Succinic Dehydrogenase and Cytochrome Oxidase Activities in Cell Cultures

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ABSTRACT

Succinic dehydrogenase and cytochrome oxidase have been assayed in permanent cell lines (HEP 1, HEP 2, and HLM), in short-term cultures of chick embryo heart cells, and in various tissues. Their activities in different cells are compared by relating them to deoxyribonucleic acid. They are very low in HEP 1, HEP 2, and HLM cells by comparison with the activities in any normal tissues examined. All the succinic dehydrogenase was shown to be located in the mitochondria of the permanent cell lines by staining with tetrazolium derivatives. Both enzymes were more active in tissues of 19-day chick embryos than in those of 11- or 14-day embryos. The increasing activities found during normal development were quickly curtailed or reversed when heart cells were grown as monolayer cultures.

Digitonin-treated mitochondria produced preparations with much higher activities of cytochrome oxidase than untreated samples. Activities measured in this way were again very much lower in HEP 1, HEP 2, and HLM cells than in the normal tissues. From the derived ratio of cytochrome oxidase:succinic dehydrogenase, it was apparent that cytochrome oxidase is diminished to a greater extent than succinic dehydrogenase in both permanent cell lines and short-term cultures, by comparison with the corresponding activities in embryonic and adult tissues. The features common to the metabolism of proliferating cells *in vitro* and malignant cells are discussed.

INTRODUCTION

The high glycolytic activity (or fermentation) of cells growing *in vitro* is well established. It has been frequently reported in studies on explant cultures (30). In monolayer cultures, higher rates of glycolysis were achieved by human foetal cells within the first few weeks of cultivation than in human malignant cells which had been maintained as permanent lines for a number of years (13).

It was clearly desirable to investigate the activities in cultured cells of the respiratory enzymes, and succinic dehydrogenase and cytochrome oxidase were selected because they are entirely located in the mitochondria of the cells. The levels of these enzymes were assayed in permanent human cell lines (HEP 1, HEP 2, and HLM), in certain adult and embryonic tissues, and in chick embryo heart cells during their first two or three passages in culture.

Digitonin has been found to increase appreciably the cytochrome oxidase activity of mitochondrial preparations (21, 26). Such increased activities probably represent more closely the total oxidase of the mitochondria, since the relatively large molecules of cytochrome *c* are unlikely to reach the oxidase of the internal membranes of intact mitochondria. The cytochrome oxidase activities of the cultured cells and various tissues have, therefore, been measured with both untreated and digitonin-treated mitochondria.

Enzyme activities have been expressed in relation to deoxyribonucleic acid (DNA), as this provides a basis for the comparison of activities in homologous organs of different species. Although the amount of DNA per nucleus varies considerably between species, the average cell mass of homologous organs is directly proportional to the characteristic DNA value for the species (16), and

enzyme activities in diploid and tetraploid lines of ascites cells have been shown to increase in direct proportion to the DNA (11). Differences between the levels of oxidative enzymes on this basis, therefore, represent changes in the metabolism of cells relative to a common unit of genetic material.

Method

Cells.—The HEP 1/56 line was derived from an epidermoid cervical carcinoma and the HEP 2/56 line from a pharyngeal carcinoma of a male patient (3, 25).[1] The HLM line (human liver male) is derived from a 20-week foetus by "transformation" of the original epithelial cell cultures (12). These permanent cell lines were maintained in culture in this laboratory for 30 months previous to this investigation. Cells grown from lung and kidney of the same foetus ceased to proliferate after several weeks.

Short-term cultures of chick embryonic heart were prepared from embryos of average incubation ages of 15 and 19 days. The tissue was chopped finely and stirred magnetically with a stainless steel rotor for 40 to 60 minutes at 37°C. in 0.25 per cent trypsin (Difco) containing 15 I.U. of hyaluronidase (Benger's hyalase) per ml. The cells were planted from suspension after washing once with the growth-promoting medium.

