THE ROLLER TUBE TISSUE CULTURE TECHNIC IN THE EVALUATION OF THE PRIMARY IRRITANCY PRODUCING CAPACITY OF TOPICAL MEDICAMENTS AND CHEMICALS*

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The purpose of this paper is to report our findings in exploring further the possible application of tissue culture technics in evaluating the potential primary irritancy producing capacities of drugs and chemicals when applied to skin and mucous membranes. In previous studies[1, 2] on this subject, we have employed the hanging-drop tissue culture technic; the roller tube method was used in the present study.

The inadequacy of present methods for testing the comparative irritant properties of new drugs is well known. It is not difficult to establish by means of patch tests that a grossly irritant substance such as bichloride of mercury is a primary irritant for intact skin in relatively low concentration, but this method is not satisfactory for detecting borderline irritant effects. Theoretically, it is possible that the comparative toxic effects of drugs and chemicals on young epithelial cells in tissue culture is a more accurate index of their primary irritancy producing potentials than patch tests applied to epithelial cells protected by keratin. However, it is important to note that, in one case, we are dealing with the intact organism, which means that many factors may modify the effect of the drug or chemical on the skin, and in the tissue culture preparation, we are testing an isolated bit of skin suspended in a constant medium.

There is no substitute for actual clinical experience in evaluating the tolerance of inflamed and denuded skin for topical medicaments. However, in the evaluation of a new drug, the accumulation of such clinical data is a slow, rather involved process, unless the drug in question is an obvious primary irritant in low concentration.

MATERIALS AND METHODS

Specimens of human skin used in this study consisted of foreskin obtained from infants following circumcision procedures. After removal of the underlying connective layer, the tissue consisted of the entire epidermis and a thin layer of dermis. 2 x 2 mm. fragments were cut for explanting. The tissue culture procedure employed was a modification of the roller tube technic, originally described by Carrel, and later by Gey;[3, 4] this method has been used extensively in Pomerat's laboratory.

The clot was made on a rectangular 12 x 50 mm. No. 1 coverslip, with two drops of heparinized chick plasma and two drops of chick embryonic extract. Two pieces of skin were

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explanted approximately 2 cm. apart on each coverslip. Usually 2 to 3 hours were required for the clot to set firmly. Thereupon, the coverslip was placed in a 16 x 150 mm. bacteriologic culture tube, and 2 ml. of supernatant nutrient fluid was added. This fluid was composed of 50 parts of Gey's balanced salt solution,[4] 45 parts of human ascitic fluid, and 5 parts of diluted chick embryonic extract. Also, in order to prevent bacterial contamination, the liquid phase of the culture medium contained 160 micrograms of neomycin sulphate per ml. of medium. In a previous study, it was demonstrated that this concentration of neomycin had no effect on epithelial cells in tissue culture. The tubes were then closed with rubber stoppers and incubated at 37° C. in an almost horizontal position. They were rotated slowly at a speed of 12 revolutions per hour, so that the fluid phase of the medium was constantly bathing the skin explants during the entire incubation period.

Drugs or chemicals to be tested were incorporated in various concentrations in the supernatant fluid. Usually, these were introduced into the system 7 to 14 days after original explantation, at a time when a uniform outgrowth of epithelial sheet was established. Each test culture, with a given drug of a particular concentration, had its own paired control. These paired cultures were carefully selected so that the initial amounts of outgrowth were comparable, and so that the other conditions of the experiment were identical at the outset and throughout the duration of the experiment. This was done in order to insure a fair comparison between control and test cultures.

For this study, arbitrarily the time of contact of drugs with the epidermal cells was set for either 18 or 42 hours. Most of the cultures were fixed at the end of either 18 or 42 hours with methyl alcohol and stained according to Jacobson's method with May-Greenwald and Giemsa stain.[5] In some, after a contact period of either 18 or 42 hours, the supernatant which contained the test substance was removed, and fresh nutrient without the substance was introduced. These cultures were kept for another week to determine their capacity to recover after they were removed from exposure to the irritant.

The end point of toxicity was determined by studying both stained preparations, as well as unfixed living cultures with phase contrast microscopy.

The following drugs and chemicals were tested:

Aureomycin hydrochloride, crystalline, Intravenous, buffered with sodium glycinate (Lederle, Lot No. D5)

Endomycin (Upjohn)

Gramicidin (Smith, Kline & French Laboratories, Lot #32650)

Mycostatin (Squibb, Lot No. St. 695-714/15-1-B)

Neomycin sulfate (Upjohn)

Penicillin G Potassium (Buffered, crystalline, Squibb)

Terramycin hydrochloride (Crystalline, Intravenous, buffered with sodium glycinate, Pfizer, WKD 537777)

Cocaine hydrochloride, U.S.P. (Merck)

Procaine hydrochloride, Sterile Crystal (Mizzy, Inc., N. Y.)

Nupercaine hydrochloride (Ciba)

Quotane (Compound SKF, No. 538-A, R.S.O. #17253, Smith, Kline & French Laboratories)

Benadryl hydrochloride (Lot No. 152976, Parke-Davis)

Thephorin 'Roche' (Hoffman-La Roche)

Boric acid, U.S.P. (Crystal, Mallinckrodt)

Resorcinol

Mercuric bichloride

Nickel chloride, Analytical Chemical $NiCl_2 \cdot 6H_2O$ (Powers-Wrightman-Rosengarten Co.)

Potassium chloride, C. P. (Coleman & Bell Co.)

Potassium dichromate, Technical (Merck)

Potassium nitrate, Reagent (Merck)

A total of approximately 2000 cultures of human skin obtained from 50 different donors

was utilized in this study. For determining final critical values, experiments were repeated at least 3 times, and in some instances, as many as 10. In this way, the comparative toxicity of the various compounds in relation to uniform explants and media could be evaluated.

