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LA THÈSE A ÉTÉ  
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EFFECTS OF TAPE STRIPPING, ETHANOL, PROPYLENE GLYCOL, AND  
VITAMIN A ACID ON HAIRLESS MOUSE SKIN:  
AN ELECTRON MICROSCOPIC CYTOCHEMICAL STUDY

by

William Roy Brown

A Thesis  
submitted to the Faculty of Graduate Studies  
through the Department of  
Biology in Partial Fulfillment  
of the requirements for the Degree  
of Master of Science at  
The University of Windsor

Windsor, Ontario, Canada  
1978

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## ABSTRACT

The response of hairless mouse skin to cellophane tape stripping, ethanol (95%), propylene glycol, vitamin A acid (0.3% in ethanol and 3% in propylene glycol) and various combinations of these treatments was investigated by electron microscopy. All treatments were applied once to the posterior dorsum and the mice were sacrificed between fifteen minutes and ten days after treatment. A total of 66 mice were examined. Chemicals were applied by pipet. Skin specimens were fixed in Dalton's potassium dichromate-buffered osmium tetroxide, dehydrated in ethanol, embedded in Epon-Araldite, sectioned and stained for transmission electron microscopy. Staining was done with uranyl acetate and lead citrate or with silver methenamine. Some sections were incubated in protease for protein digestion before staining.

Stripping four times with cellophane tape removed most of the stratum corneum and caused the development of widened intercellular spaces and microvillous cytoplasmic projections. As spaces formed, desmosomes appeared to be torn from one cell and engulfed by the other, and intramitochondrial inclusions of a type named 'corpora intra cristam', appeared. These changes began within an hour and subsided by about four days. Stripping also caused the development of occasional basal lamina breaks by 24 hours.

Ethanol (95%) without stripping produced a similar response, though less damaging. In addition, ethanol caused

the formation of lipid droplets in the cytoplasm of some keratinocytes by 24 hours and occasionally caused sloughing of the stratum corneum.

Propylene glycol (1,2-propanediol) produced no change.

Vitamin A acid (3% in propylene glycol) produced mild damaging effects with widened intercellular spaces and intramitochondrial inclusions, but no lipid droplets were found. Vitamin A acid (0.3% in ethanol) caused greater damage than vitamin A acid in propylene glycol and lipid droplets were found. With the triple combination of 0.3% vitamin A acid, ethanol and tape stripping, there was severe epidermal damage including basal lamina breaks and numerous lipid droplets, which rose to the stratum corneum producing a parakeratotic horny layer by the third day. Apart from the typical damaging effects, vitamin A acid caused inhibition of keratinization with decreased production of keratohyalin granules and tonofilaments and increased production of Golgi apparatus, endoplasmic reticulum and ribosomes.

Lipid droplets failed to stain with silver methenamine, indicating they contained no glycogen or glycoprotein. These droplets also failed to be digested by protease indicating they contained no protein. Intramitochondrial inclusions were digested by protease indicating a protein component, and they stained with silver methenamine, indicating the presence of glycogen or glycoprotein as well.

This thesis is dedicated to  
my son Jack.



## ACKNOWLEDGEMENTS

I would like to thank Dr. J.E.J. Habowsky for his unwavering support and encouragement through the two years of this study. I would also like to thank Ken Baker for his assistance in the preparation of some of the materials for this study. I would especially like to thank both Dr. Habowsky and Ken Baker for the easy-going spirit of cooperation which made work in our lab a pleasure rather than a trial. I would also like to express my appreciation to the other members of my supervisory committee, Dr. D.A. Cotter and Dr. N.F. Taylor, for their consultations regarding my work over the past two years, and for reviewing this thesis. I would also like to thank Mary Ellen McNaughton for helping me complete this work and especially for assisting me with the typing of the manuscript.

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## INTRODUCTION

### Hairless Mouse Skin.

Hairless mice were used in this study because their skin, being mammalian, is similar to that of humans in structure and function. These mice have become popular for dermatologic studies because their skin is so easy to treat and examine. Like human skin, mouse skin consists of keratinocytes arranged into basal, spinous, granular and keratinized layers, with dendritic melanocytes and Langerhans cells in the basal and spinous layers, spread regularly over the area of the epidermis. The most significant difference between mouse and human skin is the thickness. Mouse epidermis is about one third as thick as that of humans and has about eight or ten layers of corneocytes as compared to about twenty-five to thirty layers over most of the human epidermis; similarly, mouse dermis is thinner than human dermis (33).

### Tape Stripping

Stripping the skin with cellophane tape produces a mild mechanical injury to the epidermis and a fairly well-defined response. Wolf, in 1940 (54), was the first to strip the skin in this manner, but he was interested only in the layers of dead corneocytes stuck to the tape. It was Pinkus, in 1951 (36), who first described the skin's response to this treatment. Since then, many researchers (6, 15, 24, 38, 44) have studied the response to tape

stripping and agree that it induces a burst of mitotic activity in basal keratinocytes, with subsequent upward movement of these cells within the epidermis, resulting in regeneration of the corneocyte layer. This is probably the mechanism whereby the skin forms a callus in response to wear or pressure. This response is also similar to that after wounds such as cuts, scrapes, or burns (38). However, burns or penetrating wounds cause considerable cell damage and a breach of the basal lamina. There is also lateral epidermal cell migration to recover the areas and fill-in the gaps produced by cuts, scrapes and burns (23, 34). Thus, stripping is a simpler system for our studies of epidermal responses. Stripping is also useful as a pretreatment before the application of chemicals or drugs because stripping produces some of the characteristics of skin diseases such as psoriasis, with increased epidermal mitoses and a damaged corneocyte barrier.

#### Ethanol.

Ethanol has been used as a solvent for numerous chemicals in dermatological studies; in particular, 95% ethanol has been used as a solvent for the application of vitamin A acid to mouse skin in previous studies where the effect of the vitamin on the epidermal mitotic rate was examined (56). Early light microscopic studies indicated that ethanol caused some swelling of the epidermis but no cellular damage was detected (27). Ethanol has more recently been shown to cause increased epidermal mitotic activity (56)



and in another study (5), to damage epidermal cells when injected into the dermis, but there are no ultrastructural studies of possible damage from the topical application of ethanol.

#### Propylene Glycol.

Propylene glycol, like ethanol, is a popular non-polar solvent used in dermatological studies. It is known to be non-toxic and is used extensively in processed foods (32). It can be substituted in foods instead of its close relative, glycerol. Propylene glycol is used as the solvent in some common preparations of creams and ointments for such drugs as the corticosteroids used in treating various skin diseases (47). Because of its favourable properties, propylene glycol was used in this study as a solvent for vitamin A acid and the results compared with results obtained using ethanol as the solvent.

#### Vitamin A Acid.

It is well known that vitamin A is necessary for the normal differentiation of epithelial tissues (22). More than fifty years ago, dietary deficiency of vitamin A was shown to cause normal gastrointestinal epithelium to change from simple columnar to stratified squamous non-keratinizing epithelium, and the pseudostratified columnar ciliated epithelium of the respiratory tract became a stratified squamous non-keratinizing epithelium too (53). In both cases, the epithelia became more like the skin but did not form keratin. The skin also changes in response to vitamin A

deficiency with hyperkeratosis (19). Going in the opposite direction, vitamin A excess can transform embryonic skin, in tissue culture, to a ciliated mucus-secreting epithelium, as is normally found in the respiratory tract (3, 10, 30, 45, 48, 52). In studies of adult skin, excess vitamin A has demonstrated specific effects in altering and inhibiting keratinization (16, 18, 25, 55). Application of vitamin A acid can cause the benign skin tumor, keratoacanthoma, to secrete mucus (39).

Observations of the effects of deficiency and excess led to the clinical investigation of vitamin A as an anti-keratinizing factor. The early results using oral vitamin A were not outstanding and there were problems of toxicity at very high doses (37). However, over the past ten years, the acid form of vitamin A has been found effective, not orally, but in topical applications for the treatment of various skin disorders that are characterized by altered keratinization (37). Included in this list are palmar keratosis, lichen planus, lamellar ichthyosis, Darier's disease, solar keratosis, acne and psoriasis. In addition, synthetic analogues of vitamin A have shown promise in the prevention and treatment of certain epithelial cancers (46).

The mechanisms of vitamin A activity in the epidermis are not well understood and even the ultrastructural changes associated with this activity are not fully described (20). This study examined the ultrastructural changes in the skin caused by vitamin A acid in the

non-toxic solvent, propylene glycol, and these effects were compared to the damage from the mechanical injury of stripping, the chemical injury of 95% ethanol, the injury of vitamin A acid in ethanol, and other combinations of these treatments; these comparisons made it possible to distinguish those effects of vitamin A acid which were merely damaging from those peculiar to vitamin A activity.

## MATERIALS AND METHODS

MATERIALS.Chemicals.

All chemicals used were reagent grade. Ethanol (95%) was double distilled. Propylene glycol (1,2-propanediol) was obtained from Sigma Chemical Company. Vitamin A acid (all-trans-retinoic acid, Eastman) was obtained from Fisher Scientific Company. Protease from Streptomyces griseus, repurified type VI (6.5 units/mg solid) was obtained from Sigma Chemical Company.

Hairless Mice.

A colony of hairless mice was developed in Windsor by crossing hairless (hr/hr) mice from Jackson Laboratories, Bar Harbor, Maine, U.S.A., and hairy C3H mice. All of the  $F_1$  progeny from the initial cross were hairy since hairlessness is recessive. The hairless males (hr/hr) were bred again, this time with the  $F_1$  (hr/+) females. Half of the resultant  $F_2$  mice were hairless and half were hairy. Hairless mice grow hair normally at first, but at the first molt, at about one month, their hair falls out and never grows back. Only hairless males, six to ten months old, were used in this study.

TREATMENTS.Tape Stripping.

Cellophane tape was applied to the posterior dorsal

skin surface and carefully patted down, to achieve adhesion. The tape was then pulled off by doubling it back on itself, as described by Pinkus (36). This removed a fairly complete layer of corneocytes. Stripping was repeated several times to remove several layers of corneocytes. Complete stripping removed all but the last layer or two and left the skin glistening or shiny and slightly red, but still dry. After some practice, this could be achieved with about six or eight tape strips. We chose to use four strips for our experiments because it usually left two or three corneocyte layers intact and caused no visible damage to the underlying live epidermal cells, yet would consistently induce the typical response to injury.

#### Chemicals.

All chemical treatments were applied once to the posterior dorsal skin of the mice with a pipet.

Mice were killed and skin specimens surgically removed at various times after treatment, as shown in Table I.

TABLE I

TREATMENTS AND TIMES

TIME AFTER TREATMENT	TREATMENTS						
	STRIPPED	ETHANOL 95%	PROPYLENE GLYCOL	VITAMIN A ACID, 3% IN PROPYLENE GLYCOL	STRIPPED & ETHANOL	VITAMIN A ACID 0.3% & ETHANOL	VITAMIN A ACID 0.3% & STRIPPED
1/4 hr	X				X		X
1 hr	X				X		X
3 hrs	X	X	X	X	X	X	X
6 hrs	X				X		X
12 hrs	X	X	X	X	X	X	X
18 hrs	X	X			X		X
24 hrs	X	X	X	X	X	X	X
36 hrs	X	X			X		X
48 hrs	X	X	X	X	X	X	X
60 hrs	X	X			X		X
3 days	X	X	X	X	X	X	X
4 days	X	X			X		X
7 days	X	X			X		X
10 days	X				X		X

X - indicates one mouse studied

### TISSUE PROCESSING.

Mice were killed by neck fracture and skin specimens surgically removed. These specimens were sliced into 1 mm by 2 mm pieces while immersed in fixative. All specimens were fixed in Dalton's (9) potassium dichromate-buffered osmium tetroxide (1%) at 4°C, pH 7.4 for two hours (see Appendix I). The specimens were then dehydrated in graded strengths of ethanol (see Appendix II) and embedded in Epon 812 - Araldite (see Appendix III). Ultrathin sections (silver and gold) were cut on a Reichert OmU2 ultramicrotome with a Dupont diamond knife, and stained with uranyl acetate and lead citrate or with silver methenamine, before examination in an RCA EMU 3H or Philips EM 201 electron microscope. Some sections were digested with protease before staining.

