Cytochemical Localization of Certain Oxidative Enzymes[1]

JAMES L. CONKLIN, MAYNARD M. DEWEY AND RAYMOND H. KAHN
Department of Anatomy, The University of Michigan Medical School, Ann Arbor, Michigan

Employing the technique of cell fractionation, Schneider and Hogeboom ('56) described the localization of certain oxidative enzymes. They reported that cytochrome oxidase and succinic dehydrogenase were found only in the mitochondrial fraction while isocitric dehydrogenase was present in the supernatant and DPN and TPN cytochrome c reductases were observed to be in both fractions. More recently Thorne ('60) has reported a mitochondrial and a supernatant malic dehydrogenase.

In contrast to these reports on the localization of certain oxidative enzymes is the intracellular distribution as demonstrated by histochemical procedures. Scarpelli, Hess and Pearse ('58), Hess, Scarpelli and Pearse ('58), and Pearse ('60) reported that all pyridine nucleotide-linked dehydrogenases and DPN and TPN diaphorases were confined to the mitochondria. Nachlas, Walker and Seligman ('58) described the intramitochondrial localization of DPN diaphorase and extramitochondrial localization of TPN diaphorase. Becker ('61) observed the mitochondrial localization of TPN diaphorase and intra- and extramitochondrial distribution of DPN diaphorase. The obvious discrepancy between the evidence gained by the biochemical and histochemical methods might appear unresolvable except that the reliability of certain of the techniques employed in the previous histochemical studies are subject to question.

The current methods for the histochemical demonstration of oxidative enzyme activity depend upon the incubation of cells or tissues in a buffered medium containing the substrate, required cofactors, and a tetrazolium salt. In the presence of the enzyme the substrate is oxidized and the electrons made available by the oxidation are transferred (see below) to the tetrazolium salt reducing it to an insoluble formazan and thus indicating the site of enzyme activity. Of prime importance in these methods are the cofactors, the electron transferring agents and the tetrazolium salt.

Several tetrazolium compounds have been utilized for the demonstration of oxidative enzymes. Farber, Sternberg and Dunlap ('56) tested the available tetrazolium salts and reported that the best histological definition was obtained with neotetrazolium and blue tetrazolium. These compounds have subsequently been replaced by more sensitive salts. Nachlas et al. ('57) introduced the dye, Nitro-BT, (2,2′-di-p-nitrophenyl-5,5′-diphenyl-3,3′-(3, 3′-dimethoxy-4,4′-biphenylene) ditetrazolium chloride) while Pearse ('57) advocated the use of MTT (3,5-diphenyl-2-(4, 5′-dimethylthiazol-2-yl) tetrazolium bromide) chelated with cobaltous ions. Novikoff, Shin and Drucker ('61) have reported that, of the two compounds, Nitro-BT is superior for critical studies of mitochondrial morphology and oxidative enzyme activity since the deposition of MTT-Co++ is determined by physicochemical factors other than enzyme localization.

As demonstrated by Brodie and Gots ('51) the enzymatic reduction of the tetrazolium salt is dependent upon the presence of a flavoprotein, a diaphorase. In actuality with the methods employed, it is the diaphorase which is being localized and not the dehydrogenase (Farber, Sternberg and Dunlap, '56; Nachlas, Walker and Seligman, '58). Complications do not arise as long as the dehydrogenase and

[1] Research supported by USPHS Grants A-3449 and RG-6976.

diaphorase have the same distribution. However, if the dehydrogenase is located at a site which differs from the diaphorase distribution then theoretically it is impossible to demonstrate the enzyme at this site. Farber and Bueding ('56) suggested that the requirement for endogenous diaphorase could be overcome by employing another intermediate electron acceptor and utilized phenazine methosulfate for this purpose. It has been subsequently confirmed by Dewey and Conklin ('60) and Nachlas, Margulies, Goldberg and Seligman ('60) that phenazine methosulfate can be employed as a substitute for the diaphorase in histochemical and biochemical procedures.

Another difficulty which has accompanied previous histochemical studies has been that the tissues were subjected to preparative procedures such as freezing and cryostat sectioning. The mitochondrial damage which occurs as the result of freezing could lead to leaching of the enzymes and subsequent diffusion artefact. While Pearse ('60) routinely employed incubating media made hypertonic by the addition of polyvinylpyrrolidone or sucrose, the necessity of using hypertonic media has been questioned by Walker and Seligman ('61) who reported that such conditions did not improve enzyme localization. These investigators and Novikoff, Shin and Drucker ('61) have advocated the use of short fixation for accurate localization of oxidative enzymes.

A final consideration in the accurate localization of enzyme activity is the interpretation of the size and distribution of the formazan deposits. It is extremely difficult to correlate formazan particles with cellular organelles unless the distribution of these organelles is confirmed by classical histological methods (Novikoff, Shin and Drucker, '61).

