ON THE ACTION OF COLCHICINE

THE MELANOCYTE MODEL*

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PLATES 25 TO 27

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This work was begun (1, 2) in an attempt to discover if there is a common basis for the therapeutic effect of colchicine in acute gouty arthritis (3) and for its effect on the mitotic apparatus (4). Effects of colchicine have been attributed to decreased protoplasmic viscosity in both dividing (5) and resting (6, 7) cells. Experiments in cell systems hitherto employed to study colchicine action, though often elegant in design, have been technically complex and slow in operation. In the present work we have introduced a new system in which to study the action of colchicine: the frog melanocyte.[1] In this cell type, changes in cytoplasmic viscosity are effected rapidly and reversibly (8) and are easily controlled and measured (9, 10). Frog skin darkens when treated with melanocyte-stimulating hormone (MSH). Darkening, measured by reflectance, is due to dispersion of melanin granules in melanocytes, and is thought to be accompanied by a gel-to-sol cytoplasmic transformation. When washed, the skin lightens, with aggregation of melanin granules and cytoplasmic gelation.

In using this model to study the effect of colchicine, observations were made which led to a general theory of colchicine action and to the resultant possibility of using colchicine to construct biophysical models of other cell systems.

Methods

Preparation of Frog Skin.—Skin from the frog *Rana pipiens* was prepared and mounted on rings as previously described by Shizume *et al.* (9). Four samples were obtained from each frog, two from the legs and two from the thighs. Thus four frogs supplied sixteen skin samples, arranged as follows, after Wright and Lerner (10).

Group A	LL1	LT2	RT3	RL4
Group B	RL1	LL2	LT3	RT4
Group C	RT1	RL2	LL3	LT4
Group D	LT1	RT2	RL3	LL4

The first letter L or R refers to the left or right extremity of the frog. The second letter L or T refers to leg or thigh. The number 1, 2, 3, or 4 refers to the frog used. This arrangement was

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[1] This dermal melanin-bearing cell is also referred to as a *melanophore.*

designed to compensate for variations in reactivity of skin from one frog to another, as well as among different areas of a given frog. Note that each group contains one skin specimen from each frog and one from each area sampled. Thus for any single experiment one control group and up to three experimental groups were available.

All skin specimens were soaked in four changes of frog Ringer's solution over a period greater than 1 hour before any observations were made. (A change of Ringer's solution is also referred to as a "wash.")

Measurement of Melanin Granule Dispersion.—Changes in the state of dispersion of melanin granules within melanocytes were measured as changes in reflectance when the whole skin specimen was placed over a search unit attached to a photoelectric meter. The procedure was as described previously (9), except for the establishment of the scale of reflectance: for the white enamel disc and green filter (both of which accompanied the meter), the sensitivity control knobs were set at 65. All readings of skin were taken without a filter and with the skin immersed in approximately 20 ml of solution in a 50 ml beaker. The reading of a given skin specimen after initial soaking in Ringer's solution was taken as its baseline, or zero value; subsequent increase or decrease in reflectance units was measured from baseline. Sixteen specimens could be read in a 5 minute period. The graphs were prepared by totaling the changes in reflectance within each treatment group and plotting these values against time.

Preincubation with Colchicine.—Each group of skins preincubated with colchicine received the drug after the baseline readings. After the specified period of preincubation, the group was washed. Before the second set of readings, all groups (including control) were washed a second time, except for the experiments depicted in Text-figs. 2 *a* and 2 *b*, in which they were washed but once, and in Text-fig. 10 *a*, in which they were not washed.

Solutions Used.—

Frog Ringer's solution: The suspending medium was prepared as previously described (10). The pH was between 7.3 and 7.8 at the start of a given experiment. All test substances were dissolved in Ringer's solution before use.

Colchicine: Colchicine USP (Sigma Chemical Company, St. Louis) was the preparation generally used. The drug at 10^{-3} M was freshly prepared for each experiment and protected from light before use. To specified skins in 20 ml of Ringer's solution, colchicine was added to 9×10^{-5}, 5×10^{-5}, or 1×10^{-5} M. Addition of colchicine at these concentrations did not affect the pH of the Ringer's solution.

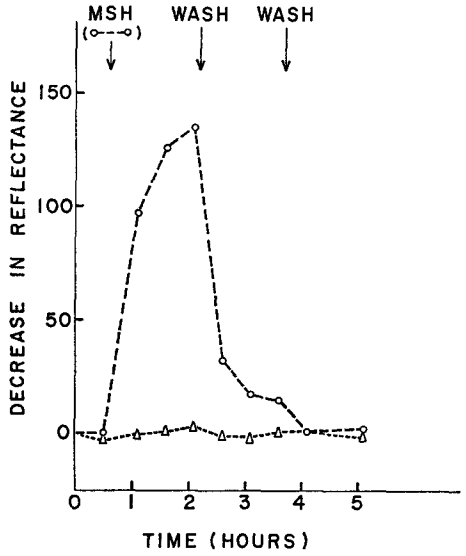
In the experiments depicted in Text-figs. 2 *a*, 2 *b*, and 10 *a*, colchicine USP is designated "C-1." For these studies approximately 1 gm of C-1 was dried *in vacuo* at 80°C for over 10 hours, yielding the sample designated "C-2." Five hundred mg of the latter sample was dissolved in triple-distilled water and lyophilized, yielding sample "C-3." In solution at 10^{-3} M, C-1 and C-2 had a faintly sweet odor; C-3 did not.

Melanocyte stimulating hormone (MSH): Beef posterior pituitary powder (Armour Pharmaceutical Company, Kankakee, Illinois) was extracted with water and lyophilized as described previously (9). One mg of the lyophilized product was diluted so that each beaker received 0.2 ml of the final product containing 10 units of MSH (9).

Other solutions: Other substances were used in final concentrations as follows: caffeine, 5.2×10^{-3} M; adenosine triphosphate disodium (ATP), 0.9×10^{-3} M; ethyl acetate, 0.8×10^{-2} M; *N*-acetyl-5-methoxytryptamine (melatonin), 4.3×10^{-10} M; hydrocortisone sodium succinate (hydrocortisone), 1×10^{-3} M; L-arterenol bitartrate hydrate (norepinephrine), 1×10^{-3} M.

RESULTS

The Behavior of Control Preparations (Text-fig. 1).—In frog skin prepared by the method described, reflectance remains constant over several hours. With further changing of Ringer's solution, reflectance may increase slightly.



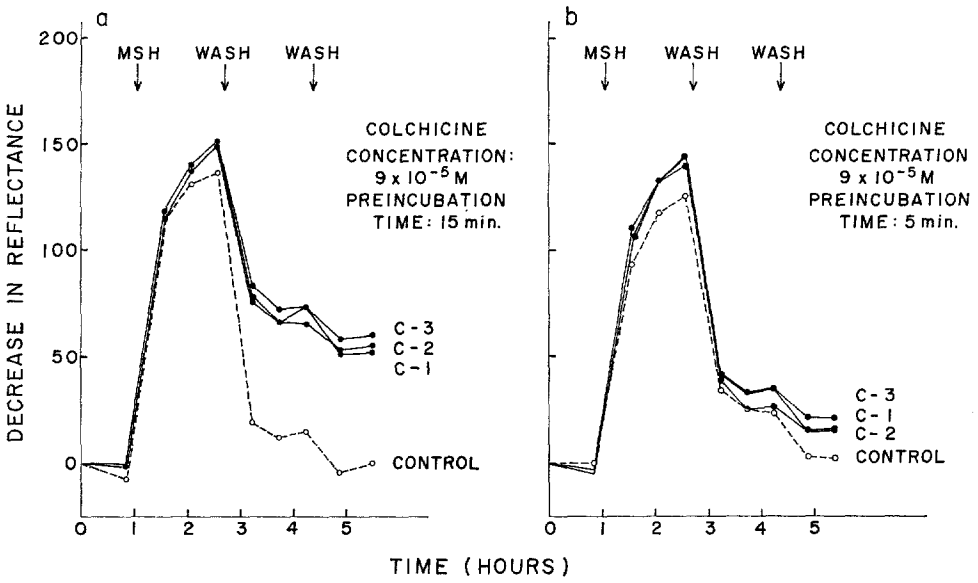
TEXT-FIG. 1. The behavior of control preparations (△ - - - △: No MSH) (see text).

The addition of a standard amount of MSH to a group of skins results in a rapid decrease in reflectance, maximal at 60 to 90 minutes. The effect is reversed by changing the Ringer's solution, whereupon the reflectance returns to near baseline.

The Effect of Preincubation with Colchicine.—
Characteristics and reproducibility (Text-figs. 2 a and 2 b):

Three different samples of colchicine (see Methods) were used, all at 9×10^{-5} M. Each sample was preincubated for 15 minutes with a group of skins, the fourth group serving as control. Then all four groups were washed, and MSH was added to each. The results for the three experimental groups were identical (Text-fig. 2 *a*). When MSH was added, groups preincubated with colchicine darkened more than did the control group. (Unlike MSH controls, darkening was not maximal at 60 to 90 minutes, but continued slowly thereafter.) Ninety minutes after the addition of MSH, all four groups of skins were washed. After washing, reversal of MSH darkening occurred readily in control skins; however, in the three groups preincubated with colchicine, inhibition of lightening was apparent. A second wash, 90 minutes after the first one, was sufficient to bring the reflectance of the control group back to baseline. In contrast, the three preincubated groups maintained comparable degrees of inhibition of lightening.