### STAINING AND CYTOCHEMISTRY.

#### Uranyl Acetate and Lead Citrate.

Uranyl acetate stain was prepared by making a saturated solution in 70% ethanol, as modified from Watson (50). Sections on copper grids were floated section-side down on the uranyl acetate solution, in the dark, for about fifteen minutes, then rinsed in distilled water and dried. These grids were then floated section-side down, in the dark, on a solution of lead citrate which was prepared as described by Reynolds (42). The grids were then rinsed in a 0.02 N solution of NaOH and then in distilled water and dried for viewing in the electron microscope.

### Silver Methenamine.

Silver methenamine stain was prepared by mixing 8 cc of 3% hexamethylenetetramine with 1 cc of 5%  $\text{AgNO}_3$ , then adding 1 cc of 2% borax, as described by Movat (29). Sections were transferred between solutions with a wire loop. Because Dalton's fixative contains potassium dichromate, we were able to omit the usual ten minute incubation in 1% periodic acid and transferred sections directly to the silver stain. The silver staining solution, with floating sections, was placed in an oven at  $55^\circ\text{C}$  until the sections and also the staining solution began to darken. This usually takes about  $\frac{1}{2}$  hour. Sections were then floated on distilled water for rinsing and transferred to 3% sodium thiosulfate for two minutes. Then they were rinsed again on distilled water before being transferred with a wire loop to copper grids for viewing.

### Protease Digestion.

Protease digestion was performed according to the method of Monneron and Bernhard (26). Ultrathin sections were floated on a 10% solution of periodic acid for thirty minutes, then rinsed by transferring them to distilled water. The sections were then floated on a 0.5% solution of protease (6.5 units/mg solid) at pH 7 and  $40^\circ\text{C}$  for periods between thirty minutes and two hours. They were then rinsed on distilled water and transferred to copper grids. Some of these digested sections were stained with uranyl acetate and lead citrate before viewing; others were left unstained.



## RESULTS

Tape Stripping.

When stripped with cellophane tape, the outer layers of corneocytes were easier to remove than the layers most recently formed over the live epidermal cells. The last corneocyte layer was particularly difficult to remove. Stripping as few as five times can remove all except the last layer (Fig. 1) while continued stripping, up to 24 times, usually failed to remove the last layer (Fig. 2). Stripping four times removed most of the stratum corneum but produced no visible damage to the live epidermal cells. However, the epidermis reacted to the stripping, a short time later, with the development of widened intercellular spaces and microvillous cytoplasmic projections (Figs. 3 & 4). Spaces began to form first in the basal layer, then the spinous layer, and finally the granular layer; they were most extensive in the basal area and decreased toward the granular layer.

As intercellular spaces formed, desmosomes appeared to be torn from one cell and engulfed by the other (Fig. 5). In some cases, holes were torn in the plasma membrane where desmosomes had been (Fig. 5). The enlargement of intercellular spaces began within an hour, reached a maximum by about 24 hours, then slowly subsided over the next few days (Table II). As the spaces decreased, the cells re-established contact through the interdigitation of

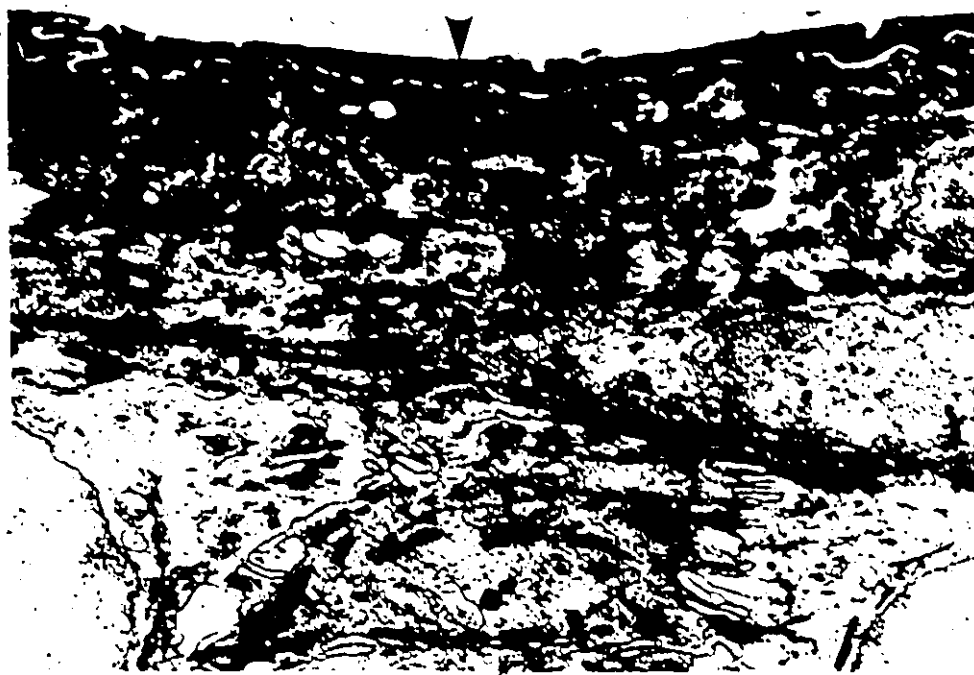


Fig. 1. Electron micrograph of hairless mouse epidermis after stripping five times with cellophane tape. The stratum corneum has been removed except for the basal corneocyte layer (arrow). (X 15,000)

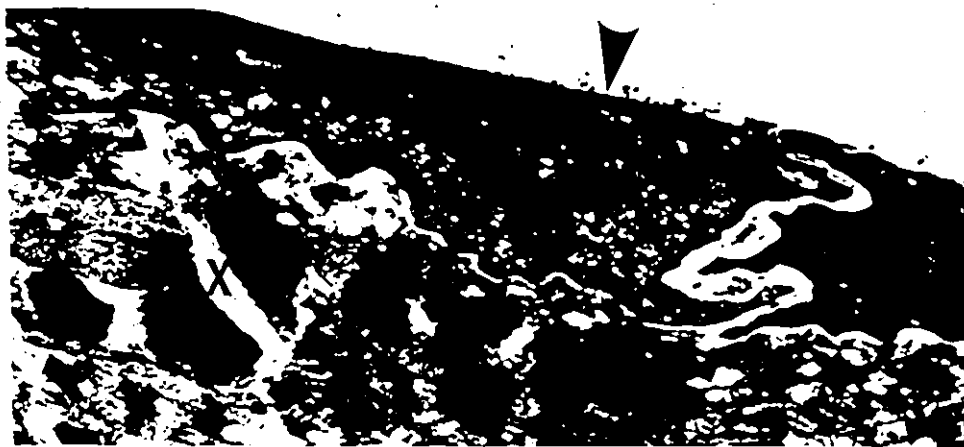


Fig. 2. Electron micrograph of hairless mouse epidermis after stripping 24 times. The basal corneocyte layer is still attached (arrow), but there is tearing (X) in the granular layer. (X 15,000).

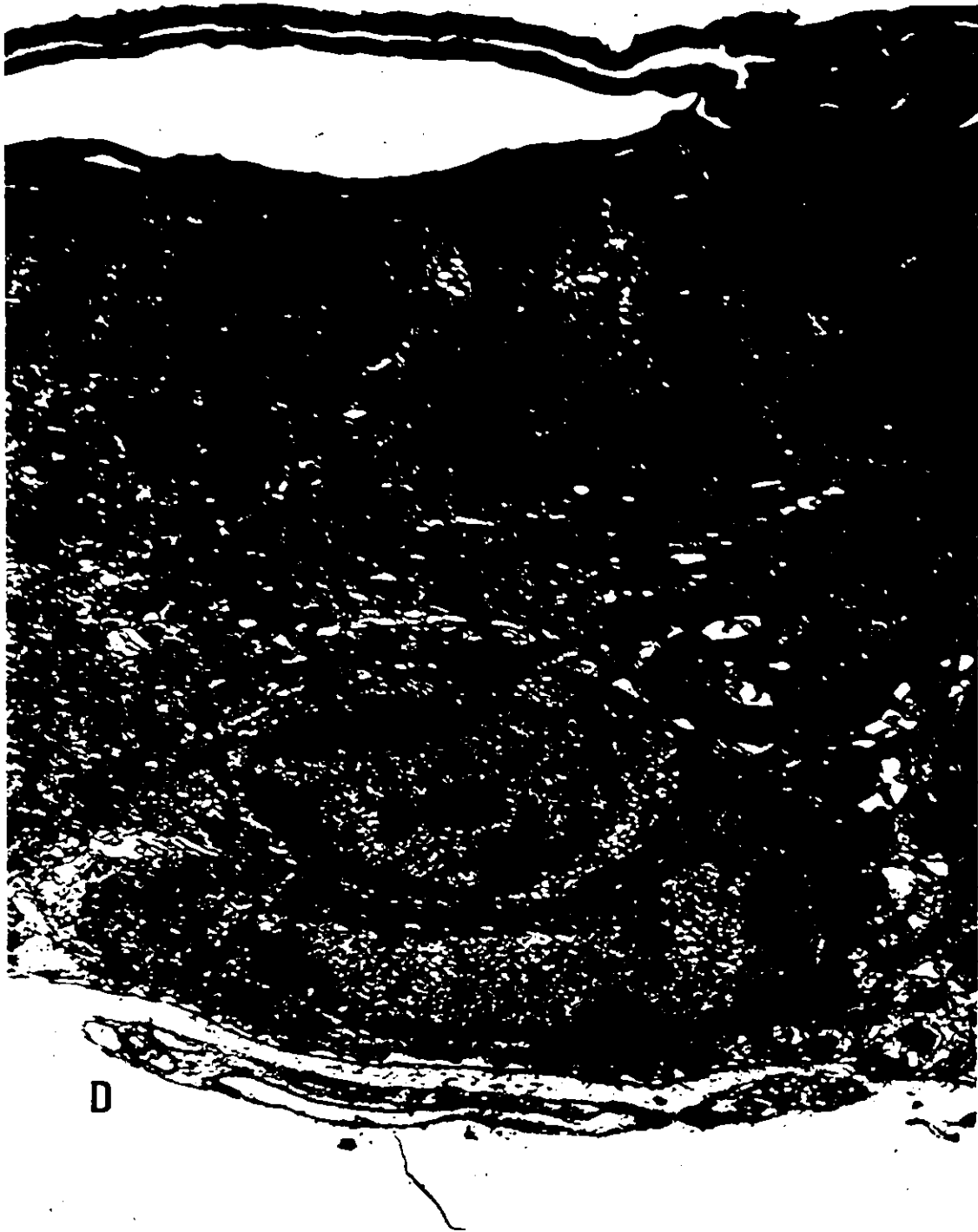


Fig. 3. Electron micrograph of intercellular spaces (arrows), 12 hours after stripping four times. D:dermis. (X 15,000)



Fig. 4. Electron micrograph of intercellular spaces (arrows), 24 hours after stripping four times. D:dermis. (X21,500)

