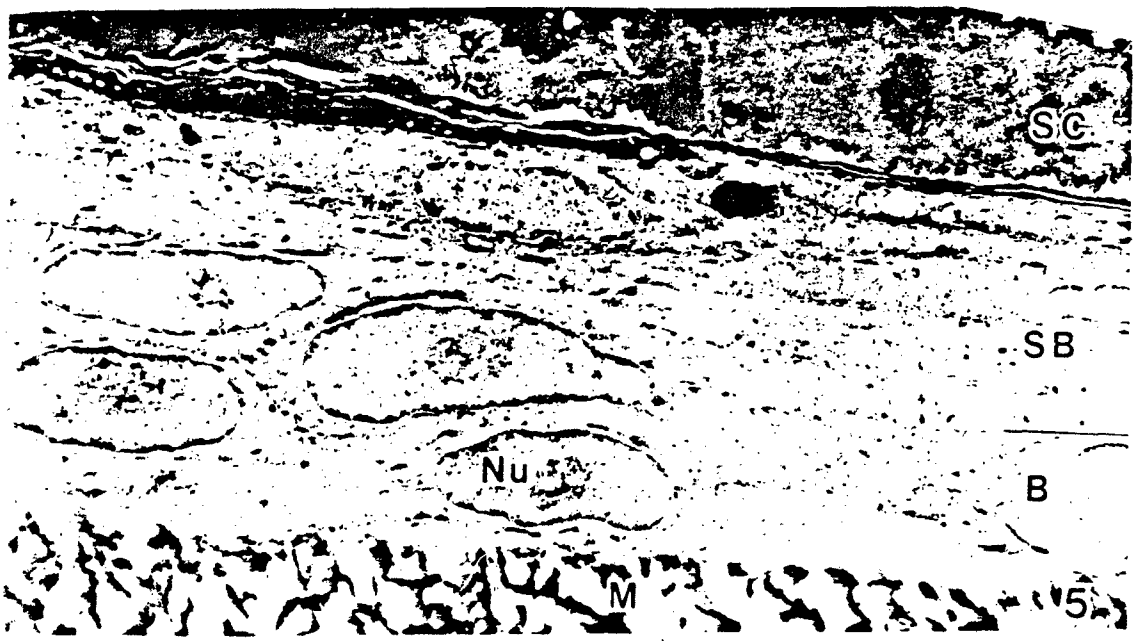
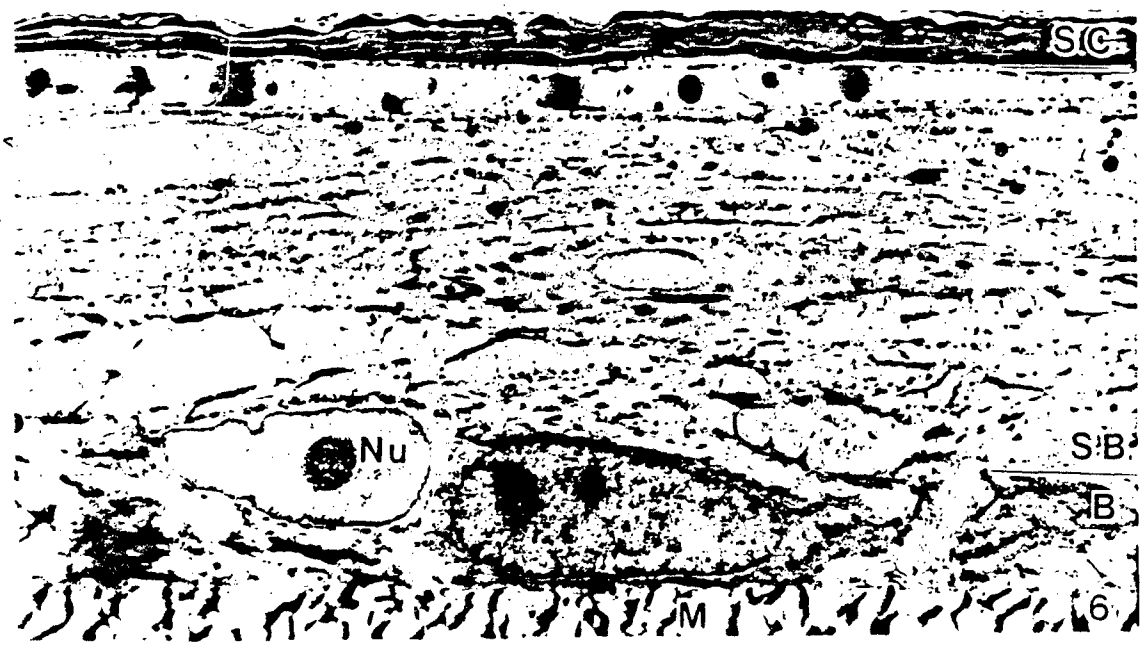


Figure 4. Cultured cutaneous epidermal cells on nylon membrane (M) for 7 days. Note the flat shape of the nuclei (Nu) and lower basal (B) cells and the increased number of thick layers in the suprabasal (SB) and immature cornified (SC) cells at the top of the culture. x5500



[illegible] Cultured cutaneous epidermal cells on nylon membrane M for 12 [illegible] to time growth at 7 days. Basal cells B , suprabasal cells SB , [illegible] SC , nucleus Nu . x5500



[illegible] cultured epidermal cells on nylon membrane M for [illegible] [illegible] layers in the stratum corneum SC , two [illegible] and the nucleus Nu in the basal layer B

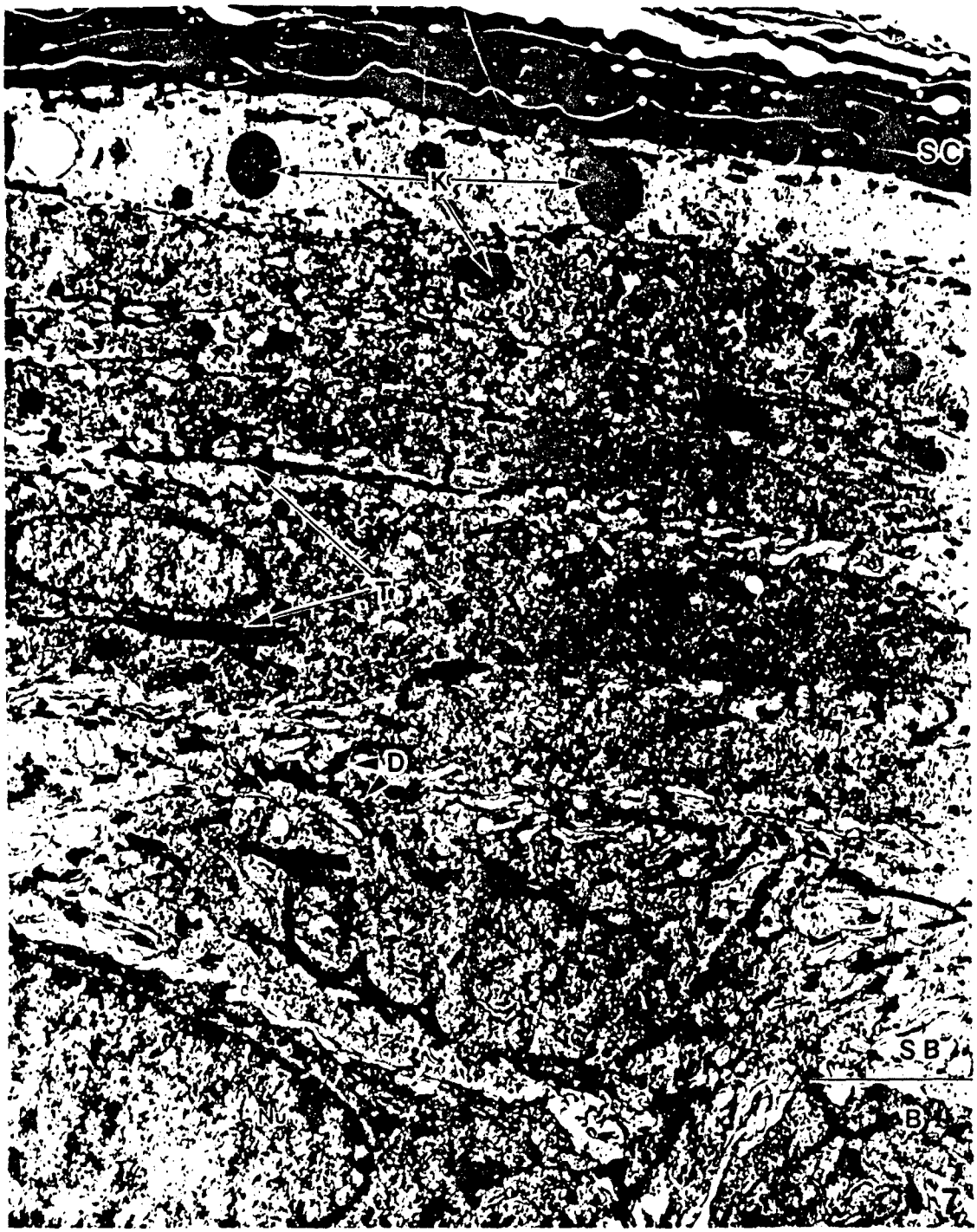


Figure 7. Cultured cutaneous epidermal cells on nylon membrane (M) for 17 days. Note the ultrastructural appearance of desmosomes (D), tonofilaments (T), and keratohyalin granules (K) in the suprabasal (SB) layers, and the stratified and cornified stratum corneum (SC) layer. x14000

Figure 8a. Human keratinocytes grown for 48 hrs on Vitrogen 100 collagen. x205

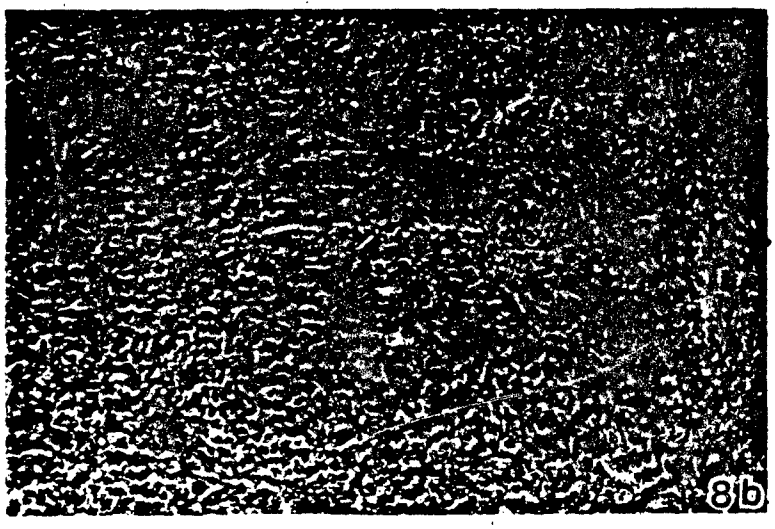


Figure 8b. Human keratinocytes grown for 5 days on Vitrogen 100 collagen. x205

35 mm dish. At this time the cultures showed several layers of nucleated cells (Figures 9a, b). No cornified layers were noted. After 13 days submerged, cultures were lifted and allowed to grow for 8 more days. Figures 10a and b are light and TEM micrographs of the resulting cultures, showing cuboidal basal cells and flat differentiated cells with typical filaments, desmosomes, and membrane-coated granules. Numerous microvilli and cytoplasmic extensions into the substratum were found. Cultures that had been grown at the air/liquid interface for 23 days showed up to 20 cornified layers (Figure 11). In contrast to the situation with murine cells, the Vitrogen 100 did not appear to have deteriorated even after 30 days of culture. Attempts to enhance the growth rate by using different media (e.g., 199 MEM, Ham's defined MEM), or by adding 10 or 20% human serum were not successful. Since the yield of viable cells is considerably lower from human foreskin as compared with rat epidermis, unsuccessful attempts were made to increase the yield by improving dermal-epidermal separation by increasing the concentration of trypsin to 1%, trypsinizing at room temperature, lengthening the time of exposure to trypsin and using collagenase plus trypsin. It is clear that lifted cultures of human epidermal keratinocytes can be grown successfully from newborn foreskin but the efficiency of the process (i.e., the yield of cells and the time to achieve the desired result) needs to be improved.