The experiment was repeated using a shorter preincubation time, 5 minutes instead of 15 (Text-fig. 2 *b*). The results were the same except for less inhibition of lightening.



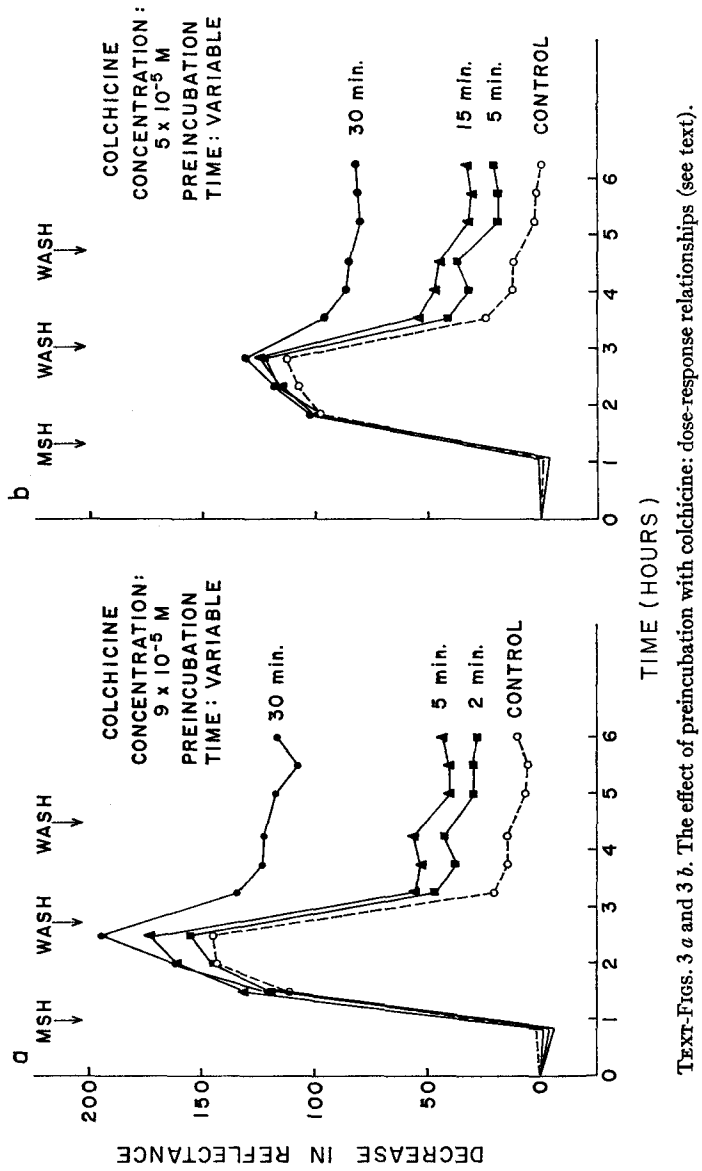
TEXT-FIGS. 2 *a* and 2 *b*. The effect of preincubation with three different samples of colchicine: characteristics and reproducibility of the effect (see text).

Summary: Preincubation of frog skin with colchicine causes an increase in darkening induced by MSH as compared to controls, and an inhibition of subsequent lightening.

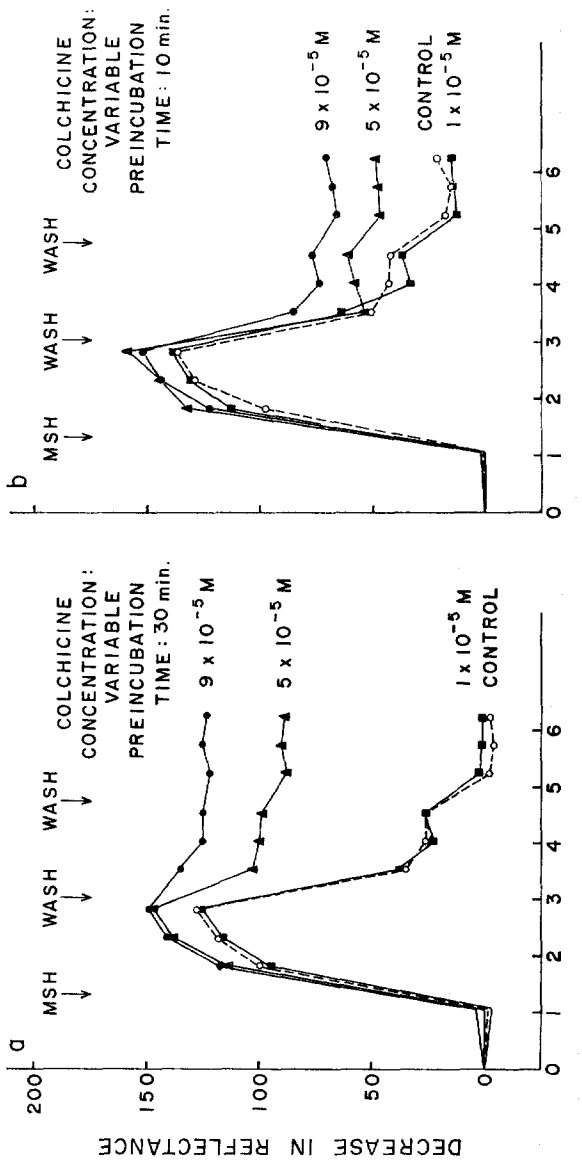
Dose-response relationships (*Text-figs. 3 and 4*): In the preceding studies, the results with two different preincubation times suggest a dose-response relationship (*i.e.*, dosage = concentration × preincubation time). However, the two experiments were done with groups of skins from different frogs and are not strictly comparable.

In the next four experiments, for a given group of skins, either concentration of colchicine or preincubation time was varied, while the other was held constant. Preliminary studies with colchicine at 9×10^{-5} M showed that a 30 minute preincubation gave strong inhibition of lightening after MSH. At the lower limit, a 30 second preincubation had virtually no effect. Varying the preincubation time in this range resulted in a commensurate variation in inhibition of lightening (Text-fig. 3 *a*). Increase in MSH darkening over controls occurred in all three preincubated groups. When the colchicine concentration was decreased to 5×10^{-5} M in a different set of skins (Text-fig. 3 *b*), the results were similar, but with less inhibition of lightening in the three preincubated groups.

In the third and fourth experiments, preincubation times were held constant at 30 and 10 minutes, respectively, while the colchicine concentration was varied from 9 ×



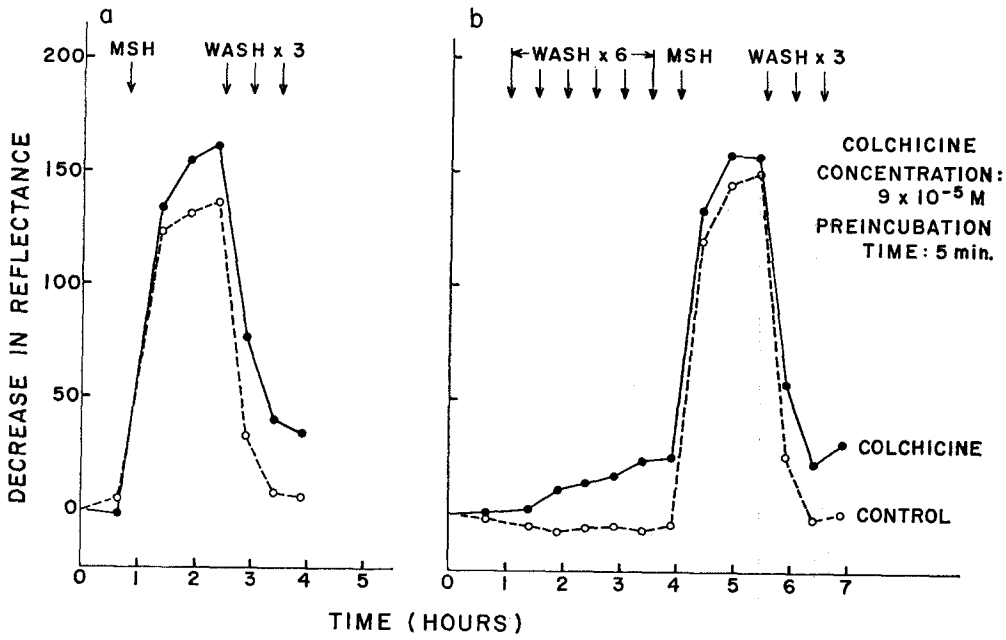
TEXT-FIGS. 3 *a* and 3 *b*. The effect of preincubation with colchicine: dose-response relationships (see text).