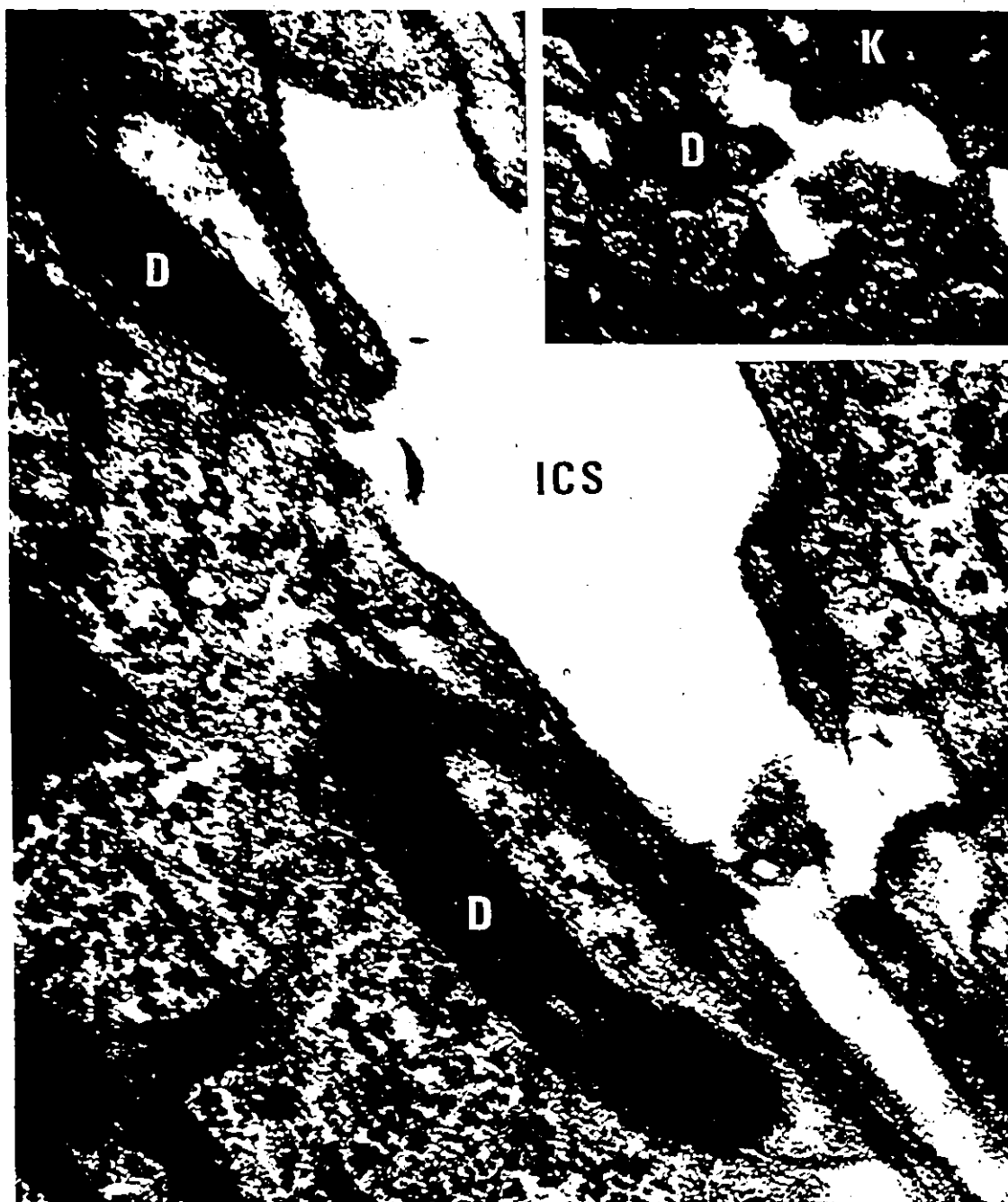


Fig. 5. Electron micrograph of desmosomes (D) engulfed, 18 hours after stripping four times. ICS:intercellular space. (X 100,000)

Inset: Desmosome (D) torn from a keratinocyte (K), 18 hours after stripping four times plus 95% ethanol. (X 60,000)

TABLE II  
CYTOLOGICAL CHANGES IN EPIDERMIS AT VARIOUS TIMES AFTER TREATMENTS

TIME AFTER TREATMENT	TREATMENTS						
	STRIPPED	ETHANOL 95%	PROPYLENE GLYCOL	VITAMIN A ACID 3% IN PROPYLENE GLYCOL	STRIPPED & ETHANOL	VITAMIN A ACID 0.3% & ETHANOL	VITAMIN A ACID 0.3% & ETHANOL & STRIPPED
1/4 hr	* -	-	-	-	* -	-	* -
1 hr	* -	-	-	-	* -	-	* -
3 hr	* -	-	-	-	* -	-	* -
6 hr	* -	-	-	-	** -	-	** -
12 hr	* -	-	-	-	** -	-	** -
18 hr	** X	-	-	-	** -	-	** -
24 hr	** -	** X	-	-	** X	X	** X
36 hr	* -	-	-	-	* -	-	** X
48 hr	** -	* -	-	* -	** -	X	** X
60 hr	* -	-	-	* -	* -	-	** X
3 days	-	-	-	-	-	-	-
4 days	* -	-	-	-	* -	-	* -
7 days	-	-	-	-	-	-	-
10 days	-	-	-	-	-	-	-

Symbols used: \* intercellular spaces; X lipid droplets; 0 intramitochondrial inclusions;  
- no change. An increase in the number of symbols indicates an increase in the element.

cytoplasmic projections and the formation of new desmosomes (Fig. 6).

Occasional breaks were found to develop in the basal lamina at 24 hours after stripping (Fig. 7). In one area, a 'ballooning' cytoplasmic projection was found, with very diffuse contents (Fig. 8). It appeared to have expanded to fill the available intercellular space. At the same time as spaces formed, i.e. beginning by one hour and continuing to day four (Table II), dense intramitochondrial inclusions and large amounts of glycogen were found to be produced in some keratinocytes (Fig. 9). It was found that the inclusions could be digested with protease (Fig. 10); this indicates that these inclusions contain a protein component. They also stained with silver methenamine (Fig. 11), indicating that they also contain glycogen or glycoprotein. A lipid droplet (Fig. 12) was found in a keratinocyte 18 hours after stripping. This droplet failed to be digested by protease therefore it appears to contain no protein.

### Ethanol

Ethanol, like stripping, produced intercellular spaces (Fig. 13). However, these spaces were sometimes largest in the upper layers of the epidermis, decreasing toward the basal layer, instead of the other way around (Fig. 14). Spaces were found at 18 to 48 hours after ethanol application (Table II). The basal lamina showed distinct areas of disruption but not complete breaks. Basal lamina material appeared to be disintegrating and floating away, but

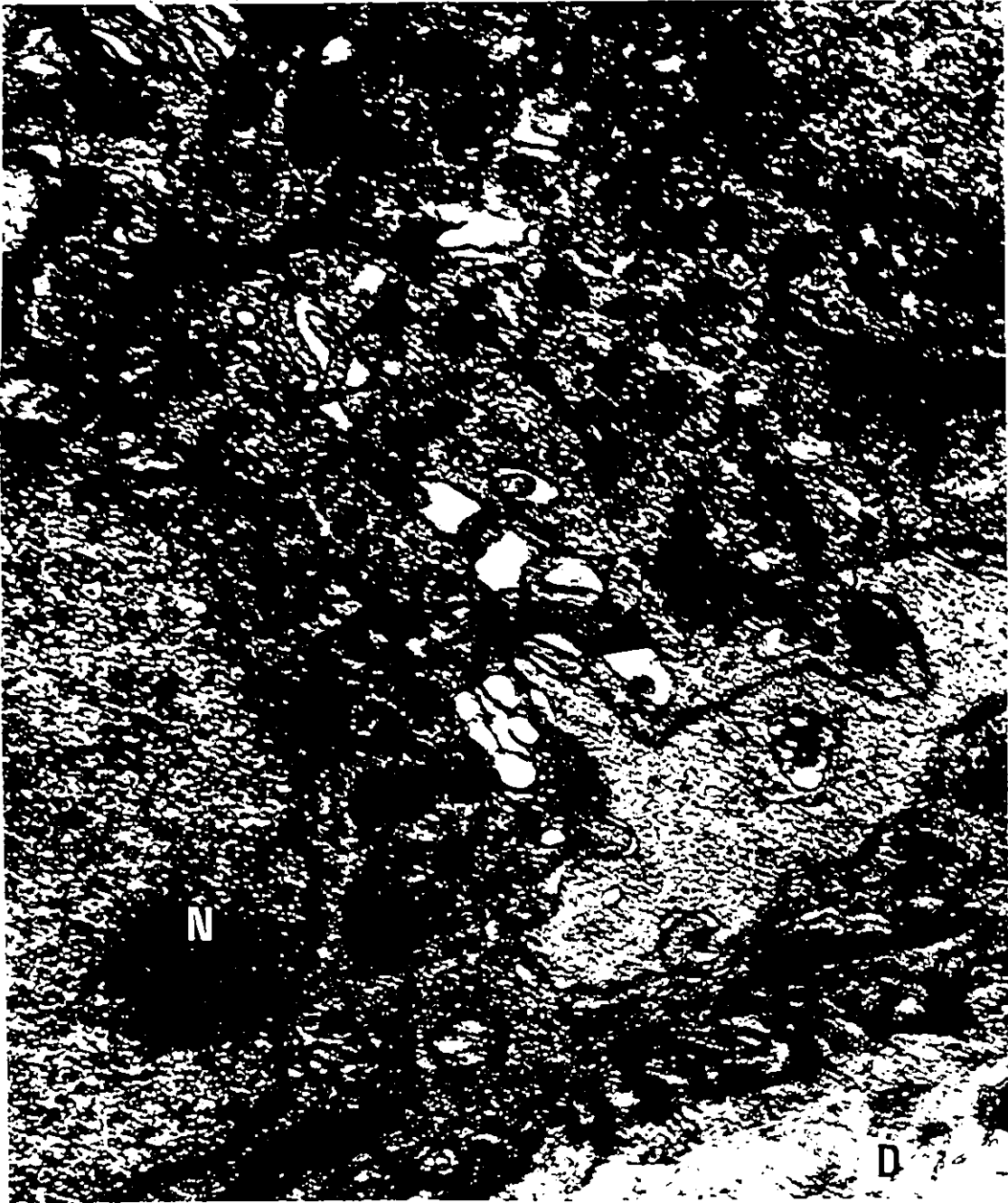


Fig. 6. Electron micrograph of epidermal cells re-establishing contact with interdigitation of cytoplasmic projections (arrows), four days after stripping four times. D:dermis, N:nucleus. (X 40,000)



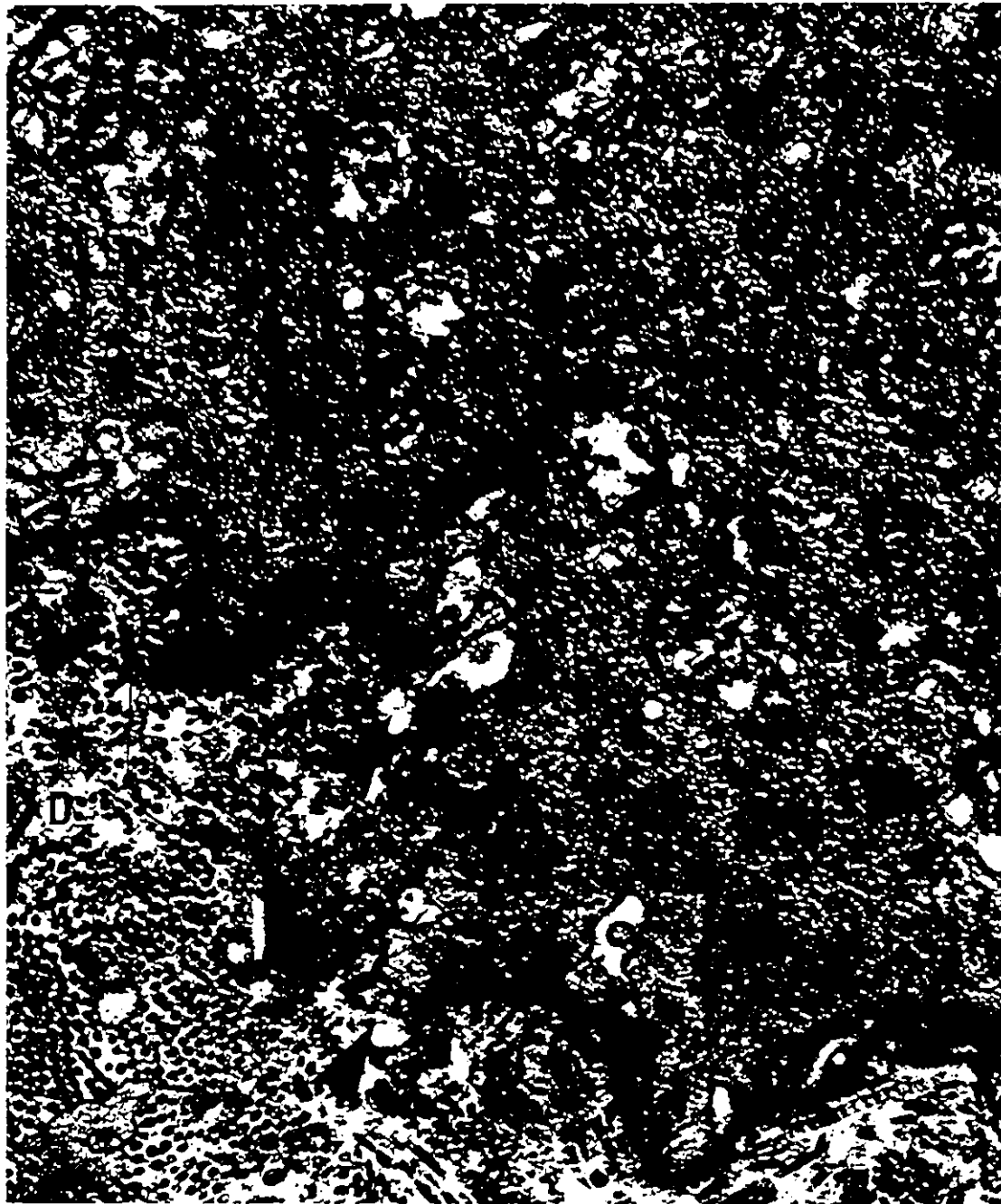


Fig. 7. Electron micrograph of basal lamina breaks (arrows), 24 hours after stripping. D:dermis. (X 40,000)