4) The effect of various solvents on cell viability.

Experiments have recently been initiated to identify the best solvents for applying BCES to the surface of the keratinocyte culture. The results of this study will be reported later.

Figure 9a. Electron micrograph of human keratinocytes grown submerged for 3 wks on Vitrogen 100 collagen (C). No cornfied layer is evident. x640

Figure 9b. Electron micrograph of human keratinocytes grown submerged for 3 wks on Vitrogen 100 (C). Note desmosomes (D), tonofilaments (T), absence of cornified layers. x60000

Figure 10a. Electron micrograph of human keratinocytes grown submerged for 2 wks and lifted for 8 days on Vitrogen 100 collagen (C). Note keratohyalin-like granules (K) and appearance of cornified layers (SC). x640

5) Provision of cultures (from the rat) for other subprojects.

During the period covered by this report, most of the cultures used in the other subprojects were grown submerged on plastic. Some of the data generated in Subproject 4, DNA Repair, were obtained by lifted cultures grown on collagen. Near the end of the reporting period, cultures on nylon membranes became available for use in the subprojects. This report does not include data obtained in the other subprojects for cultures grown on nylon membranes.

e. Discussion and conclusion

Although the epidermis developed in vitro is not identical to the tissue in situ, we are encouraged by the results obtained so far. Both collagen and nylon membranes serve as excellent substrata for the attachment and growth of human and rat keratinocytes, and incubation at the air/liquid interface results in stratification and differentiation of the cells with time. At present we have no information as to which of the two substrata supports growth and differentiation better. It appears, however, that cultures grown on nylon membranes should be the system of choice for the studies of the other subprojects. As noted above, membranes eliminate the heterogeneity in quality of the substratum which occurs when collagen is used. Use of membranes has the advantage of convenience and economy but the disadvantage of not allowing the continuous monitoring of growth because of the opacity of the substratum. On balance, however, the advantages outweigh the disadvantages, particularly because the morphology of the culture on membranes is more similar to that of the tissue in situ.

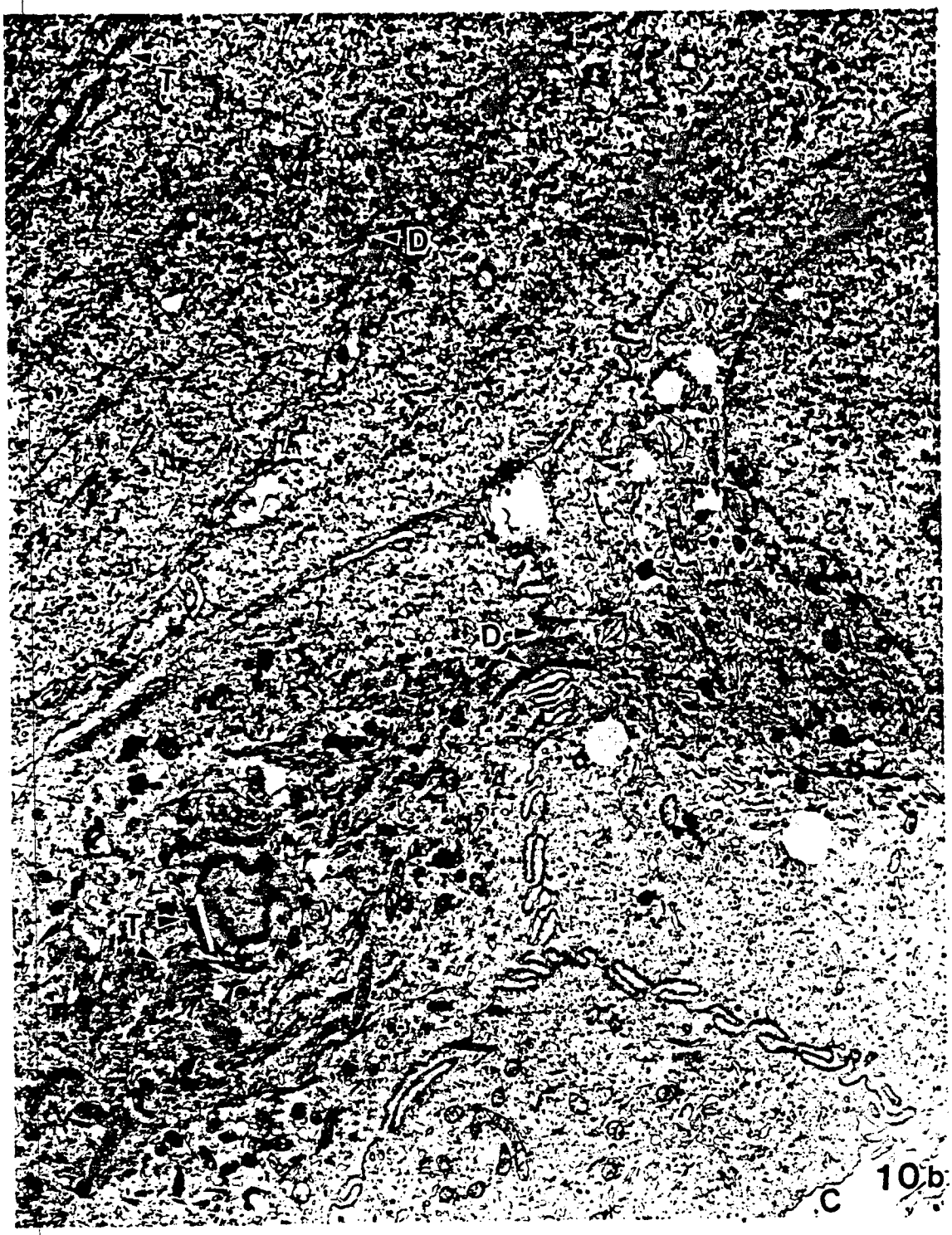


Figure 10b. Electron micrograph of human keratinocytes grown submerged for 2 weeks and lifted for 8 days on Vitrogen 100 collagen (C). Note tonofilaments (T) and desmosomes (D). x10000

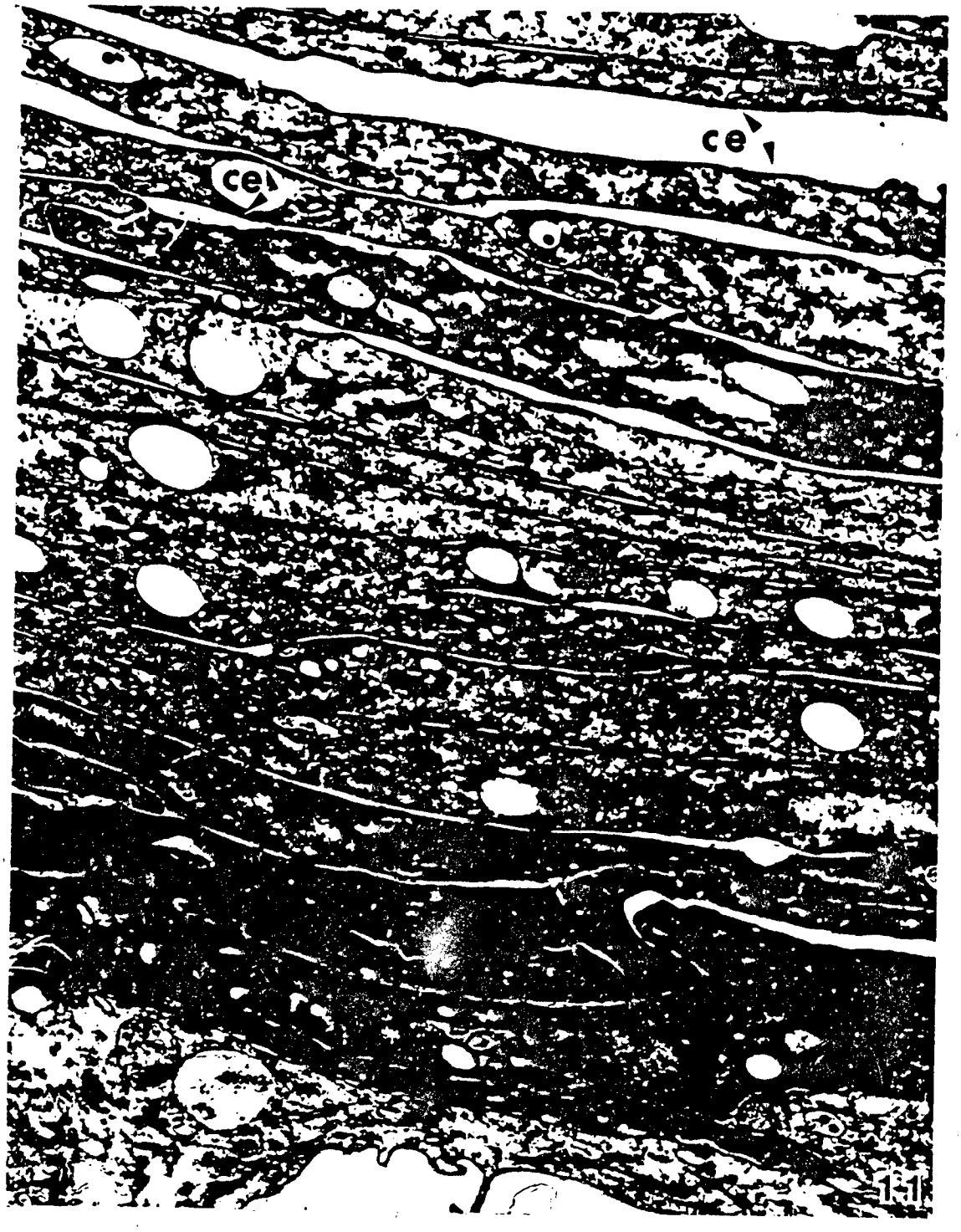


Figure 11. Electron micrograph of cornified layers of human keratinocytes, grown submerged for 2 wks and lifted for 23 days. x16000

In Subproject 3 techniques are being developed to obtain a purified population of basal cells, starting with the isolated epidermis or with a culture. These techniques can be used in the present project to compare the effects of exposure to BCES on biochemical parameters of germinative and differentiated cells. Purified populations of basal or differentiated cells can be exposed directly to BCES and the effects noted relatively quickly as compared with the case when exposing a culture and separating the populations after exposure.