RESULTS AND DISCUSSION

With human foreskin cultivated *in vitro*, epithelial outgrowth in sheets usually appeared in 5 to 6 days, and for a period of 4 weeks, continued to increase in size. The rate of outgrowth varied with different skin specimens, and also, with different batches of ascitic fluids used in the culture medium. Usually, a sheet of epithelial cells, approximately 2–3 mm. in width, was obtained evenly around the explant at the end of 7 to 14 days' cultivation (Fig. 1), its thickness varying from one to a few cell layers. Active proliferation of these cells was evidenced by the presence of numerous mitotic figures (Fig. 6), especially during the first two weeks. The number of dividing cells gradually decreased during the third and fourth weeks. The cultures began to degenerate after four weeks, and the additional of fresh nutrient fluid usually did not prolong the life of the cultures. No attempt was made to subculture these cells. This four-week period of observation of actively proliferating and apparently healthy epidermal cells provided sufficient time for the purpose of our studies. The viability of these cells was further confirmed with the use of time-lapse microcinematographic recordings. With this technic, one was able to observe the characteristics of these cells in the living state, including membrane activity, pinocytosis[6], and the presence of long filamentous mitochondria (Fig. 4, 5, 19), as reported by Lewis, Pomerat and Ezell[7].

Although the outgrowth from these skin explants was essentially epithelial, sometimes small numbers of fibroblasts also were present, especially in cultures kept longer than 3 to 4 weeks. In prolonged cultivation, and occasionally, in earlier stages, fibroblasts were found to be the predominant cellular elements. In this event, one found compact sheets of fibroblastic cells in parallel or interwoven patterns. Since our present interest lies in the epidermal cells, the fibroblasts will not be described.

EFFECTS OF DRUGS ON HUMAN SKIN IN CULTIVATION

A. Examination of Stained Preparations

The degree of injury induced by the test compounds was determined by the presence of the following changes:

1. Sloughing and destruction of the epithelial sheet (Fig. 2, 3).
2. Loss of staining properties and cellular outlines.
3. Changes in the nuclear elements. Injured nuclei were hyperchromatic, pyknotic, relatively small, and often bizarre in shape. Usually, these changes were associated with disintegration of the nuclear contents into small fragments, either with or without disruption of the nuclear membrane (Fig. 7, 9).
4. Changes in the cytoplasm. The cytoplasm was stained poorly, or not at all. In most cases, the cellular outlines were lost. In isolated areas where cells occurred singly (not in sheets), there was formation of pseudopod-like processes at

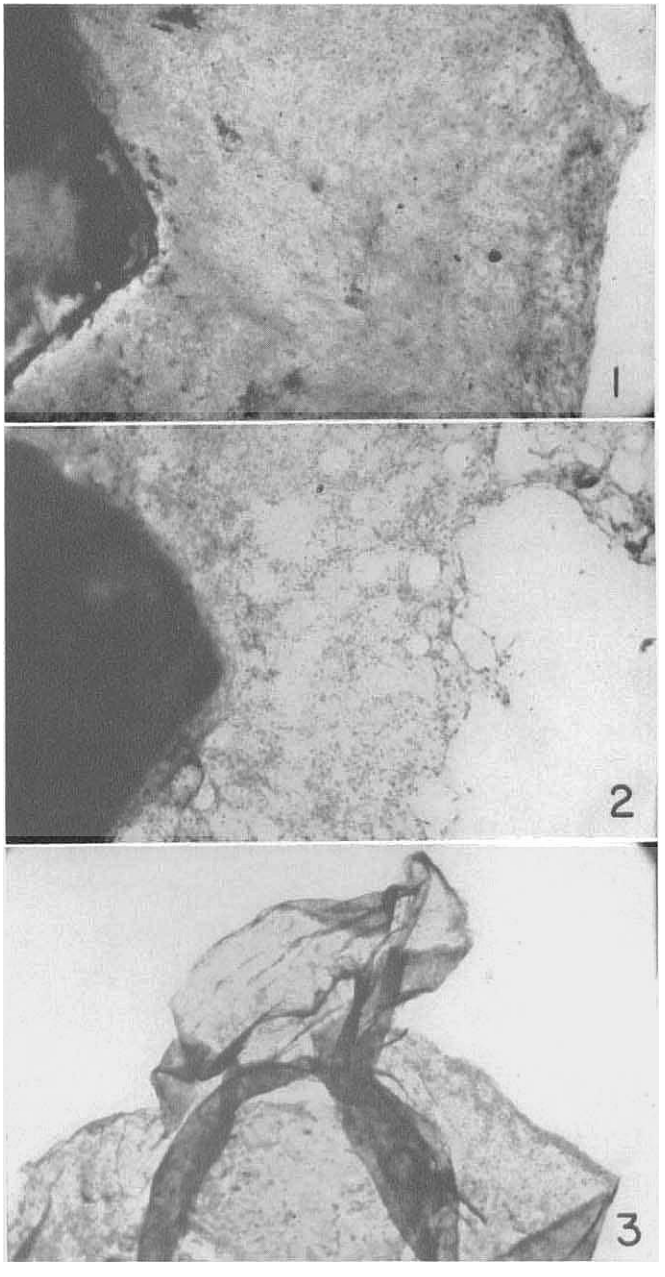
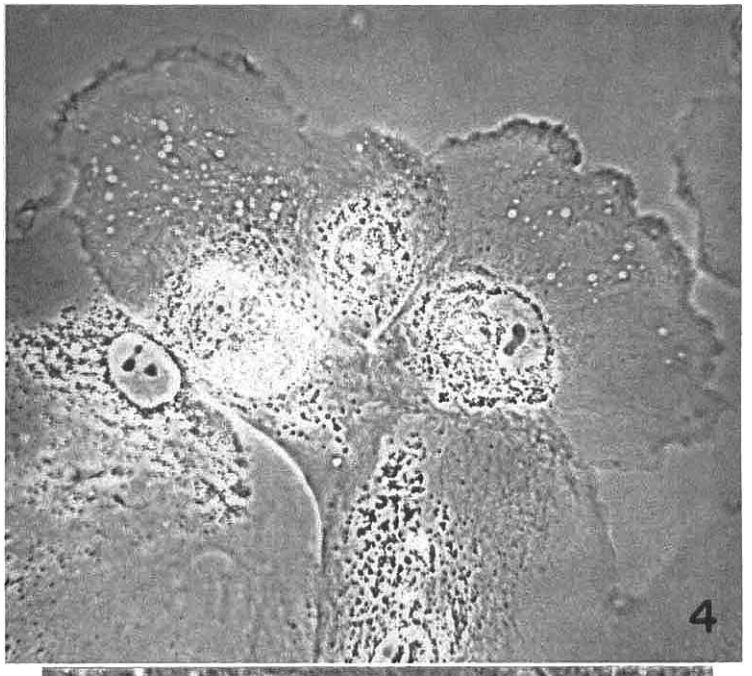


FIG. 1–3. Epithelial outgrowth from human foreskin explants. 15 days' cultivation in roller tube. May-Greenwald and Giemsa stain. ×17.5

FIG. 1. Normal sheet.