The L strain fibroblast has been demonstrated to possess certain morphological characteristics which make it suitable material for the study of the distribution of oxidative enzymes. Under the conditions employed, the mitochondria are localized in a particular area of the cell and are clearly distinguishable from other components of the cytoplasm. (Kahn, Conklin and Dewey, '62; Dewey, Kahn and Conklin, '62.) In addition the cells may be grown as monolayers thus providing material which may be studied by phase optics and supravital staining. Furthermore, oxidative enzymes may be readily demonstrated in cells which are untreated and thus adverse preparative procedures are avoided.

In the present study an attempt has been made to accurately localize several of the oxidative enzymes by employing Nitro-BT as the terminal electron acceptor and utilizing phenazine methosulfate as an intermediate electron transferring agent. The distribution of the oxidative enzymes was correlated with the distribution of cytoplasmic organelles as demonstrated by phase optics and supravital staining. In addition, the effect of hypertonic media on enzyme activity and localization was evaluated.

MATERIALS AND METHODS

Stock NCTC 929 (L strain) fibroblasts[2] maintained in stationary bottle cultures in medium 199 (Morgan, Morton and Parker, '50) supplemented with 0.5% Bactopeptone were subcultured on coverslips and grown for 48 hours as a monolayer. For the demonstration of enzyme activity, coverslips were removed from the culture medium, rinsed in balanced salt solution, and either placed directly into the substrate or frozen at −70°C prior to incubation. Oxidative enzyme activity was demonstrated by incubation in the following substrates:

Glucose-6-phosphate Dehydrogenase (G6DH).-dipotassium glucose-6-phosphate (0.2 M), triphosphopyridine nucleotide (TPN) (0.3 mg/ml), cyanide (0.1 M), sodium fluoride (0.1 M), tris hydroxyaminomethylmethane (Tris) buffer (0.25 M, pH 7.4), phenazine methosulfate (PMS) (1.0 μg/ml), Nitro-BT (0.5 mg/ml).

Glycerophosphate Dehydrogenase (GDH).-sodium (DL) a-glycerophosphate (0.25 M), diphosphopyridine nucleotide (DPN) (0.3 mg/ml), cyanide, PMS, Nitro-BT, Tris buffer.

Hydroxybutyric Dehydrogenase (HDH).-B-hydroxybutyrate (0.33 M), DPN, cysteine (0.01 M), nicotinamide (0.05 M), PMS, Nitro-BT, cyanide, Sorenson's phosphate buffer (0.25 M, pH 7.4).

[2] Generously supplied by Dr. Donald L. Merchant.

Lactic Dehydrogenase (LDH), *Isocitric Dehydrogenase* (IDH), *Malic Dehydrogenase* (MDH).-sodium (DL) lactate (0.1 M), disodium (DL) isocitrate (0.25 M), and sodium (L) malate (0.25 M) respectively, DPN, cyanide, PMS, Nitro-BT, phosphate buffer.

Succinic Dehydrogenase (SDH).-sodium succinate (0.25 M), cyanide, PMS, Nitro-BT, phosphate buffer.

Diphosphopyridine Nucleotide Diaphorase (DPND) and *Triphosphopyridine Nucleotide Diaphorase* (TPND).-reduced DPN (DPNH) and reduced TPN (TPNH) (0.5 mg/ml) respectively, Nitro-BT, phosphate buffer.

Cytochrome oxidase was localized by the method of Burstone ('60) employing N-phenyl-p-phenylenediamine monohydrochloride, 1-hydroxy-2-naphthoic acid and cytochrome C. Cells were also incubated in substrates containing either 7.5% polyvinylpyrrolidone K-30 (PVP), 0.44 M or 0.88 M sucrose in order to determine the effect of osmolar protection on enzyme activity and localization.

Since the accurate localization of the various enzymes was the primary purpose of the study, the distribution of cellular organelles was confirmed by examining viable cells with phase optics. In addition, mitochondrial morphology and distribution were revealed by supravital staining with Janus green B (0.01% in isotonic sodium chloride).

RESULTS

The intracellular localization of oxidative enzymes in the L strain fibroblast is facilitated by the morphological characteristics of this cell. When viewed with phase optics (fig. 1) the cytoplasm of the L cell can be divided into three distinct areas: a juxtanuclear zone, a granular zone, and an outer nongranular zone. The juxtanuclear zone consists of vacuoles embedded in a homogenous cytoplasm. The granular zone surrounds both the nucleus and the juxtanuclear zone and contains numerous, moderately large (ca. 2 μ), dense granules. Extending from the granular zone to the outer limits of the cells is a thin layer of nongranular cytoplasm, i.e. the nongranular zone. When the cells are supravitally stained with Janus green B, the dye is localized only in the large dense granules of the granular zone (fig. 2) indicating that mitochondria are confined exclusively to this area. The consistent size of the mitochondria and their localization in the granular zone of the cytoplasm facilitated the interpretation of the formazan deposits produced by the various enzyme procedures. The deposits varied with the procedure employed and could be arbitrarily categorized (table 1) as to size and site of deposition and correlated with cytoplasmic structures demonstrated by other procedures. Large formazan granules corresponded in size, number, and distribution to the mitochondria (figs. 4–10 and fig. 12) and were confined to the granular zone. Finer formazan granules were also observed and were present in the juxtanuclear, granular and nongranular zones (figs. 5–12). These formazan granules were considerably smaller and more numerous than the mitochondria. After certain procedures the smaller formazan granules appeared to be associated with a fine reticulum which was present throughout the cytoplasm (figs. 6–12) and which was clearly separable from the larger, gran-