2. Plans for Year 3

a. To make available lifted murine cultures on membranes (or on collagen, if requested) for the subprojects.

b. To ascertain the degree of biochemical similarity between the lifted culture and the tissue *in situ* (both animal and human).

The presence of the following biochemical markers for epidermal differentiation *in vivo* will be ascertained in the lifted culture grown on a nylon membrane:

1) Cell surface labeling by lectins (10, 11).

In situ, murine basal cells bind the I-B_4 isolectin of *Griffonia simplicifolia* (I-D-galactoside specificity) but do not bind agglutinin I from *Ulex europeus* (L-fucoside specificity). On the other hand, the differentiated layer immediately above the basal layer binds both of these lectins. Granular cells bind the A_2B_2 isolectin from *G. simplicifolia* (N-acetyl-D-galactosamine specificity), and cornified cells bind the II lectin from *G. simplicifolia* (N-acetyl-D-glucosamine specificity).

2) Immunocytochemical labeling for keratin filaments and histidine-rich protein.

Keratohyalin, containing histidine-rich protein (12) as a major component (13), appears in the granular cells *in situ*. Different species of keratin peptide can be visualized immunologically using monoclonal antibodies (14). An antibody which recognizes a keratin found only in the basal cell and another antibody which interacts with a keratin found in differentiated cells will be tested.

c. To develop a means of applying BCES on the surface of the culture that produces penetration like that for topical *in situ* application.

A satisfactory technique must prevent BCES from running off the edge of the culture and penetrating the lower cell layers from the side of the culture. The technique must also deliver a reproducible dose. [^{14}C]BCES will be used to develop a satisfactory application method of the toxicant. Experiments will be designed to show whether or not BCES applied topically to the culture reaches the basal cell by intracutaneous transit from the surface or from the sides of the culture. These experiments will also attempt to measure the rate and degree of penetration from the surface.

D. Subproject 3, Alkylation

Subproject Director: R. S. Mitra

1. Progress Report

a. Objective

This subproject seeks to understand the molecular mechanisms by which topically applied BCES causes the destruction of epidermal cells and to elucidate at which stage of differentation the epidermal keratinocyte is most susceptible to toxicity from BCES.

b. Background

Alkylation of DNA may occur on one strand or may lead to cross-linking of the chains (15). Procaryotic and eukaryotic cells (16, 17) are generally capable of repairing such lesions, provided that the damage is not extensive. Cell death probably results from a high level of DNA cross-linking or monoalkylation of DNA.

This report deals with the following aspects of the project: determination of DNA cross-links and single strand breaks after BCES exposure, and separation of populations of basal and differentiated cells.

c. Methods

1) Determination of DNA alkylation

Cultures of keratinocytes from newborn rat skin are prepared as described above. The cultures are exposed to 25 ug of [^{14}C]BCES per ml of medium 2 days after seeding. The cultures are incubated at 35°C for 1 hr and are then washed and harvested by the method of Liu et al. (18). DNA is extracted by treatment of the cell suspensions with SDS, proteinase K, and buffer-saturated phenol, and is then dialyzed exhaustively against 10 mM Tris-HCl and 1 mM EDTA, pH 7.5. Cesium chloride is added to the aqueous phase to yield a refractive index of 1.400 and the suspension is centrifuged at 48,000 x g for 24 hr at 20°C. Approximately 50 fractions are collected from each tube, and samples from each fraction are assayed for absorbance at 260 nm and for radioactivity.

2) Determination of DNA cross-links

The radioactive fractions from the lysates are pooled, and samples of the DNA are assayed for cross-linking using ethidium bromide as outlined by Brent (19). This assay requires that the fluorescence (excitation at 525 nm and emission at 600 nm) of a DNA solution be assayed before and after heat-denaturation. Ethidium bromide binds preferentially to double-stranded DNA (20). Therefore, covalent cross-linking will result in an insignificant loss of fluorescence, while native DNA will lose nearly all fluorescence after the heat-denaturation step.

3) Quantification of single-strand breaks induced by BCES as a function of BCES exposures

The presence of single-strand breaks in the DNA as a result of labilization of alkylated bases is assayed using a 5-20% alkaline sucrose gradient (0.7 M NaCl, 10 mM EDTA and 0.1 N NaOH). Because earlier experiments had failed to show an increase in single-strand breaks induced by BCES, it was thought that inappropriate lysis conditions (temperature and/or duration) had been used. In previous experiments, lysis incubations for 15 min at 60°C or for 5 min at 100°C had been tried. In the present experiments, cultures are exposed to BCES at 2.5 fg and 10 fg per ml in complete medium for 1 hr. BCES-treated and control cultures are harvested, and a sample of each cell suspension is treated with SDS (1% final concentration) and incubated for 1 hr at 37°C. The lysates are then exposed to alkali (0.25 N final concentration) and incubated for an additional 30 minutes in reduced light at room temperature, and then layered on alkaline sucrose gradients for analysis.

4) Relative sensitivity of the different epidermal cell types to BCES

In order to study the relative sensitivity to BCES of the cells in three epidermal nucleated strata, attempts have been made to separate different cell types in Percoll gradients. A continuous self-forming Percoll gradient is used to fractionate discrete populations of cells obtained by the trypsinization of epidermis from 1-2 day old newborn rats. The dissociated cells are resuspended in 38% Percoll and centrifuged in an SS34 rotor (Sorvall) at 30,000 x g for 15 min at 4°C. In the centrifugal field the Percoll particles sediment, forming a continuous density gradient in which the cells align themselves according to their densities. These gradient conditions have been optimized to separate what appear to be two distinct populations of cells based on density and morphology, i.e., basal and differentiated cells.

d. Results and discussion

1) Alkylation of DNA

The exposure of young (2-3 days) cultures of keratinocytes to labeled mustard resulted in an incorporation of 125 cpm per fg of DNA. When the labeled DNA was subjected to centrifugation in a neutral cesium chloride gradient, the radioactivity was found to be associated with the DNA (Figure 12). However, a considerable amount of radioactivity was found to be associated with 260 nm-absorbing material, which appeared at the bottom of the gradient. Since BCES is known to bind to other cellular components such as proteins and RNA and it is known that alkylated RNA is much more stable than alkylated DNA (21), it is probable that the high-density radioactive material was RNA.

An increase in single-strand breaks in DNA after exposure of keratinocytes to both 2.5 and 10 μg/ml of BCES was demonstrated when the lysate was heated to 37°C for 1 hr, with subsequent analysis in a 5-20% sucrose gradient (Figure 13). In spite of the absence of an internal marker which would have eliminated the possibility of variability in the gradient, the magnitude of the changes in the position of the peak indicates that the

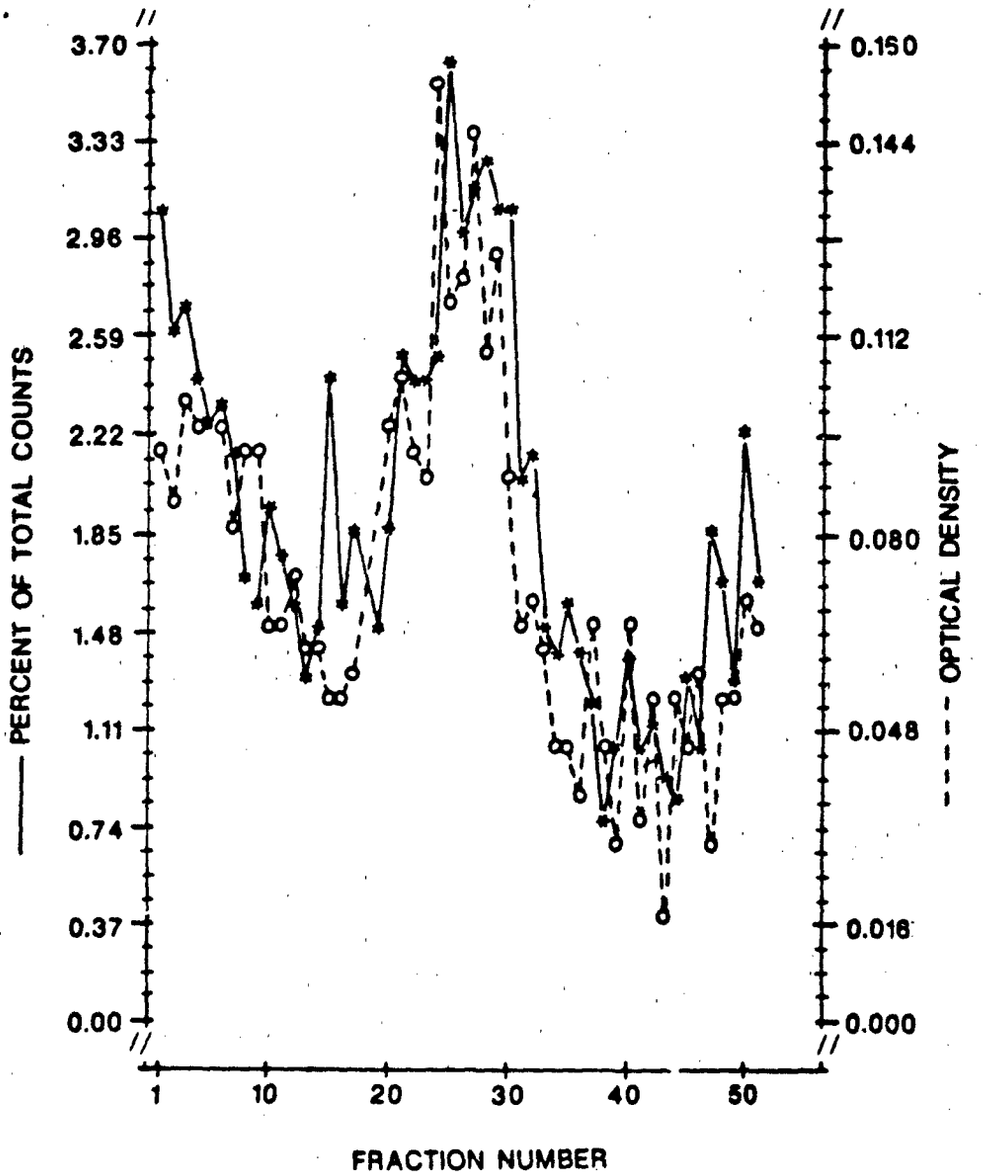


Figure 12. Neutral cesium chloride gradient centrifugation of DNA obtained from a culture of keratinocytes exposed to ^{14}C-BCES. The keratinocyte culture was exposed to 5μc of ^{14}C-BCES (25 μg/ml) and after the exposure period, the cellular lysate was prepared and lysate was subjected to CsCl gradient centrifugation. Details available in the Methods section (D.l.c.l.).

