INHIBITION OF EHRLICH ASCITES TUMOUR CELLS BY SKELETAL MUSCLE EXTRACTS*

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Received for publication January 8, 1968

ALTHOUGH skeletal muscle constitutes approximately 40 per cent of body mass, and receives a correspondingly large blood supply, distant metastasis of malignant tumours to skeletal muscle is rare. Neoplastic cells were found in the peripheral blood of up to 55 per cent of patients with malignancy (Griffiths and Salsbury, 1965), and metastatic tumour emboli or growths in 6 of 38 autopsies of patients with malignant disease, 3 in patients with lymphoma and 3 with carcinoma (Pearson, 1959). However, the ability of skeletal muscle to support tumour growth appears to be low, since macroscopic metastasis to muscle is a rarity (Muslow, 1943). Willis (1952) observed such metastasis in only 4 of 500 consecutive autopsies of patients with malignancy.

In experimental animals, successful implantation of some tumours following intra-arterial (Coman, Eisenburg and McCutcheon, 1949; Coman, DeLong and McCutcheon, 1951) or intramuscular (DeLong and Coman, 1950) injection has led some authors to attribute the organ distribution of metastases solely to circulatory differences. Others, employing tumours which spare muscle and some other tissues, have attributed the distribution of metastases to the ability of the tissues to sustain the growth of particular tumour types (Schmähl, 1961; Sugarbaker, 1952).

In the present study, the effect of muscle extracts on the growth and survival of Ehrlich ascites tumour cells in culture medium was compared with the effect of extracts of other tissues.

MATERIALS AND METHODS

Ehrlich ascites tumour.—A stock of this tumour was maintained by weekly i.p. transfers into male Swiss mice, weighing 20–25 g., of 0·2 ml. of a suspension containing 2×10^6 tumour cells in isotonic saline. This tumour is highly malignant in mice following parenteral administration by any route. Intramuscular injection of 0·02 ml. of a suspension containing 1614 cells resulted in death of 20 mice in 8–47 days, with a mean ($\pm$ S.E.) of 22·4 $\pm$ 2·5 days.

Culture of tumour cells.—Ehrlich ascites tumour cells were suspended in a culture medium containing 40 per cent (v/v) of bovine serum and 60 per cent (v/v) of medium 199 with Hanks' balanced salt solution, adjusted to pH 7·2. Chick embryo extract was not employed as it was found to be toxic for Ehrlich ascites tumour cells in culture (Ely and Gray, 1960). Five ml. of cell suspension were incubated at 37° in a 30 ml. sterile bottle which was sealed by rubber stopper and rotated, at 45 degrees tilt, once every 5 min. After 12 hr. of incubation, 1 ml. of tissue extract, or isotonic saline as a control, was added. One ml. amounts of the

* Supported by Public Health Service Grants NB 03464 from the National Institute of Neurological Diseases and Blindness and CA 05588 from the National Cancer Institute.

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suspension were withdrawn immediately, and 24, 48 and 72 hr. after addition of the tissue extract or saline, for determination of cell population, in a counting chamber 0·6 mm. deep, and cell morphology. Initial cell counts immediately after addition of tissue extract or isotonic saline were 90–120 per μl.

Cell morphology.—Tumour cells obtained from the culture media were observed unstained by phase contrast microscopy, or stained with acridine orange. Staining was performed by mixing equal volumes of fresh cell suspension and acridine orange solution (10 mg. in 100 ml. isotonic saline), and observing fluorescence of the stain microscopically with an ultraviolet light source.

Tissue extracts.—Skeletal muscle was obtained from Swiss mice, Wistar rats and New Zealand rabbits, and heart muscle, thymus, liver, kidney and spleen from Swiss mice. Animals were killed by bleeding, and tissues were processed immediately. Four g. of sliced tissues were homogenized with 25 ml. of isotonic saline for 2 min. in a Lourdes homogenizer. The homogenate was centrifuged at 27,000 $\times$ ***g*** for 20 min., and the supernatant recovered.