Medium.—All the cultures were growing rapidly at the time of sampling for assay. The growth-promoting medium was prepared from the following:

(*a*) A chemically defined portion, medium NCTC 109 (15). This was supplemented by the addition of 0.72 mg. ferric nitrate, 0.20 mg. *n*-butyl-*p*-hydroxybenzoate, 50 mg. penicillin, and 50 mg. streptomycin per litre of medium. The salt components for the human cells were those of Earle's solution, and for the chick cells, those of Tyrode solution.

(*b*) Chick embryo extract, as prepared by Leslie, Fulton, and Sinclair (13).

(*c*) Pooled AB human serum or horse serum (for the fowl cells).

The medium consisted of 20 volume embryo extract and 20 volume serum added to 60 volume of the modified NCTC 109.

Cultures were grown in large flasks (Roux pattern) or in specially designed pyrex roller tubes, 11 cm. long, 4 cm. in diameter, and having an internal surface area of approximately 130 sq. cm. The flasks required 50 ml. medium containing 2 million cells as an initial inoculum and produced 30 to 50 million cells each. The roller tubes were inoculated with 1 million cells in 10 ml. medium and finally contained 20 to 30 million cells. About 300 million cells were required for each test on the permanent cell lines.

For cytological purposes, cells were grown on single coverslips (4.4 × 1.25 cm.) in universal containers (volume 25 ml.). About 0.5 million cells were inoculated in 2 ml. medium and the containers were kept at a slight incline at 37°. After 24 hours, each coverslip bore a fine sheet of cells suitable for staining.

Preliminary Treatment of Cells for Analysis.—Cells and tissues were disrupted by a teflon homogeniser (Arthur Thomas Co., Philadelphia) in 0.44 M sucrose, and diluted to give a 10 per cent homogenate. A small portion of this was used for DNA determination by the indole reagent (1). A portion of the homogenate, suitably diluted, was kept for the assay of cytochrome oxidase. The remainder was centrifuged at 20,000 *g* for 15 minutes and the pellet of mitochondria and nuclei was resuspended in the same volume of 0.44 M sucrose as used to prepare the 10 per cent homogenate. A portion was taken for the DNA determination, and the remainder used for the assay of succinic dehydrogenase. The homogenate could not be used because it contained endogenous substrates and other dehydrogenases which produced appreciable non-specific reduction of the tetrazolium derivative.

Measurement of Cytochrome Oxidase Activity.—The method was essentially that of Simon (21) with minor modifications.

A 20 μM solution of cytochrome *c* was prepared in 0.44 M sucrose: 0.05 M phosphate buffer (pH 7.1). This was reduced with 7 mg. sodium dithionite per 45 ml., and the excess reducing agent was removed by a stream of oxygen. 0.1 ml. of cell homogenate was added to 2.9 ml. reduced cytochrome *c* in a 1 cm. cuvette and a zero time reading at 550 mμ was taken as quickly as possible. The temperature was maintained at 37° throughout the reaction period by using the warm cell attachment for the Unicam SP 500. The change in optical density (O.D.) caused by the oxidation of reduced cytochrome *c* was recorded every 10 seconds for 2 minutes; the O.D. at complete oxidation was obtained after adding a small drop of saturated potassium ferricyanide to the cuvette.

In order to demonstrate the effect of digitonin on the reaction, varying amounts of digitonin were dissolved in 0.5 ml. homogenate and, within 30 seconds, 0.1 ml. was removed and assayed as described above. It was found that amounts of digitonin greater than 5 mg. per 0.5 ml. produced opalescent solutions, while less than 1 mg. per 0.5 ml. homogenate produced no increase in the reaction velocity. 2.0 mg. digitonin per 0.5 ml. produced the optimal effect.

The 10 per cent homogenates were diluted, as necessary, in order to ensure that the rate of change in O.D. did not exceed 0.02 in 10 seconds with a cytochrome *c* concentration of 20 μM (9). The dilutions were considerably greater when assays were carried out in the presence of digitonin.