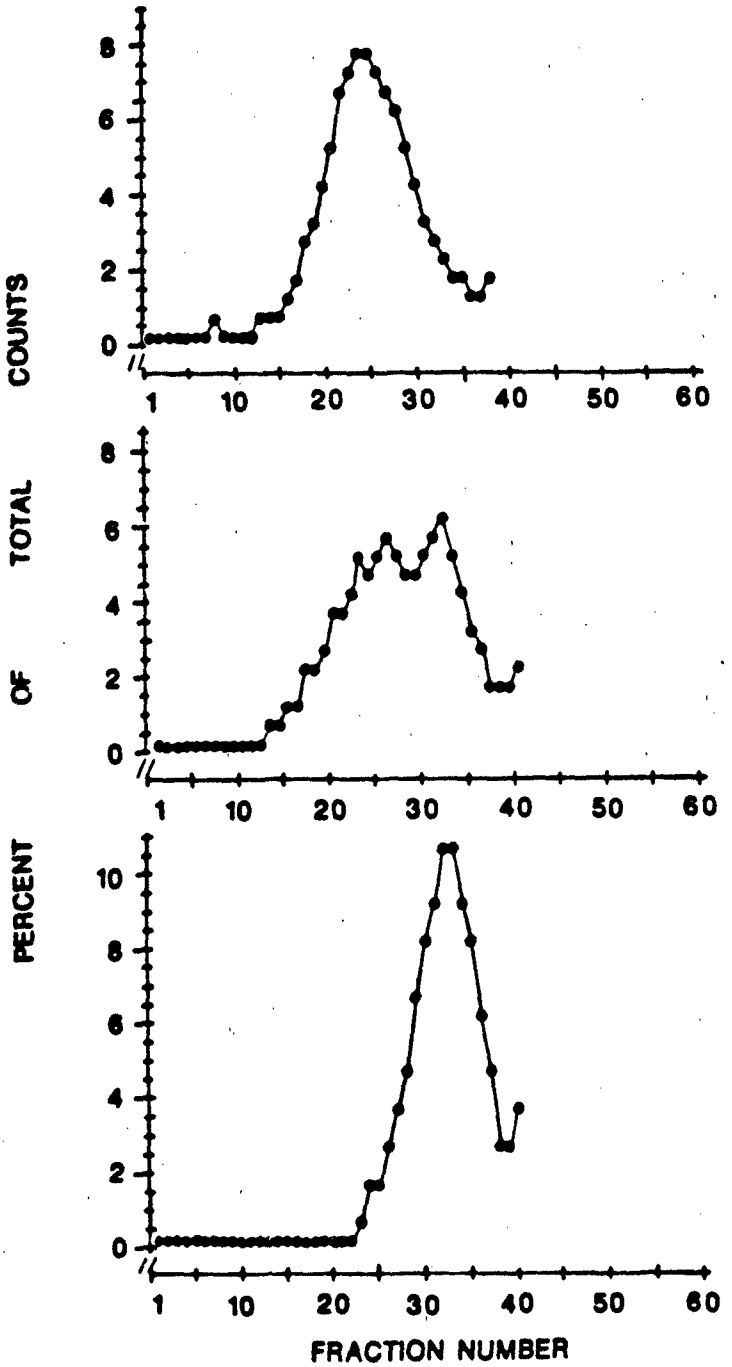


Figure 13. Alkaline sedimentation profiles of DNA in sucrose gradients, showing the appearance of single-stranded breaks after BCES exposure. The 3-day-old keratinocytes prelabeled with [^{3}H]thymidine were exposed to BCES per ml at 0 ug (a), 2.5 ug (b) and 10 ug (c) for 1 hr. BCES-exposed cells were lysed with SDS and incubated for 1 hr at 37°C and were then subjected to centrifugation in a 5-20% alkaline sucrose gradient. Details available in the Methods section (D.1.c.3).

observed shifts were not artifactual but represented a significant change in the number of single-strand breaks. Furthermore, this shift appears to be related to the dose of BCES, since the position of the peak from cultures treated with 10 ug/ml BCES was nearer to the top of the gradient than that from cultures treated with 2.5 ug/ml BCES. Unfortunately, heating the lysate at 100°C for 5 min resulted in extensive DNA degradation in both BCES-treated and control cultures, making interpretation of the data difficult. Hence, heating the lysate at 100°C for 5 min cannot be used for the estimation of the total number of apurinic sites after exposure to BCES followed by alkali.

When a population of dissociated keratinocytes from a trypsinized epidermis was centrifuged in a 38% self-forming Percoll gradient, the most dense band of cells at the bottom, which corresponded to a density exceeding 1.062 g/cc, consisted of at least 95% spherical cells (Figure 14) that exhibited a high nuclear to cytoplasmic ratio, a high percentage of exclusion of trypan blue (>95%) and a high efficiency of plating.

A less dense band of cells located at the top of the gradient, covering a density range between 1.033 and 1.049 g/cc, consisted of a heterogeneous population of cells with respect to size and shape. In comparison (Figures 15a and b) with the cells which predominate in the denser band, these less dense cells are much larger and appear flat and irregular in shape. The nuclear to cytoplasmic ratio exhibited by these less dense cells was much lower than that shown by cells in the denser band. In addition, these cells did not exclude trypan blue. In this population there was a cohort of cells, roughly 5-10%, which appeared to contain basophilic keratohyalin-like granules and probably represented granular cells.

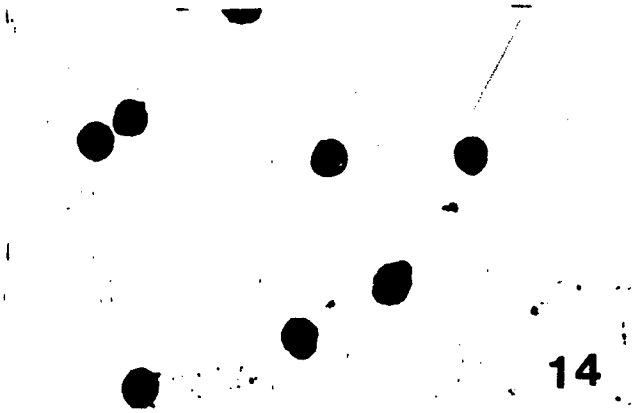


Figure 14. A smear of cells from the lowest band of a 38% Percoll gradient (α = 1.062 + g/cc). Stained with Weigert's Hemotoxylin. Dark cell clumps of basal cells. Based on these morphological characteristics, these cells were designated as basal cells. x 100

Figure 15a. A smear of cells from the upper band of a 38% Percoll gradient (α = 1.033 - 1.049 g/cc). Hemotoxylin stain. Note the lightly stained differentiated cells. x 100

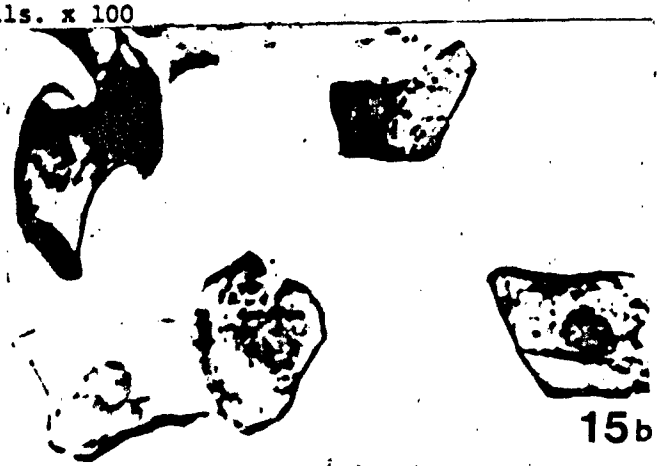


Figure 15b. A smear of cells from the upper band of a 38% Percoll gradient. Note the granular cells. x 100

2. Plans for Year 3

a. To estimate the total number of apurinic sites in DNA which result from exposure of a culture to BCES.

As noted above, heating of control cellular lysate at a high temperature resulted in the extensive degradation of DNA. Therefore, heating the BCES-treated cellular lysate at the high temperature necessary to convert all of the alkylated sites to apurinic sites introduces an error in the estimation. An effort is being made to optimize the lysis conditions (e.g., length and temperature of heating and the duration of incubation in alkali) in order to minimize artificial breakage. Once optimal conditions are determined, the number of alkylated sites on the DNA of basal and differentiated cells will be determined after "topical" exposure of the whole culture and after exposure of the two cell types separately. In order to define the results better, cellular DNA will be labeled by exposure to [^{3}H]TdR for exposed cells and [^{14}C]TdR for control cells. The [^{14}C]DNA will be used as an internal marker during subsequent experiments.

b. To determine the degree of alkylation.

As noted above, [^{14}C]BCES was found to be associated with purified DNA, which indicates that DNA was alkylated after BCES exposure. The degree of alkylation will be evaluated.

The alkylation of DNA should cause denaturation at the site of alkylation, and the degree of denaturation should be proportional to the degree of alkylation. S_1 endonuclease, an enzyme which, under appropriate conditions, degrades single- but not double-stranded regions of DNA, will be used to quantify the amount of single-stranded DNA. Keratinocytes in culture will be exposed to BCES as described earlier and the DNA from the treated culture will be extracted using phenol. The kinetics of hydrolysis of alkylated DNA samples by S_1 nuclease will be monitored by incubating a reaction mixture (22) containing the enzyme in 15 umoles of NaCl, 40 umoles of sodium acetate, pH 5.0, and 0.2 umole of $ZnCl_2$ per ml for 1 hr at 37°C. After incubation, non-digested DNA will be precipitated with perchloric acid. Samples will be centrifuged and absorbance at 260 nm will be determined in the supernatant solution as a measure of the single-stranded DNA in the reaction mixture. The degree of alkylation in DNA will be determined by thermal denaturation and by the kinetics of formaldehyde unwinding.

c. To determine the degree of cross-linking in DNA after BCES exposure

In previous experiments, the ethidium bromide assay failed to demonstrate the presence of cross-linking in the DNA. Lack of apparent cross-linking in the presence of demonstrated alkylation suggests that the BCES-induced cross-links may be heat-labile (15) or that the time of incubation used was not adequate for the formation of cross-links. A modification of the ethidium bromide assay which involves alkali denaturation and subsequent renaturation of BCES-treated DNA will be tried to assay the degree of cross-linking. In this method, DNA in a solution of 0.015 M NaCl-0.0015 M sodium citrate will be denatured with 0.08 M NaOH. After incubation for 10 min at 37°C, the solution will be neutralized to pH 6.7 with 0.017 M citric acid.

d. To separate epidermal cell types

While morphological characterization indicates that an isolated population of cells has been fractionated into an apparent basal cell fraction and a differentiated cell fraction, cell populations will be further characterized using biochemical markers such as lectin binding and the incorporation of [^{3}H]TdR. Efforts will be made to separate differentiated cells into spinous, granular and cornified populations bound to immobilized matrices (e.g., nitrocellulose membranes). Once these populations have been obtained, they may be directly exposed to BCES to investigate which cell type is preferentially affected. The degree of alkylation of the basal, spinous and granular cells will also be studied in vitro using exposure conditions equivalent to those required in situ to produce cytotoxicity.