TEXT-FIGS. 4 *a* and 4 *b*. The effect of preincubation with colchicine: dose-response relationships (see text).

10^{-5} to 1×10^{-5} M (Text-figs. 4 *a* and 4 *b*). For a given preincubation time, the degree of inhibition of lightening varied directly with the concentration of colchicine. With colchicine at 1×10^{-5} M, there was neither increased MSH darkening nor inhibition of subsequent lightening, even with a 30 minute preincubation.

Summary: Inhibition of lightening by colchicine is dependent on both its concentration and preincubation time.



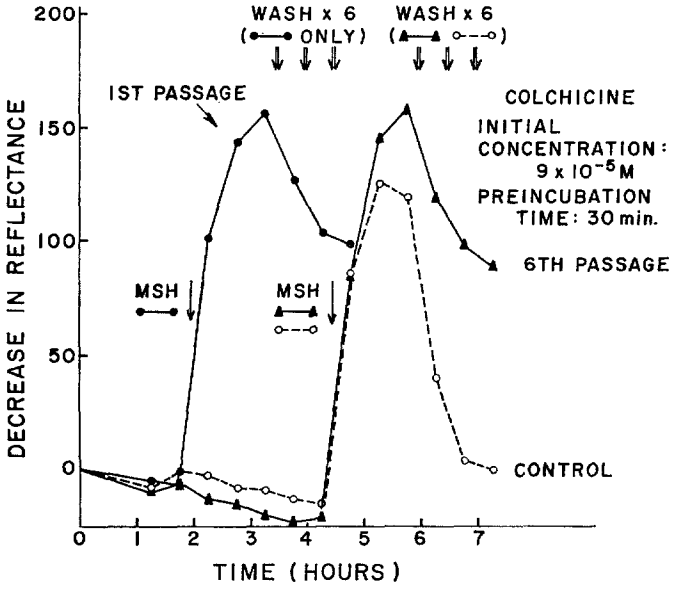
TEXT-FIGS. 5 *a* and 5 *b*. Attempt to "wash out" the colchicine effect from preincubated preparations (see text).

Attempt to "wash out" the colchicine effect from preincubated preparations (Text-figs. 5 a and 5 b): In the preceding studies, both preincubated and control groups were washed twice before addition of MSH. The question arose whether additional changes of Ringer's solution might reverse the effect of preincubation with colchicine.

In the first part of the next experiment (Text-fig. 5 *a*) the design was the same as in Text-figs. 3 *a* and 3 *b*, using a colchicine concentration of 9×10^{-5} M and a 5 minute preincubation time. However, in the other two groups of skins (Text-fig. 5 *b*), after the same preincubation conditions and double wash, MSH was not added immediately. Instead, the groups were rewashed at 30 minute intervals for 3 hours, and only then was MSH added. Subsequent reading and washing were carried out at the same in-

tervals as in Text-fig. 5 *a*. Despite the frequent washing, a gradual darkening of the experimental group in Text-fig. 5 *b* was noted, resulting in a higher take-off point when MSH was finally added (see Text-figs. 10 *a* and 10 *b*). Otherwise, the curves in Text-figs. 5 *a* and 5 *b* are virtually superimposable; *i.e.*, the conditions of washing were not sufficient to alter the preincubation effect. Similarly, after MSH darkening was effected and the groups had been washed twice over the subsequent 3 hours (Text-figs. 2 to 4) subsequent washing was not found to diminish the established degree of inhibition of lightening.

Summary: The effect of preincubation with colchicine could not be "washed out" under the conditions described.



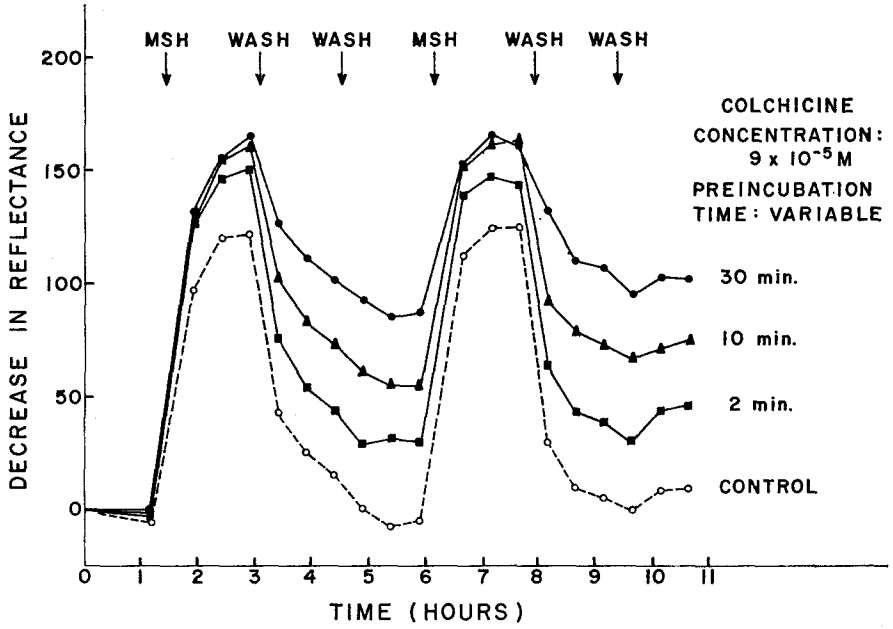
TEXT-FIG. 6. Attempt to exhaust the colchicine solution by successive preincubations (see text).

Attempt to exhaust the colchicine solution by successive preincubations (Text-fig. 6):

Initially a single group of skins was preincubated for 30 minutes in Ringer's solution containing colchicine at 9×10^{-5} M. Several other groups contained only Ringer's solution. After 30 minutes, the beakers of another group were emptied, and the fluid from each beaker of the first group was transferred to the corresponding beaker of the second group. The first group was then washed twice and MSH was added, as in Text-figs. 3 *a* and 3 *b*. The second group was allowed a 30 minute preincubation, and then its fluid contents were transferred to an emptied third group, and so on. In this manner,

six groups of skins were preincubated consecutively for 30 minutes in the *same* colchicine solution. The three groups plotted in Text-fig. 6 all came from the same four frogs. The two experimental curves describe the first and the sixth groups of skins passed through the same colchicine solution. The two curves are essentially the same, as was the curve describing the third passage (not shown in Text-fig. 6).

Summary: The power of a 9×10^{-5} M colchicine solution was not diminished by passage through it of six successive skins, each for 30 minutes.



TEXT-FIG. 7. Attempt to alter the colchicine effect by a second cycle of darkening and lightening (see text).

Attempt to alter the colchicine effect by a second cycle of darkening and lightening (Text-fig. 7):

The experimental design was as in Text-figs. 3 *a* and 3 *b* except that, after the degrees of inhibition of lightening were determined, the experiment began again, with addition of MSH to all skins. The preincubations with colchicine occurred only once, at the beginning of the experiment. After the initial cycle of darkening and lightening had been measured, further addition of MSH resulted in a comparable degree of darkening to that obtained previously in each group. Moreover, subsequent washing again established inhibition of lightening, once more in proportion to the original colchicine preincubation time. The consecutive curves are superimposable until about the tenth hour, when all the groups, including the control, began slowly to darken.

Summary: After inhibition of lightening following a single exposure to colchicine had been demonstrated, a further addition of MSH and washing resulted in essentially the same curves of increased darkening and dosage-dependent inhibition of lightening that had been obtained in the first cycle.

The effect in relation to darkening agents other than MSH (Text-figs. 8 a to 8 c):

Substances other than MSH are known to darken frog skin (8). The design of these experiments is as in Text-figs. 3 *a* and 3 *b*, except that, instead of a standard dose of MSH, the darkening agent used was caffeine, adenosine triphosphate (ATP), or ethyl acetate (see Methods). Preincubation with colchicine resulted in an increase in darkening compared to controls and a dosage-dependent inhibition of lightening with each of these darkening agents substituted for MSH.

Because the colchicine was prepared commercially by precipitation from ethyl acetate, we were particularly interested in the darkening power of this organic solvent. However, 0.8×10^{-2} M was required to produce the degree of darkening seen in the control group of Text-fig. 8 *c*. In another experiment (not shown in Text-fig. 8 *c*), we assumed that 100 per cent by weight of the highest concentration of colchicine used (9×10^{-5} M) was actually ethyl acetate (an extreme allowance, as one is a white, odorless powder, the other a clear, odorous liquid). Ethyl acetate in this range produced no significant darkening. Moreover, purification procedures (see Methods) resulted in no change in colchicine effect (Text-figs. 2 *a*, 2 *b*, and 10 *a*).

Summary: The effect of preincubation with colchicine was independent of the darkening agent employed.