The effect of heat and of dialysis on muscle extract was studied. Heated muscle extract was prepared by incubating the extract at 56° for 30 min., and recovering the supernatant following removal of resulting precipitate by centrifugation at 10,000 $\times$ ***g*** for 10 min. Dialyzed muscle extract was prepared by dialyzing the extract in cellophane tubing against continuously changing isotonic saline for 24 hr. The dialysate of muscle extract was obtained by dialyzing the extract in cellophane tubing against an equal volume of isotonic saline for 24 hr. The volume of the dialysate was reduced to half by evaporation under reduced pressure so that it contained the same concentration of dialyzable substances as the original muscle extract.

All preparations were carried out aseptically at 4°, and extracts were filtered through a sterile Millipore filter type GS (pore size 0·22 $\pm$ 0·02 μ) before use.

RESULTS

Effects on cell counts.—The cell counts in cultures to which mouse skeletal muscle or thymus extract had been added were significantly lower at 24, 48, and 72 hr. than in cultures containing added saline as a control, or kidney or liver extract. ($P < 0{\cdot}05$) (Fig. 1). The cell counts of cultures containing heart muscle extract were lower than those containing added saline, kidney extract or liver extract at 24 hr. ($P < 0{\cdot}05$), and lower than those containing added saline, liver extract or spleen extract at 48 hr. ($P < 0{\cdot}01$), but no significant differences were noted at 72 hr. The cell counts in cultures containing spleen extract were lower than in those containing added saline, kidney extract or liver extract only at 24 hr. (P 0·05). The results indicate that extracts of mouse skeletal muscle, thymus and, to a lesser degree, heart muscle and spleen contained a substance or substances which reduced the population of Ehrlich ascites tumour cells in culture medium. The effect of skeletal muscle extract derived from rats and rabbits was the same as that from mice (Fig. 2).

Dilution of mouse skeletal muscle extract decreased the inhibitory effect on tumour cells (Fig. 3). Heating the extract at 56° for 30 min., or dialyzing the extract, resulted in a decrease in inhibitory activity ($P < 0{\cdot}001$ and $P < 0{\cdot}01$, respectively) (Fig. 4). The dialysate reduced cell population to the same degree as untreated muscle extract, and to a greater degree than the dialyzed or heat treated extract ($P < 0{\cdot}05$). The activity of the dialysate was not significantly affected by heating at 56° for 30 min., which did not result in any precipitate.

Some of the chemical components of the extracts studied are listed in the Table. Skeletal muscle extract, its dialysate, and spleen extract contained higher concentrations of potassium and phosphorus than the culture medium or the other extracts. The concentration of calcium in all extracts was negligible, compared to the culture medium. However, there was no effect on cell growth,