E. Subproject 4, DNA Repair

Subproject Director: Rory Conolly

1. Progress Report

a. Objective

The overall purpose of this subproject is to investigate the role of DNA repair in the epithelial toxicity of BCES. This subproject will attempt to correlate the extent of repair of BCES-induced strand breakage in DNA with the severity of changes in biochemical and morphological indices of BCES toxicity that are being investigated in the other subprojects.

b. Background

During the first year of this project, the alkaline unwinding/hydroxylapatite chromatographic assay (23) for single-strand breaks in DNA was adapted for use with cultures of epidermal keratinocytes. The procedure was tested using N-methyl-N-nitro-N-nitrosoguanidine (MNNG) as the alkylating agent. Repair of MNNG-induced lesions in the DNA was also evaluated.

c. Methods

1) Alkaline unwinding/hydroxylapatite chromatographic assay

The following protocol has been developed for the alkaline unwinding/hydroxylapatite chromatographic assay for DNA damage in lifted cultures:

The 13 mm membrane containing a 7-9-day culture is transferred from the prefilter pad to a new culture dish and the culture is submerged in EBSS medium. Toxicant is applied at an appropriate dilution to the medium. At the end of the exposure, the medium is removed and the cells are washed with phosphate-buffered saline (PBS). Two ml of lysis solution (0.03 M NaOH, 0.01 M Na_2HPO_4, 0.13% SDS, 0.03 M EDTA Na, adjusted to pH 12.8 with 5 N NaOH) are added to initiate alkaline unwinding. Lysis proceeds at room temperature in the dark for 30 min. One ml of neutralizing solution (dilute HCl) is added to terminate unwinding. All the neutralized cellular suspension is chromatographed on hydroxylapatite to separate double-stranded and single-stranded DNA. DNA is quantified fluorometrically.

2) Assay for DNA-Protein cross-links

The following protocol has been developed for assay of DNA-protein cross-links:

After the toxicant has been applied and the cells washed with PBS, the cells are lysed for 5 min in 2 ml of lysis solution and neutralized. The lysate is then incubated with proteinase K (0.5 mg/l of Tris buffer, pH 7.4) for 30 min followed by 15 min in another 2 ml of lysis solution. After neutralization, double-stranded DNA is quantified as described above. Table 1 indicates the results of such an assay performed on a culture exposed to formaldehyde to generate DNA-protein cross-links.

Table 1. Assay of DNA-Protein Cross-links in Cultured Keratinocytes

Exposure	% Apparent Double-stranded DNA	
	- Proteinase K	+ Proteinase K
Control	56.4 ± 3.8	57.4 ± 1.7
300 μM Formaldehyde	61.2 ± 2.4	50.8 ± 2.4
500 μM Formaldehyde	68.6 ± 2.1	55.7 ± 3.3

d. Results

1) Determination of DNA damage in lifted cultures

Using the procedure described in Section c1, the percentage of double-stranded DNA was determined in lifted cultures exposed to 0.126 mM BCES for 90 min. The data in Table 2 show that exposure to BCES results in a decreased unwinding of double-stranded DNA in the alkaline lysis solution when compared with DNA from unexposed cells. Presumably this difference reflects cross-linking of DNA strands or DNA-protein cross-links in BCES-treated cells.

Table 2. Determination of Double-stranded DNA in Lifted Cultures

Exposure	% Apparent Double-stranded DNA
Control	26.3 ± 3.3
126 μM BCES*	43.5 ± 2.1

* Lysis time: 90 min.

2) Characterization of BCES-induced DNA damage and repair in submerged culture grown on plastic

The purpose of this study was to obtain a dose-response relationship for BCES-induced DNA damage and to investigate how changes in the duration of incubation of cells with BCES affected the amount of resulting DNA damage. This experiment also tested for repair of BCES-induced DNA damage. Figure 16 shows that, at 50, 100, and 200 uM BCES, 30 min. of incubation increased the percentage of double-stranded DNA, indicating cross-link damage. Conversely, 3 hr of incubation with BCES at these concentrations consistently yielded less double-stranded DNA than that in controls. It appears that at 3 hr, cross-links are converted into single-strand breaks and alkali-labile sites. An alternative explanation for these data is that at 3 hr, cytotoxic effects of BCES result in secondary DNA damage. The data may also be explained by some combination of these two effects. At 300 uM BCES, the percentage of double-strandedness is decreased after both the 30 min and 3 hr incubations. This probably reflects cytotoxic effects of BCES at this relatively high concentration.

Figures 17 and 18 illustrate the repair of BCES-induced lesions in cells incubated for 30 min and 3 hr, respectively. In Figure 17 cross-link damage predominates immediately after challenge and 12 hr later, single-strand breaks and alkali-labile lesions predominated. These data are consistent with a time-dependent repair of cross-link damage giving rise to single-strand breaks and alkali-labile lesions. It is also possible that cytotoxic effects of BCES contribute to or are responsible for this effect. Comparison of 12 hr repair in Figures 17 and 18 shows that the 3 hr exposure to BCES results in much greater loss of double-strandedness than does the 30 min exposure. This difference is interpreted as suggesting that the 3 hr exposure to BCES is cytotoxic to the cells. The percentage of double-strandedness decreases dramatically at all BCES concentrations tested 12 hr after termination of challenge.

3) Evaluation of cytotoxic effects of MNNG and BCES by exclusion of trypan blue

Since changes in the percentage of double-stranded DNA can be secondary to cytotoxic effects of chemicals, cytotoxic effects of BCES were monitored. The cytotoxic effects of the monofunctional alkylating agent MNNG were also assessed.

Cells were exposed to 50 uM BCES or 500 uM MNNG for 30 min. Control cultures received vehicle only. A solution of 0.075% trypan blue in PBS was added to the cells immediately after exposure. Exclusion of trypan blue was evaluated by light microscopy. There was no difference between control and toxicant-treated cells immediately after exposure. For both control and toxicant-treated cultures, the basal cells took up no dye. Some cornified cells appear to become stained. These data indicate that exposure to 50 uM BCES or 500 uM MNNG is not cytotoxic, as judged by this parameter, immediately after challenge. Determinations of cytotoxicity by this method for higher concentrations of BCES have not been made as yet.

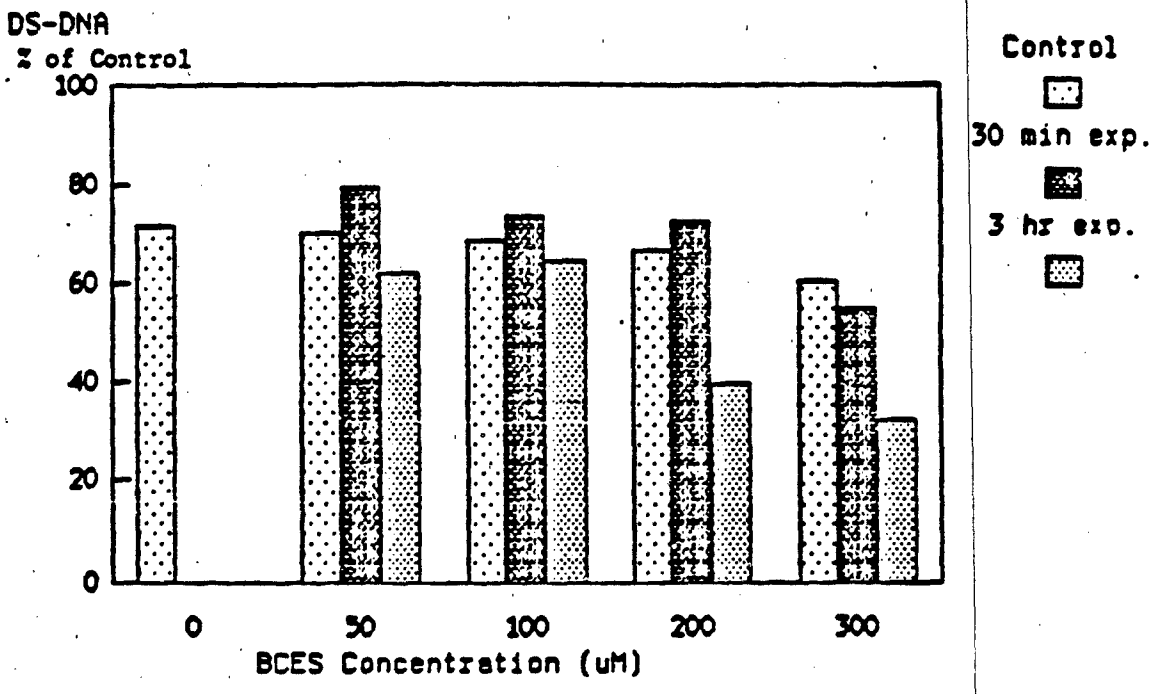


Figure 16. DNA damage resulting from exposure to BCES for 30 min and for 3 hr, with control. Cultures were exposed only to ethanol and methylene chloride. DS-DNA = double-stranded DNA. N=4 for control and 6 for cultures exposed to BCES from two experiments. Final concentrations of ethanol were as follows: 2.5% for 50 uM BCES; 5% for 100 uM BCES; 10% for 200 uM BCES and 15% for 300 uM BCES. Control cultures for each BCES concentration were exposed to ethanol at the same final concentration as in the experimental cultures. No significant effects on the % DS-DNA were observed with the ethanol-methylene chloride mixtures used in the controls.

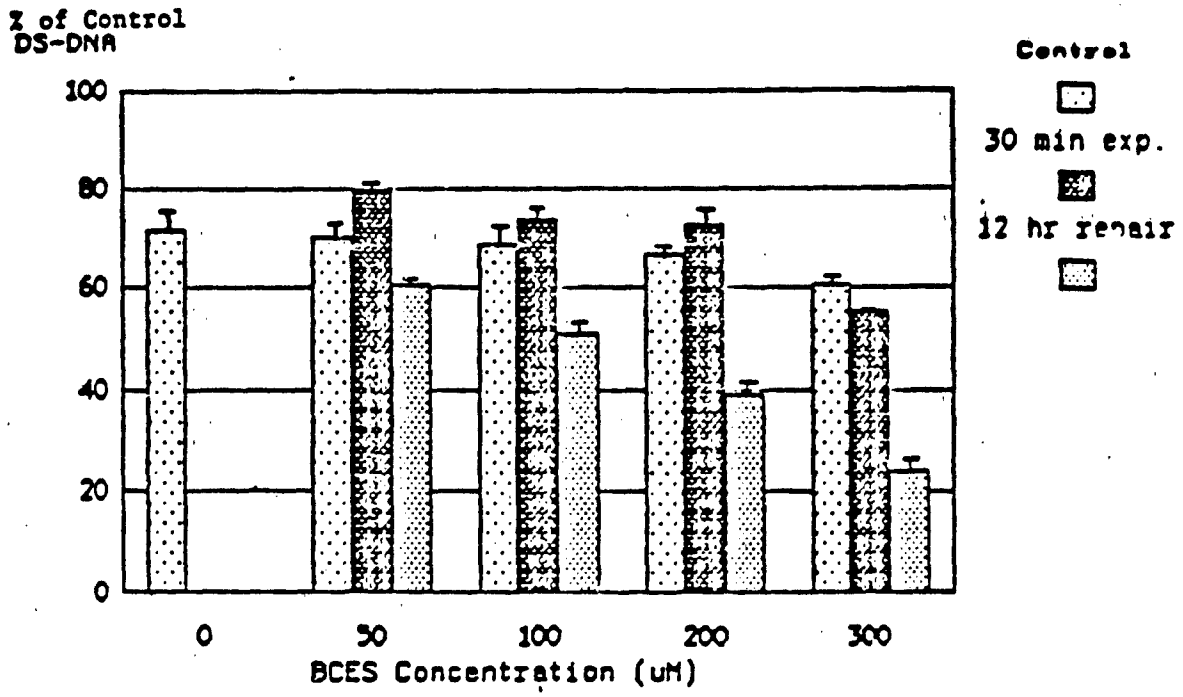


Figure 17. DNA repair during 12 hr of incubation exposure to BCES for 30 min. For experimental details see legend for Figure 16. Error bars indicate S.E.