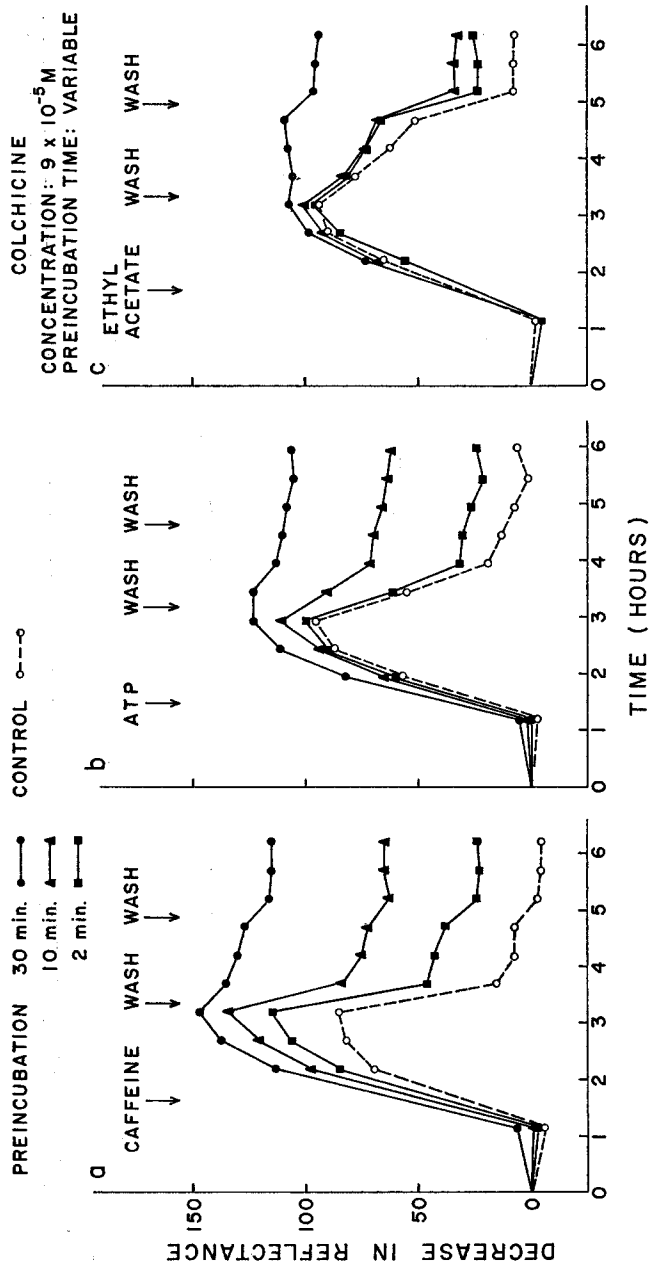
The effect in the presence of lightening agents (Text-figs. 9 a to 9 c):

Certain substances are known partially to antagonize the darkening effect of MSH. These include melatonin, hydrocortisone, and norepinephrine, in concentrations as low as 5×10^{-12}, 6×10^{-7}, 6×10^{-7} M, respectively (11). The design of these experiments is the same as in Text-figs. 3 *a* and 3 *b* except that, instead of attempting to reverse darkening by washing, we added a lightening agent—either melatonin, hydrocortisone, or norepinephrine (see Methods). With each substance, the groups preincubated with colchicine again showed a dosage-dependent inhibition of lightening.

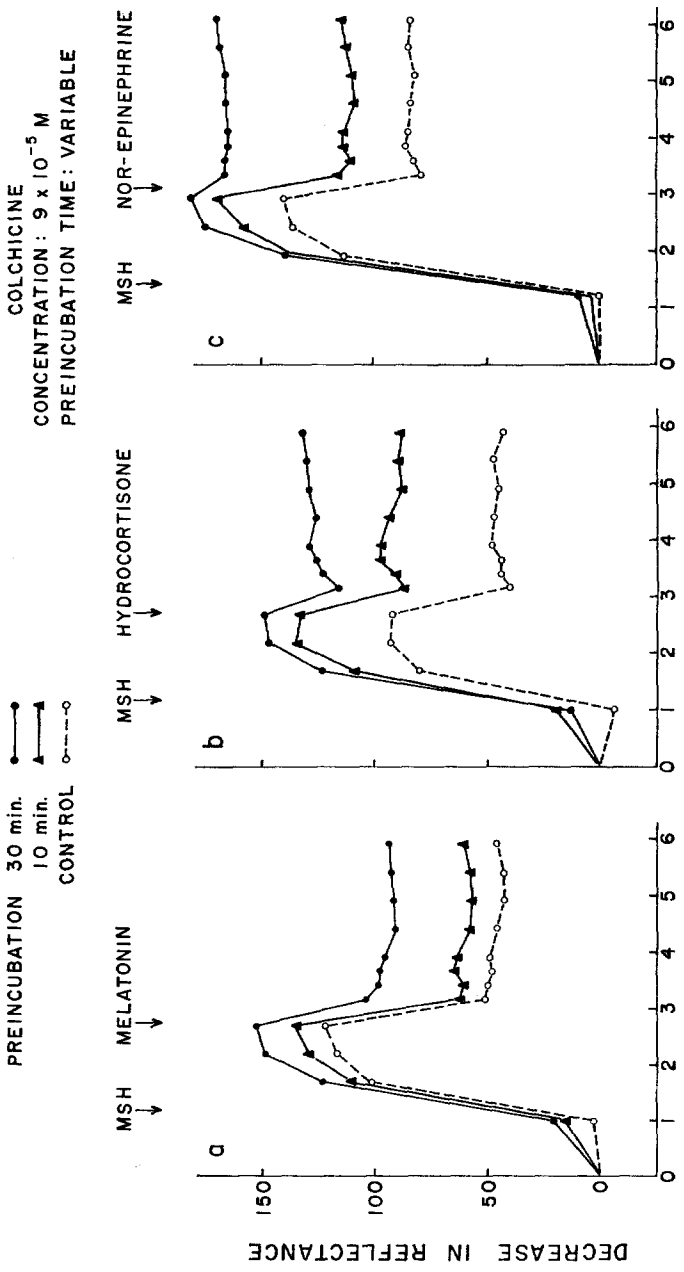
Summary: The effect of preincubation with colchicine was independent of the method used to reverse darkening by MSH.

The effect in the absence of a darkening agent (Text-figs. 10 a, 10 b, and 11):

In the next experiment (Text-fig. 10 *a*) the same three preparations of colchicine as in Text-figs. 2 *a* and 2 *b* were added to three groups of skins, all at 9×10^{-5} M. No



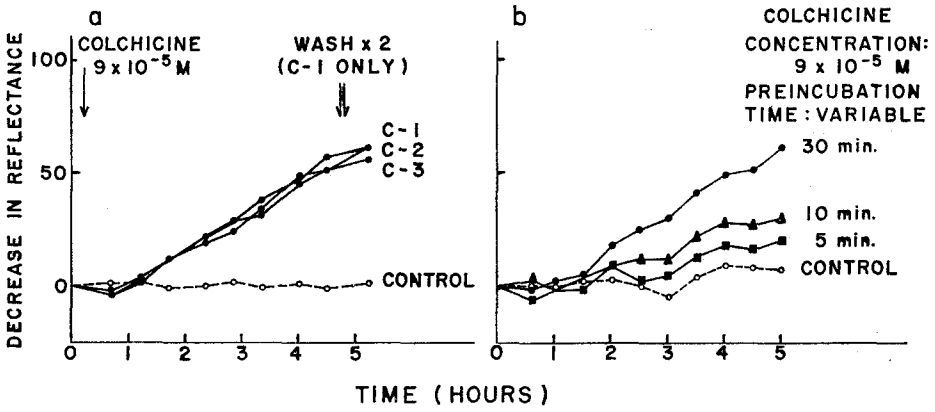
TEXT-FIGS. 8 *a* to 8 *c*. The colchicine effect in relation to darkening agents other than MSH (see text).



TEXT-FIGS. 9 *a* to 9 *c*. The colchicine effect in the presence of lightening agents (see text).

darkening agent was added, and the groups were not washed. No difference from the control group was noted for about 1 hour. Then the colchicine treated groups began gradually to darken, all at comparable rates. The darkening was not reversed by washing one group (C-1) 4½ hours after addition of colchicine. Nine hours after addition (not shown in Text-fig. 10 *a*), the three treated groups showed a net decrease in reflectance of between 61 and 70 units.

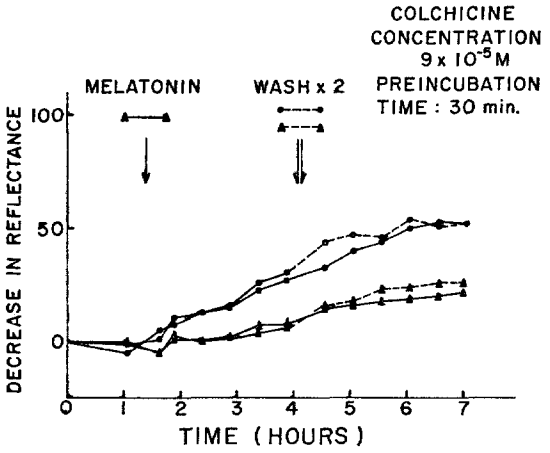
In the experiment depicted in Text-fig. 10 *b*, three groups of skins were preincubated with colchicine, 9×10^{-5} M, for variable periods of time. No darkening agent was added. Again, no effect was noted for about 1 hour. Then the preincubated groups began gradually not only to darken but to do so in proportion to the time of preincubation.