[1] Dr. A. E. Moore, Sloan-Kettering Institute for Cancer Research, New York, kindly sent these cells in January, 1956.

The velocity constant (k) of the first order reaction for the oxidation of cytochrome c was calculated in the usual way from the change in optical density with time (23). From the values for "k," oxygen uptake as μl. per 0.1 ml. homogenate sample was calculated according to the procedure of Fritz and Beevers (5). From the determination of the amount of DNA phosphorus (DNAP), the final results were expressed as μl. O_2 μg. DNAP/hour.

Measurement of Succinic Dehydrogenase Activity.—The procedure was based on the method of Shelton and Rice (20). Each 25 ml. Erlenmeyer flask contained 0.5 ml. of 0.2 M tris buffer in 0.7 M sucrose, adjusted to pH 8.4, 0.1 ml. of 0.5 M sodium succinate (or water in controls), 0.1 ml. of 2 mM potassium cyanide, and 0.1 ml. of 1 per cent 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride (INT), obtained from British Drug Houses, Poole. The reaction was initiated by adding 0.2 ml. of suitably diluted mitochondrial and nuclear suspension, and the flasks were shaken mechanically for 10 minutes at 37°C. 0.5 ml. of 20 per cent trichloracetic acid was added to stop the reaction, and the red colour of the formazan was extracted with 3 ml. ethyl acetate, as described by Shelton and Rice (20). Under the standard assay conditions, formazan production was linear up to 10 minutes. The digitonin treatment, applied in the cytochrome oxidase assay, inhibited succinic dehydrogenase activity, as measured by the tetrazolium method. It has no effect upon succinic dehydrogenase assayed by the spectrophotometric method.

The O.D. of the ethyl acetate extract was measured at 490 mμ with a Unicam SP 500. This value was converted into "T" (tetrazolium) units, which were defined as "the succinic dehydrogenase activity which will produce an optical density of 0.010 at 490 mμ after 10 minutes incubation at 37°C. under standard assay conditions." The activities were expressed as "T" units per μg. DNAP.

Cytochemical Demonstration of Succinic Dehydrogenase.—Coverslips bearing sheets of cells were washed with Earle's saline and frozen at −20°C. to destroy the activity of non-specific dehydrogenases. The material was incubated under the same conditions as for the assay of succinic dehydrogenase. The coverslips were mounted on slides, sealed with paraffin wax, and photographed.

As INT is unsuitable for making permanent cytological preparations, nitro-blue tetrazolium (NBT), obtained from Dajac Laboratories, Philadelphia, was used instead, according to the method described by Nachlas, Tsou, Souza, Cheng, and Seligman (17). The coverslip cultures were rinsed, frozen, and incubated for 90 minutes at 37°C. in a solution containing per ml., 0.5 ml. 0.2 M tris buffer, at pH 8.4, 0.1 ml. of 0.5 M sodium succinate (or water for control), 0.1 ml. of 2 mM potassium cyanide, and 0.3 ml. of 0.16 per cent NBT. After washing with Earle's saline, the cells were fixed in a solution of formaldehyde in this saline. In order to show the nuclei in relation to the cytoplasmic staining, Feulgen staining was carried out by treating the cells with N HCl at 60°C. for 6 minutes and producing the Schiff's base by exposure to the reagent for ½ to 1 hour at room temperature.

RESULTS

Table I shows the similar activities of succinic dehydrogenase and of cytochrome oxidase per unit DNAP in the permanent cell lines. The values for each enzyme are much smaller than the corresponding activities in certain adult and foetal rat tissues. They are also very much lower than any of the levels obtained for embryonic chick organs (Table II).