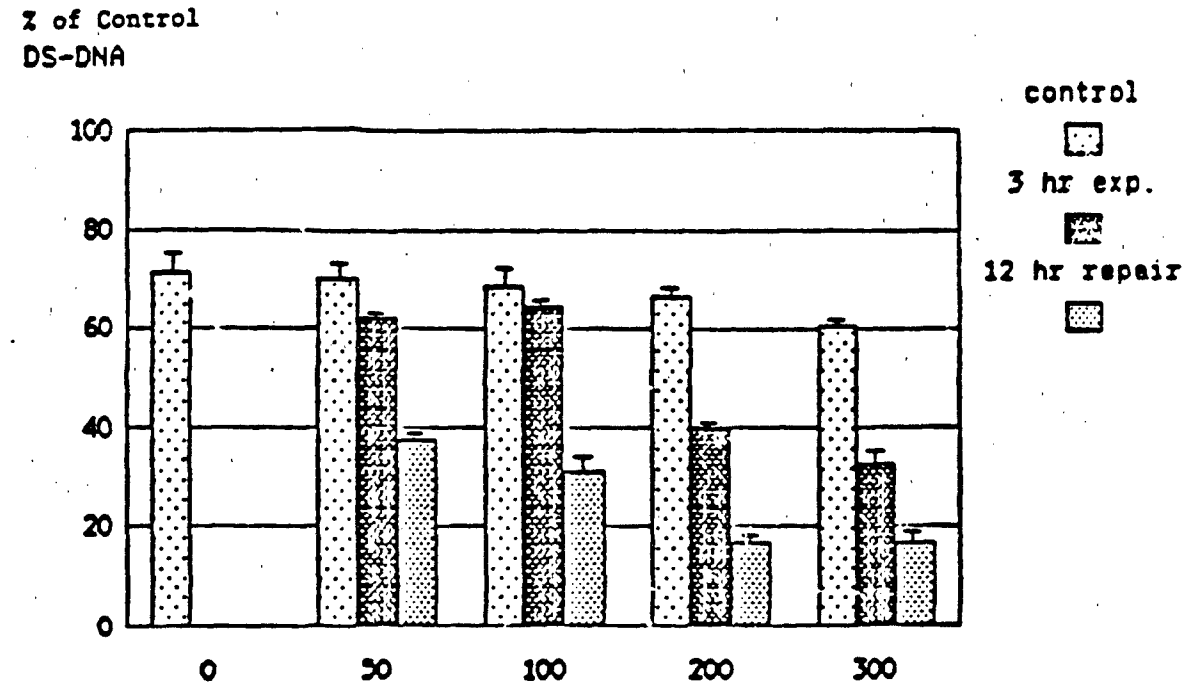


Figure 18. DNA repair during 12 hr of incubation after damage caused by exposure to BCES for 3 hr. For experimental details see legend for Figure 16. Error bars indicate S.E.

4) Comparison of basal and differentiated cells with respect to DNA damage and repair after exposure to MNNG and BCES

Cultures grown in low Ca^{2+} (0.06 mM) comprise basal and spinous cells in monolayer, while cultures grown in high Ca^{2+} (1.8 mM) contain cells which have differentiated further and have stratified.

Cells grown in submerged culture in high and low levels of Ca^{2+} were compared for sensitivity to BCES-induced DNA damage and for their ability to repair this damage. Figures 19 and 20 show that challenge of either high or low Ca^{2+} cells results in a similar degree of cross-link damage and that there was little difference between cell types in the rate of decrease in double stranded DNA in the 4 hr immediately after challenge. The similarity of basal and differentiated cells with respect to BCES-induced DNA damage contrasts with the differential sensitivity of these cell types to MNNG-induced damage. Figures 21 and 22 show that the percent of double-stranded DNA from exposed vs control cells was less in the more differentiated than in the less differentiated cultures when measured after 30 min of exposure to different doses of MNNG. This difference may represent a greater susceptibility in the more differentiated cells but there is a higher rate of repair in the less differentated cultures (grown in 0.06 mM Ca^{2+}). Whereas in the less differentiated cultures (Figure 22) there was an increase of double-stranded DNA from 37% of normal to 68% in 12 hr of incubation subsequent to exposure to 30 uM MNNG, the recovery was only to 40% of normal from 23% in the more differentiated culture (Figure 21).

5) Factors affecting susceptibility to DNA damage: MNNG pretreatment

Pretreatment with a low level (0.05 fM) of MNNG affects sensitivity of keratinocyte cultures to a DNA-damaging challenge dose of MNNG or BCES. To date, these studies have been conducted in submerged culture.

In the experiment depicted in Figure 23, keratinocytes, 6-7 days old, were pretreated (PE) with 0.05 uM MNNG for 1 hr at 8 AM, 2 PM and 8 PM for 2 consecutive days. Control (CPE) cultures received the vehicle only. At 8 AM on the third day, PE and CPE cells were exposed to a dose of 30 uM MNNG. Typically, 1/2 hr exposure to 30 uM MNNG decreased the amount of double-stranded DNA from 88% to 36% in both PE and CPE cultures. DNA repair was inferred from time-dependent return to the control level of double-strandedness after challenge. One hr after challenge, double-stranded DNA increased 22% in PE but only 10% in CPE culture. Two hr after challenge, PE cells had a further increase of 8% in double-stranded material, while in CPE the increase was 16%. No further increases in percent of double-strandedness were observed 6 hr after MNNG challenge.

In the preliminary experiment reported in Table 3, the data suggest that cross-linking, measured immediately after a 1 hr exposure to BCES, is diminished in MNNG-pretreated cultures. It is possible that pretreatment with 0.05 uM MNNG accelerates repair of BCES-induced DNA cross-links.

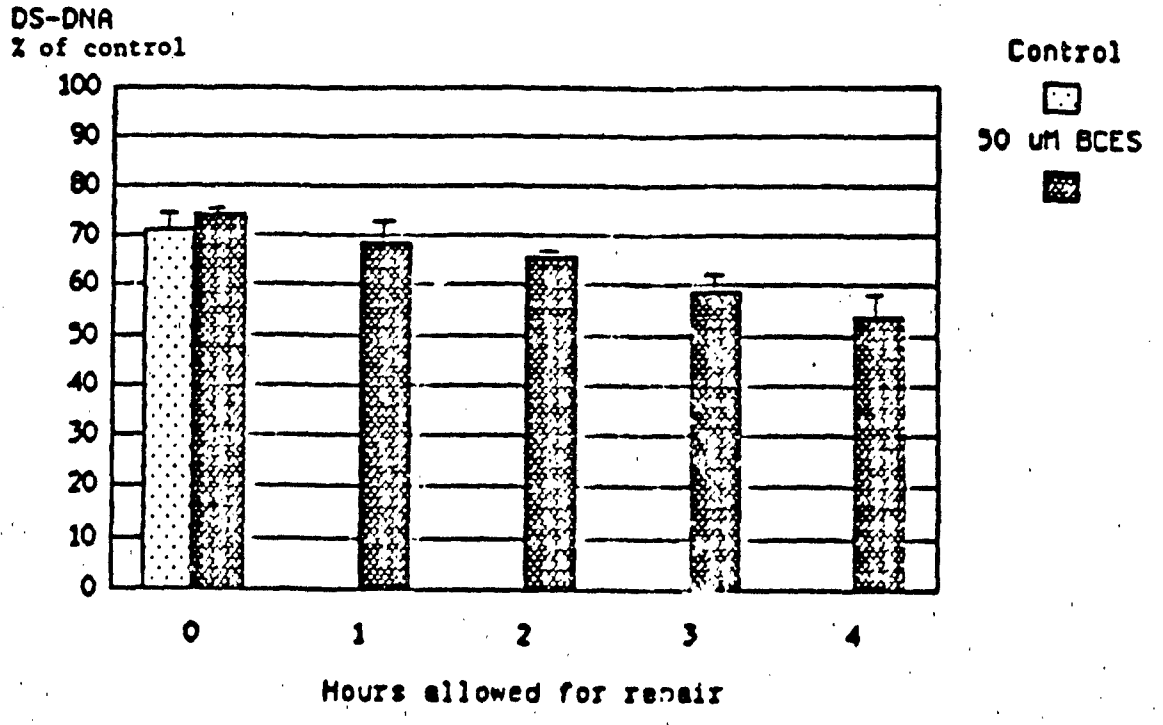


Figure 19. DNA damage and repair in basal keratinocytes exposed to 50 μM BCES for 30 min. Cells were washed to terminate exposure and refed with medium. Error bars indicate S.E. N=8 from two experiments.

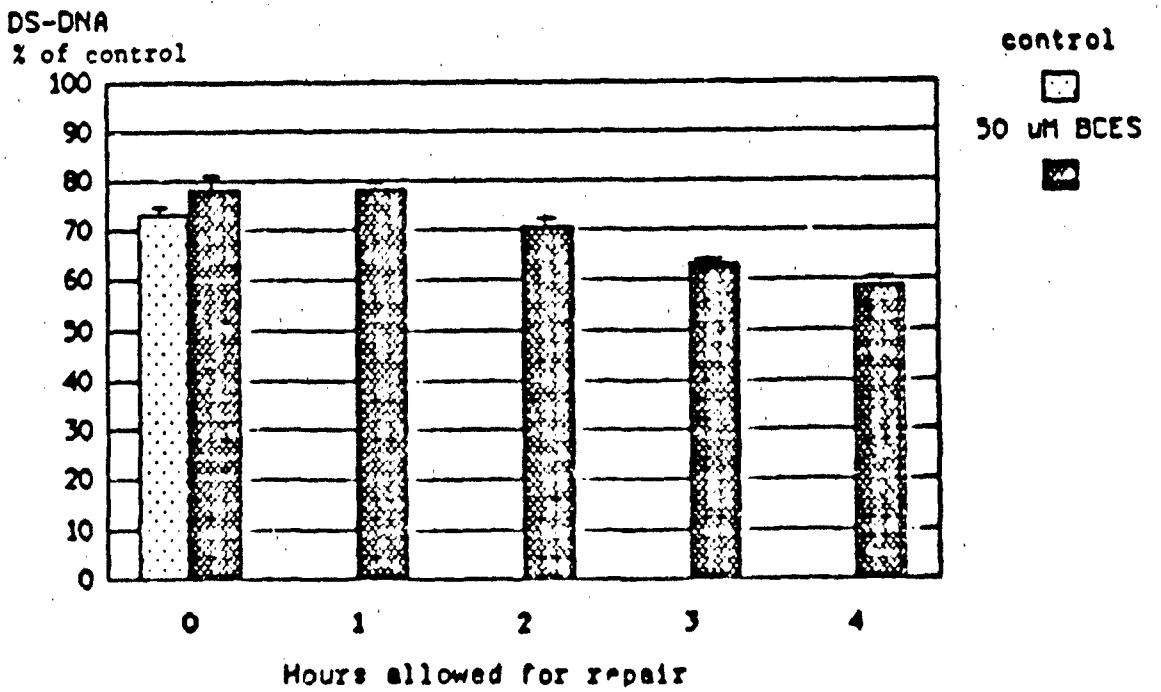


Figure 20. DNA damage and repair in differentiated keratinocytes exposed to 50 μM BCES for 30 min. Error bars indicate S.E. N=8 from two experiments.