TEXT-FIGS. 10 *a* and 10 *b*. The colchicine effect in the absence of a darkening agent: Text-fig. 10 *a*. Three different samples of colchicine. Text-fig. 10 *b*. Dose-response relationships (see text).

In the experiment depicted in Text-fig. 11, all four groups of skins were preincubated with colchicine, 9×10^{-5} M for 30 minutes. Then the lightening agent, melatonin, was added to two groups. The two groups so treated showed less subsequent darkening than the two other groups. Two and one-half hours after the addition of melatonin, one group that had received melatonin and one group that had not were washed twice. No reversal of darkening occurred in the washed groups as compared to their unwashed controls. Six hours after the addition of melatonin, MSH was added to all four groups (not shown in Text-fig. 11). Continued activity of melatonin in the melatonin-treated, unwashed group was indicated by less darkening than in the other three groups after addition of the standard dose of MSH.

Summary: Colchicine alone produces a gradual, irreversible, dosage-dependent darkening over several hours. This darkening was inhibited by addition of the lightening agent, melatonin.



TEXT-FIG. 11. The colchicine effect in the absence of a darkening agent: addition of melatonin (•—•: No melatonin, no wash) (see text).

The effect shown in consecutive photomicrographs (Figs. 1 to 3):

Figs. 1 *a* to 1 *d*. Two of four samples of skin from a single frog were preincubated with colchicine, 9×10^{-5} M for 30 minutes (samples a and c). After these preincubations, all four samples still appeared light. Microscopically, one saw black dots, each of which depicted melanin granules aggregated about the nucleus of an otherwise invisible melanocyte.

Figs. 2 *a* to 2 *d*. Next, MSH was added to one preincubated skin and to one control skin (samples a and b). One and one-half hours later, the samples so treated appeared dark; microscopically, melanin granules were dispersed, outlining the cells in which they resided (Figs. 2 *a* and 2 *b*). Sample c, which had been preincubated with colchicine but not incubated with MSH, was darkened somewhat; dispersion of melanin granules in melanocytes was not marked (Fig. 2 *c*). Sample d was unchanged in the gross and microscopically (Fig. 2 *d*). At this stage, the decrease in reflectance of the individual samples a to d was, respectively, 45, 35, 13, and 3.

Figs. 3 *a* to 3 *d*. All skin samples were washed both after the second set of photomicrographs and again 1½ hours later. About 1 hour after the second wash, sample a was still dark; granules in melanocytes remained dispersed (Fig. 3 *a*). In contrast, sample b, which had not been preincubated with colchicine, was light again, its granules aggregated (Fig. 3 *b*). Sample c remained darkened somewhat, its granules partially dispersed (Fig. 3 *c*). Sample d continued unchanged (Fig. 3 *d*). Decrease in reflectance of samples a to d was, respectively, 37, 2, 19, and 4.

DISCUSSION

The Melanocyte Model.—The melanocyte model fulfilled every expectation. Its first attribute is simplicity; one or more experiments could be carried out in a single day. Next, the colchicine effect was both reproducible and dosage-

dependent within the specified limits of concentration and exposure time. Finally, after the period of preincubation, colchicine was no longer required in the ambient Ringer's solution. This last attribute is of special importance, as it allowed the effects of other agents to be *separated* from that of colchicine.

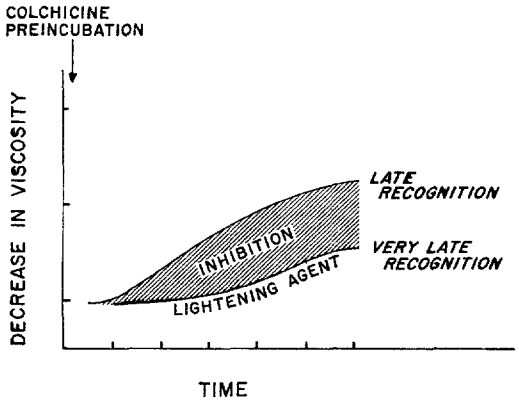
Recognition of Colchicine Action.—After preincubation with colchicine, the melanocytes were altered. However, alteration was not immediately apparent, at least by reflectance measurements. That it had occurred was demonstrable in two ways: by addition of a darkening agent and washing, or simply by waiting. Addition of a darkening agent resulted in an increase of darkening in frog skins pretreated with colchicine compared to controls, even though colchicine was no longer present in solution. Furthermore, the subsequent washing of these skins revealed a dosage-dependent inhibition of lightening, an inhibition whose potential was established *before* addition of the darkening agent (Text-figs. 3 and 4). Thus, use of the darkening agent provided *early recognition* of a cellular change produced *previously* by colchicine. So long as it could produce a reversible darkening, the chemical structure of the darkening agent seemed irrelevant; the agents used included a peptide hormone (MSH) (Text-figs. 3 and 4), a pharmacologically active purine (caffeine), a nucleotide present in all living cells (ATP), and a simple organic solvent (ethyl acetate) (Text-figs. 8 *a* to 8 *c*). Nor did the colchicine effect depend upon the method employed to reverse darkening by MSH; dosage-dependent inhibition of lightening occurred whether lightening was achieved by the hormones, melatonin, hydrocortisone or norepinephrine (Text-figs. 9 *a* to 9 *c*); or simply by removal of MSH from the medium (Text-figs. 3 and 4).

The second way to demonstrate alteration in the melanocyte by preincubation with colchicine was simply to wait. After an interval of about 1 hour during which no change was noted, the preincubated skins began gradually and *irreversibly* to darken, again in a dosage-dependent manner (Text-fig. 10 *b*). This method provided *late recognition* of the cellular change produced by preincubation with colchicine, a change eventually expressed as dosage-dependent inhibition of lightening. Addition of the lightening agent melantonin delayed recognition of this change still further (Text-fig. 11).

Colchicine Action in Terms of Cytoplasmic Viscosity.—As noted earlier, dispersion of melanin granules in melanocytes is thought to be accompanied by a reversible gel-to-sol cytoplasmic transformation (8). In such a system, reflectance becomes a measure of cytoplasmic viscosity as well as of granule dispersion. If we assume that colchicine does not disturb the relation between viscosity and dispersion, we can substitute the word "viscosity" for "reflectance." The resultant schema is depicted in Text-figs. 12 and 13. It is best explained by a single effect of colchicine on the dynamic equilibrium between protoplasmic sol and gel, where the most viscous conditions can no longer be developed. By

adding agents that affect the equilibrium, we alter the speed with which we can *recognize* the action of colchicine; we do not alter the basic action itself.

Analogous Action of Colchicine in Other Systems.—We thus arrive at the concept that the action of colchicine, *i.e.* its binding to cellular targets, is rapid and dosage-dependent, but that the recognition of that action depends on the state of the protoplasm and its alteration by active agents. If this concept can be applied to other cell systems, it may allow plausible explanations for heretofore apparently unrelated colchicine effects. Such effects have been noted in regard to (*a*) the "activity" and "efficiency" of colchicine, (*b*) the relation of colchicine requirement to degree of gelation, and (*c*) agents that appear syner-

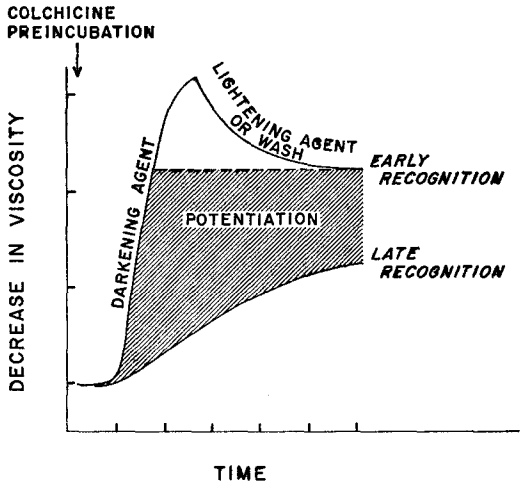


TEXT-FIG. 12. The effect of colchicine on the frog melanocyte in terms of cytoplasmic viscosity: Inhibition of recognition. Preincubation with colchicine produces a dosage-dependent decrease in cytoplasmic viscosity. The effect, at least in the short run, is irreversible. The process requires time; we refer to its appreciation as "late recognition." Addition of a lightening agent inhibits the irreversible decrease in viscosity, and thus retards further the appreciation of colchicine action. We refer to the result as "very late recognition" (or, as a limit, "no recognition").

gistic or antagonistic to colchicine. Each of these effects in other systems has its counterpart in the melanocyte model.

Activity and efficiency: In studying threshold colchicine-like effects of many substances on root tips of the onion bulb, Allium Cepa, Ostergren (12) saw the need to distinguish between activity, measured by the inverse of the "activity threshold," and efficiency, representing the ability of a substance to produce a *visible* effect. In the melanocyte model a simpler approach is possible: the activity of colchicine is directly dosage-dependent, while the efficiency (or "recognition," in the model) is a *variable* which can be increased, at a given time prior to the maximal effect, by adding a darkening agent and washing (Text-figs. 2 *a*, 2 *b*, and 13), or decreased by adding a lightening agent (Text-figs. 11 and 12).