Fig. 8. Electron micrograph of ballooning cytoplasmic projections (BP), 18 hours after stripping. D:dermis.  
(X 40,000)



Fig. 9. Electron micrograph of intramitochondrial inclusions (arrows) and glycogen (G) in a keratinocyte, 18 hours after stripping. N:nucleus. (X 50,000)

Inset: intramitochondrial inclusions (arrows), one hour after stripping. (X 50,000)

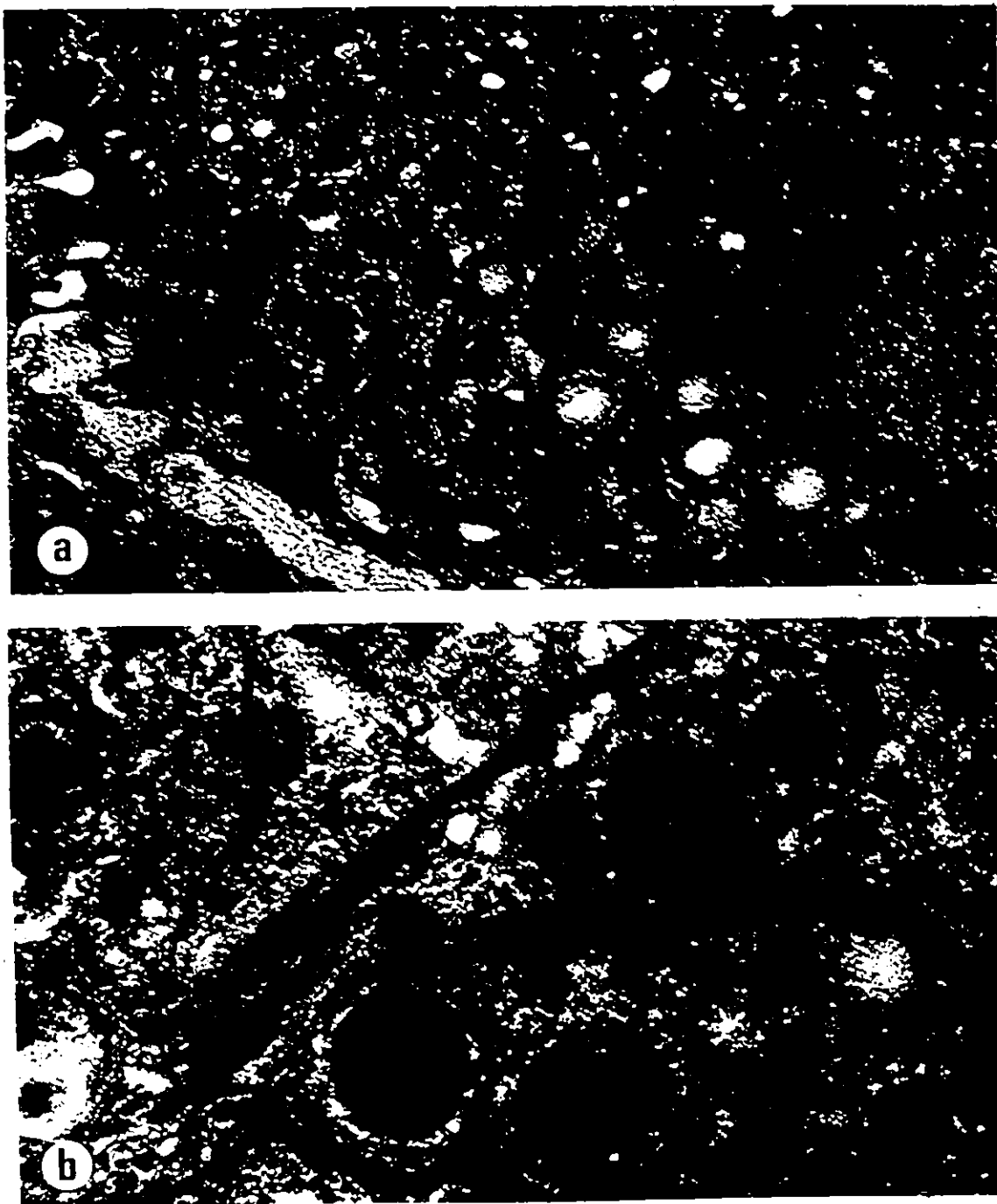


Fig. 10. Electron micrograph of protease digested intra-mitochondrial inclusions (arrows), 18 hours after stripping. After half an hour of protease digestion at 40 C, the inclusions stain less densely. (a) (X 25,000) (b) (X 50,000)

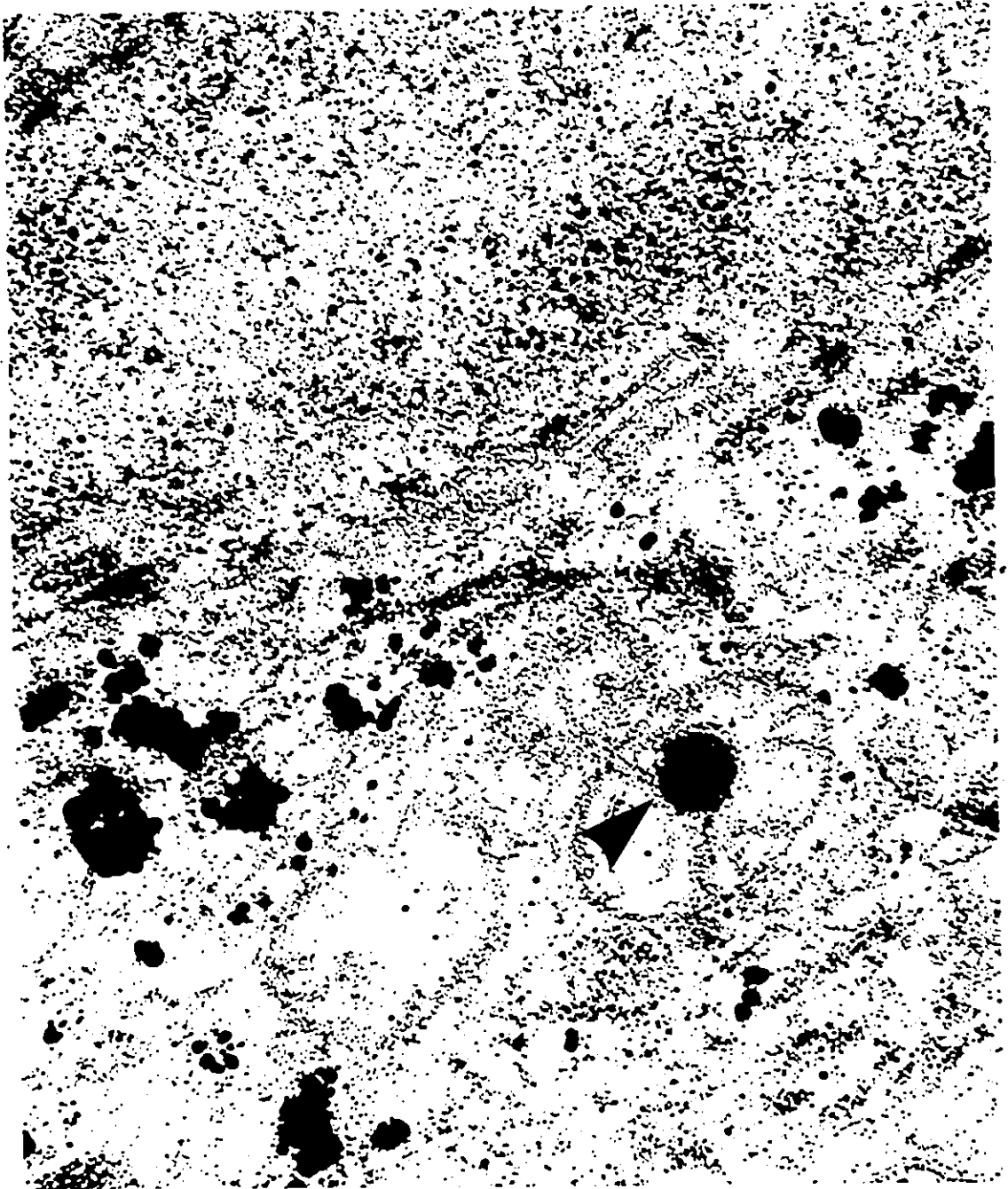


Fig. 11. Electron micrograph of a silver methenamine stained intramitochondrial inclusion (arrow), 24 hours after 95% ethanol. (X 80,000)

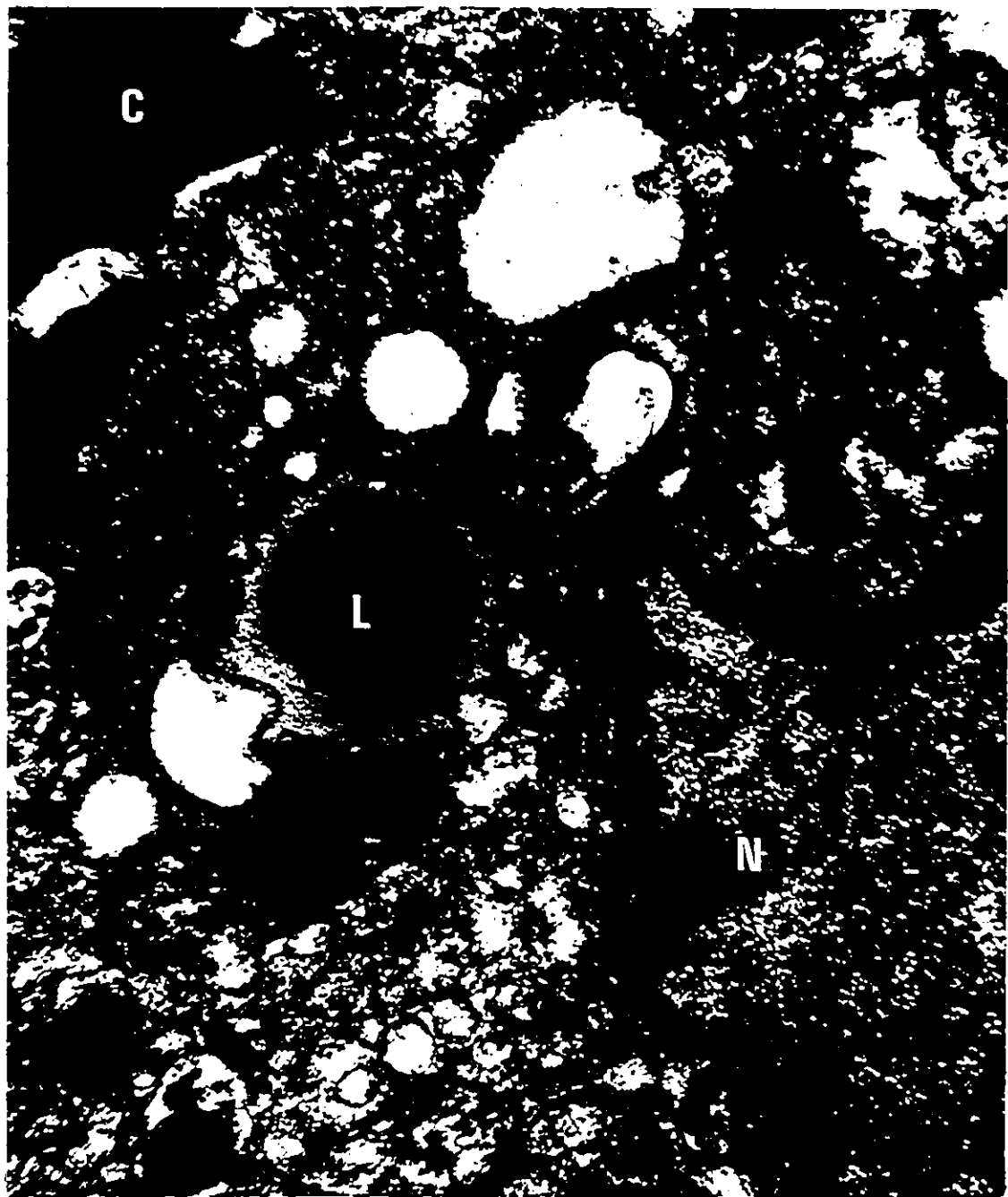


Fig. 12. Electron micrograph of a lipid droplet (L) in a granular layer keratinocyte, 18 hours after stripping. After half an hour of protease digestion at 40°C, the lipid droplet appears unaffected. N:nucleus. C:corneocyte. (X 50,000)

