ANTAGONISTIC EFFECTS OF 6-MERCAPTOPURINE AND COENZYME A ON MITOCHONDRIA AND MITOSIS IN TISSUE CULTURE*, ‡

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PLATES 37 TO 39

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Two substances related to adenine and both bearing —SH groups have recently moved into prominence because of their biological effects: coenzyme A, as the coenzyme of biological acetylations or 2-carbon transfers (1), and 6-mercaptopurine (2), as an antagonist of physiological purines and nucleic acids and as a potential anti-cancer agent (3, 4).

Coenzyme A has been found to be highly beneficial to tissue cultures and to prolong their survival in incomplete media (5).

The agent 6-mercaptopurine is reported to decrease mitotic incidence in tissue cultures (6). In cultures of Crocker mouse sarcoma 180 exposed for 24 hours to 1 mM 6-mercaptopurine, mitotic incidence is about 15 per cent of that in untreated cultures. Embryonic mouse skin cultures are less affected. In them, similar treatment reduces mitotic incidence to about 50 to 70 per cent of the incidence in control cultures. The partial mitotic inhibition in embryonic skin cultures caused by 6-mercaptopurine is readily blocked by simultaneous administration of any one of a number of physiological purines, nucleosides, and nucleotides. However, these substances are not so effective in protecting sarcoma 180 cultures against 6-mercaptopurine. On the other hand, the more complex material, coenzyme A, readily blocks 6-mercaptopurine–induced mitotic inhibition, and it is more effective in this respect for sarcoma 180 cultures than for embryonic skin cultures. Thus in its effect the coenzyme is complementary to that of the simpler adenine-containing metabolites. As little as 0.02 mM coenzyme A can block the mitotic inhibition of sarcoma 180 cells caused by 1.0 mM 6-mercaptopurine (6).

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The work reported earlier (6) was carried out with a commercial preparation assaying 30 per cent coenzyme A. The present paper reports results with commercial preparations assaying about 75 per cent coenzyme A. The antagonism between 6-mercaptopurine and coenzyme A with respect to mitotic incidence is confirmed. In addition, the two agents are found to have what may be interpreted as opposite effects on mitochondrial morphology and on cellular lipogenesis.

Methods and Materials

Tissue cultures of embryonic mouse skin and Crocker mouse sarcoma 180 were planted on coverslip inserts held to the flattened walls of roller tubes with a plasma clot. Two coverslips were in each tube. Each coverslip bore one explant of sarcoma 180 and two explants of embryonic skin under a thin clot of plasma. In each roller tube was 1.0 ml. of fluid nutrient medium consisting of 4 parts Gey's balanced salt solution, 2 parts 50 per cent chick embryo extract, 3 parts horse serum, and 1 part human cord serum. Penicillin G (25 units) and streptomycin (25 μg.) were added in the salt solution.

The cultures were incubated at 37°C. in a drum rotating at 6 revolutions per hour for 24 hours. The cultures were then dosed with 6-mercaptopurine and coenzyme A in saline solutions, 0.1 ml. of the fluid medium in each tube being replaced with a like volume of saline containing the agent. Incubation then continued for another 24 hours.

On the 3rd day, coverslip inserts were removed from the tubes for phase contrast photomicrography. The plasma clot was carefully wiped from the back of the coverslip. The coverslip was set, cultures down, in a drop of the fluid medium on a microscope slide and examined under oil immersion. Several photographs were rapidly made, and the next coverslip was then removed for examination. Mitochondrial lengths were measured in photographs of ten cells for each of the four experimental conditions: (*a*) control, (*b*) treated with 6-mercaptopurine, (*c*) treated with coenzyme A in addition to 6-mercaptopurine, and (*d*) treated with coenzyme A alone.

Other cultures were fixed with alcohol-acetic acid 3:1, stained with the Feulgen nucleal reaction and light green, and mounted in diaphane on microscope slides as permanent mounts. One thousand nuclei in the outgrowth of each culture were counted in order to determine mitotic incidence under the several experimental conditions.

Still other coverslip cultures were fixed in 10 per cent neutral formalin, stained with Sudan black B in 70 per cent alcohol, and mounted in glycerogel for a demonstration of lipid to supplement the phase contrast observations.