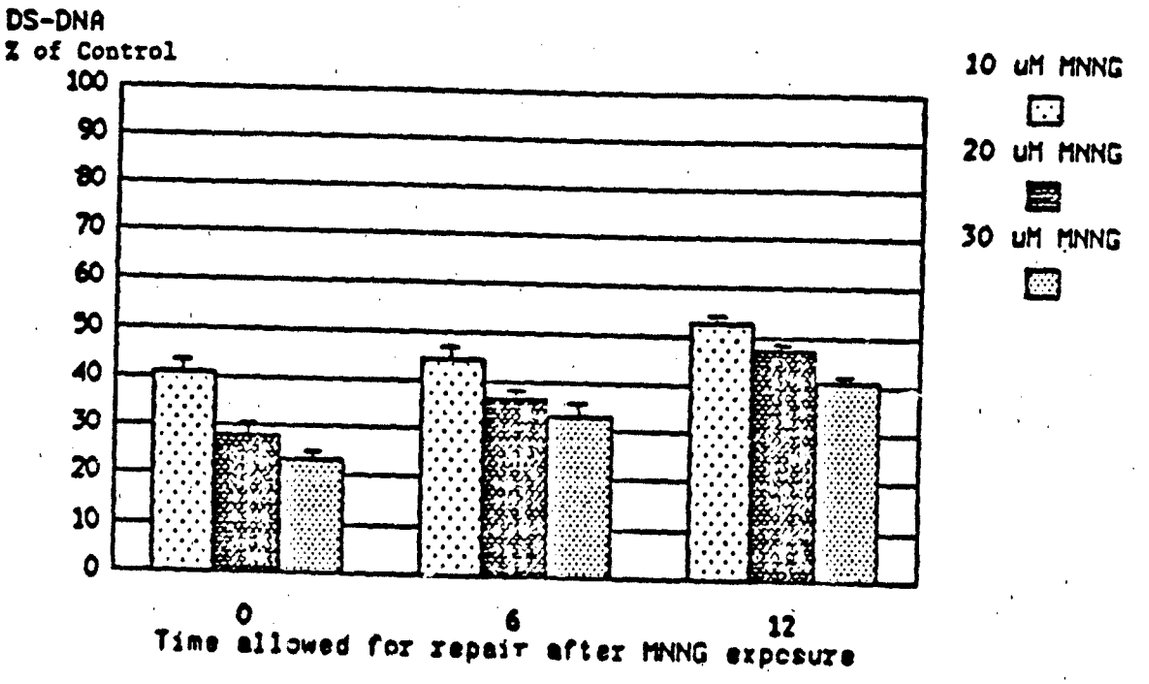


Figure 21. DNA damage and repair after exposure of keratinocytes to MNNG for 30 min. N=4 from two experiments. Differentiated cells from a 9-day-old culture grown in 1.8 mM Ca^{2+} were used.

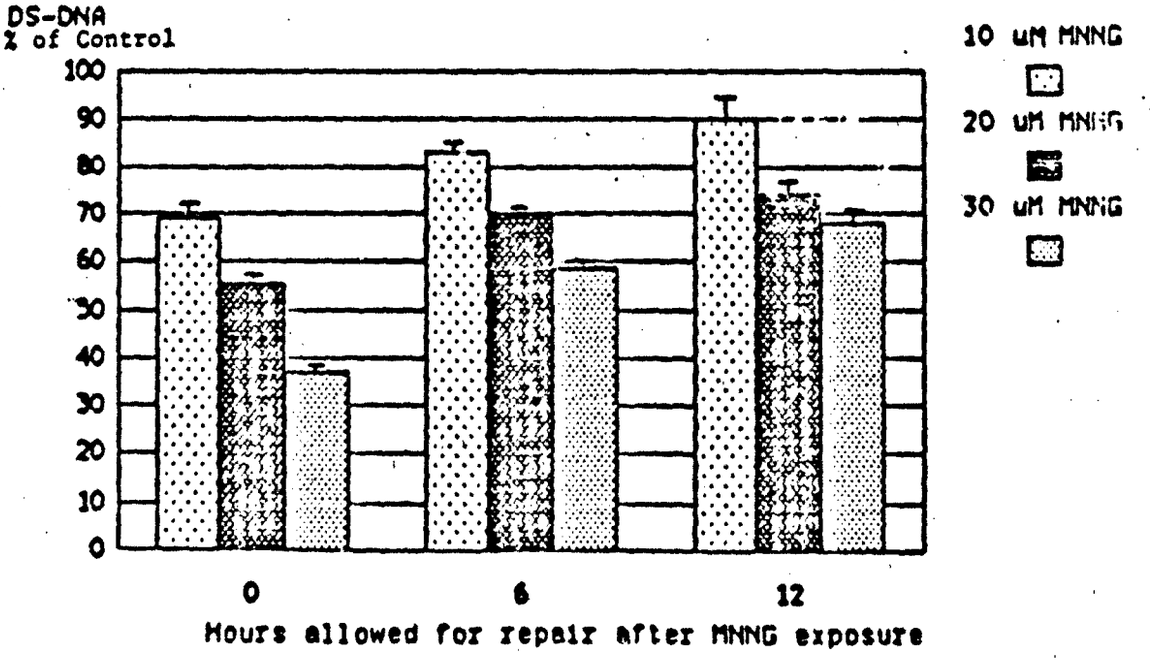


Figure 22. DNA damage and repair after exposure of keratinocytes to MNNG for 30 min. N=4 from two experiments. Basal cells from a 9-day-old culture grown in 0.06 mM Ca^{2+} were used.

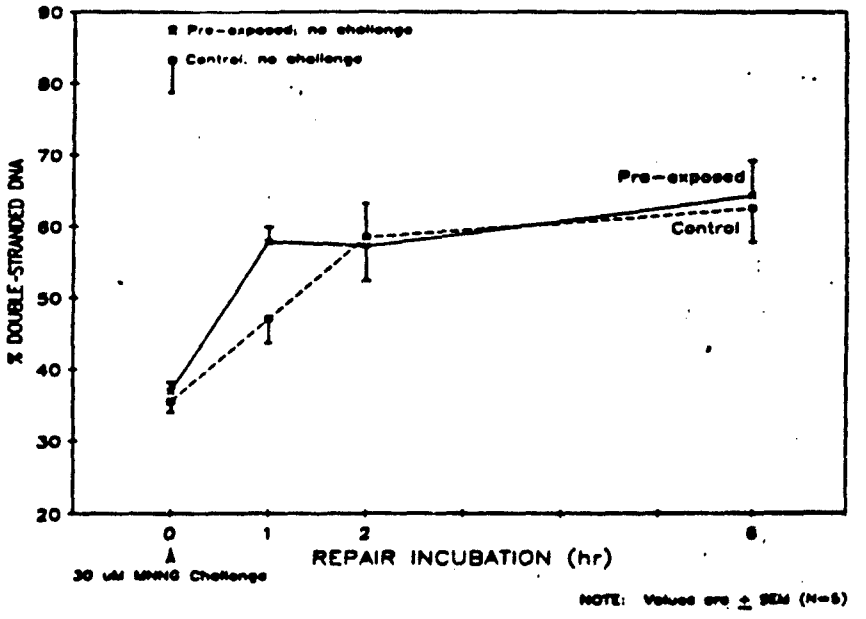


Figure 23. Effect of pretreatment with MNNG on repair of lesions in DNA resulting from exposure to BCES.

Table 3. Effect of MNNG Pre-exposure on BCES-induced DNA Cross-linking

Pre-exposure	Challenge (1 hr)	% Double-stranded DNA
Control	Control	28.2 ± 1.3
MNNG	Control	38.3 ± 9.1
Control	63 μM BCES	58.1 ± 0.5
MNNG	63 μM BCES	45.3 ± 5.0

2. Plans for Year 3

   a. To identify the reason for the pattern of DNA damage after BCES treatment, i.e., sites of apparent cross-linkage turning into alkali-labile sites presumably having single-strand break-sites

   b. To compare basal cells and differentiated cells with respect to DNA damage and repair

c. To identify the biochemical mechanism responsible for the pattern of DNA repair seen in BCES-treated cultures after MNNG pretreatment

d. To study the influence of cellular polyamine levels on susceptibility to BCES-induced DNA damage.

F. Subproject 5, Mitochondrial Metabolism

Subproject Director: Michael Brabec

1. Progress Report

a. Objective

The objective of this subproject is to determine whether BCES affects mitochondrial metabolism in a manner that contributes to the vesicant action of this compound.

b. Background

Chemicals that inhibit mitochondrial function are often potent and devastating toxic agents. Work conducted during the first year of this project examined the respiration of cultured keratinocytes exposed to putative mitochondrial inhibitors. Attempts to measure the respiration of keratinocytes were complicated by the fact that these cells grow only when affixed to a substratum. Cells were suspended by careful scraping of the plate with a rubber policeman and rinsing into a sterile 35 ml electrode chamber (2.5 ml assay volume) and respiration monitored with a Clark electrode The measured rates of respiration were highly variable. This was attributed to unresolved difficulties of obtaining a uniform cell suspension (clumps of keratinocytes were visible in the assay chamber) and adding a known cell concentration to the chamber. The operation of the electrode required that the solution be stirred with a magnetic stirring bar. The movement of the bar also appeared to be destructive as the observed rates of respiration declined if the incubation continued past several minutes. Therefore, alternative methods of monitoring mitochondrial function in situ intact cells were examined. The production of lactate and the utilization of glucose by the cultures were identified as methods of indirectly determining mitochondrial function. The efforts of the past year have concentrated on examining the response of keratinocytes grown in low Ca^{2+} (0.06 fM) to low-level exposures to BCES.

c. Methods

1) Incubation with BCES

BCES stock solution (10 mg/ml in methylene chloride) is diluted with absolute alcohol immediately before use to a final concentration of 2 mg BCES/ml. The appropriate volume of diluted BCES (4-25 ul, 10-300 uM final concentration) is applied to the culture. After 10 min of exposure, the medium is drawn off into 5% hypochlorite solution and fresh medium applied. The cells are incubated at 34°C and samples of medium are withdrawn at the appropriate times for determination of lactate and glucose concentrations.

2) Lactate determination

Lactate concentrations in the medium of cell cultures are determined as described by Brabec et al. (24).