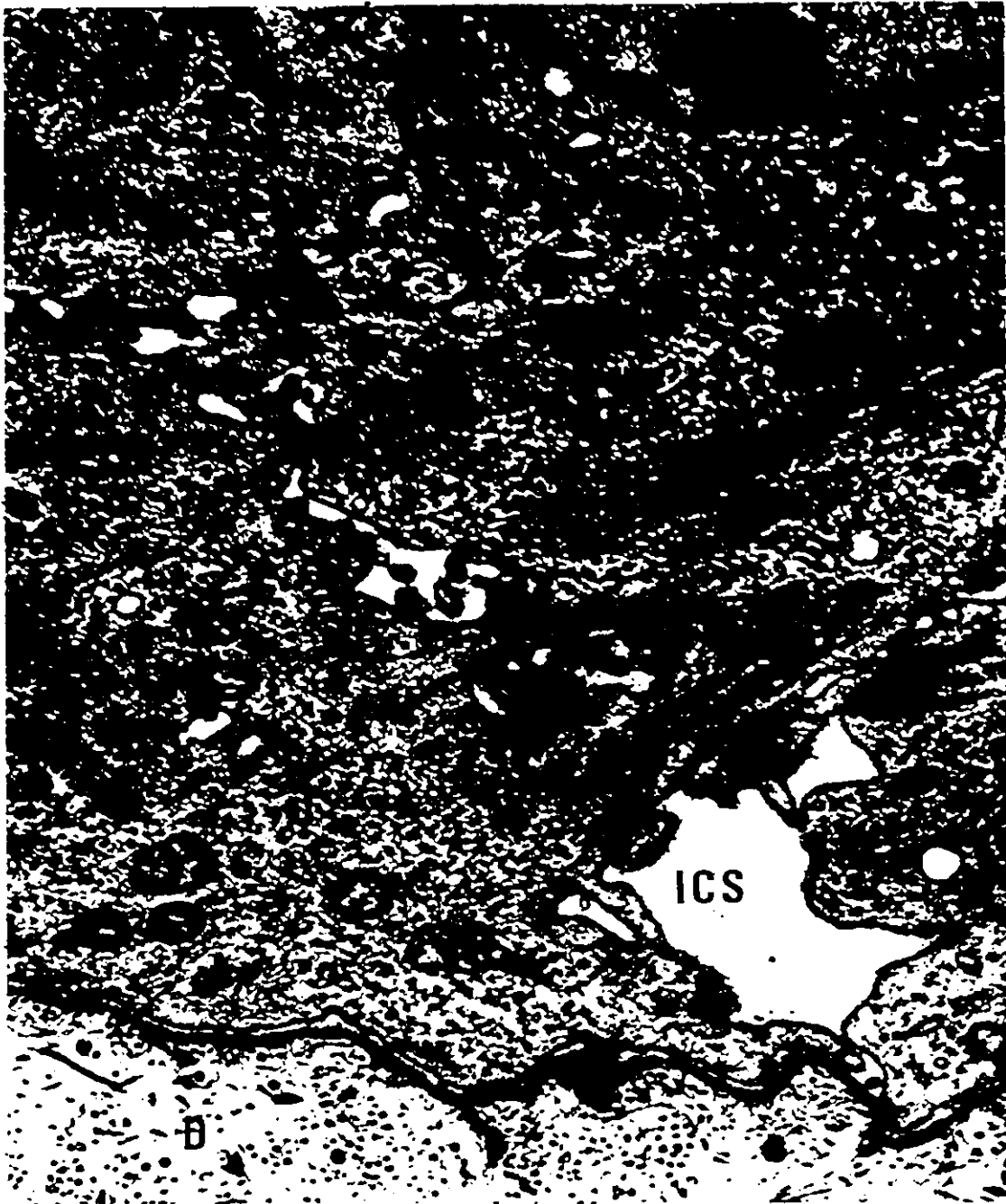


Fig. 13. Electron micrograph of intercellular spaces (ICS),  
24 hours after 95% ethanol. Didermis. (X 25,000)



Fig. 14. Electron micrograph of intercellular spaces (ICS), 24 hours after 95% ethanol. The spaces are larger in the upper portion of the epidermis and the stratum corneum has been sloughed off (arrow). D:dermis. Silver methenamine stained only. (X 20,000)



there was always a thin layer of intact basal lamina adjacent to the basal epidermal cells (Fig. 15).

Dense intramitochondrial inclusions were found after ethanol treatment, but to a lesser extent than with stripping (Fig. 16 and Table II). Also like stripping, ethanol occasionally caused the accumulation of glycogen in some keratinocytes. These glycogen deposits were identified cytochemically with silver methenamine stain (Fig. 17). Another effect of ethanol was the sloughing of the stratum corneum (Fig. 17); often most or all of the dead cell layers were lost. Ethanol induced the appearance of many lipid droplets in some tissue samples (Fig. 17). Attempts to stain this ~~material~~ with silver methenamine resulted in a non-specific spotted pattern of silver precipitation (Fig. 18), indicating that there was no glycogen or glycoprotein present or it was isolated in small discrete areas. Similar droplets in stripped skin (Fig. 12) did not digest with protease, therefore there appears to be no protein present.

#### Propylene Glycol.

Propylene glycol produced no detectable change in the epidermis (Fig. 19 and 20).

#### Vitamin A Acid.

Since propylene glycol produced no detectable changes in the skin, the effects of vitamin A acid in propylene glycol are described in this study as vitamin A acid effects.

Vitamin A acid (3%) in propylene glycol caused the



Fig. 15. Electron micrograph of the basal lamina (BL) showing some dispersion (arrows), 24 hours after 95% ethanol. C: collagen. (X 50,000)

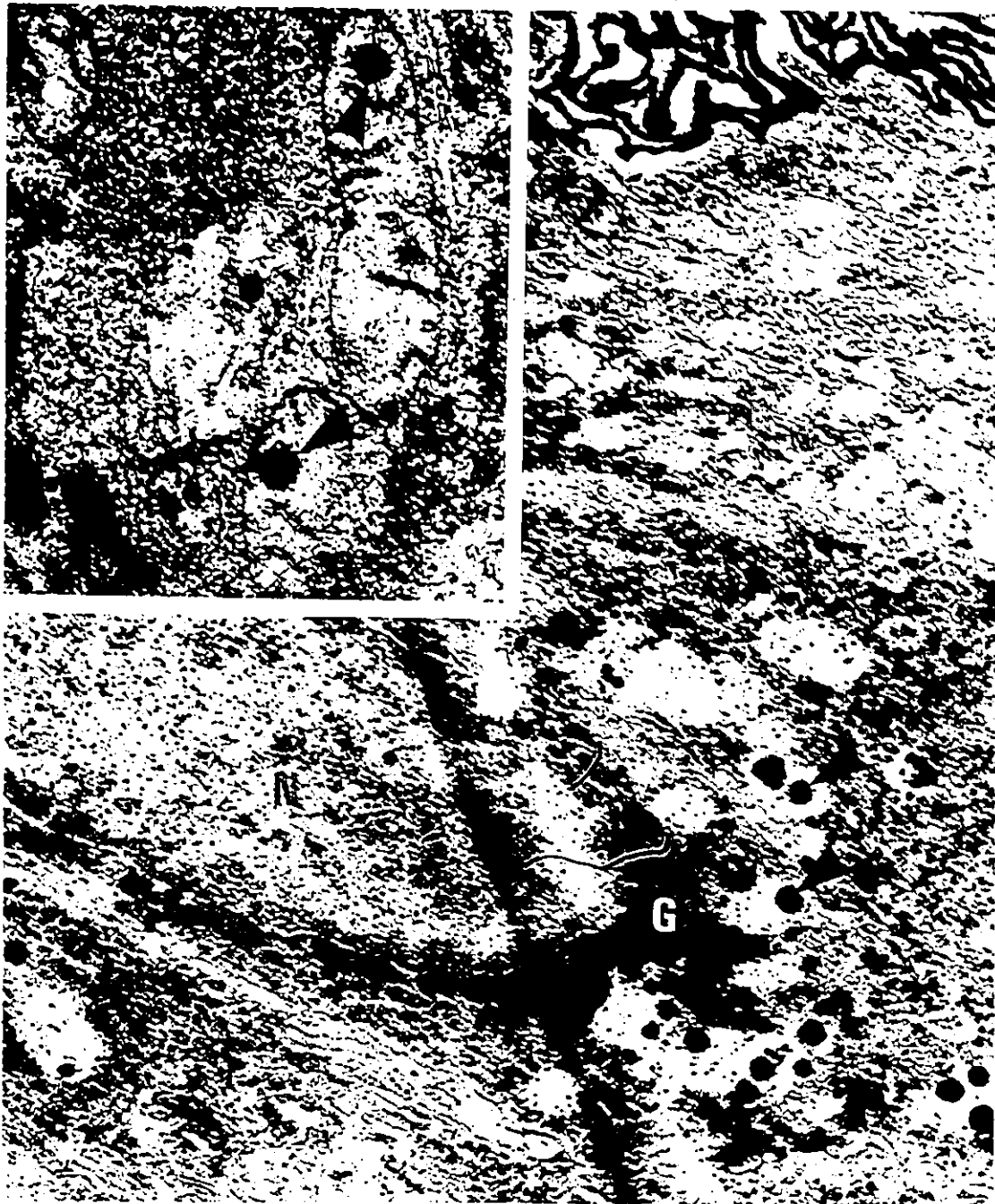


Fig. 16. Electron micrograph of intramitochondrial inclusions (arrows) and glycogen (G), 24 hours after 95% ethanol. Silver methenamine stained. N:nucleus. (X 25,000)  
Inset: Intramitochondrial inclusions (arrows) from same tissue. (X 50,000)



Fig. 17. Electron micrograph of lipid droplets (L),  
24 hours after ethanol. C: cornucyte. (X 50,000)