The 6-mercaptopurine was kindly furnished by Dr. G. H. Hitchings of the Wellcome Research Laboratories, Tuckahoe, New York. The 75 per cent coenzyme A preparations were obtained commercially as fluffy white powders.

RESULTS

Mitotic counts in the outgrowths of stained cultures confirmed the partial mitotic inhibition caused by 6-mercaptopurine and its blocking by coenzyme A. Results of an experiment with 75 per cent coenzyme A are presented in Table I.

The mitotic inhibition caused by 1 mM 6-mercaptopurine is prevented in sarcoma cells by 0.2 or 0.02 mM coenzyme A. Skin cells are protected by the higher but not the lower concentration of coenzyme A. These results are in complete accord with those obtained with a 30 per cent coenzyme A preparation (6).

Phase contrast photomicrographs of fibroblasts of the embryo mouse skin are presented in Figs. 1 to 5 in order to show effects of 6-mercaptopurine and coenzyme A on mitochondria. In Fig. 1 are fibroblasts of a control culture. The mitochondria are filamentous and of moderate length. After 24 hours' exposure to 1 mM 6-mercaptopurine (Figs. 2 and 3), the mitochondria of embryo skin fibroblasts are shorter. In some treated cells they appear thinner. Fig. 4 shows fibroblasts treated for a day with a combination of 1 mM 6-mercaptopurine and 0.2 mM coenzyme A. The mitochondria resemble those in untreated fibroblasts. In embryonic fibroblasts treated with 0.2 mM coenzyme A alone for 24 hours (Fig. 5), the mitochondria are thicker and longer than in the

TABLE I

Effects of 6-Mercaptopurine and Coenzyme A on Mitotic Incidence in Tissue Cultures from a Single Experiment

Concentration		Mitoses per 1000 nuclei			
		Sarcoma 180		Embryo skin	
6-Mercaptopurine	Coenzyme A	Mean M	Standard deviation σ'	Mean M	Standard deviation σ'
mM	*mM*				
—	—	129.5	2.5	110.5	2.5
1	—	17.5	3.5	55.0	2.0
1	0.2	116.5	26.5	105.0	24.0
1	0.02	93.5	10.5	62.0	10.5
—	0.2	141.5	8.5	110.5	6.5

control cells. The mitochondria in Fig. 5 provide an extreme example of the coenzyme A effect. On the average, mitochondria are longer by about 50 per cent under coenzyme A treatment than under control conditions.

A summary of the measurements of mitochondrial lengths in photographs is presented in Table II. Lengths of all mitochondria evident in each of ten cells under the four experimental conditions stated were measured and the arithmetic mean for each cell was determined. The mean of these individual cell means for each group of ten cells, with its standard deviation calculated as for a small sample, is entered in the table. Results such as these have been obtained in each of five separate experiments.

The mean mitochondrial length is near 2 μ in control fibroblasts, approximately half that value in cells treated with 6-mercaptopurine, near the control value in fibroblasts treated with coenzyme A in addition to 6-mercaptopurine, and about 3 μ in cells treated with coenzyme A alone. The difference in mean mitochondrial length between any two of these groups is statistically highly significant, with one exception: fibroblasts treated with 6-mercaptopurine plus

coenzyme A have mitochondria not significantly different in length from those in untreated fibroblasts.

Table II also contains the average number of mitochondria available for measurement in the photographed optical sections of the cells. These numbers by no means represent the total numbers of mitochondria per cell, but they must bear some relation to total numbers per cell.