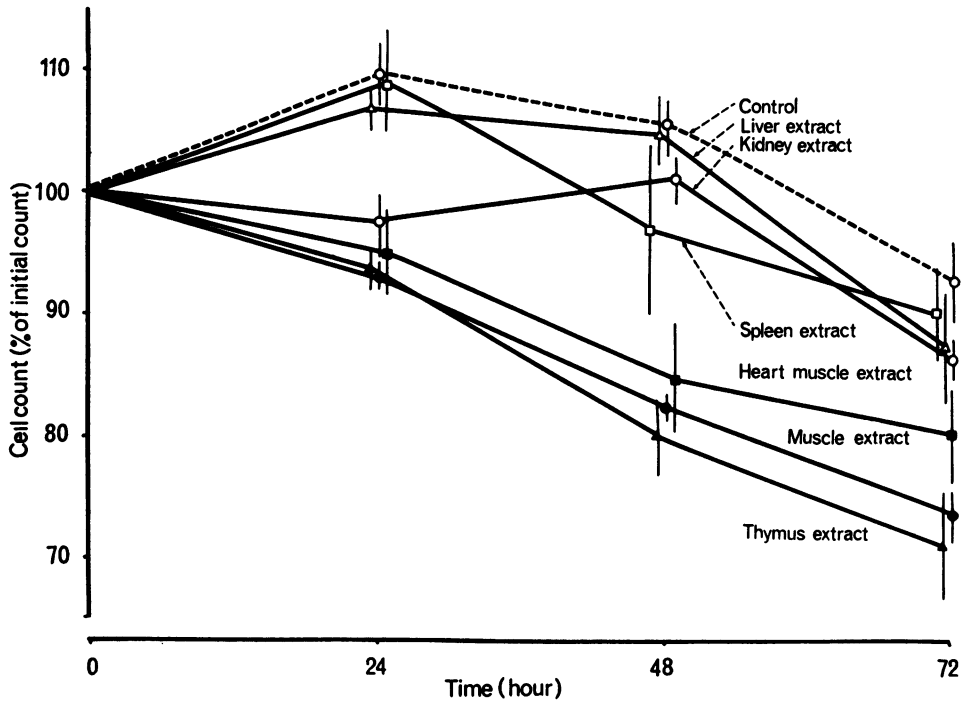

FIG. 1.—Effect of extracts of mouse tissues on the cell count of Ehrlich ascites tumour cells in culture. The vertical lines indicate the standard error of the mean. The number of experiments were 22 with saline (control), 8 with kidney extract, 8 with liver extract, 11 with spleen extract, 7 with heart muscle extract, 58 with skeletal muscle extract, and 7 with thymus extract. Cell counts of cultures with muscle extract or thymus extract were significantly lower at 24, 48, and 72 hr. than cell counts of control cultures, or of cultures with kidney or liver extract ($P < 0.05$).

TABLE.—*Chemical Components of Tissue Extracts* (*mg. per* 100 *ml.*)

Extracts	Protein nitrogen*	Non-protein nitrogen†	Na	K	Ca	P
Skeletal muscle, Untreated	59·6	18·6	354	42·1	0·0	11·3
Dialyzed	35·9	1·9	354	0·8	0·0	0·1
Dialysate	0·0	18·4	358	36·0	0·0	9·0
Heart Muscle	64·6	10·0	358	21·4	0·0	4·4
Liver	77·0	10·6	358	28·5	0·0	3·9
Kidney	52·5	12·5	353	22·6	0·0	4·5
Spleen	119·0	19·0	354	39·0	0·0	5·8
Saline	0·0	0·0	354	0·0	0·0	0·0
Culture medium	361·1	30·5	324	24·4	5·3	4·1

* Nitrogen precipitable by 10 per cent (w/v) trichloracetic acid.
† Nitrogen remaining in solution with 10 per cent (w/v) trichloracetic acid.

survival, or morphology by changes in the concentration of these ions in the culture medium comparable to changes produced by addition of these extracts. The dialysate was free of protein.

Morphological changes.—Changes appeared in tumour cells in culture concomitant with reduction in cell count. In the control suspensions or in the presence of tissue extracts which did not affect cell population, these began after 72 hr. of incubation, and did not become pronounced until after 96 hr. of incubation. In the presence of skeletal muscle extract, or its dialysate, similar cellular alterations occurred much earlier, concomitant with the fall in cell count. Phase contrast microscopy revealed the appearance of many vacuoles and granules in the cytoplasm, and of a more distinct border in the nucleus, after 24 hr. of incuba-