The greater cytochrome oxidase activities found in all the samples after digitonin treatment are believed to be the result of the disruption of intact mitochondria and the access of substrate to the oxidase of the internal membranes (21). The permanent cell lines required more prolonged homogenisation than the tissues in order to break down the cells, and their homogenates probably contained some intact cells. With these preparations, the increased cytochrome oxidase obtained by digitonin treatment may have arisen from the disruption of both intact cells and mitochondria. Nevertheless, the maximal values remain far lower than those for tissue homogenates similarly treated with digitonin.

The two enzymes are known to be located entirely in the mitochondria of animal tissues. Cytochemical procedures based on the enzymic reduction of INT and NBT were used to confirm that succinic dehydrogenase was likewise located in the mitochondria of the permanent cell lines. Figs. 1 *a*, 1 *b*, and 1 *c* show the formazan staining is confined to the cytoplasmic particles of HLM and HEP 1 cells when both INT and NBT were used as hydrogen acceptors. Fig. 1 *d* confirms that succinic dehydrogenase is responsible for the staining, since no formazan was produced from NBT in the absence of succinate.

Lower activities of oxidative enzymes in foetal or embryonic tissues, as compared with those of adult tissues, have frequently been reported (2, 4, 19, 22) and they are apparent in the results for chick embryo tissues reported in Table II. The activities of the two enzymes were measured in chick heart, liver, and brain taken from embryos of average incubation ages of 11 (10 to 12) days, 15 (14 to 16) days, and 19 (18 to 20) days. From Table II, it

TABLE I

Activities of Succinic Dehydrogenase and Cytochrome Oxidase in Permanent Cell Lines (HEP 1, HEP 2, and HLM) and in Certain Adult and Embryonic Tissues

Origin and cell type	Succinic dehydrogenase* *T* units‡/μg. DNAP	Cytochrome oxidase* without digitonin treatment	Cytochrome oxidase with digitonin treatment
		μl. O_2/μg. DNAP/hr.	
Human			
HEP 1/56	1.73 ± 0.11	1.19 ± 0.14	9.28
HEP 2/56	1.57 ± 0.12	1.10 ± 0.12	5.28
HLM	3.12 ± 0.29	1.53 ± 0.27	4.25
Rat embryo§			
Kidney	3.45 ± 0.14	3.37	19.64
Liver	11.25 ± 1.18	19.85	94.40
Rat adult			
Kidney	54.43 ± 2.03	55.42 ± 0.95	301.60
Liver	70.30 ± 3.55	64.17 ± 2.45	460.00

* Where standard errors of the means are given, they are based on 6 to 21 separate observations. Other results are the means of 2 to 3 observations.

‡ The *T* (Tetrazolium) unit is defined as "the succinic dehydrogenase activity which will produce an optical density of 0.010 at 490 mμ after 10 minutes incubation at 37°C. under standard assay conditions."

§ The litters were near full-term and each embryo used weighed about 6 gm.

For succinic dehydrogenase assays the pellet of mitochondria and nuclei was used; for cytochrome oxidase, assays were carried out on the homogenate, diluted as necessary.

TABLE II

Succinic Dehydrogenase and Cytochrome Oxidase Activities during Development of Chick Embryo Organs at Average Incubation Ages of 11, 15, and 19 Days

Chick embryo organ	Average incubation age	Succinic dehydrogenase* *T* units‡/μg. DNAP	Cytochrome oxidase* without digitonin	Cytochrome oxidase with digitonin
			μl. O_2/μg. DNAP/hr.	
	days			
Heart	11	17.20 ± 1.00	27.75 ± 2.15	226.50
	15	29.81 ± 2.75	63.83 ± 4.02	354.80
	19	47.79 ± 1.24	103.12 ± 8.21	424.80
Liver	11	36.90 ± 0.58	37.53	429.80
	15	30.61 ± 0.66	34.62	368.40
	19	49.92 ± 1.72	74.44 ± 9.84	507.20
Brain	11	16.24 ± 1.36	3.36	116.40
	15	25.82 ± 1.18	8.04	343.20
	19	40.67 ± 3.18	13.57 ± 3.44	306.50

* and ‡ as in Table I.

can be seen that in all three tissues they are larger at 19 days than at 11 days. Succinic dehydrogenase activity increases 2.8 times in heart, 1.4 times in liver, and 2.5 times in brain, while the maximal cytochrome oxidase activity (*i.e.* after digitonin treatment) increases 1.9, 1.2, and 2.6 times respectively in the same period.