TABLE II

A. Effects of 6-Mercaptopurine and Coenzyme A on Mitochondrial Lengths and Number of Mitochondria per Fibroblast in Optical Section

Treatment	No. of cells	Mean mitochondrial length		Mean mitochondrial No.	
		Mean M	Standard deviation σ'	Mean M	Standard deviation σ'
		μ	μ		
(*a*) None	10	1.88	0.07	38	5.7
(*b*) 6MP* 1 mM	10	0.96	0.08	60	5.5
(*c*) 6MP 1 mM plus CoA‡ 0.2 mM	10	1.73	0.11	53	6.5
(*d*) CoA 0.2 mM	10	2.94	0.21	29	3.7

B. Statistical Significance of Differences between Mean Mitochondrial Lengths

Treatments compared	Probability that sampled populations are not different
(*a*) and (*b*)	$P < 0.0001$
(*a*) and (*c*)	$P = 0.25$
(*a*) and (*d*)	$P = 0.0003$
(*b*) and (*c*)	$P < 0.0001$
(*b*) and (*d*)	$P < 0.0001$
(*c*) and (*d*)	$P = 0.0001$

* 6MP, 6-mercaptopurine.

‡ CoA, coenzyme A.

The number of mitochondria observed per cell stands in a roughly inverse relationship to the mean mitochondrial length. Treatment with 6-mercaptopurine increases the mean number of mitochondria visible per cell, and treatment with coenzyme A decreases the number visible per cell. Hence it may be suggested that mitochondria fragment under the influence of 6-mercaptopurine but coalesce end-to-end under the influence of coenzyme A alone. The change of importance may be one of mitochondrial surface area relative to volume.

Observations with phase contrast microscopy have repeatedly shown a diminution in the quantity of refractile lipid droplets in cells treated with 6-mercaptopurine, and conversely, an increase over the control in cells treated with coenzyme A alone. In cells treated with a combination of 6-mercapto-

purine and coenzyme A, lipid droplets have usually been found intermediate in quantity between control cells and those treated with 6-mercaptopurine alone. These changes are evident in Figs. 1 to 5, and they are also seen in cultures stained with Sudan black B (Figs. 6 to 9).

DISCUSSION

Mitochondria are notoriously sensitive to unfavorable conditions of cell life, and the specificity of any morphological change in them is therefore open to question. However, an impressive beginning in the interpretation of morphological changes in mitochondria has been made by the Belgian school. Chèvremont and Chèvremont-Comhaire (7) have described changes in tissue cultures treated with adrenochrome or its isomer, trihydroxy-*N*-methylindole, resembling to some extent the effects of 6-mercaptopurine. Cells treated with trihydroxy-*N*-methylindole suffer preprophasic mitotic inhibition and mitochondrial shortening, thickening, and rounding. Frederic (8) has described mitochondrial changes with a number of agents. Notable among these agents is 2,4-dinitrophenol, well known for its apparent ability to dissociate oxidation from phosphorylation. Dinitrophenol increases mobility of the mitochondria and causes them to become thicker and to lengthen by terminal fusion with one another. Their number per cell is thus decreased. These changes are suggestive of those caused by coenzyme A in our material.

There is reason to look on the mitochondria as the principal sites of respiration in the cell (9) and on coenzyme A as being significantly involved therein. Enzymes of the Krebs cycle are relatively concentrated in the mitochrondrial fraction of normal liver homogenates (10), although several enzymes of the cycle occur in other fractions (11). The mitochondrial fraction contains about half the coenzyme A and pantothenic acid of rat liver homogenates (12). Coenzyme A is known to participate in the enzymatic condensation of acetate and oxalacetate to citrate (13, 14) and thus to play a leading role in the introduction of 2-carbon units into the tricarboxylic acid cycle.

Coenzyme A is involved in a number of other enzyme systems localized in the mitochondria, including that catalyzing the formation of succinate from alpha-ketoglutarate in the citric acid cycle (15). It is instructive for our present purposes to note the mitochondrial localization of enzyme systems making use of coenzyme A in the metabolism of fatty acids (16–19).

It is generally conceded that 6-mercaptopurine interferes with nucleic acid metabolism and that its toxicity in a variety of biological systems may be counteracted by a number of physiological purines, nucleosides, and nucleotides (2, 20–24). It has been reported that 6-mercaptopurine inhibits "*de novo*" synthesis of nucleic acid and the incorporation of hypoxanthine but not of adenine into polynucleotides (25). There is suggestive evidence that isotopically labelled 6-mercaptopurine is itself incorporated into tissue nucleic acids after

injection into mice (26). However, purine analogs are recognized to interfere in a variety of processes, and there is such a considerable variation in pharmacological effect among a series of purine analogs studied as to render unsatisfactory all explanations of their mechanisms of action based solely on antagonism to the metabolism of purines or nucleic acids (27).