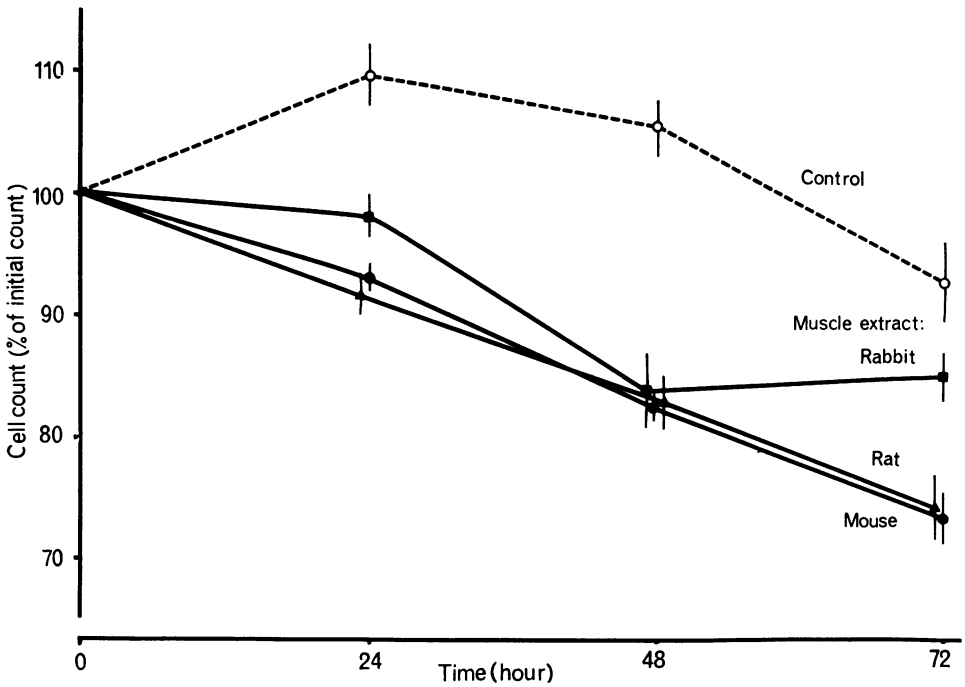

FIG. 2.—Effect of extracts prepared from skeletal muscle of rabbits, rats, and mice on the cell count of Ehrlich ascites tumour cells in culture. Vertical lines indicate the standard error of the mean. The number of experiments were 22 with added saline (control), 58 with mouse muscle extract, and 8 each with rabbit or rat muscle extract. Cell counts in cultures with muscle extracts were significantly lower than that of the control ($P < 0{\cdot}01$), except for the value of the culture with rabbit muscle extract at 72 hr.

tion (Figs. 5 and 6). At 48 hr., the cells became shrunken and nucleoli larger and more distinct (Fig. 7). Finally, the cells appeared as an accumulation of granules (Fig. 8.)

Fresh tumour cells stained with acridine orange showed a green fluorescence in the nucleus, nucleolus, cytoplasm and cytoplasmic granules (Fig. 9). After 24–48 hr. of incubation in the presence of skeletal muscle extract, the granules turned to red-orange and the nucleoli appeared yellow. The cytoplasm then became diffusely orange, and the nucleus light green with distinct yellow-orange nucleoli (Fig. 10). After 72 hr. of incubation, many cells stained entirely orange. Similar changes occurred much later in the control suspensions, or in the presence

of tissue extracts which did not affect cell population. The pattern of staining reflects the viability of the cells, and is in accord with the findings of Wolf and Aronson (1961) in cultured embryonic cells.