Since heart tissue showed consistently increasing activities for the oxidative enzymes during development (Table II), it was selected for studying

TABLE III

Succinic Dehydrogenase and Cytochrome Oxidase Activities in Chick Heart Cells Brought into Suspension by Trypsin-Hyaluronidase Treatment and Grown in Cultures for Short Periods

Chick embryo heart	Average incubation age	Succinic dehydrogenase* *T* units‡/μg. DNAP	Cytochrome oxidase* without digitonin	Cytochrome oxidase* with digitonin
			μl. O_2/μg. DNAP/hr.	
	days			
Fresh tissue	15	29.81 ± 2.75	63.83 ± 4.02	354.80
Trypsinized		22.11 ± 0.58	68.57	—
Passage I, 7 days		21.52 ± 0.57	8.31	102.60
Passage III, 10 days		30.61 ± 0.57	21.92	117.00
Fresh tissue	19	47.79 ± 1.24	103.12 ± 8.21	424.80
Trypsinized		26.58 ± 1.21	34.92	251.60
Passage I, 7 days		Very small	27.31 ± 0.75	217.40
Passage II, 12 days		—	65.13	234.40

* and ‡ as in Table I.

TABLE IV

The Digitonin Factors and the Ratios of Maximal Cytochrome Oxidase (i.e. Obtained by Digitonin Treatment) to Succinic Dehydrogenase Activity for Permanent Cell Lines and for Chick Embryo Cells

Origin	Cell type or incubation age	Digitonin factor*	Ratio $\frac{\text{Cytochrome oxidase‡}}{\text{Succinic dehydrogenase§}}$
	days		
Permanent human cell lines	HEP 1/56	7.8	5.36
	HEP 2/56	4.8	3.51
	HLM	2.8	1.36
Rat liver:—foetal adult	—	4.8	8.40
	—	7.2	6.55
Cultures of 15-day embryo chick heart	Passage I, 7 days	12.4	4.75
Cultures of 15-day embryo chick heart	Passage II, 10 days	5.3	3.87
Cultures of 19-day embryo chick heart	Passage I, 7 days	8.0	—
Chick embryo:—			
Heart	11	8.2	13.18
	15	5.6	11.91
	19	4.1	8.89
Liver	11	11.5	11.65
	15	10.7	12.05
	19	6.8	10.17
Brain	11	34.7	7.17
	15	42.7	13.54
	19	22.6	11.65

* The "digitonin factor" is defined as "the ratio of cytochrome oxidase activity of 0.5 ml. homogenate, obtained after treatment with digitonin to that obtained before digitonin treatment" (see Tables I, II, and III).

‡ Activity expressed as μl. O_2/μg. DNAP/hour.

§ Activity expressed as *T* units/μg. DNAP (see footnote, Table I).

changes in enzyme activities during the cultivation of cells for periods up to 3 weeks. Tissue from 15- and 19-day embryos was brought to a cell suspension by trypsin-hyaluronidase treatment and maintained in a state of rapid proliferation in groups of roller tubes by subculturing at 5 to 7 day intervals.

The results of two experiments are given in Table III. With the 15-day heart tissue, trypsinisation had little effect on the activities of the two enzymes, but both were substantially reduced at this stage with the 19-day tissue. Carbon dioxide formation is reduced and fermentation increased in growing cultures of mesenchyme cells by prolonging the exposure to trypsin when setting up the cultures (10). Whether the reduction in oxidative activity is solely the result of toxic effects or whether it is also an inevitable outcome of rapid uncontrolled growth *in vitro* has still to be decided.