FIG. 2. Partial injury following treatment. Note the retracted and poorly stained areas.

FIG. 3. Complete injury, showing folding of epithelial sheet and its detachment from clot.



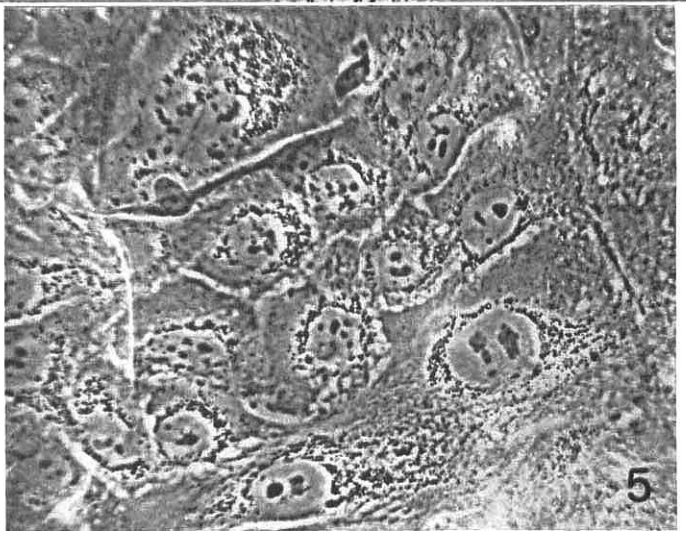


FIG. 4–5. Human epidermal cells *in vitro*. 14 day living cultures. Dark phase contrast microscopy ×430.

FIG. 4. Cells at the periphery of sheet. Note the undulating membrane, long filamentous mitochondria, perinuclear granules, and vacuoles formed in pinocytosis.

FIG. 5. Cells in the middle portion of epithelial outgrowth. Note the smaller sizes and intercellular spaces and connections.

the periphery of the degenerated cells (Fig. 8). Marked vacuolization occurred in some cells (Fig. 9); often this phenomenon was seen when the destruction was not complete.

B. Examination of Unfixed Cultures under Phase Contrast Microscope

1. Retraction of membrane. In contrast to the undulating membrane of normal epidermal cells at the periphery of the sheets, the free margin of injured cells had a ragged, torn appearance with thready processes (Fig. 10, 20). Most of the cells throughout the sheet had a contracted outline with loss of details and condensation of the contents (Fig. 10, 24).

2. Formation of coarse granules. In some, the nuclei exhibited no apparent change; but immediately surrounding the nuclei, and filling the entire cytoplasm, there were large collections of coarse granules (Fig. 14, 15). Probably this change is an early sign of degeneration. It was seen occasionally in untreated cultures, but was most prominent in those treated with toxic concentrations of nickel chloride, nupercaine, and less frequently, benadryl.

3. Vacuole formation in the cytoplasm. This picture was more frequent in the case of procaine- or cocaine-treated specimens. The vacuoles accumulated predominantly in the perinuclear zone; there were no vacuoles at the periphery of the cytoplasm (Fig. 13, 14). In normal epidermal cells, there were granules in the perinuclear zone (Fig. 4, 5, 19), which seemed to be replaced by vacuoles in the treated cells.

4. Separation and widening of intercellular spaces. This predominant change was seen in cells subjected to the toxic action of resorcinol, mercuric bichloride, and potassium nitrate, more marked in the latter two (Fig. 16, 17, 18, 20, 22). There were large vacuoles and spaces between cells when the drug concentration was high, or collections of small vacuoles in lower concentrations.

Not all of the above-mentioned changes were observed in every specimen; some changes were more prominent with the use of one compound, and very minimal with others. Also, the changes varied according to the degree of damage inflicted upon the cells by different concentrations of a given compound. For the sake of convenience, it is preferable to discuss the results of these experiments according to separate groups of compounds.

Mercuric bichloride and Potassium dichromate

These two chemicals are very toxic, and both produced complete damage of epidermal cells with no recovery after 18 hours exposure at a concentration of 50 micrograms per ml.

Benadryl, Quotane, Nupercaine and Thephorin

Drugs in this group were found to be moderately toxic. Their toxic dosage range was between 125 to 500 γ/ml. At a concentration of 500 γ/ml., all four compounds produced complete damage of the epithelial cells after 18 hours' exposure. At 250 γ/ml., there was complete damage in Benadryl and Quotane treated cultures, but only partial injury in some of the Nupercaine and The-