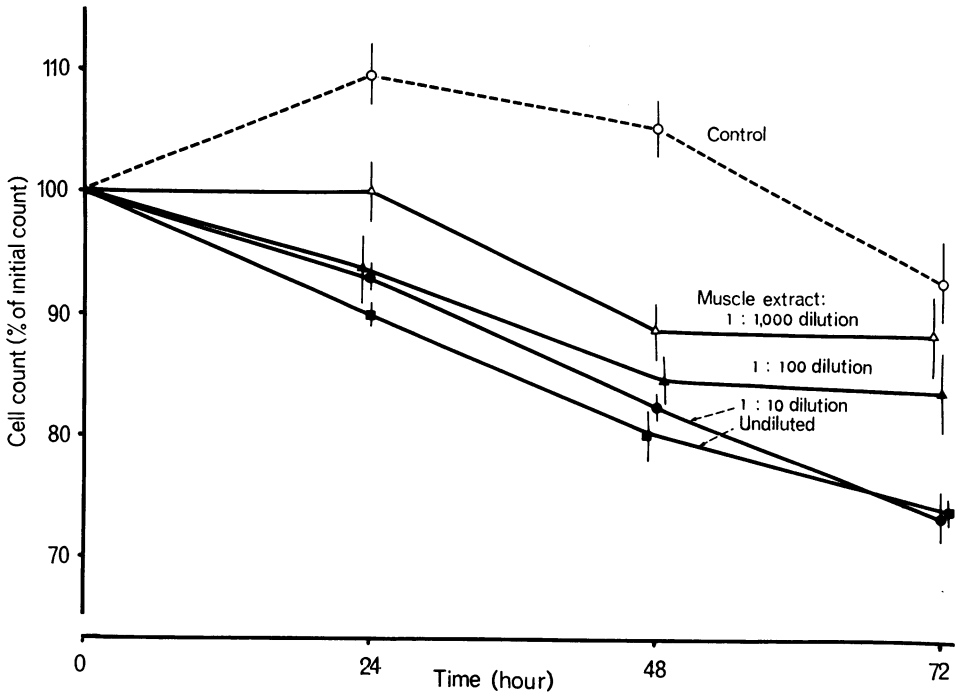

FIG. 3.—Effect of different concentrations of mouse skeletal muscle extract on the cell count of Ehrlich ascites tumour cells in culture. Vertical lines indicate the standard error of the mean. The number of experiments were 22 with added saline (control), 58 with undiluted muscle extract, and 6 with each dilution of muscle extract. Cell counts of cultures with undiluted muscle extract or with 1 : 10 dilution of muscle extract were significantly lower throughout the experiments than cell counts of the control ($P < 0{\cdot}01$).

DISCUSSION

Extracts of skeletal muscle of mice, rats, and rabbits inhibited the growth and survival of Ehrlich ascites tumour cells in culture medium. Extracts of mouse heart muscle, thymus, and spleen also had an inhibitory effect, while extracts of liver and kidney had no effect. It is of interest that distant metastases in patients with carcinoma are relatively uncommon not only in skeletal muscle, but also in the spleen (Warren and Davis, 1934), heart, and thymus (Willis, 1952).

The inhibitory effect of skeletal muscle extract on tumour cells in culture was reduced following heating of the extract at 56° for 30 min. and removal of the resulting precipitate, which was largely protein, or following dialysis. The inhibitory activity of the dialysate, which contained no protein, was as great as that of the original muscle extract, and was not affected by heating. These findings suggest that the inhibitory activity of skeletal muscle extract is due to a heat-stable substance of small molecular size which is loosely bound to protein.

The inhibitory activity of the tissue extracts could not be ascribed to differences in the concentration of sodium, potassium, calcium, or phosphorus.

Extracts and fractions of skeletal muscle and other tissues have been found to contain factors which inhibit or promote the growth of a variety of tumour cells in culture. Parshley found nondialyzable inhibitory substances in muscle, as well as in aorta and tendon, following mild tryptic digestion (Parshley, 1965). Szent-Gyorgi, Hegyeli and McLuaghlin (1963) extracted a growth inhibitory