It was difficult to obtain sufficient mitochondria from the growing cultures for succinic dehydrogenase assay, and no consistent trend is apparent in the cultures from 15-day heart. The levels of cytochrome oxidase were always lower in the cultured cells, the values being about 30 per cent of the original in the 15-day explants, and about 50 per cent in those from 19-day heart. These losses all occurred within 3 weeks from the time of preparation of the initial cultures.

Table IV summarises two aspects of the preceding results. The "digitonin factor" (*i.e.* the ratio of cytochrome oxidase activity after digitonin treatment to oxidase activity without digitonin treatment) might indicate the extent of internal mitochondrial structure, provided that homogenisation completely disrupted the cells without damaging the mitochondria. Significant differences for this factor between cultured cells and "*in vivo*" tissues were not found. The only consistent trend was the decreasing values for the factors during the 11 to 19 days of development of all three chick tissues. This could be a reflection either of increasing numbers of mitochondria of smaller average size, or of increasing fragility of the mitochondria to homogenisation.

Differences between mitochondria in the various cell types might also be revealed by the ratio of cytochrome oxidase activity to succinic dehydrogenase activity. When these are calculated, using the maximal oxidase values, the lower ratios for cultured cells, as compared with embryonic, become apparent. (Table IV). These results suggest that the adaptation of cells to tissue culture conditions produces a greater depression of cytochrome oxidase activity than of succinic dehydrogenase activity.

DISCUSSION

This investigation shows that the oxidative enzymes, succinic dehydrogenase, and cytochrome oxidase have unusually low activities in the permanent cell lines HEP 1, HEP 2, and HLM. With embryonic heart cells, the steady increase in activity of these enzymes found during development *in vivo* is abruptly curtailed or reversed when the cells are made to proliferate rapidly in cell cultures.

In studies on monolayer cultures of monkey kidney cells, it was found that succinic dehydrogenase and the cytochrome system were less active in cells cultured for 7 to 12 days than in the original tissue (6). Using similar culture material, Warburg, Gawehn, and Geissler (29) reported increasing rates of aerobic and anaerobic fermentation after cultivation for 5 to 6 days after explantation. Other authors have drawn attention to the high glycolytic activity in cultures of proliferating embryonic cells and to the fact that these values could exceed the rates in cell cultures of malignant origin (8, 12, 13).

Glycolytic rates in cell cultures thus reach or exceed the levels which Warburg (27) has for many years considered to be inseparable from the damaged respiration of malignant cells. Warburg (29) has modified his views in some respects, because he found that intense glycolysis could be associated with undiminished respiration in cultured cells which were not malignant (and would not necessarily transform to malignant cells). Instead of carcinogenesis *in vitro* beginning with respiratory damage and being completed when fermentation is fully established (27), he now considers that it commences in cell cultures with intensive glycolysis, that respiration is reduced at a later stage, and that malignancy occurs when (and if) the cells become resistant to the "cytolysin" of the host (28).

While the initial trypsin treatment of tissues can reduce the oxidative capacity of cells, the extent to which the respiratory enzymes may be reduced by continued growth *in vitro*, can be judged from their activities in the HEP 1, HEP 2, and HLM cells. In the absence of measurements made on comparable normal tissues, the nearest comparison which can be made is between the transformed cells from human foetal liver (HLM) and the livers of

foetal and adult rats. The use of DNA as a standard of reference narrows the species gap, since the average cell mass and certain enzymic activities are directly proportional to the DNA per cell in homologous organs (16) and in related cells (11).

On this basis, the HLM cells have succinic dehydrogenase activities which are about 28 and 4 per cent of those in foetal and adult rat liver respectively, while their cytochrome oxidase levels are about 8 and 2 per cent respectively. The activities of the carcinoma lines (HEP 1 and HEP 2) are similar to those of HLM cells. The metabolism of these cell lines has been found previously to have certain features in common, which distinguish them from short-term cultures of human foetal cells (12).