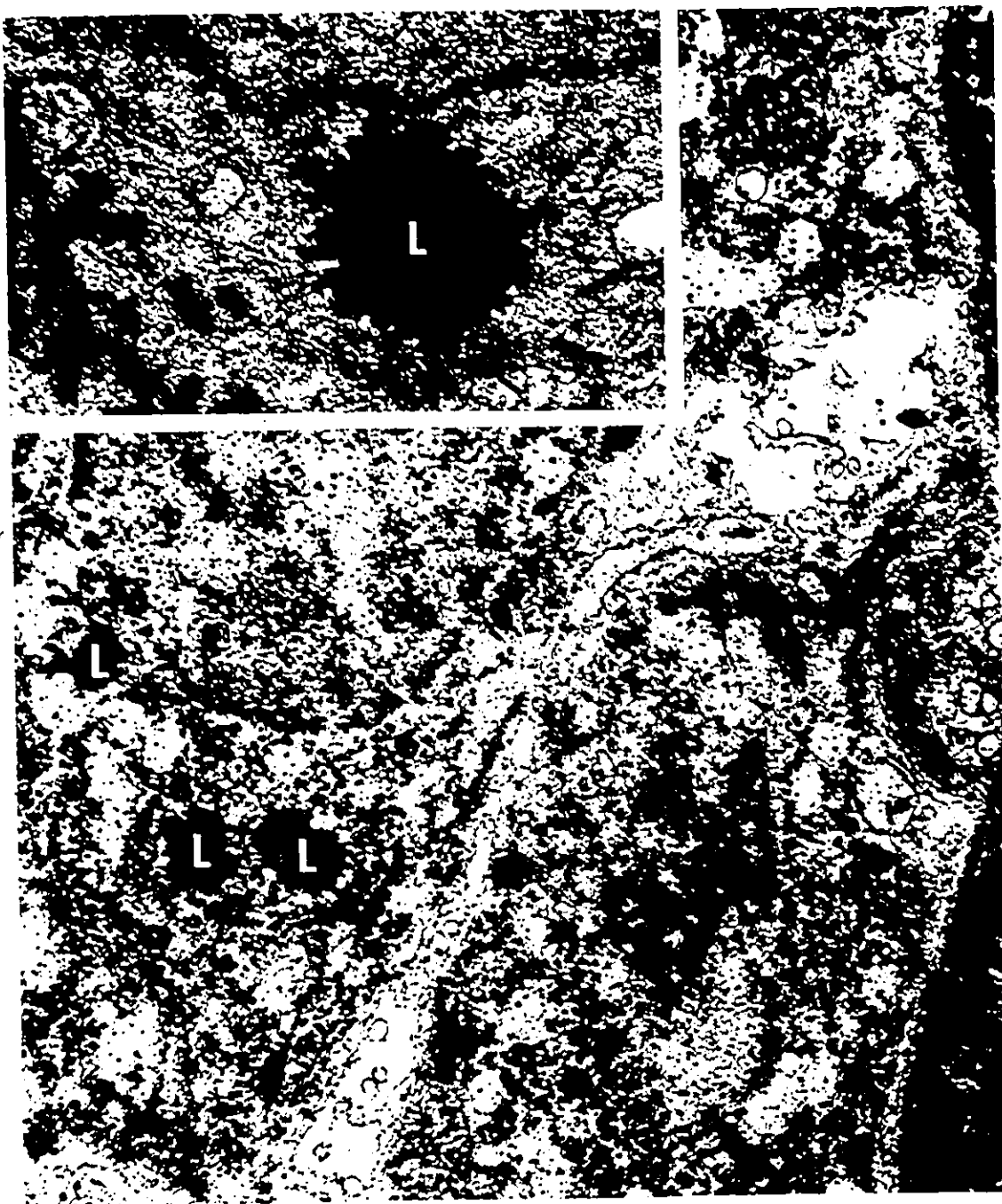


Fig. 18. Electron micrograph of lipid droplets (L), 24 hours after 95% ethanol. Silver methenamine stained. (X 20,000)  
Inset: lipid droplet (L) from same tissue. Silver stained. (X 40,000)



Fig. 19. Electron micrograph of propylene glycol treated epidermis, 48 hours after treatment. The skin appears normal.  
(X 11,500)



Fig. 20. Electron micrograph of normal hairless mouse epidermis. (X 11,500)

development of intercellular spaces (Fig. 21). These spaces were first detected at 48 hours and persisted until at least 72 hours (Table II). They were smaller than those found after stripping or 95% ethanol. Dense intramitochondrial inclusions also appeared at 48 and 72 hours (Fig. 21 and Table II). The basal lamina seemed to be normal and there was no sloughing of the stratum corneum. No glycogen could be detected in these vitamin A acid treated tissues, even when stained with silver methenamine only. Lipid droplets were not detected in these tissues either. Overall, the damaging effects of 3% vitamin A acid appeared to be less than that of stripping or 95% ethanol.

The elements of keratinization, keratohyalin granules and tonofilaments were reduced (Fig. 22). Keratohyalin granules were decreased in size and number and usually appeared round instead of irregularly shaped. In addition they did not appear to be associated with tonofilaments as they usually are. There were only a few scattered bundles of tonofilaments in each cell instead of the usual abundance. Instead of keratinization elements, these cells showed increased amounts of ribosomes, endoplasmic reticulum and Golgi apparatus. Golgi apparatus can be found right up to the uppermost cell layers of the epidermis of these tissues; this is never possible in normal skin.



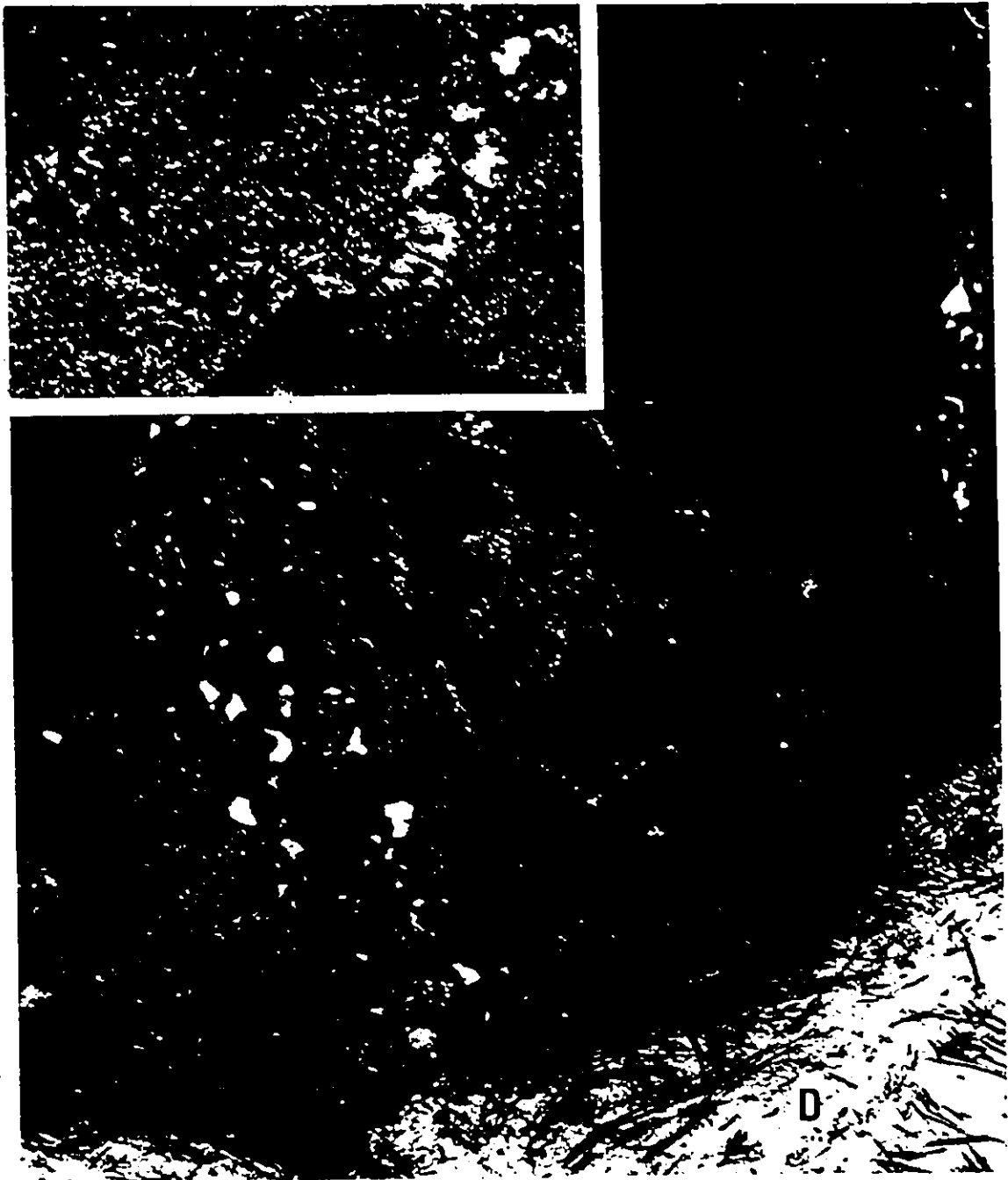


Fig. 21. Electron micrograph of intercellular spaces (large arrows) and intramitochondrial inclusions (small arrows), 48 hours after 3% vitamin A acid in propylene glycol. D: dermis. (X 15,000)

Inset: Intramitochondrial inclusions (arrows), from same tissue. (X 30,000)



Fig. 22. Electron micrograph of the upper layers of the epidermis, 24 hours after 3% vitamin A acid in propylene glycol. Note the decrease in keratohyalin granules (large arrows) and tonofilament bundles (small arrows).

C:corneocytes. (X 15,000)

### Combined Treatments.

#### (i) Stripping and Ethanol.

The combined mechanical damage of tape stripping and chemical damage of 95% ethanol produced greater damage than either alone. Intercellular spaces (Fig. 23 and Table II) and intramitochondrial inclusions (Fig. 24 and Table II) were increased. The basal lamina also showed greater damage (Fig. 25) than stripping or ethanol alone. The moderately dense lipid droplets found in the skin of one animal after this double treatment were very large and numerous (Fig. 26). Small dense areas were found in some of these droplets (Fig. 27). These denser areas were not detected in the droplets of any other tissue samples and are of unknown significance. Some small droplets, apparently just beginning to form, had a membranous internal structure that suggested a possible derivation from smooth endoplasmic reticulum (Fig. 28).

#### (ii) Ethanol and 0.3% Vitamin A Acid.

The combination of ethanol and vitamin A acid caused more damage than either alone as evidenced by the increased intercellular spaces (Fig. 29 and Table II).

#### (iii) Stripping, Ethanol and 0.3% Vitamin A Acid.

This triple combination produced the most severe damage of any treatment. Intercellular spaces were largest after this treatment (Fig. 30 and Table II) and there were numerous intramitochondrial inclusions (Fig. 31 and Table II).



Fig. 23. Electron micrograph of intercellular spaces (arrows), 24 hours after stripping and ethanol. D:dermis (X 8,500)



Fig. 24. Electron micrograph of intramitochondrial inclusions (arrows), 48 hours after stripping and ethanol. N:nucleus. (X 50,000)



Fig. 25. Electron micrograph of broken basal lamina (arrows), 18 hours after stripping and ethanol. D:dermis (X 50,000)



Fig. 26. Electron micrograph of a lipid droplet (L), 24 hours after stripping and ethanol. N: nucleus. (X 100,000)



Fig. 27. Electron micrograph of a lipid droplet with small dense areas (arrows), 24 hours after stripping and ethanol. (X 100,000)





Fig. 28. Electron micrograph of a lipid droplet beginning to form (arrow), 24 hours after stripping and ethanol.

(X 100,000)



Fig. 29. Electron micrograph of intercellular spaces (arrows), 48 hours after 0.3% vitamin A acid in ethanol. D: dermis. (X 6,000)