Relation of colchicine requirement to degree of gelation: In studying formation of the (gelated) mitotic spindle in the grasshopper neuroblast, Gaulden and Carlson noted that the more completely the spindle is developed at the time of exposure to colchicine, the greater is the concentration of colchicine required to destroy it or to prevent its further development (13). Here we compare the gelation that occurs with cell division to the gelation that occurs when melanin granules aggregate. Godman (7) obtained analogous results in an entirely different system. He observed the disruption by colchicine of sarcoblast ribbons grown in tissue culture of striated muscle. They progressed (reversibly!) from organized, straplike, birefringent, pulsatile units to segmented, homogeneous,



TEXT-FIG. 13. The effect of colchicine on the frog melanocyte in terms of cytoplasmic viscosity: Potentiation of recognition. (See Text-fig. 12 *re* "late recognition"). Addition of a darkening agent potentiates the recognition of colchicine action; after subsequent washing or addition of a lightening agent, what would have been a reversible decrease in viscosity is maintained in a dosage-dependent manner attributable to *prior* treatment with colchicine. We refer to the resultant rapid appreciation of colchicine action as "early recognition."

expanded globules. The effect was directly proportional to the concentration of colchicine and inversely related to the degree of differentiation of the sarcoblast. Here the less differentiated sarcoblast fiber may be compared to the less viscous cytoplasm of the dark melanocyte. Restating the findings of these authors, one may say that they were able to *recognize* the action of colchicine *earlier* when dealing with less gelated structures.

Supporting this contention is Godman's observation that the maximal colchicine effect occurred in 4 to 5 days *whether or not* colchicine was present in the medium after the standard 48 hour treatment. That is, the optimal dosage (concentration × time of exposure) was reached after 2 days but the maximal

effect was seen only after 4 or 5 days. In the melanocyte model, this delayed response corresponds to "late recognition" (Text-figs. 10 *a*, 10 *b*, and 12).

Synergism and antagonism: Working with explants or chicken fibroblasts, Lettré (14) found that a variety of substances, with little or no antimitotic activity of their own, can increase the antimitotic activity of colchicine. The mechanism of action of these synergists was obscure. In the present studies, the reversible darkening agents were "synergists" of colchicine; their use was followed, after a fixed interval, by more inhibition of lightening than was apparent with colchicine treatment alone. Moreover, in the melanocyte model an explanation of synergism is possible in terms of early recognition of the action of colchicine (Text-fig. 13). Since the specific action of colchicine must have taken place in the preincubation period, we have said that the darkening agents only allowed the prompt *recognition* of *previous* colchicine action. Conversely, any substance with a transient ability to darken frog skin—or, in general terms, to reduce protoplasmic viscosity—would, on the addition of colchicine, have this ability amplified and maintained.

"Antagonism" may be viewed in a similar light: any substance capable of increasing or maintaining protoplasmic viscosity would appear, after a fixed interval, to "antagonize" the action of colchicine. By this definition, melatonin was such a substance, whether it was *inhibiting* (Text-fig. 11) or *permitting* (Text-fig. 9 *a*) recognition of colchicine action.

Reversibility.—One of the most striking and significant aspects of the antimitotic effect of colchicine is reversibility (15); on removal of an optimal dosage of colchicine, the spindle will ultimately reform and the cell will divide. Similarly, reversibility of colchicine effects on resting cells has been demonstrated. Godman studied the ability of colchicine, in concentrations as low as 10^{-7} to 10^{-8} M, to cause disruption of sarcoblast ribbons in tissue culture (7). The result was segments which were markedly shortened and widened, and of diminished consistency. On withdrawal of the drug, the nucleated segments were capable of regeneration, usually requiring a fortnight for restitution. Mizurski studied the effects of colchicine, 10^{-3} to 10^{-7} M, on hanging drop cultures of fibroblasts (6). He watched the initially organized colony of radially arranged cells become disrupted, the cells rounded or polygonal and independent of each other. Colchicine was thought to have affected the sol-gel equilibrium of the cortical layer of the resting cell. Again the effect was reversible. Fortunately, in the few hours during which the melanocyte model operates, the effect of colchicine is not reversible (Text-figs. 5 *a* and 5 *b* and text). As has been noted, this fortuitous circumstance allows an analysis of colchicine action that would not otherwise be possible.

Colchicine in Acute Gouty Arthritis.—

Recent work has allowed a tentative approach to understanding the therapeutic and prophylactic action of colchicine in acute gouty arthritis. Prerequisite to this

approach were the findings that (*a*) sodium urate crystals are very frequently present in the synovial fluid of gouty patients (16), and (*b*) intraarticular or subcutaneous injections of such crystals in gouty and non-gouty patients and in dogs results in an inflammatory reaction (17, 18). In the synovial fluid during both spontaneous acute attacks and those induced by injected crystals, there is leukocytosis and phagocytosis of crystals (17, 19). Furthermore, the degree of phagocytosis can be correlated with the clinical severity of the attack, spontaneous or induced (17). The following events have been suggested in gouty inflammation (17, 20): (*a*) Urate microcrystals precipitate from supersaturated body fluids. (*b*) Under certain conditions, an inflammatory response occurs, manifested by leukocytosis and phagocytosis of crystals. (*c*) The metabolic products of the inflammatory response are thought to favor further precipitation of urate, and a vicious cycle occurs, a cycle *driven* by the products of inflammation.

Colchicine is thought to break the inflammatory cycle (20, 21). Administration of colchicine cured severe inflammatory reactions elicited by the intraarticular injection of microcrystalline sodium urate (17). Pretreatment with therapeutic amounts (for gout) of colchicine resulted in a diminished inflammatory response to urate crystals injected intra-articularly or subcutaneously in gouty volunteer subjects (21).

Of the various elements of the inflammatory response that might be affected by colchicine, the polymorphonuclear leukocyte is an especially likely target. It is the predominant cell type present in the synovial fluid of inflamed gouty joints. It is a cell type known to be sensitive to the larger doses of colchicine that inhibit mitosis (22); a colchicine analogue, desacetylmethylcolchicine, is used in the treatment of chronic granulocytic leukemia (23). Finally, the ameboid functions of the polymorphonuclear leukocyte demand frequent, reversible changes in cytoplasmic viscosity: in order to reach the synovial fluid, it must stick to the wall of a blood vessel, undergo diapedesis, wander through the tissues, and eventually arrive in the target area, where its phagocytic function comes into play. Results from the melanocyte model suggest that transient, reversible gel-to-sol reactions will be amplified and maintained by colchicine action. If colchicine were to affect the cytoplasm of the polymorphonuclear leukocyte in a similar manner, the result would be interference with the consecutive ameboid activities listed above. Such interference might slow the inflammatory reaction enough to break the propagated cycle.

There is already some evidence in support of this hypothesis. Under phase microscopy at 37 to 39 degrees centigrade, treatment with colchicine (10^{-3} M) resulted in rounding up of human polymorphonuclear leukocytes as compared to controls, with decrease in formation of pseudopods and cessation of mobility (2). By using as a measure of mobility the numbers of leukocytes that crossed a millipore filter in response to a chemotactic stimulus, inhibition by colchicine could be detected in very low concentration (about 10^{-7} M) (24). In addition, treatment with colchicine (2.5×10^{-6} M) interfered with some biochemical functions associated with phagocytosis of urate crystals by human leukocytes *in vitro* (25).

Colchicine Action in Relation to ATP and Contractile Protein.—The mitotic spindle is an oriented, gelated, birefringent structure that responds to low concentrations ($<10^{-3}$ M) of colchicine by dissolution and contraction (4, 26). Inoué has postulated that oriented and non-oriented material form a labile

equilibrium in the mitotic apparatus. Colchicine is thought to disorganize the orientation of the micelles in the astral rays and spindle fibers. Similar events are thought to occur in the anaphase movement of chromosomes, with local reduction in the quantity of oriented material and the consequent shortening of chromosomal fibers.

From "fiber models" (27), the mitotic apparatus has been viewed as a contractile system of the actomyosin type. In explants of chicken fibroblasts, colchicine was thought to inhibit the action of ATP (14), but this conclusion has been challenged (28). In melanocytes, Lerner and Takahashi (8) have summarized the evidence, by no means complete, for a birefringent, gelated and contractile network imbedding the melanin granules, and for the participation of ATP in the reversible gel-to-sol reaction effected by MSH. In the present studies, ATP was employed as just one of a number of reversible darkening agents (Text-fig. 8 *b*); as such it *potentiated* the colchicine effect. However, since we did not examine the *biochemical* effects of exogenous ATP, or of any other darkening agent, we cannot speculate usefully on the possibility of an "actomyosin-like" target.