Hence in considering the actions of 6-mercaptopurine on tissue cultures, one may well be wary of any unitary hypothesis of the mechanism of action. Blocking of the 6-mercaptopurine–induced mitotic inhibition by purines, nucleosides, and nucleotides, at least in embryo mouse skin cultures, suggests that an interference in nucleic acid metabolism is responsible for the mitotic inhibition. On the other hand, the greater effectiveness of coenzyme A in blocking 6-mercaptopurine–induced mitotic inhibition casts some doubt on the primary importance of an upset in nucleic acid metabolism, unless coenzyme A is itself in some way involved in nucleic acid metabolism.

Possibly of greater significance in the mitotic inhibition may be action by 6-mercaptopurine as an antimetabolite of coenzyme A, especially an interference in the introduction of 2-carbon units from glycolysis into the respiratory cycle. Energy production through the tricarboxylic acid cycle is essential for mitotic activity of the mouse ear epidermis *in vitro*, according to work of Bullough and Johnson (28). One locus of attack by 6-mercaptopurine might therefore be in the mitochondria, in enzyme systems that provide energy or material needed for mitosis or steps leading to mitosis. Supporting evidence on this point is the observation by Mihich that both oxygen consumption and carbon dioxide production by tumor slices from animals treated with 6-mercaptopurine are only about half of control values (29). The contrary effects of 6-mercaptopurine and coenzyme A on lipogenesis *in vitro* may perhaps also be considered as evidence of interference by 6-mercaptopurine in a mitochondrial function of coenzyme A, participation in the biosynthesis of fatty acids. Whether interference in lipogenesis by 6-mercaptopurine bears more than a roughly parallel relation to mitotic inhibition is problematical.

The alternative hypothesis may also be entertained that 6-mercaptopurine interferes in the synthesis of coenzyme A; Hamilton and Elion suggest that 6-mercaptopurine may even be incorporated into the structure of various coenzymes (30). If this interference does occur, all processes dependent on coenzyme A would be affected in cells treated with 6-mercaptopurine. However, tissue culture experiments now under way with the known precursors of coenzyme A indicate that 6-mercaptopurine may not interfere in coenzyme A biosynthesis.

SUMMARY

The partial mitotic inhibition caused by 6-mercaptopurine in tissue cultures of Crocker mouse sarcoma 180 and embryonic mouse skin is blocked by co-

enzyme A. 6-Mercaptopurine and coenzyme A also have opposite effects on mitochondrial morphology. Mitochondria in cells treated with 6-mercaptopurine become thin and fragmented. Coenzyme A blocks this effect, and alone coenzyme A makes for longer and thicker mitochondria. 6-Mercaptopurine inhibits lipogenesis in embryo skin fibroblasts, and this inhibition is partly counteracted by coenzyme A, which by itself makes for a greater accumulation of lipid droplets in the cytoplasm.

It is suggested that at least one part of the action by which 6-mercaptopurine decreases mitotic incidence in tissue cultures may be an interference on the part of 6-mercaptopurine, acting as an antimetabolite of coenzyme A, in mitochondrial function related to cell division.