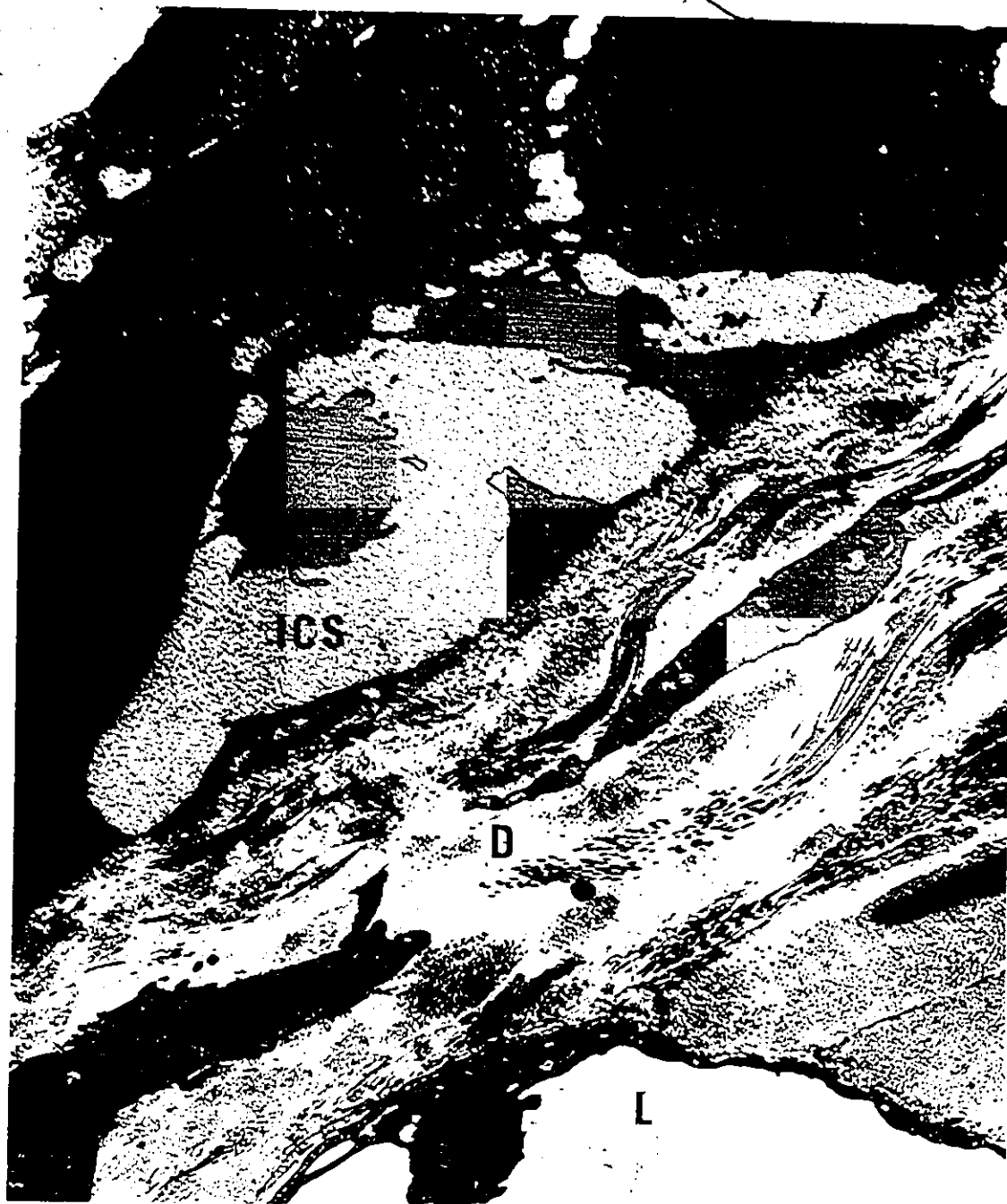


Fig. 30. Electron micrograph of intercellular spaces (ICS), 24 hours after stripping, ethanol, and 0.3% vitamin A acid. D: dermis. L: lymphatic vessel. (X 9,000)

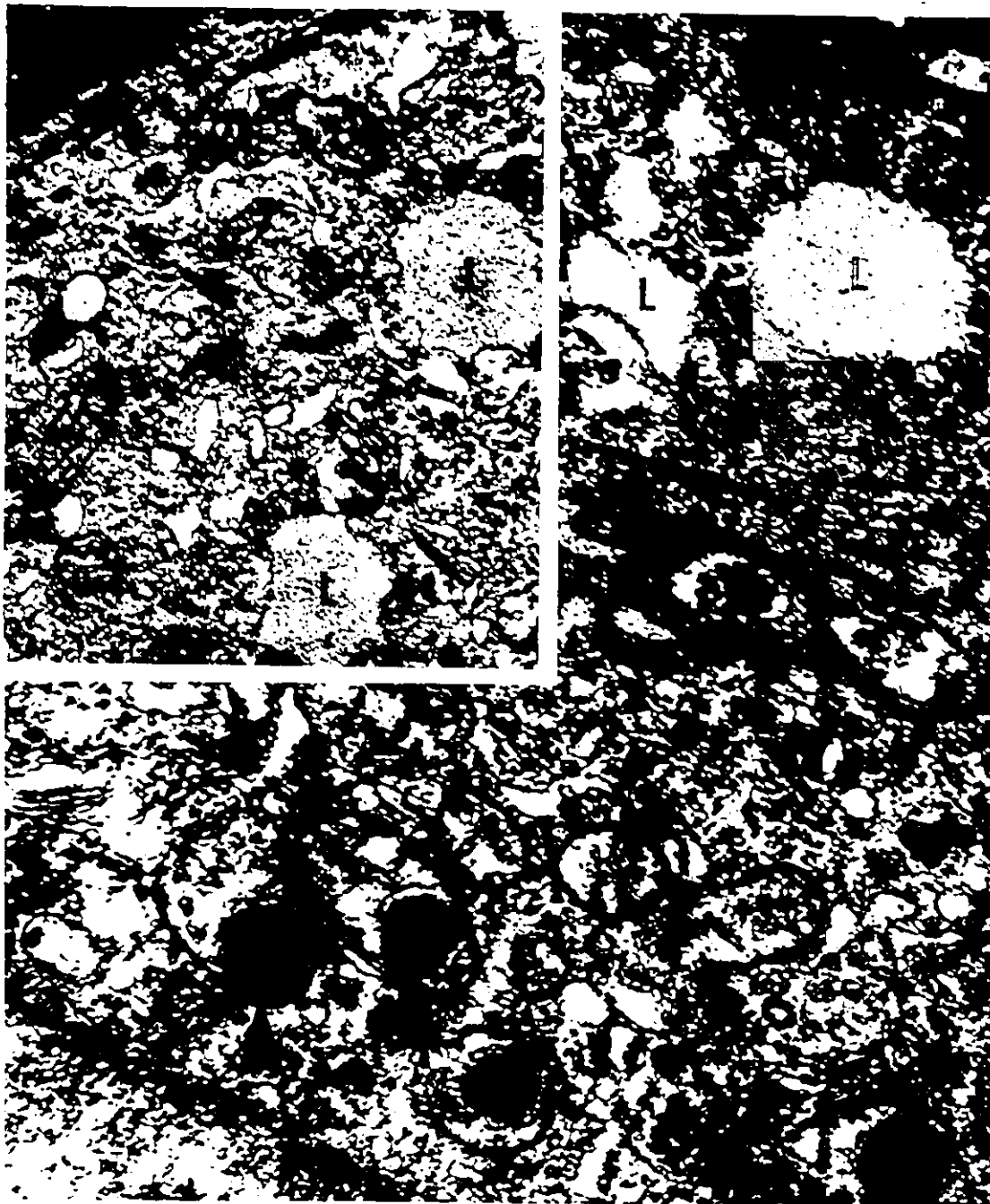


Fig. 31. Electron micrograph of intramitochondrial inclusions (arrows) and lipid droplets (L), 36 hours after stripping, ethanol, and 0.3% vitamin A acid. (X 60,000)

Inset: Lipid droplets (L), from same tissue. (X 40,000)

Homogeneous droplets again appeared at 24 hours (Fig. 31) and were passed up to the stratum corneum over the next two days (Table II and III) so that they were very extensive in the corneum by day three, producing a parakeratotic horny layer (Fig. 32). There was also some sloughing of the stratum corneum as shown in Fig. 30. Basal lamina breaks were also more extensive than those found with any other treatment (Fig. 33).

TABLE III

LOCATION OF LIPID DROPLETS AT VARIOUS TIMES AFTER COMBINED  
STRIPPING, ETHANOL, AND 0.3% VITAMIN A ACID

TIME AFTER. TREATMENT	LOCATION				
	BASAL LAYER	SPINOUS LAYER	GRANULAR LAYER	NEW CORNEOCYTES	OLD CORNEOCYTES
½ hr	-	-	-	-	-
1 hr	-	-	-	-	-
3 hr	-	-	-	-	-
6 hr	-	-	-	-	-
12 hr	-	-	-	-	-
18 hr	-	-	-	-	-
24 hr	-	X	X	?	?
36 hr	X	X	X	X	-
48 hr	-	-	X	?	?
60 hr	-	-	-	X	-
3 days	-	-	-	X	X
4 days	-	-	-	-	X
7 days	-	-	-	-	X
10 days	-	-	-	-	-

Symbols used: X lipid droplets  
 - no lipid droplets  
 ? corneum missing



Fig. 32. Electron micrograph of stratum corneum with many lipid droplets (arrows), 3 days after stripping, ethanol, and 0.3% vitamin A acid. (X 8,500)



Fig. 33. Electron micrograph of the basal lamina broken in several places (arrows) with cytoplasmic contents of damaged keratinocytes pouring into the dermis (D), 36 hours after stripping, ethanol, and 0.3% vitamin A acid. (X 15,000)



## DISCUSSION

Tape Stripping.

Tape stripping four times produced the typical regenerative response to wounding (38) even when two to five layers of corneocytes remained attached. This regeneration has been characterized, in part, by the development of enlarged intercellular spaces, in both hairless mice (38) and in humans (24). A similar enlargement of spaces has been found after incision wounds in human skin (34) and rat gingiva (23); after excessive ultraviolet irradiation in humans and Guinea pigs (31); after topical application of vitamin A acid in Guinea pigs (55); after vitamin A in tissue culture of Guinea pig ear skin (2) and embryonic mouse skin (48); and in certain skin diseases such as pemphigus (51) and some viral skin diseases (21). This study revealed the early development of these intercellular spaces and found them to begin within an hour after stripping of hairless mice. Spaces were previously reported to begin only by four hours in stripped human skin (24). It may be that mouse skin reacts to stripping more quickly than human skin because it is so much thinner. The mechanisms involved in the formation of intercellular spaces are unknown, thus the fact that spaces begin to form very early may be useful in discovering these mechanisms.

It was found that as intercellular spaces formed, desmosomes were torn from plasma membranes of keratinocytes.

This is in agreement with the findings of Mishima and Pinkus (24). However, Allen and Potten (1) did not find tearing of the plasma membrane when desmosomal connections were broken. They proposed a model where the plasma membrane pinches off without tearing. Perhaps desmosomes are removed without tearing in some cases and with tearing in others. In any case it is believed that a desmosome, once removed, is invaginated into a keratinocyte where it is broken down, presumably in a phagolysosome (24). When cells re-establish contact, it is thought that new desmosomes are formed 'de novo' (24). The fact that plasma membranes can be torn by the forces separating the keratinocytes suggests that the cell separation is not merely due to a lack of adhesiveness between cell surfaces but probably involves fluid pressures.

The dense intramitochondrial inclusions found in damaged epidermal cells appear to be part of the response to injury since they accompany the formation of enlarged intercellular spaces. Morphologically similar inclusions have been found in the epidermis or in the epithelial cells of mucous membranes in the following situations: after local application of carcinogens, croton oil, podophyllin, benzene and turpentine; in cases of vitamin A deficiency; after wounding of mouse skin; in normal epithelium of the tongue, rectum and stomach and in the stomach of embryonal mice (43). The ultrastructure, composition, and occurrence of this type of intramitochondrial inclusion has recently been examined and reviewed by Rupec and Bruhl (43). The name 'corpora intra

cristam' was accepted for this type of inclusion as originally proposed by Frie and Sheldon (11). It had been reported by Kakefuda et al. (17) that these inclusions were not digested after incubation with DNase, RNase and pronase (protease). However, Rupec and Bruhl did achieve protease digestion of this type of inclusion in mouse skin fixed in glutaraldehyde and osmium tetroxide. When they duplicated Kakefuda's fixation with just glutaraldehyde, they too failed to get digestion by protease. We achieved digestion of intramitochondrial inclusions with Dalton's fixative (9) which also has osmium tetroxide but not glutaraldehyde; it is buffered with potassium dichromate. Thus our findings support those of Rupec and Bruhl who found these inclusions to contain protein.

We also found that these inclusions could be stained with silver methenamine. Silver methenamine is known to stain glycogen and glycoprotein but it is not entirely specific, especially in osmium-fixed tissues (7). Nevertheless, this staining at least suggests a carbohydrate component in these inclusions. Because these inclusions are digestable with protease and stain with silver methenamine, there is a possibility that these inclusions contain glycoprotein. Rupec and Bruhl also found that these inclusions could be extracted in chloroform-methanol when they were fixed in glutaraldehyde (43). From that finding, Rupec and Bruhl concluded that these inclusions had a lipid component as well.

The finding of glycogen deposits in keratinocytes has not been reported in previous ultrastructural studies of stripping or wounding. However, biochemical assay of epidermal tissue from regenerating wounds has shown an increasing gradient of glycogen from the marginally affected area to the advancing epithelial tip, with a maximal nine times normal glycogen at the tip (28). Glycogen is also known to accumulate in keratinocytes in psoriatic skin (13) which, like stripped or wounded skin, has an increased mitotic rate. A five-fold increase in glycogen was found in the psoriatic lesions compared to the uninvolved epidermis. Several other epidermal proliferative diseases such as eczema also produce glycogen accumulation (49). In the human fetus, less than four months old, basal as well as peridermal layers are laden with glycogen. However, from six months to birth, when keratinization takes place, there is a sudden and complete reduction of the glycogen (49). Thus, glycogen in adult epidermis appears to be a sign of damage or disease.