Perhaps a parallel may be drawn between our results and those of Angevine (29), who studied nerve destruction by colchicine in mice. If ATP (10^{-1} M) was added to the colchicine (10^{-2} M) solution, there was a pronounced augmentation of colchicine neuropathy as compared to the effect of the colchicine solution alone. One wonders if the author was observing disorganization by colchicine of axonal elements, with potentiation by ATP similar to that seen in the melanocyte model (Text fig. 8 *b*). Indeed, Hoffman (30), studying an inhibitory effect of colchicine on axon sprouting from the partially deneurotized sciatic plexus of the rat, concluded that colchicine reduced the viscosity of the axogel (the cortical layers of the axoplasm).

The Action of Colchicine in Gelated, Protoplasmic Cell Systems: A General Formulation.—The findings in the melanocyte model have led us to suggest that colchicine opposes forces within the cell which support protoplasmic viscosity, *i.e.* promote the organized state of elements concerned with structure and movement; while it allows free rein to those forces which reduce viscosity, *i.e.* promote the disorganized state of such elements. Our data do not indicate whether colchicine affects the organized molecules directly or whether it affects indirectly the maintenance of the gelated condition. In the former case, however, the direct effect would have to be such that it interferes not with efficient solation, but only with subsequent recovery (Text-fig. 13). Our findings support the view of Godman (7) that "whatever the mechanism of the action of colchicine on anisotropic systems, it appears to result finally in a more random arrangement of particles. This evidently may be elicited in cell components of fibrillar arrangement concerned with structure and movement, whether in the

hyaloplasm or ectoplasm, the atractoplasm (achromatic figure) or the muscle proto-fibrils."

With the findings in the melanocyte model added to what has been learned in other systems, a general formulation may be drawn for the action of colchicine:

1. Living cells contain organized, labile, fibrillar systems concerned with structure and movement.

2. The viscosity, or degree of gelation, in these systems is determined through a dynamic equilibrium between gel and sol, organization and disorganization, whose setting at any given time is established by cellular homeostatic mechanisms.

3. Colchicine, in low concentration and in a dosage-dependent manner, lowers the potential limit of gelation.

4. The effect *per se* is not lethal, and is eventually reversible, provided that the cell does not succumb to some other influence during the period of colchicine action.

5. The binding of colchicine to its cellular targets is rapid.

(*a*) If the initial protoplasmic viscosity is low, inhibition of gelation will be seen rapidly.

(*b*) If the initial protoplasmic viscosity is high, time will be required for a new equilibrium to be established. In the melanocyte model, this time accounts for the "late recognition" of the colchicine effect.

(i) The time required to reach the new equilibrium will be decreased by any agent that interferes, directly or indirectly, with maintenance of the gelated state. In the melanocyte model, such an agent promotes "early recognition" of the colchicine effect; it appears "synergistic" to colchicine.

(ii) In contrast, the time required to reach the new equilibrium will be increased by any agent that augments or maintains, directly or indirectly, the gelated state. In the melanocyte model, such an agent promotes "very late recognition" of the colchicine effect; it appears "antagonistic" to colchicine.

Significance of the Melanocyte Model and of the New Interpretation of Colchicine Action.—Colchicine interferes with the reversible control of granule movement by a number of substances of physiologic and pharmacologic interest, and does so in an orderly, predictable and reproducible fashion. Furthermore, it operates in a consistent manner, *regardless* of the particular darkening or lightening agent used. By this virtue, colchicine becomes a tool with which to study, in live cells, biological effects of controlled physical alteration; until now, this use of colchicine has been formally pursued only in the special case of the mitotic apparatus. Though the melanocyte is convenient for such studies, similar application may be possible in other cell systems. If such models can be uti-

lized, then colchicine, beyond its unique and prestigious career in cytogenetic studies and in gout, would become a biophysical tool of considerable power.

SUMMARY

The effect of colchicine was studied on the rapid, reversible darkening of frog skin under the influence of melanocyte-stimulating hormone (MSH). Darkening is due to dispersion of melanin granules in melanocytes and is thought to be accompanied by a gel-to-sol cytoplasmic transformation. After subsequent washing, the skin lightens, with aggregation of melanin granules and cytoplasmic gelation.

Preincubation of skin with colchicine had the following effects:
1. Darkening induced by MSH was increased in comparison to control skins, and on removal of MSH, lightening was inhibited. Inhibition was a function of both concentration (1×10^{-5} to 9×10^{-5} M) and exposure time (2 to 30 minutes). Once established, inhibition was maintained throughout the remainder of the experiment. 2. The same effects were noted (*a*) when darkening was effected by agents other than MSH (ATP, 0.9×10^{-3} M; caffeine, 5.2×10^{-3} M; ethyl acetate, 0.8×10^{-2} M), and (*b*) when lightening was effected by addition of chemical agents (melatonin, 4.3×10^{-10} M; hydrocortisone, 1×10^{-3} M; norepinephrine, 1×10^{-3} M), instead of by washing. 3. Colchicine alone produced a gradual, irreversible, dosage-dependent darkening over several hours. This darkening was inhibited by melatonin, 4.3×10^{-10} M.

The melanocyte model is used to construct a general theory of colchicine action on living cells, an action resulting in decreased protoplasmic viscosity. In this formulation colchicine lowers the potential limit of protoplasmic gelation, and does it rapidly, reversibly, in low concentration, in a dosage-dependent manner, and without killing the cell. The theory allows interpretation of "synergism" and "antagonism" to colchicine by other substances. It suggests a tentative approach to the understanding of colchicine action in acute gouty arthritis, where interference with ameboid activities of polymorphonuclear leukocytes is one possible aspect of the anti-inflammatory effect of colchicine. Finally, the colchicine-treated melanocyte is viewed as a good, live physical model that can be used to elucidate some fundamental biological properties.

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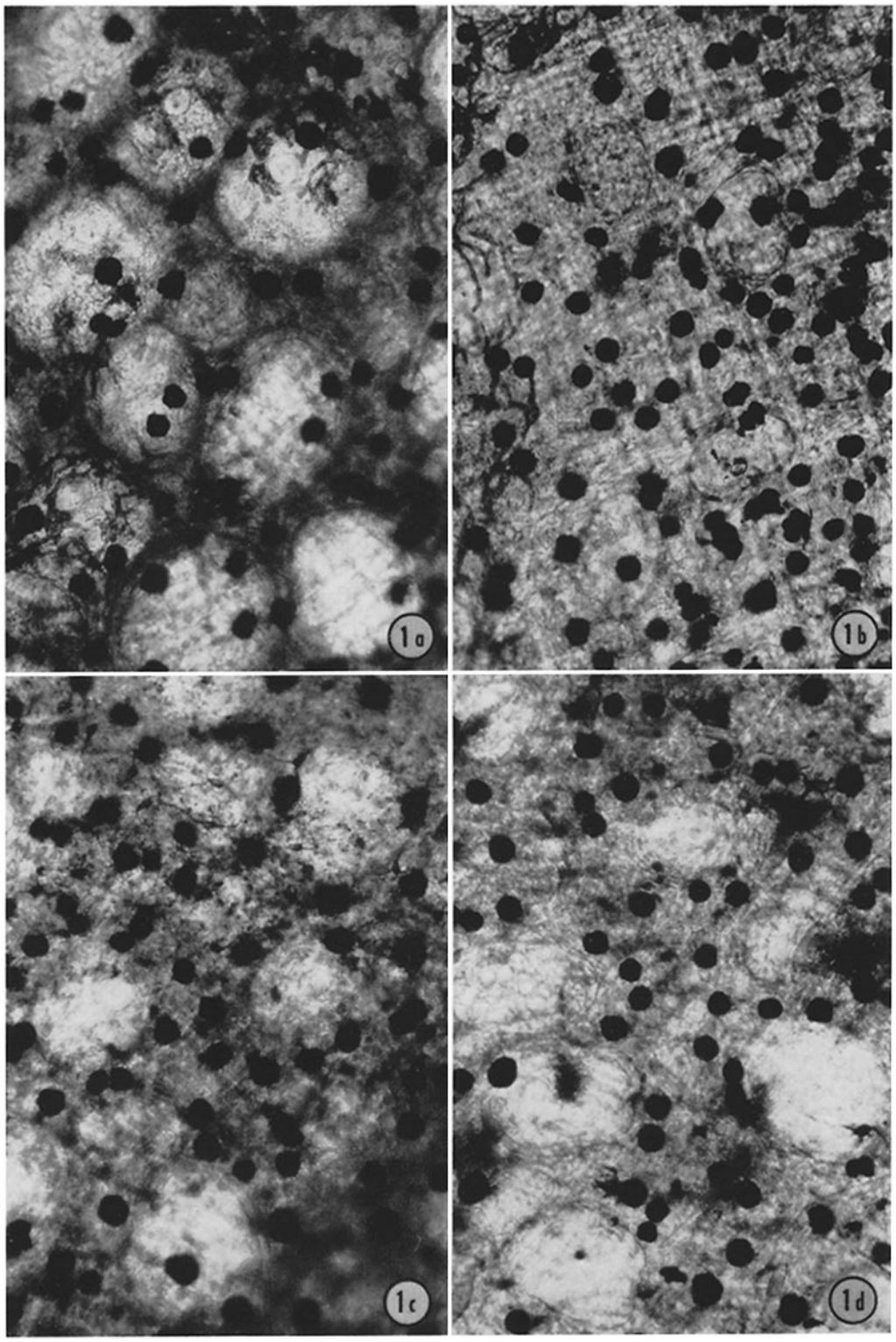
EXPLANATION OF PLATES

The colchicine effect is shown in consecutive sets of photomicrographs. The position of melanin granules in melanocytes is seen in representative fields.