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EXPLANATION OF PLATES

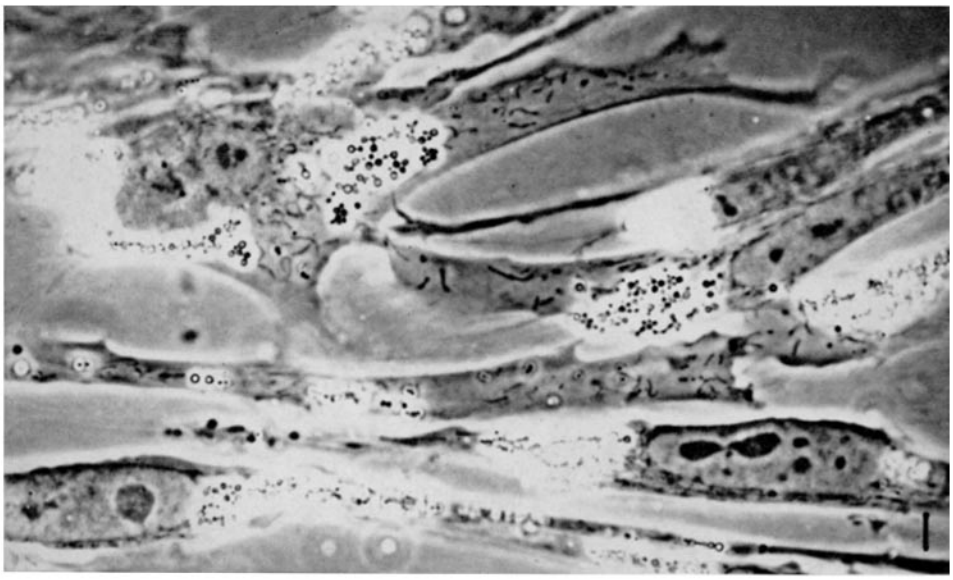
PLATE 37

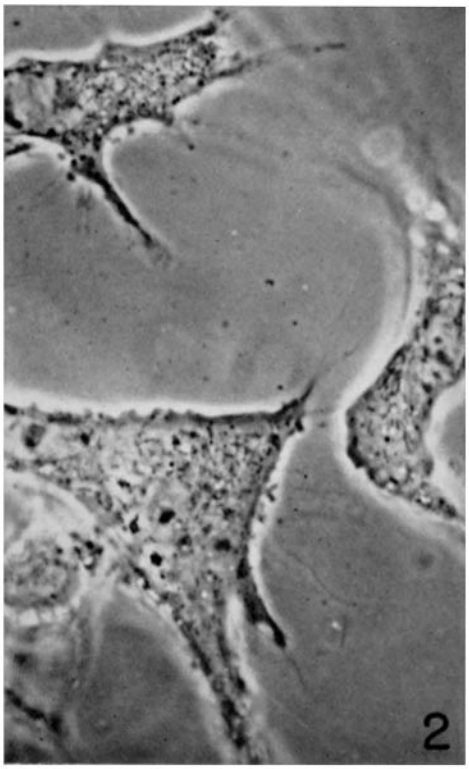
Photomicrographs of embryonic mouse skin fibroblasts 2 days in culture. Oil immersion, dark medium phase contrast. × 1090.

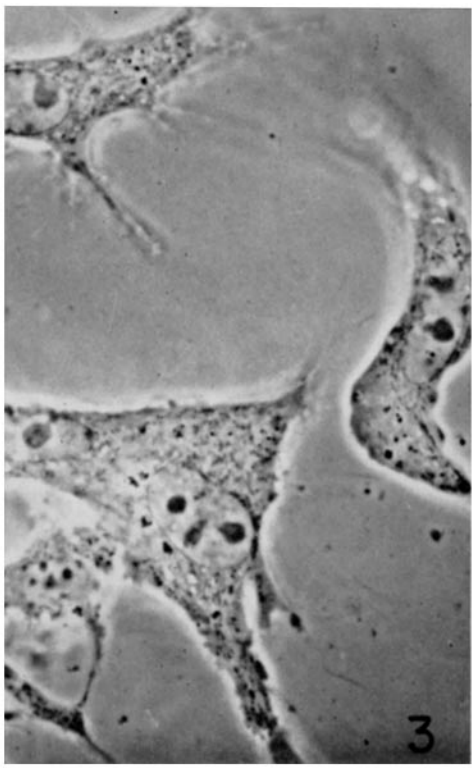
FIG. 1. Cells of control culture. Mitochondria appear as opaque rods or filaments of moderate length. Cells also contain masses of lipid droplets.

FIG. 2. Cells of culture treated 1 day with 1 mM 6-mercaptopurine. Mitochondria are short, sometimes beaded, rods and granules of intermediate density. Cells contain little fat.

FIG. 3. Different focal level of cells in Fig. 2.