In these cell lines, it is possible that there are relatively few mitochondria per cell and that these mitochondria are essentially different from those in cells growing *in vivo*. As evidence of this, lower ratios for cytochrome oxidase to succinic dehydrogenase activities were found in chick heart cells in culture than in embryonic heart, liver, and brain; similarly, the ratios for HLM cells (and for HEP 1 and HEP 2) were very low by comparison with the ratios for foetal and adult rat liver (Table IV). Other indications that mitochondria become altered in cells growing *in vitro* are the reversal of the Pasteur effect in monolayer kidney cell cultures (6), the qualitatively different response of chick fibroblasts and HeLa cells to changing glucose concentrations (24), and the failure of thyroid hormones to inhibit the growth of HEP 1, HEP 2, and HLM cells at concentrations which inhibited that of short-term cultures of human lung fibroblasts (14).

The important finding that early mitosis of the micronuclei of *Stentor coeruleus* may be induced by removal of the ciliated half of the organism (7) may have some bearing on the metabolic nature of cells in culture. The authors suggest that, in the absence of ciliary activity, an increased ratio of ATP to ADP produces a shift towards a more reduced state of the cytochrome system, and towards higher concentrations of reduced pyridine nucleotides. These conditions would favour the production of other triphosphates, of deoxyribose, and of sulphydryl groups—all of which are essential agents in DNA synthesis and mitotic activity.

In the cultured cells, it is seen that cytochrome oxidase activity is reduced to a greater extent than that of succinic dehydrogenase—a change which should produce relatively high ratios of the reduced to oxidised forms of pyridine nucleotides. At the same time, the intensive aerobic glycolysis of these cells should ensure a constant regeneration of ATP. Together, these conditions produce the metabolic pattern required for rapid mitotic activity. The fact that many normal cells, with this type of metabolism cease to proliferate after some weeks or months *in vitro*, and that not all "transform" nor become malignant (18), indicates that essential differences underlie the common features of metabolism of cultured cells.

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Explanation of Plate 135

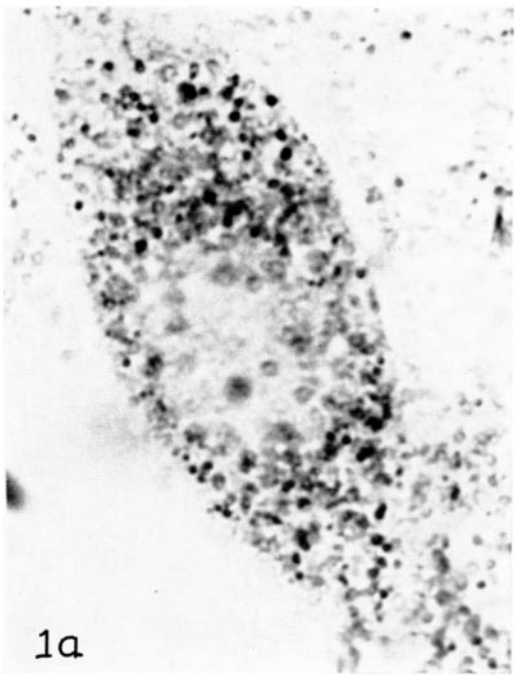
Fig. 1. HLM and HEP 1 cells cultivated on coverslips for 24 hours.