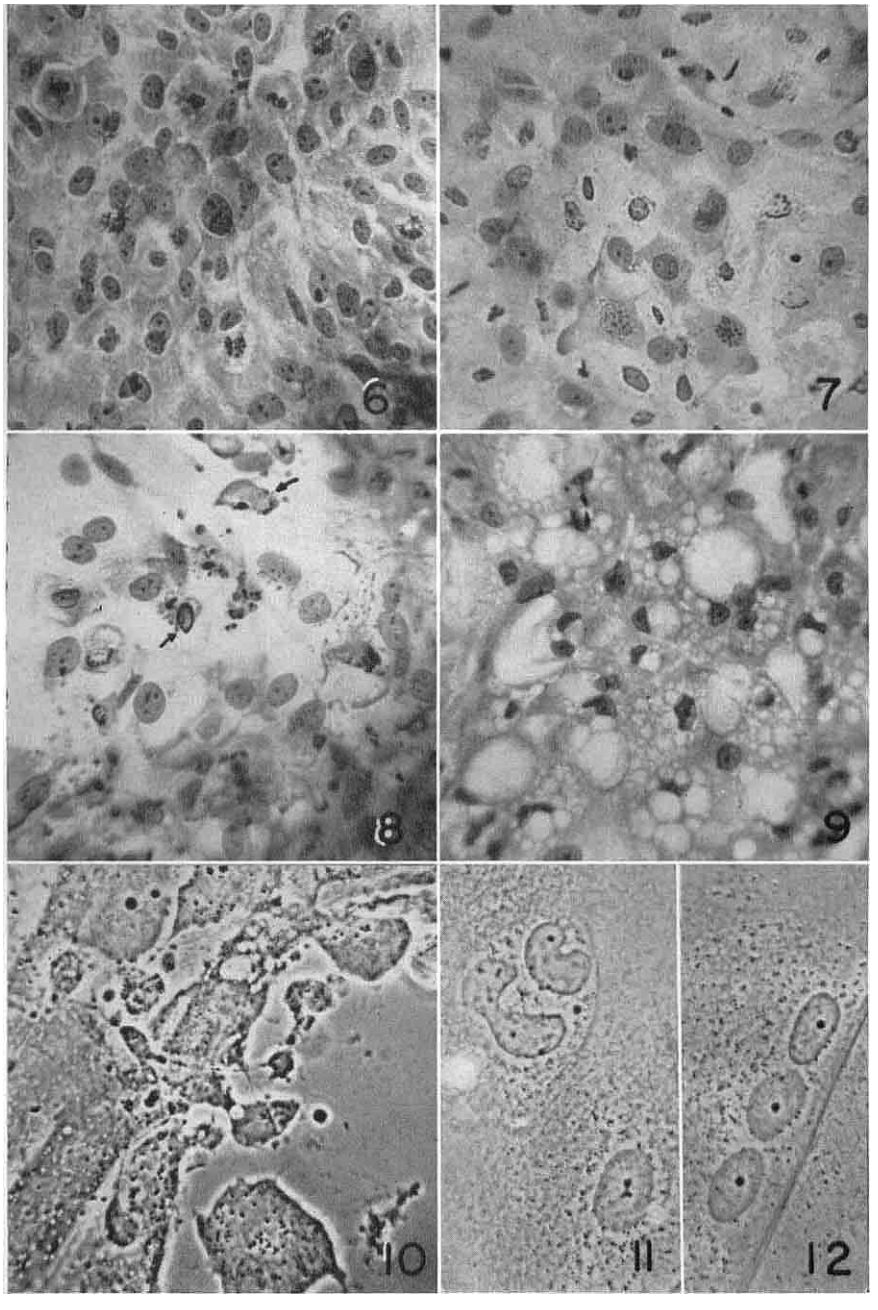


FIG. 6–9. Stained preparations. May-Greenwald and Giemsa stain ×215.

FIG. 6. Normal epidermal cells, 14 days in cultivation, showing 7 mitotic figures in the field.

FIG. 7. Epidermal cells showing poor staining and loss of cellular outline, pyknosis, and karyorrhexis as result of toxic drug effect.

FIG. 8. Degenerating cells showing pseudopod-like processes of cytoplasm.

FIG. 9. Marked vacuolization of cytoplasm and pyknotic nuclei in injured human epidermal cells.

FIG. 10–12. Unfixed cultures showing toxic effects of test compound. Dark phase contrast microscopy ×430.

FIG. 10. Periphery or free margin of epithelial sheet. Note the granularity of cells, loss of details, and condensation of the contents.

FIG. 11–12. Loss of cellular outline and bizarre-shaped nuclei.