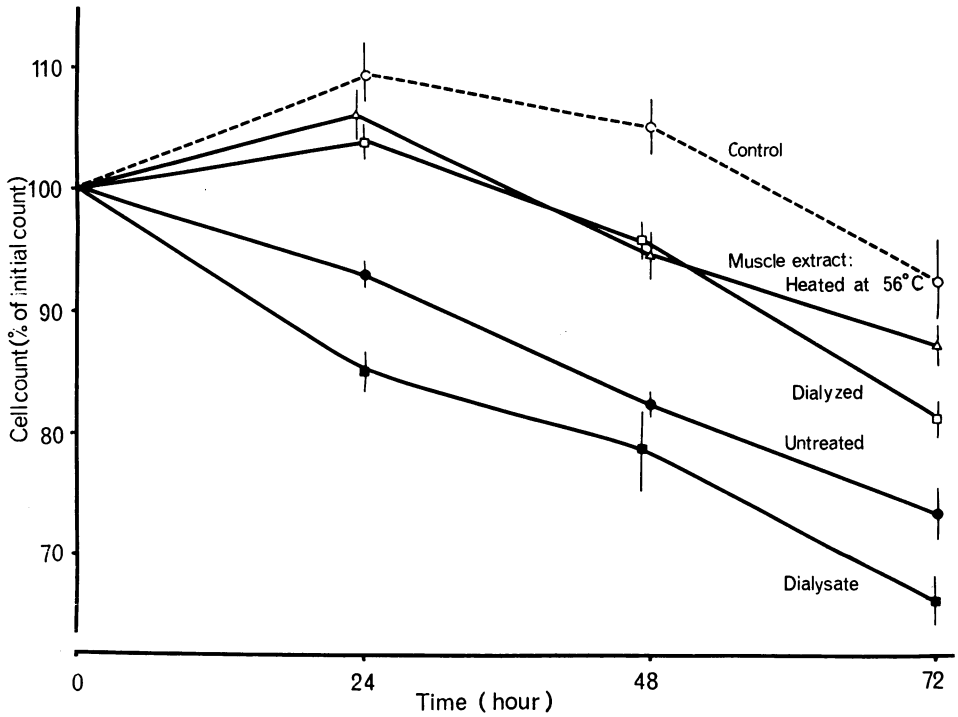

FIG. 4.—Effect of heated or dialyzed mouse skeletal muscle extract on the cell count of Ehrlich ascites tumour cells in culture. Vertical lines indicate the standard error of the mean. The number of experiments were 22 with added saline (control), 58 with untreated muscle extract, 18 with muscle extract heated at 56° for 30 min., 24 with dialyzed muscle extract, and 8 with the dialysate. Cell counts of cultures with untreated muscle extract or muscle dialysate were significantly lower throughout the experiments than those of the control or those with heated or dialyzed muscle extract ($P < 0{\cdot}05$).

substance, termed retine, and a growth promoting substance, termed promine, from all tissues tested, including skeletal muscle. These substances, which were both of low molecular weight, approximately 450, affected the growth of Krebs 2 ascites tumour in mice. Kanao (1965) isolated a heat-stable dialyzable polypeptide from skeletal muscle which inhibited mitosis of sea urchin eggs.

The growth of transplanted neoplastic cells was significantly slower in exercised experimental animals than in control animals, and in some instances, there was complete tumour regression in exercised animals (Hoffman, Paschkis, DeBias, Cantarow and Williams, 1962; Rusch and Kline, 1966). Hoffman *et al.* (1962) found that the bath fluid of electrically stimulated muscle contained a non-

dialyzable substance which inhibited the growth of Walker 256 and Murphy lymphosarcoma in rats. Although these observations suggest that the tumour inhibiting substance of skeletal muscle is a metabolic product of muscle, the relation of the active component to the distribution and growth of tumours and metastases remains to be elucidated.

SUMMARY

The effect of extracts of skeletal muscle and other tissues on the growth of neoplastic cells in tissue culture was studied in order to investigate the cause of the extremely low incidence of distant metastasis to skeletal muscle in patients with malignancy. Forty-eight hr. after addition of saline extract of mouse tissues, the following changes in the number of Ehrlich ascites tumour cells in culture were found: control, + 15·4 per cent; liver, + 5·2 per cent; spleen, + 1·1 per cent; kidney, — 2·9 per cent; heart muscle, — 15·2 per cent; skeletal muscle, — 17·6 per cent; and thymus — 20·0 per cent. Inhibitory effect was of the same degree when extracts of muscle of mice, rats, or rabbits were utilized. The inhibitory factor in skeletal muscle extract was dialyzable through cellophane membrane and was removed by heating the original extract but not the dialysate. These properties suggest that it is a heat stable small molecule which is loosely bound to protein. The viability of tumour cells estimated by phase contrast microscopy and vital staining with acridine orange was in accord with the cell population in culture.

We are indebted to Dr. Kanematsu Sugiura, Walker Laboratory, Sloan-Kettering Institute, Rye, New York, who kindly provided a line of Ehrlich ascites tumour.

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EXPLANATION OF PLATE

FIGS. 5–8.—Ehrlich ascites tumour cells. Compressed fresh sample. Phase contrast microscopy. × 730.

FIG. 5.—Cells freshly obtained from ascites.

FIG. 6.—Cells from the culture 24 hr. after addition of mouse skeletal muscle extract. Numerous vacuoles and bullae have appeared in the cytoplasm.