A single lipid droplet was found in a keratinocyte 18 hours after stripping and this droplet was unaffected by half an hour of protease digestion; thus it did not contain protein.

#### Ethanol.

Although ethanol has been widely used as a solvent for applying various drugs and chemicals to the skin, there have been few studies of possible damage caused by the ethanol. Some swelling of the epidermis was reported from light

microscopic studies, but it was concluded that the cells were not damaged (27). Ethanol (95%) has also been shown to cause increased mitosis when applied to the skin (56), but there are no previous ultrastructural studies of the effects of high concentrations of ethanol applied to the skin. There is, however, one related ultrastructural study, by Bruckner and Guess, in which high concentrations of ethanol were injected into the dermis, with resultant damage to the overlying epidermal cells (5). They reported that ethanol caused epidermal cell necrosis with 'alcoholic protein denaturation' and 'cytoplasmic coagulation', as demonstrated by the aggregation of ribosomes and tonofibrils. The present study revealed cell damage but not aggregation of ribosomes and tonofibrils after topical application of 95% ethanol. Like Bruckner and Guess, this study showed broken cell membranes and widening of intercellular spaces, but did not find swollen endoplasmic reticulum and mitochondria as they did. In general, it appears there was more epidermal damage in the intradermal injection study. This may be related to the inflammatory reaction produced in the dermis by the injection of ethanol.

After topical application of ethanol, some keratinocytes developed many droplets of moderately dense material in their cytoplasm that are identical in morphology to the lipid droplets found in fat cells and rarely in normal keratinocytes (33). Such droplets have not been previously reported in ethanol treated epidermis. This study failed

to determine the composition of this material. However, since it did not stain with silver methenamine and was not digested by protease, it was concluded that it did not contain carbohydrate or protein. The small dense areas within the droplets of some of the tissue samples is also of unknown composition. Considering the damage caused by high concentrations of ethanol, it may be unwise to use it as a solvent for skin application studies, and previous work that used ethanol should be re-evaluated in the light of our findings.

#### Vitamin A Acid.

The ultrastructural effects of vitamin A acid application to normal adult mammalian skin 'in vivo' have been studied previously in Guinea pig ear skin by Wolff, Christophers and Braun-Falco (55). Since this vitamin has become important in the treatment of various skin diseases, it was considered appropriate to study the effects of this compound further, using a different animal and two different solvents. This study also investigated the effects of vitamin A acid on tape stripped skin, which more closely approximates diseased skin such as psoriasis, where vitamin A acid has been found somewhat effective as a treatment. Wolff et al. used concentrations of 1% and 3% vitamin A acid with daily applications for eight days, while this study involved a single application of either 0.3% or 3%.

In this study, a single application of 3% vitamin A acid produced changes in the skin, but not as pronounced as those reported by Wolff et al. after daily applications.

As in the study of Wolff et al., this study showed epidermal damage in the form of intercellular spaces. Dense intramitochondrial inclusions were found after vitamin A acid treatment in this study but were not reported previously. Glycogen was found in this study, as it was in that of Wolff et al., but only in vitamin A acid-treated epidermis that had first been tape stripped, or when ethanol was used as the solvent. Perhaps the single application of 3% vitamin A acid in propylene glycol was insufficient to induce glycogen accumulation. Wolff et al. found glycogen in their samples but used acetone as a solvent; this may have been partly or fully responsible for the glycogen accumulation. No control experiments with acetone were reported in their work.

Moderately dense homogeneous droplets were also found by Wolff et al., but these structures were not detected in this study after a single application of 3% vitamin A acid in propylene glycol. However, these or similar structures appeared after stripping or ethanol treatment; after 0.3% vitamin A acid in ethanol, these droplets were abundant in the corneum, as well as the live epidermal cells. Wolff et al. reported that the droplets in their preparations stained with silver methenamine, and claimed they contained some mucosubstances, but they did not illustrate this finding. Attempts to stain similar appearing droplets in this work gave negative results. Thus, the question whether vitamin A causes adult mammalian epidermal cells to produce

mucus could not be resolved.

Vitamin A was first described by Harris et al. in 1932 (14) to have an anti-keratinizing effect. This concept has been confirmed in adult mammalian skin by Wolff et al. in Guinea pig ear skin (55); and the present study reaffirms this finding in mouse back skin. The keratinization elements, keratohyalin granules and tonofilaments were reduced while the synthetic elements, ribosomes, Golgi apparatus and endoplasmic reticulum, were increased with a resultant abnormal or parakeratotic stratum corneum. Logan, in 1972 (20), suggested that there was no inhibition of keratinization in mammalian skin, but merely a 'disturbance' in the process. However, this study and the work of Wolff et al., published two years before Logan's review, shows specific inhibition of the production of keratin and keratin precursors.

Precisely how vitamin A is able to produce its epithelial effects is largely unknown. However, it is known that vitamin A (retinol) and vitamin A acid (retinoic acid) each have target epithelial tissues, and each has its own specific binding protein in these tissues (8, 35). The tissue binding proteins are distinct from the serum binding protein for vitamin A, which carries it through the circulatory system to its target tissues. Some target tissues have a binding protein for retinol only and some have both, but no tissues have yet been found with only retinoic acid binding protein (35). Muscle tissue, which is



not responsive to vitamin A, has no vitamin A binding proteins, while skin does have the binding proteins (35). In this respect, the retinoids are similar to the steroids, which are known to have specific binding proteins in their target tissues.

There is evidence that the retinoids, like steroids, pass from the cytoplasm to the nucleus, where they appear to bind to the DNA. In 1970, Prutkin and Bogart (41), using electron microscopic autoradiography, showed that carbon 14-labeled retinoic acid, applied to the skin, enters epidermal cells and is found mostly in the cytoplasm at 24 hours. At 48 and 96 hours, it was found mainly in the nucleus, associated with the euchromatin, the site of RNA synthesis. This suggests vitamin A may be inhibiting or stimulating RNA synthesis.

In 1971, Prutkin did further studies, applying actinomycin D to the skin before retinoic acid (40). Actinomycin D binds to DNA and inhibits RNA-polymerase, thus preventing RNA synthesis (12). This treatment resulted in the inhibition of retinoic acid effects. Specifically, actinomycin D prevented the mucus production usually caused by retinoic acid in Prutkin's system, the keratoacanthoma, a squamous cell carcinoma that grows to a certain extent then spontaneously regresses. Thus, it appears retinoic acid affects a region of the genome involved in transcription of messenger-RNA coded for the translation of mucoprotein. Perhaps messenger-RNA coded for the production of keratinization

elements are also inhibited in this way, and the synthetic elements of Golgi apparatus, endoplasmic reticulum and ribosomes stimulated.

It should be noted that the keratoacanthoma is a cancerous growth, so vitamin A acid may affect these cells differently than normal epidermal cells. Vitamin A can transform early embryonic mammalian skin to a mucus-secreting epithelium, but from the studies reported here, it is not certain vitamin A can cause normal adult mammalian skin to produce mucus. Typically, cancerous cells are similar to embryonic cells in many ways and this may account for the mucus production in the keratoacanthoma, when stimulated with vitamin A acid. While these considerations in no way deny Prutkin's finding that retinoic acid-induced mucus production in the keratoacanthoma is mediated by RNA-polymerase activity, it would be interesting to know if RNA-polymerase activity is necessary for the induction of the anti-keratinizing effects of vitamin A acid in normal mammalian skin.

Zil, in 1972 (56), studied the mechanism of vitamin A acid stimulation of epidermal cells using a different approach. He found that retinoic acid caused an increase in epidermal mitoses within two hours and had a prolonged effect as well. Retinol, however, did not produce the early increase in mitoses but did induce the later, prolonged increase. Zil concluded that retinoic acid stimulated  $G_2$  (gap 2) cells to enter mitosis while retinol stimulated

only those cells in earlier phases of the mitotic cycle. Perhaps this difference in epidermal cell susceptibility to stimulation by retinol or retinoic acid is part of the explanation why retinoic acid is more effective than retinol in producing certain epidermal changes and in treating some skin diseases.

To understand the mechanisms of vitamin A activity in epidermal cells, the precise nature of the ultrastructural changes must be known. The biochemical nature of the materials produced in the mitochondria and cytoplasm of vitamin A treated epidermis must be analysed. Autoradiographic tracer studies may be used to follow the production of these materials over time and to follow the path of the various vitamin A analogues through the cells. It is established that vitamin A affects embryonic, adult and metaplastic (keratoacanthoma) epidermal tissues differently, and it may be that vitamin A also acts differently on diseased epidermal cells. These are some of the areas that need further study in order to use vitamin A most effectively in the treatment of skin diseases.

## SUMMARY

1. Stripping the skin four times with cellophane tape removed most of the stratum corneum and caused the development of widened intercellular spaces. As the spaces formed, desmosomes were torn from one cell and engulfed by the other, and intramitochondrial inclusions, glycogen and basal lamina breaks appeared.
2. Ethanol (95%) caused similar damage and also induced the formation of lipid droplets in some keratinocytes. Ethanol also caused sloughing of the stratum corneum.
3. Propylene glycol produced no detectable damage.
4. Vitamin A acid produced mild damage with widened intercellular spaces and intramitochondrial inclusions, but no lipid droplets or glycogen were detected. It also caused inhibition of keratinization.
5. Combinations of treatments caused increased damage.
6. Lipid droplets failed to stain with silver methenamine, indicating they contained no glycogen or glycoprotein. They also failed to be digested by protease, indicating they contained no protein.
7. Intramitochondrial inclusions were digested with protease indicating a protein component, and they also stained with silver methenamine, indicating the presence of glycogen or glycoprotein as well.

## APPENDIX I

DALTON'S FIXATIVEPreliminary

Prepare 2.5 N KOH solution

(14.0 gm KOH plus water to make 100 ml)

Prepare 3.4% NaCl solution

(8.5 gm NaCl plus water to make 250 ml)

Buffer

Add 10.0 gm potassium dichromate to 200 ml water.

Adjust to pH 7.4 with 2.5 N KOH.

Add 250 ml of 3.4% NaCl solution.

Add 250 ml of distilled water.

Store at 4°C. (Stable for several months)

Fixative

Dissolve 1.0 gm osmium tetroxide to 50 ml distilled water.

Add 50 ml dichromate buffer.

## APPENDIX II

DEHYDRATION AND EMBEDDING SCHEDULE

- 1) Fix tissue.
- 2) Rinse in 50% ethanol at 4°C.
- 3) 50% ethanol            15 min            4°C            Agitate
- 4) 70% ethanol           15 min           4°C            "
- 5) 90% ethanol           15 min           4°C            "
- (May be left in refrigerator overnight without agitation)
- 6) 100% ethanol          30 min          room temp.      Agitate
- 7) 100% ethanol          30 min          "                "
- 8) 100% ethanol          30 min          "                "
- 9) propylene oxide       20 min          "                "
- 10) propylene oxide       20 min          "                "
- 11) 1:1 propylene  
oxide - Epon              30 min          "                "
- 12) 1:1 propylene  
oxide - Epon              30 min          "                "
- 13) 1:2 propylene  
oxide - Epon              60 min          "                "
- 14) Epon                   90 min          "                "
- 15) Embed in capsules with fresh Epon.
- 16) Polymerize (35°C - 40°C) overnight.
- 17) Polymerize (60°C - 65°C) for two days.

## APPENDIX III

EPON EMBEDDING MEDIUM

- 1) Tare plastic cup on top-loading balance.
- 2) Add Araldite (34.5 gm).
- 3) Add Epon (48 gm).
- 4) Add DDSA (dodecenyl succinic anhydride) (90 gm).
- 5) Mix very well with a teflon stirrer avoiding froth.
- 6) Add 3.9 ml DMP-30 using a 5 ml disposable syringe & mix.

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VITA AUCTORIS

Born: June 26, 1945. Kenora, Ontario, Canada.

Son of Mr. and Mrs. Roy Brown.

Primary Education:

North Ward School, Kenora, Ontario. 1951 - 1959.

Secondary Education:

Lakewood High School, Kenora, Ontario.

1959 - 1963, 1964 - 1966.

University Education:

University of Waterloo, Waterloo, Ontario.

1966 - 1971, 1974 - 1975.

(B.I.S. Bachelor of Independent Studies)