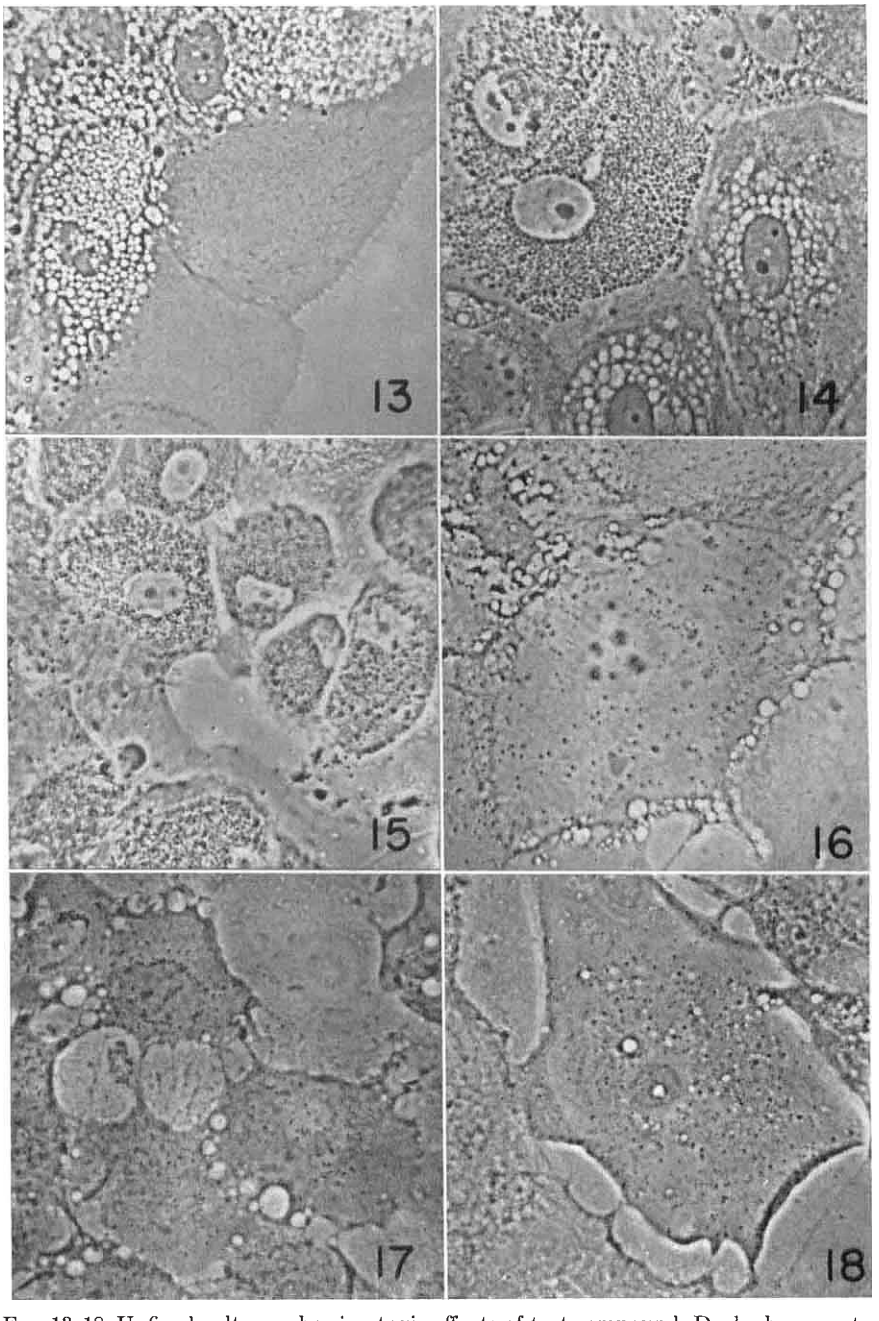


FIG. 13–18. Unfixed cultures showing toxic effects of test compound. Dark phase contrast microscopy ×430.

FIG. 13. Cells at the periphery of sheet, showing slight retraction of free margin and perinuclear vacuoles. Toxic effects of procaine.

FIG. 14. Cells showing vacuoles and granules. Toxic effects of procaine.

FIG. 15. Cells filled with granules, following toxic concentration of nickel chloride.

FIG. 16. Small vacuoles in intercellular spaces. Toxic effects of resorcinol.

FIG. 17. Vacuoles in intercellular spaces following toxic concentration of mercuric bichloride.

FIG. 18. Large intercellular spaces due to toxic effect of potassium nitrate.

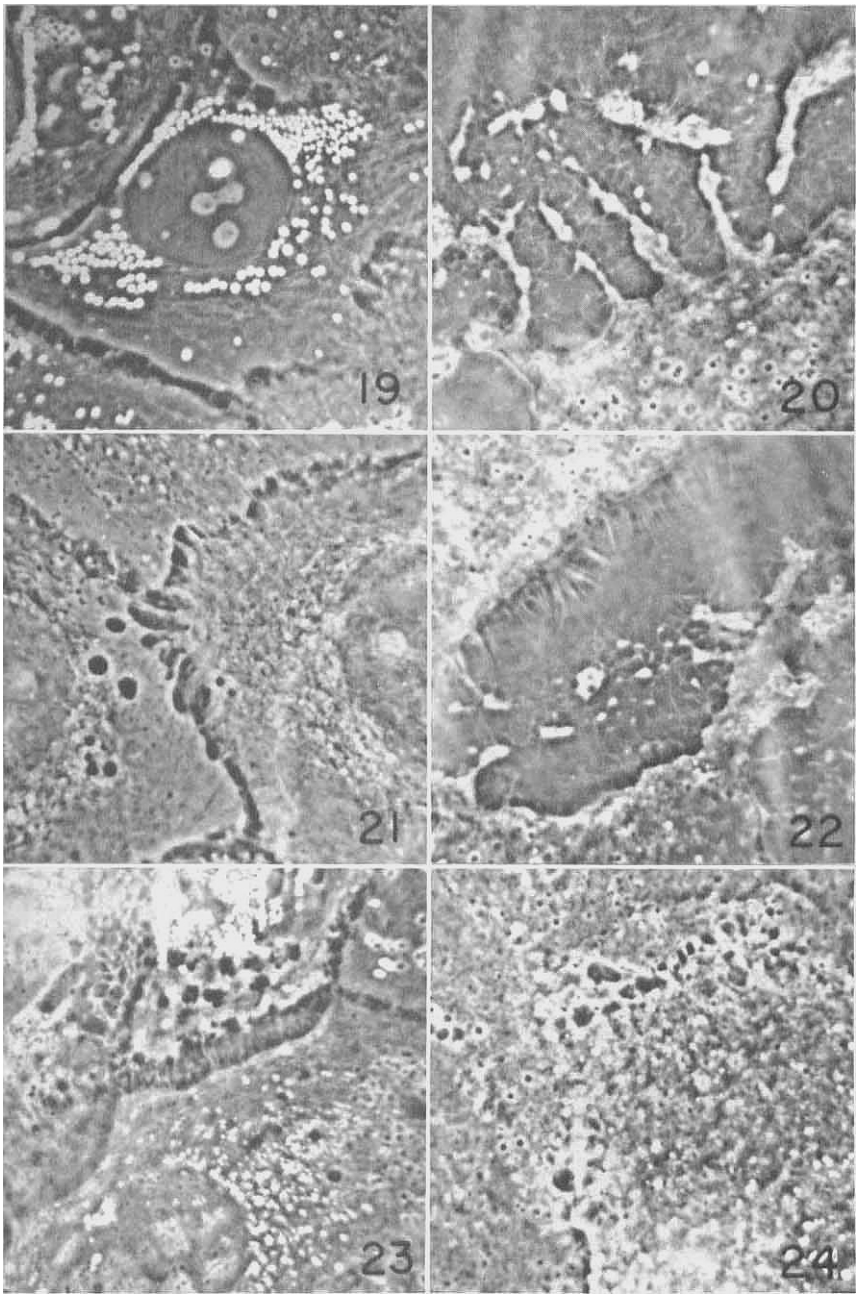


FIG. 19–24. Unfixed cultures. Bright phase contrast microscopy ×970.