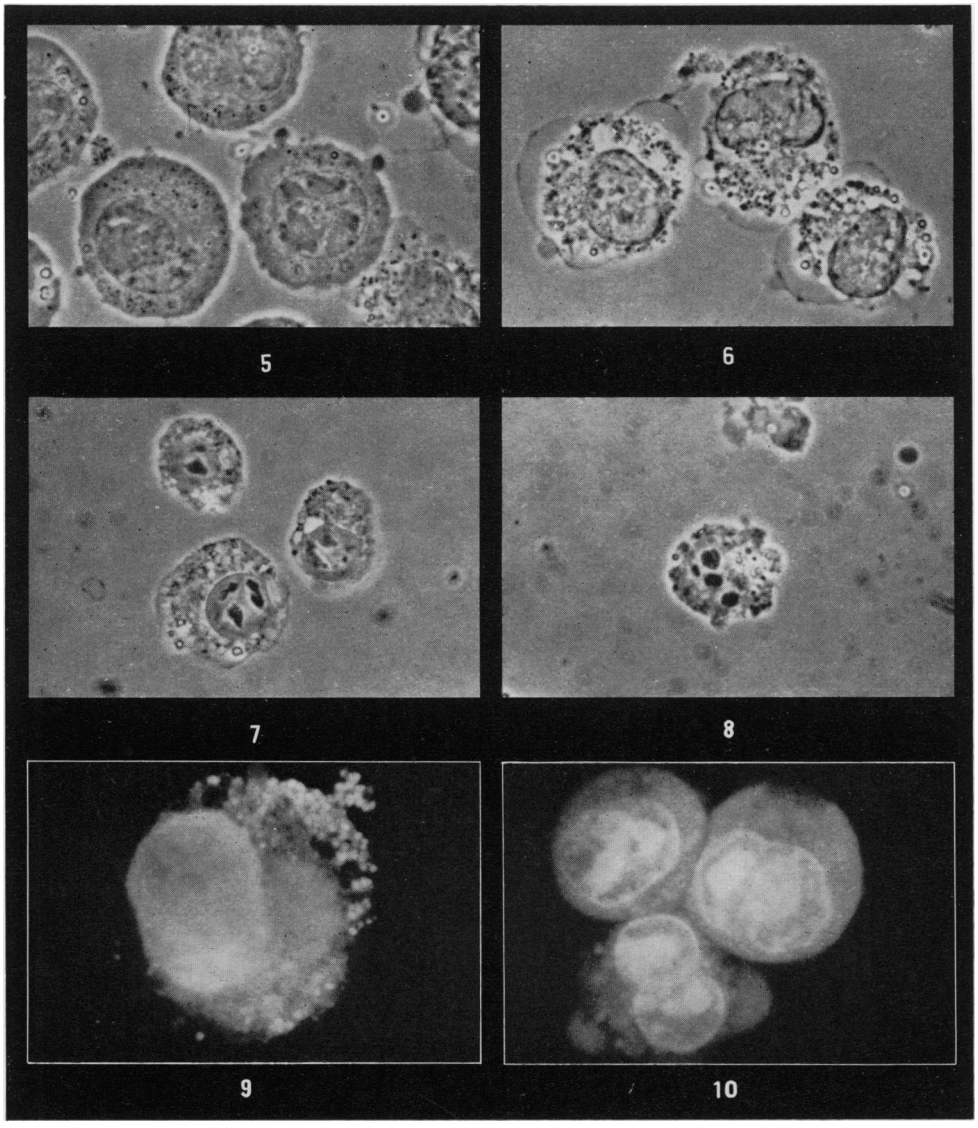
FIG. 7.—Cells from the culture 48 hr. after addition of mouse skeletal muscle extract. The size of the cell is smaller, and prominent nucleoli are visible.

FIG. 8.—Cell from the culture 48 hr. after addition of mouse skeletal muscle extract. The cell is shrunken, granular, and the border of the nucleus is no longer clear.

FIGS. 9 and 10.—Ehrlich ascites tumour cell. Vital staining with acridine orange. Fluorescence microscopy. × 1,050.

FIG. 9.—Cell freshly obtained from ascites. All cellular structures, including the nucleus, cytoplasm, and cytoplasmic granules, stain with green fluorescence.

FIG. 10.—Cells from the culture 48 hr. after addition of mouse skeletal muscle extract. The nucleoli and nuclear membrane stain with yellow-orange fluorescence on the light green nuclear substance, and the cytoplasm with orange-red fluorescence.



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