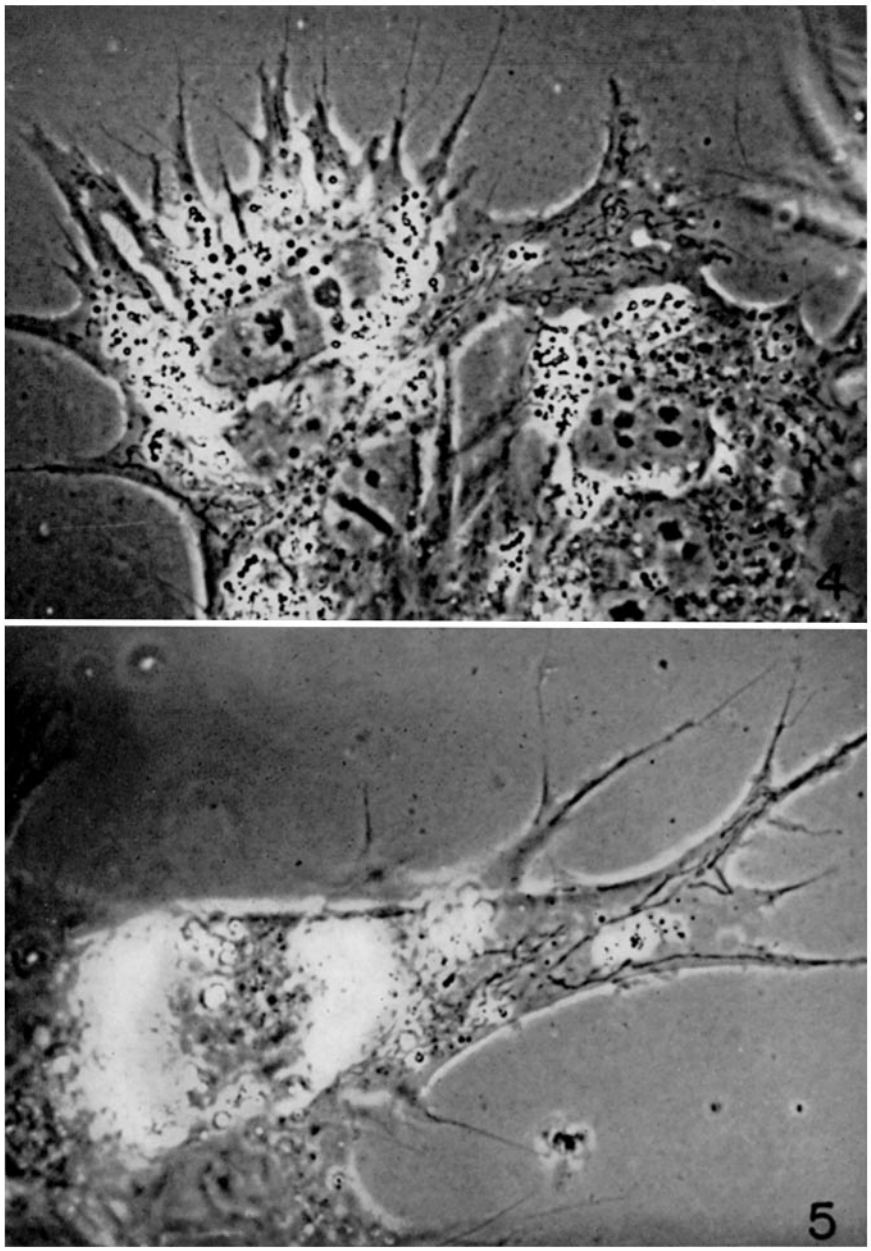
(Biesele: Coenzyme A and mitochondria)

PLATE 38

Photomicrographs of embryonic mouse skin fibroblasts 2 days in culture. Oil immersion, dark medium phase contrast. × 1090.

FIG. 4. Cells of culture treated 1 day with 1 mM 6-mercaptopurine and 0.2 mM coenzyme A together. Mitochondria are opaque filaments of about same size as in control cells of Fig. 1. These cells also contain many lipid droplets.

FIG. 5. Cells of culture treated 1 day with 0.2 mM coenzyme A alone. Mitochondria, as in distal cytoplasm, are long filaments. There are several large masses of lipid.