FIG. 19. Normal epidermal cell showing nucleoli, perinuclear granules, filamentous mitochondria, and intercellular connections.

FIG. 20, 22 AND 24. Toxic effects of drugs showing retraction, loss of cellular details, disturbances in the intercellular relations.

FIG. 21 AND 23. Normal intercellular connections.

phorin treated cultures. Perhaps the latter two, Nupercaine and Thephorin, are slightly less toxic than Benadryl and Quotane. However, the difference is within the range of experimental error, and, therefore, was not considered significant.

Resorcinol and Nickel chloride

Complete injury of the epithelial cells was noted at a concentration of 2000 γ/ml. of resorcinol and nickel chloride after a contact period of 18 hours. Except for a few instances, recovery was not observed after cultivation in fresh medium for 1 week after removal from the medium containing the test compound.

Cocaine and Procaine

Cocaine and Procaine were found to be of approximately the same degree of toxicity for human epithelium, perhaps with the former slightly less toxic than the latter. The effects of these compounds on human epidermal cells cultivated *in vitro* were the same, *i.e.*, both produced marked vacuolization of the cytoplasm. Apparently, the process was reversible at lower concentrations of 1000 γ/ml., and occasionally, with concentrations as high as 4000 γ/ml. Even at a concentration of 8000 γ/ml. the injury was never complete, since with removal of the test compound and introduction of fresh nutrient medium, normal epithelial sheets could be obtained with one week's continuous incubation. One cannot be certain whether this 'new' sheet formation was actually formed from outgrowth of the original explant (after complete destruction of the primary sheet by toxic action) or whether it was part of the innermost protected layers of the primary sheet, which were less accessible to the toxic effect of the test compound in the 'circulating' fluid medium (Fig. 25). It was concluded that this concentration definitely was not high enough to injure the entire skin explant to the extent of preventing new growth or recovery.

Potassium chloride and potassium nitrate

Both compounds were only slightly toxic. At a concentration of 8000 γ/ml. of culture medium, potassium chloride produced complete damage in one half of the cultures tested and partial injury in the others. Potassium nitrate was somewhat less toxic, producing partial damage in all cultures at this concentration following contact of either 18 or 42 hours. Both compounds produced complete damage at 16000 γ/ml. in all cultures, and there was neither recovery nor new sheet formation after the removal of the test substance. One has the impression that these salts have the same toxicity range, with potassium chloride slightly more toxic than potassium nitrate.

Boric acid

This is the least toxic compound among those included in this study, with the exceptions of some antibiotics which will be discussed separately. A concentration of as high as 64000 γ/ml. was necessary to produce complete injury of epithelial cells.

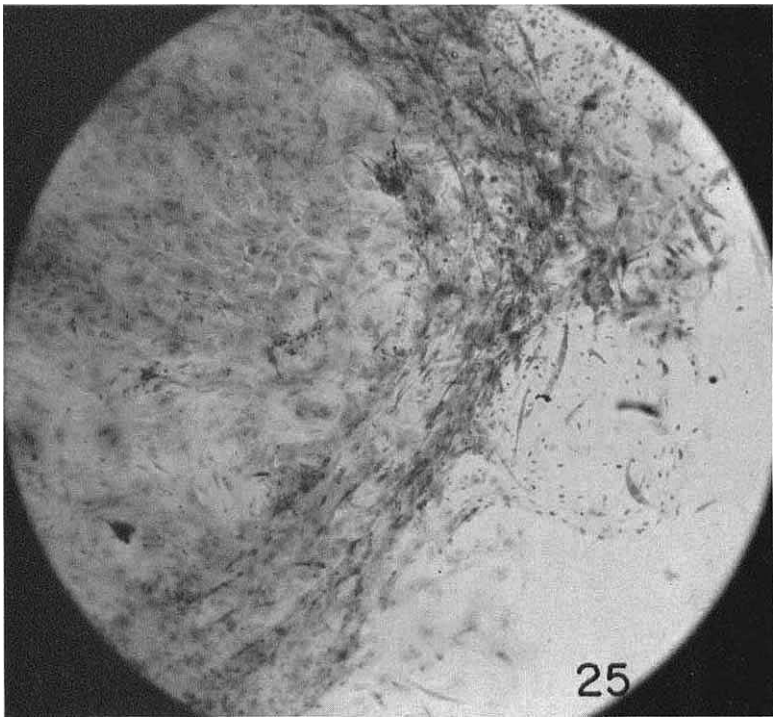


FIG. 25. Normal or 'new' epithelial sheet formation with mitosis on the left close to explants, and completely degenerated epithelium with scattered fibroblasts on the right.

Antibiotics: Gramicidin, Aureomycin, Mycostatin, Neomycin, Penicillin, and Terramycin

The low solubilities of some of the antibiotics, and their precipitation when introduced into culture medium, made it difficult to determine their toxicity.

Neomycin and penicillin at concentrations of 20,000 and 10,000 γ/ml. of culture medium respectively produced no injury to human epithelium after contact for 18 to 42 hours.

Aureomycin and terramycin are much more toxic although their low solubility and tendency to precipitate prevented accurate evaluation of their effects on the cells. In their partially soluble state, concentrations of 1250 to 2500 γ/ml. of these antibiotics produced results varying from no damage to complete damage in the cultures. By rough estimation, one would estimate that the degree of toxicity of these two antibiotics lies somewhere between the resorcinol-nickel-chloride and the procaine-cocaine groups.

Endomycin is soluble in water, but tends to precipitate when incorporated in the culture medium at a concentration of 1000 γ or more per ml., thus presenting technical difficulty for accurate evaluation. At 1250 γ/ml., endomycin did not have an injurious effect on epithelial cells.

Gramicidin is insoluble in water, but soluble in alcohol. However, its solubility in alcohol was such that this solvent could not be used. Therefore, a suspension

was used. The crystals seemed to dissolve to a certain extent when incorporated in the ascitic fluid-embryonic-extract medium. Complete injury of the epithelial sheet was observed at concentration of 200 γ/ml. for a contact of 18 hours with no recovery after its removal. This indicated that the degree of toxicity was somewhat less than the mercuric bichloride, potassium dichromate group, but more than the Benadryl-Quotane group.