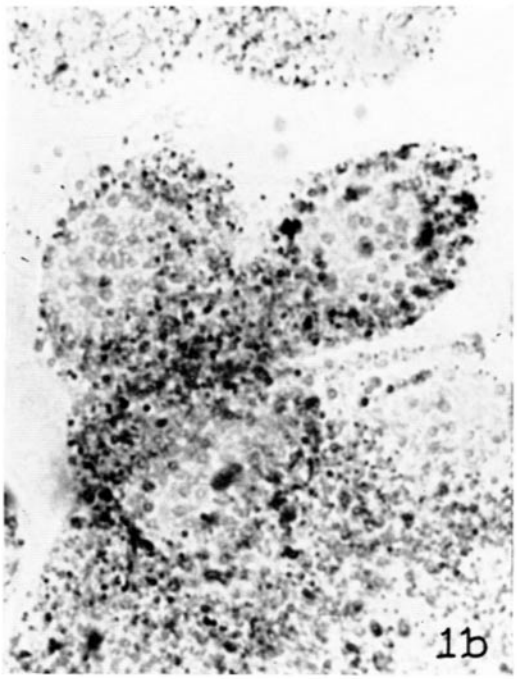
1 *a*. HLM cells incubated in sodium succinate buffer containing 0.1 per cent iodonitro-tetrazolium chloride, unfixed. × 1750.

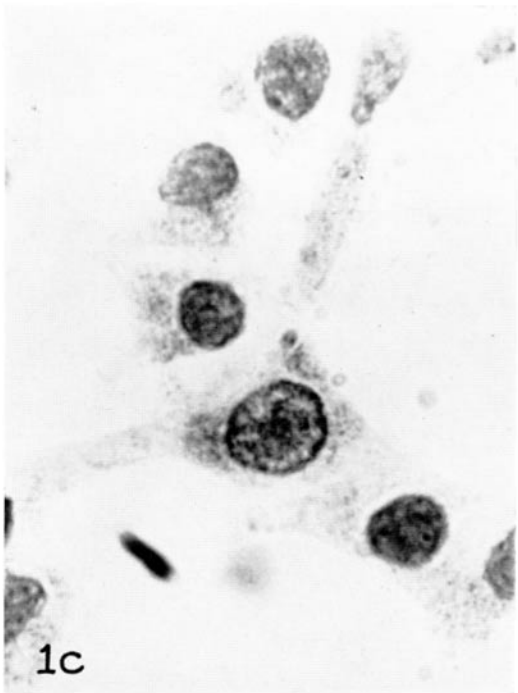
1 *b*. HEP 1 cells incubated in sodium succinate buffer containing 0.1 per cent iodonitro-tetrazolium chloride, unfixed. × 1750.

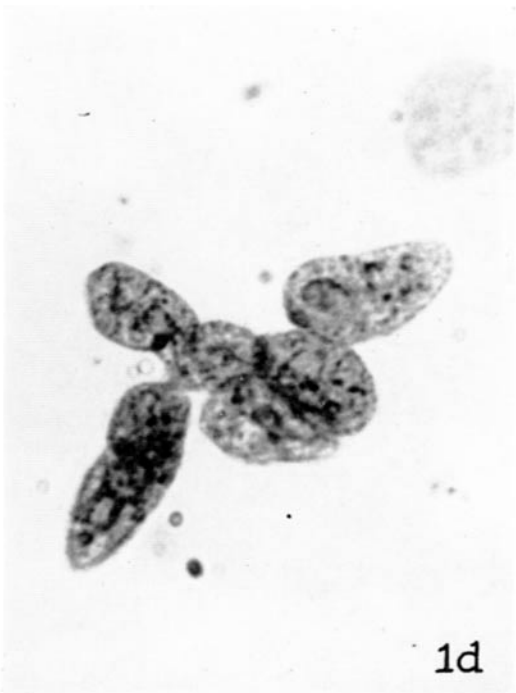
1 *c*. HEP 1 cells incubated in succinate buffer containing 0.05 per cent nitro-blue tetrazolium chloride, fixed in formol saline with the nuclei Feulgen-stained. × 1300.

1 *d*. As in 1 *c*, but incubated in succinate-free buffer, nuclei Feulgen-stained. × 1750.









(Leslie and Yarnell: Oxidase activities in cell cultures)