(Biesele: Coenzyme A and mitochondria)

PLATE 39

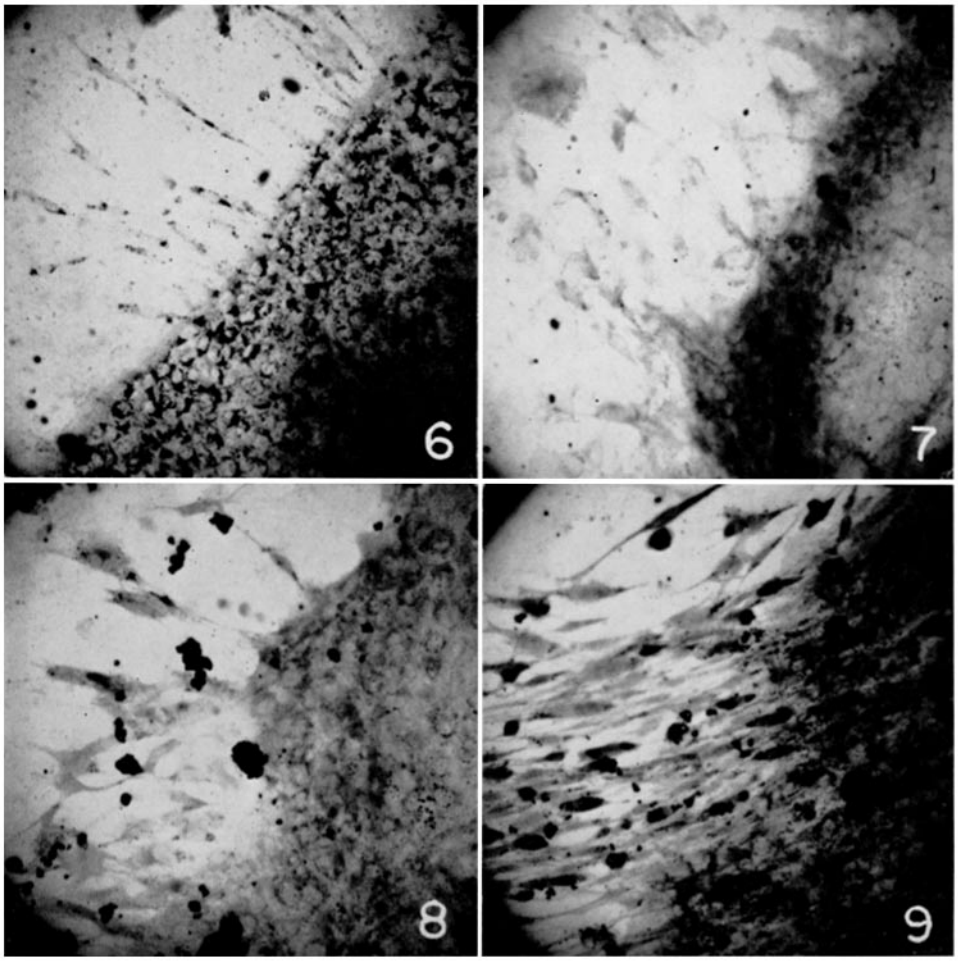
Photomicrographs of embryonic mouse skin cultures fixed with formalin and stained with Sudan black B. Epithelial cells and explant are to the right, fibroblasts are to the left. Standard light microscopy. × 150.

FIG. 6. Cells of a control culture. Sudanophilic lipid is seen as opaque cytoplasmic rings in epithelial cells and as masses at either end of nucleus in fibroblasts.

FIG. 7. Cells of culture treated 1 day with 1 mM 6-mercaptopurine. The cells show little sudanophilia.

FIG. 8. Cells of culture treated 1 day with 1 mM 6-mercaptopurine and 0.1 mM coenzyme A together. Cellular sudanophilia is intermediate between that of controls and that of 6-mercaptopurine-treated cultures. Large opaque objects are masses of precipitated stain, not cells.

FIG. 9. Cells of culture treated 1 day with 0.1 mM coenzyme A alone. Many cells are strongly sudanophilic.



(Biesele: Coenzyme A and mitochondria)