Mycostatin is also insoluble in water.[8] Therefore, it was incorporated in the culture medium as an aqueous suspension. Since its potency per milligram was known to vary with different batches, its concentration was expressed in term of units rather than micrograms. At 5000 units per ml., there was complete injury of the epidermal cells after contact of 18 hours. Since this antibiotic rapidly loses its activity in aqueous solution as well as in plasma or blood, the 42-hour contact test was not done. There was either partial recovery or new sheet formation when the culture was continued in incubation for 1 week following the removal from contact with the compound and introduction of fresh nutrient fluid. Therefore, its toxicity is approximately of the same order as that of the nickel-chloride-resorcinol group.

A comparison between the toxicity results obtained from the two tissue culture technics, the hanging drop slides and roller tubes, is summarized in Table I. It was found, in most instances, that the concentration of a particular compound required to produce complete injury of human epithelial outgrowth in the roller tubes was higher than the necessary concentration in the hanging drop cultures. In analyzing data obtained from hanging drop cultures of skin, one noticed a significant difference in the degree of toxicity between Nupercaine, Thephorin, and Quotane; *i.e.*, the minimal inhibitory dose in γ/ml. (MID)[1] was 25–30 for the former two, and 125–250 for the latter. This observation is in disagreement with that obtained from patch testing as well as clinical experience[9], which indicates that the primary-irritant effects of these above-mentioned drugs are approximately of the same order. The roller tube results, however, correspond fairly well with observations derived from *in vivo* experiments. Similarly, in hanging drop cultures, nickel chloride was found to be more toxic than clinical observations indicated[9], whereas in roller tube cultures, its toxicity corresponded to that of the Resorcinol group. These facts support the assumption that the roller tube technic simulates more closely the conditions of the *in vivo* test. It is conceivable that the amount of a substance required to inhibit the initiation of outgrowth of epidermal cells *in vitro* would differ from that required to produce complete injury of living cells which are already in existence. This partly explains the difference in results obtained with these two technics, because, in the roller tube cultures, a well-established epithelial sheet is present before the compound is introduced into the medium; this condition is more comparable to the circumstances one encounters in actual experience. Still another variable factor in tissue culture experiments is the donor of the skin used for explants. With the hanging drop technic in previous experiments by the authors[1, 2] adult human skin was utilized, whereas newborn preputial skin was employed in this study.

In regard to the order of irritancy producing capacities of the compounds

TABLE I

Toxicity in gamma per ml. of culture medium of various drugs and chemicals to explants of human skin

Drug or Chemical	Hanging Drop Slide		Roller Tube		
	LID	MID	NI	PI	CI
Mercuric bichloride	1.0–2.5	6.25–12.5	1	12.5–25	50
Potassium dichromate				12.5–25	50
Gramicidin*	0.1–0.2	0.5–1.0	50	100	200
Quotane	2.5–3.0	125–250		125	250
Benadryl	30–40	165–330		125	250
Nupercaine	1.25–2.5	25–30	125	250	500
Thephorin	2.0–2.5	25–30	125	250	500
Nickel chloride	–6	50–100	250	500–1000	2000
Resorcinol	2.5–3.0	500–1000		1000	2000
Mycostatin†			500	1250–2500	5000
Terramycin‡	50–100	520–1040		1250	
Aureomycin‡	105–210	830–1250		2500	
Endomycin‡	50–100	250–500	1250		
Procaine	12.5–25	1000–2500		500–2000	4000
Cocaine	205–415	1665–3330		500–4000	8000
Potassium chloride			2000	4000–8000	16000
Potassium nitrate			4000	8000	16000
Boric acid	12.5–25	312–625	8000	16000–32000	64000
Penicillin	1500–1875	2000–3750	10000		
Neomycin	830–1015	4165–6250	20000		

* Insoluble, suspension was used in the medium.

‡ Precipitation when mixed with culture medium.

† Insoluble, suspension used in the medium. Concentration in units per ml. (2 units approximately equivalent to 1 microgram).

LID—Least injurious dose.

MID—Minimal inhibitory dose.

NI—No injury.

PI—Partial injury.

CI—Complete injury.

tested, excepting those for which data are not available, there is gross correlation of the results of the present experiment with the patch-testing concentrations of substances recommended by Rostenberg and Sulzberger[10]. Similar correlation exists with the concentrations found in the various topical preparations marketed by pharmaceutical houses. It is important to note, in this connection, that the strengths of substances, as observed by the afore-mentioned authors, do not

TABLE II

Concentrations of drugs in gammas per ml. of medium

Compound	Complete Injury	Recommended Patch Testing Concentrations (Rostenberg & Sulzberger)
Mercuric bichloride	50	0.1% Aq.
Potassium dichromate	50	0.5–1% Aq.
Nupercaine	500	1% Pet.
Nickel chloride	2000	5% Aq. Nickel nitrate 5–10% Aq. Nickel sulfate
Resorcinol	2000	3% Aq.
Procaine	4000	2% Aq.
Cocaine	8000	1% Aq.
Potassium chloride	16000	3–10% Aq.
Potassium nitrate	16000	25% Aq.
Boric acid	64000	Powder pure or Boric acid ointment

necessarily indicate that stronger concentrations would not also have been innocuous. In other words, they have listed concentrations which are not primarily irritating, but not the maximum concentrations which will not irritate. Therefore, one cannot expect to obtain exact correlation with our *in vitro* results, which represent the least injurious dose on the one hand, and the minimal inhibiting dose on the other.

On the basis of toxicity data obtained *in vitro*, 2 sets of patch test solutions were prepared for all the compounds tested in this study, except those which were not soluble and those which were soluble but of low toxicity. The lower and higher concentrations of these solutions represented those which were expected, respectively, to be non-irritating or irritating. The concentrations and pH of the solutions are listed in Table III.