3) Protein synthesis

Protein synthesis by cells in culture is estimated by adding either [^{14}C]leucine (5 uCi/plate, 30 Ci/mole) or [^{3}H]leucine (10 uCi/plate, 180 Ci/mMole) to the culture media for 30 min prior to the addition of one volume of 10% trichloroacetic acid (TCA). The precipitated protein is collected on filters and processed to remove soluble radioactivity as previously described (25). Radioactivity is determined by liquid scintillation spectrometry.

4) Glucose determination

Glucose concentration is determined in the culture media by the method of Bergmeyer et al. (26).

d. Results

1) Rates of lactate accumulation

Confluent 6-day-old cultures produced lactate in a linear manner for at least 10 hr (the longest period of incubation assayed). Dinitrophenol (50 uM) stimulated the rate of lactate production 170% over a 10 hr experiment. Addition of carrier solvent (ethanol:methylene chloride, 4:1) also stimulated the rate of lactate production. When the rates of lactate accumulation in the presence of BCES were corrected for the presence of solvent, BCES appeared to inhibit the rate of lactate accumulation. In a typical experiment, BCES at concentrations of 100 fM and 300 fM inhibited lactate accumulation by 20% and 33%, respectively. The pattern of lactate production at concentrations below 100 uM was not consistent. This was attributed to the volatility of the solvent and the difficulty of maintaining a constant concentration of BCES and solvent in the media while samples were being taken.

2) Protein synthesis

BCES did not alter the rate of protein synthesis in the cultures under our incubation conditions at concentrations up to 100 uM. A depression of protein synthesis was noted at 300 uM that appeared to depend on the time after the addition of BCES. Measurement of leucine incorporation 3.5-4 hr after BCES exposure revealed a decrease of about 50% in the rate of leucine incorporation. If the incubation period was prolonged another 2 hr, the rates of protein synthesis appeared to be similar to that of control.

3) Rate of glucose utilization

Cultures exhibited a linear rate of glucose consumption. A confluent culture, 15 mm dia., 5 days post-plating, utilized 0.05 mmole/l of glucose per hour. The effect of BCES of glucose metabolism by keratinocyte cultures is given in Table 4. As can be seen, rates of glucose consumption were increased by the carrier solvent. This effect of the solvent will require further examination.

Table 4. Glucose Consumption by Cultures of 9-Day-Old Rat Keratinocytes.

Treatment	Glucose Consumption (mmoles/l/hr)
Control	0.013
20 uM BCES	0.158
50 " "	0.167
100 " "	0.246
300 " "	0.089
Solvent Control	0.104

Concentration of glucose was determined in the culture media two hrs after 10 min of exposure to BCES. Three 15 mm plates were assayed per group.

e. Discussion and conclusion

Short exposures to low concentrations of BCES can inhibit mitochondrial metabolism, as revealed by the stimulation of the rate of lactate production in 7-9-day-old cultures grown in 0.06 mM Ca^{2+}. However, comparison of our results with those of others in the group (see Section E) indicate that the inhibition of mitochondrial function required a higher concentration of BCES than was required to produce DNA damage. The exposure conditions of these two studies were not equivalent in that in the present study, exposures were for a shorter time period (10 min vs. 30 min or 3 hr) and rates of lactate production at 12 and 24 hr post-exposure were not studied. Significant changes in DNA integrity and repair were reported at these longer periods of incubation (cf. Section E). Preliminary evidence also indicated that morphological changes occurred in cultures reared in the low calcium medium under conditions of prolonged exposure but low concentrations. The rate of lactate production in these cells in response to BCES has not been extensively examined. It is possible that the inhibition of mitochondrial function may not be an acute effect of BCES at these low concentrations (below 100 uM) but does progressively develop with time. The sensitivity of nuclear DNA to alteration by low concentrations of BCES raises the question whether mitochondrial DNA may be a target of BCES.

2. Plans for Year 3

a. To examine the effects of BCES on mitochondrial function in cells reared in low $Ca2^{+}$ medium and in basal, spinous and granular cells freshly prepared by density gradient centrifugation (cf. Section 2).

The parameters to be studied are listed below. The exposure conditions will be extended to cover the range of 10-300 uM BCES for 30 min. The cultures will be examined for periods up to 24 hr post-exposure. Preliminary work on the development and application of some of these procedures occurred during the past year. Since mitochondrial DNA may be a target of BCES, additional work will focus on the effect of BCES on the biogenesis of mitochondrial components in the separate cultures.

1) Mitrochondrial metabolism

Mitochondrial metabolism will be examined by monitoring the following parameters: Lactate production, glucose utilization and protein synthesis. These techniques and their application to basal cell cultures were described above.

2) Membrane energization

The ability of the mitochondrial membrane to localize the fluorescent dye Rhodamine 123 *in situ* will be examined in the resolved keratinocyte populations. Rhodamine 123 has the unusual property of binding only to the energized mitochondrial membrane. Application of this vital stain to keratinocyte populations produces a bright fluorescence outlining the mitochondrial profiles within the cells. Inhibition of mitochondrial function leads to a loss of fluorescence. Some preliminary work on this technique has been accomplished. The technique has been described by others (27).

3) Distribution of calcium

The distribution of calcium within the keratinocyte will be studied as a function of exposure to BCES in the resolved populations of keratinocytes. The distribution of calcium within the cell depends, in part, on mitochondrial functional integrity. Alterations in mitochondrial integrity lead to a redistribution of calcium, often associated with the expression of a cellular pathology (28). Preliminary work with this technique has occurred in test systems of non-keratinocyte cell lines. The technique will be calibrated by the addition of known metabolic inhibitors to the incubation system. The technique has been described previously (29).

4) Mitochondrial biogenesis

The effect of BCES exposure on the synthesis of mitochondrial DNA and protein in the keratinocytes will be examined. The extraction, purification and analysis of mitochondrial proteins from the keratinocyte cultures will be developed, with the basic procedures as outlined in De Wit and Brabec (30). Synthesis of mitochondrial DNA will be examined by adding 10 uCi/ml of [^{3}H]thymidine to the culture media and extracting the mitochondrial DNA as previously described (31).

G. Subproject 6, Metabolism

Subproject Director: A. Kulkarni

1. Progress Report

a. Objective

The results of an in vivo study reported by Davison et al. (32) suggest that the initial glutathione-BCES adduct undergoes further degradation to generate different metabolites. During this reporting period, efforts were focused on the study of keratinocyte enzymes that are likely to be involved in the metabolism of BCES. To understand and demonstrate the possible involvement of glutathione S-transferase (GSHTr) and peroxidase in BCES biotransformation, the use of purified enzymes was felt to be necessary. The progress made so far on this aspect is outlined below.

b. Background

Limited data are available on the various biochemical pathways involved in xenobiotic metabolism in keratinocytes. Therefore, during the first year, activities of several enzymes were measured in the appropriate subcellular fractions of the skin from 3-5-day-old rats. Substantial levels of GSHTr, peroxidase, esterase, alcohol dehydrogenase, aldehyde dehydrogenase, and lactate dehydrogenase were observed.

c. Methods and materials

1) Isolation of enzymes

Both GSHTr and peroxidase are being isolated from the initial 10% (w/v) crude homogenate of whole skin (epidermis and dermis) of 4-day rats (n=20-40/preparation) in 50 mM Tris buffer, pH 7.2, containing 0.25 M sucrose. The homogenate is centrifuged at 39,000 x g for 30 min at 4°C. The particulate material (pellet) which contains peroxidase, and the supernatant solution, which contains GSHTr, are used as starting materials and processed separately during enzyme purification.

d. Results

1) Purification of GSHTr

Although the presence of GSHTr in keratinocytes is documented, there are no reports on the purification and characterization of the enzyme. Significant GSHTr activity is observed (Table 5) in each whole skin preparation. Using glutathione coupled with epoxy-activated Sepharose 6B, affinity chromatography alone results in about 1,000-fold purification. An examination of the enzyme preparation at this stage by polyacrylamide gel electrophoresis (PAGE) revealed the presence of several proteins. The limitations imposed by relatively small sample volumes and failure of other conventional methods (e.g., hydroxylapatite column chromatography) led to the development of a new rapid high performance liquid chromatography (HPLC) method for further enzyme purification. Two different HPLC columns (Synchrom AX-300 and Protein-Pak DEAE) were tested. The HPLC conditions are being

optimized at present. The preliminary data (Table 5) are promising and it appears that with the use of HPLC, a substantial purification of GSHTr is possible.

2) Purification of peroxidase

The particulate fraction of the tissue is suspended in an extraction medium (homogenization buffer containing 0.5 M $CaCl_2$) and centrifuged at 39,000 x g for 30 min at 4°C. This "Ca-shock" treatment dissociates peroxidase from the membranes. The supernatant solution containing peroxidase is subjected to affinity chromatography using a Con A Sepharose column. The activity is eluted as a single peak when a mannitol gradient is applied. This procedure results in a 19-fold enrichment in specific activity with about 70% enzyme (Table 6) recovery. The enzyme can be further purified on a P-150 column, yielding an overall 82-fold purification and about 11% enzyme recovery (Table 6).

3) Estimation of BCES

The prescribed colorimetric method of BCES estimation based on the alkylation of 4-(p-nitrobenzyl) pyridine was standardized. This method will be used to estimate the amount of BCES applied or metabolized by the keratinocytes.

e. Discussion and conclusion

From the data given in Tables 5 and 6, it is clear that we have established the presence of GSHTr and peroxidase in the whole skin of the rat. The tissue levels are high enough to permit the isolation and purification of these enzymes. The purification methods employed appear to be satisfactory.

2. Plans for Year 3

a. To complete purification of cutaneous GSHTr and peroxidase

b. To examine the purified GSHTr and peroxidase preparations for their ability to metabolize BCES (The necessary methodology for the quantitation of metabolites will be developed.)

c. To estimate the relative distribution of GSHTr and peroxidase in epidermal keratinocytes and dermal fibroblasts as well as their activities in cultured keratinocytes

d. To quantify BCES metabolism by the keratinocytes in culture.

Table 5. Purification of Cutaneous GSHTr*

Purification Step	No. of Expts.	Specific (μmoles/min/mg)	Fold Purification	% Recovery
Crude extract	4	0.034 ± 0.007	-	100
Affinity chromatography	4	29.88 ± 7.00	1021 ± 368	106 ± 20
HPLC				
Synchrom AX-300	1			
Applied		12.5	-	100
Recovered		35.0	2.8	122
Protein Pak DEAE	1			
Applied		13.2	-	100
Recovered		216.0	16.4	15

* Fold purification reflects a change over previous step. Recovery is based on enzyme yield for a given step. GSHTr activity in all the fractions was monitored according to the procedure of Habig et al. (33), using 1-chloro-2,4-dinitrobenzene as a substrate.

Table 6. Purification of Cutaneous Peroxidase*

Purification Step	Specific Activity (Units/mg)	Fold Purification	% Recovery
Con A Sepharose			
Applied	0.63	-	100
Recovered	11.89	19	75
P-150			
Applied	12.0	-	-
Recovered	56.6	4.3	15

* The data represent the results of a typical experiment. Peroxidase activity is measured spectrophotometrically using guaiacol as a substrate, according to Chance and Maehly (34). One unit is defined as a change of 1.0 absorbance unit at 470 nm/min due to enzyme activity.

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