PLATE 25

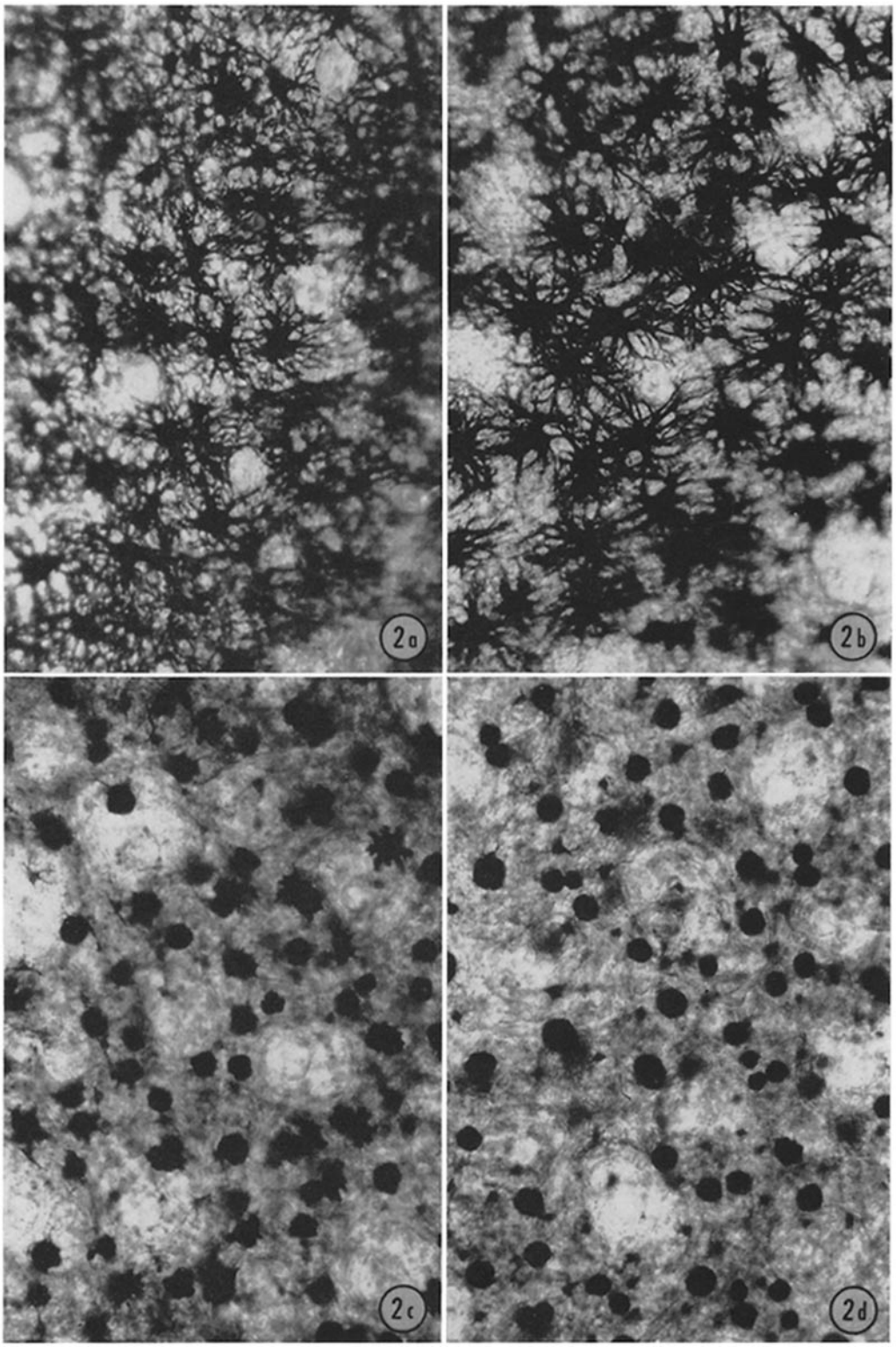
FIGS. 1 *a* to 1 *d*. Four samples of skin from a single frog, a few minutes after preincubation of samples a and c with colchicine, 9×10^{-5} M for 30 minutes (see text). Water immersion. Approximately × 140.



(Malawista: Action of colchicine)

PLATE 26

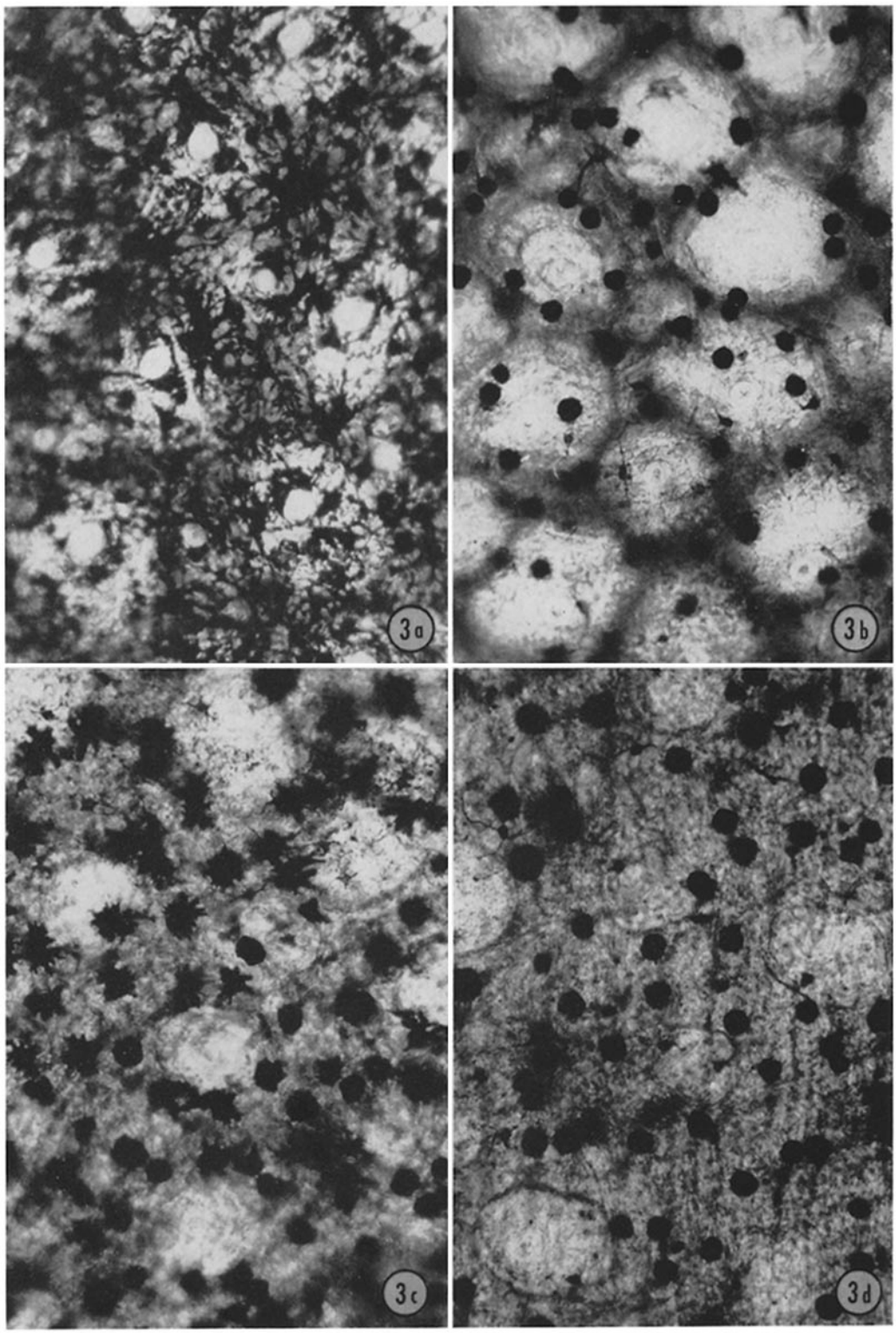
FIGS. 2 *a* to 2 *d*. The same four samples of skin as in Figs. 1 *a* to 1 *d*, 1½ hours after MSH was added to samples a and b (see text). Water immersion. Approximately × 140.



(Malawista: Action of colchicine)

PLATE 27

FIGS. 3 *a* to 3 *d*. The same four samples of skin as in Figs. 2 *a* to 2 *d*, after all samples have been washed twice over a period of 2½ hours (see text). Water immersion. Approximately × 140.



(Malawista: Action of colchicine)