Patch tests were done on 12 patients who were hospitalized for treatment of dermatitis. As would be expected, the results were not informative. In general, there were less reactions observed in the lower concentration than in the higher concentration of a given compound, but the results varied so much that no conclusions could be drawn. This perhaps may serve as another indication of the numerous variations and difficulties encountered in collecting information by means of patch testing. It is known that factors such as temperature, humidity and trauma often modify the reactions of skin, so that uniform results may not be obtained even when the tests are done on the same individual at different times. These factors are easily controlled under the experimental conditions of the *in vitro* test. Furthermore, in the case of patch-testing, a protective skin barrier must be passed before the test solution can exert any effect on the viable epithelial cells. One cannot be certain whether such a barrier exists in epithelium in cultivation.

TABLE III

pH values of solutions used for patch testing*

Compounds	% of Solution Concentration	pH
Mercuric chloride	0.5%	5.25
	1.0%	4.72
Potassium dichromate	0.5%	4.85
	1.0%	4.65
Benadryl	1%	7.25
	5%	6.65
Quotane	1%	6.00
	5%	5.20
Nupercaine	1%	6.35
	5%	5.50
Thephorin	1%	4.00
	5%	3.60
Nickel chloride	5%	6.70
	15%	6.75
Resorcinol	5%	6.70
	15%	5.85
Procaine	10%	5.62
	30%	5.60
Cocaine	10%	4.50
	30%	4.40
Potassium chloride	25%	9.15
	34.7% (Saturated)†	9.40
Potassium nitrate	25%	9.25
	31.6% (Saturated)†	9.32

† Primary irritation is not expected at this concentration.

* Aqueous solution except 5% Thephorin which was made in Gey's balanced salt solution due to its precipitation at this concentration in water.

If present, it probably is not as efficient. It is possible, therefore, that the *in vitro* test eventually may prove to be a more sensitive measure of the order of irritancy of new compounds.

Proof or disproof of this thesis can be derived only from the testing of numerous compounds, and correlation with results of both large scale patch-testing and prolonged clinical trial of such compounds. Such data are not available at the present time. However, our experience to date clearly indicates that there is gross correlation; *i.e.*, the relative order of toxicity of the substances tested corresponds well with actual clinical experience. For instance, neomycin in powder form and saturated solution of boric acid, both of which have been used on inflamed and denuded skin without evidence of primary irritation, were found to be relatively non-toxic to cells *in vitro*. On the other end of the list, mercuric bichloride and potassium dichromate are well-known primary irritants. Penicillin, although well known as a sensitizer, also has a low primary irritant effect both *in vitro* and *in vivo*. Cocaine is tolerated by human nasal mucosa in concentrations as high as 10 per cent. Its toxicity on human skin grown in tissue culture likewise was of low order.

The microscopic changes observed in this study represent toxic effects of these compounds on the human epidermis. Similar changes, though not necessarily identical, may also occur *in vivo*. With the elimination of many of the complex intrinsic and extrinsic factors involved in testing the intact human organism, these changes must represent a purely epidermal response to the effects of toxic agents.

The site of the injurious effect was found to vary with different compounds. Some predominantly involved the cell proper, *i.e.*, cytoplasm and nucleus, and others, the intercellular spaces, although all changes eventually lead to death. Other than in the evaluation of primary irritant effects, perhaps this method also will be useful in the study of the action of a drug on epidermal cells and in postulating its effectiveness in the treatment of dermatologic conditions which involve the epidermis primarily. With the aid of time-lapse movie recordings, the complete sequence of events may be followed in the cells.

The method proposed at present has definite advantages, in that it is an objective test, the conditions of experiments are relatively uniform, and the results are readily reproducible. However, there are certain important limitations. The substances to be tested must be water-soluble. When the test compound is only sparingly soluble in water, or tends to precipitate in the supernatant serous fluid medium, accurate concentration cannot be obtained for fair comparison with those compounds which are completely in solution. In some cases other solvents, such as alcohol, acetone, and certain glycols may be used, since these can be incorporated in the culture medium in considerable concentrations without injury to the cells[11].

Even with its limitations, it is believed that this method can be advantageously employed in the pre-clinical evaluation of irritancy of topical medicaments. Also, this approach may have a place in the determination of potential toxicity for skin and mucous membrane of new chemicals used in industry.

SUMMARY

1. The comparative toxic effects of 20 compounds, including chemicals, antibiotics, antihistaminics, local anesthetics, and antiseptics for human skin, cultivated with the roller tube tissue culture technic are reported.

2. Various types of toxic effects of the test compounds on human epidermal cells are described.

3. There was a very wide range in toxicity of these 20 compounds tested; the most toxic ones, mercuric bichloride and potassium dichromate, produced complete injury of epidermal cells in a concentration of 50 mcg. per ml., whereas the growing epithelium tolerated neomycin in a concentration as high as 20,000 mcg. for 42 hours without evidence of injury.

4. Correlation between the *in vitro* and the *in vivo* effects of test compounds when applied to the skin and mucous membrane is discussed.

5. The advantages and limitations of the presented method are considered.

6. This approach is proposed as a desirable laboratory procedure for preclinical

evaluation of the primary irritant effects of new topical therapeutic agents as well as for the determination of potential toxicity of chemicals used in industry.

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DISCUSSION

Dr. Victor H. Witten (New York, N. Y.): I would like to ask the presenters at what concentration or dilution of bichloride of mercury, resorcin, or of the other substance being tested do you consider them "toxic" to cells? Certainly resorcin in concentrations of two per cent and higher as used in dermatologic therapy only very rarely proves to be "toxic" or to act as a primary irritant clinically.

Dr. Funan Hu (in closing): The toxic concentrations represent relative values. For example, 50 gamma of mercuric bichloride per ml. completely inhibits the growth of human epithelial cells in tissue culture, while *in vivo* the concentration which is tolerated by normal epithelium is of the order of magnitude of 0.1%; other new compounds which have this same order of toxicity *in vitro*, would be expected to have approximately the same degree of primary irritancy-producing capacity when applied to skin.