TABLE 1

Distribution of oxidative enzymes

Enzyme	Juxtanuclear zone	Granular zone
Cytochrome oxidase	Absent	Large granules
SDH	Absent	Large granules
IDH	Small granules, reticular	Large granules, reticular
MDH	Small granules, reticular	Large granules, reticular
HDH	Reticular	Reticular, few large granules
DPND	Small granules, reticular	Large granules, reticular
LDH	Small granules, reticular	Large granules, reticular
G6DH	Small granules, reticular	Reticular, few large granules
TPND	Reticular	Reticular
GPDH	Reticular	Reticular, few large granules

ular mitochondria. Cytochrome oxidase activity was present only in the large granules of the granular zone (fig. 3).

The exclusive deposition of formazan within the mitochondria after certain procedures, and the presence of formazan both intra- and extra-mitochondrially after others, would indicate a dual localization of certain oxidative enzymes. IDH, MDH, LDH, HDH, G6DH, GDH and DPND (figs. 5–10 and fig. 12) are present within the mitochondria and also associated with extramitochondrial structures. SDH and cytochrome oxidase (figs. 3 and 4) are located only within the mitochondria while TPND (fig. 11) was observed to be exclusively extramitochondrial in localization.

The localization of the various oxidative enzymes was reproducible under all conditions of incubation. While the addition of PVP and sucrose appeared to increase the granular nature of the formazan, the distribution was identical to that obtained without the utilization of these additives. The only apparent effect of the hypertonic media was to prolong the incubation time. Likewise freezing the cells prior to incubation in the substrate did not alter the distribution of formazan. Freezing did, however, markedly increase the activity of all enzymes except cytochrome oxidase which was inhibited. (Activity was interpreted visually from the amount of formazan deposited in a 15 minute period and is a function of both enzyme concentration and enzyme turnover rate.) Freezing was least effective in the activation of DPND, MDH, LDH, G6DH and TPND and facilitated the demonstration of SDH, GPDH, HDH and IDH. However, the latter enzymes could be demonstrated without prior freezing if the incubation period was prolonged.

When enzyme activity was demonstrated under comparable conditions a difference in the level of activity of the various enzymes was apparent. In decreasing order of relative activity the enzymes could be grouped as follows: DPND≅LDH>MDH> IDH≅G6DH≅TPND≅HDH>GPDH≅SDH. The activity of cytochrome oxidase could not be directly correlated with dehydrogenase and diaphorase activity since a different cytochemical procedure was employed.

In considering the relative activities of the various enzymes, it was of interest that variation in enzyme activity paralleled variation in density of cell population. When grown as monolayers, under the present conditions, the L strain fibroblasts occur as colonies of closely grouped cells with single cells scattered between the colonies. The activity of DPND, LDH and MDH was greater in colonies of cells than in single isolated cells. This variation in activity was less evident with respect to the other enzymes studied.

DISCUSSION

In the present study an attempt has been made to accurately localize certain of the oxidative enzymes in the L strain fibroblast. Localization was greatly facilitated by several factors: 1. The use of viable cells which permitted study by phase optics and supravital staining; 2. The characteristic distribution of organelles in the L cell; 3. The omission of preparative procedures thus minimizing the production of artefact; 4. The use of phenazine methosulfate as an intermediate electron acceptor for the demonstration of dehydogenase activity; and 5. The employement of Nitro-BT as the terminal electron acceptor.

The selection of the L strain fibroblast for the study of enzyme localization was most fortuitious because of the characteristic distribution of mitochondria in the granular zone of the cell (figs. 1 and 2). When oxidative enzyme activity was demonstrated in these cells it was readily apparent that some enzymes were confined to the mitochondria while others were also present extramitochondrially. An analysis of table 1 and figures 3 and 4 illustrates that only cytochrome oxidase and SDH were restricted to the mitochondria. Figures 5 through 10 and figure 12 demonstrate the dual localization of IDH, MDH, HDH, DPND, LDH, GDH, and G6DH within the mitochondria as well as elsewhere in the cytoplasm. TPND activity was present only extramitochondrially (fig. 11). The inability to demonstrate the multiple localization of certain of the oxidative enzymes in previous histochemical studies may have occurred, in part, because of the failure to compare the distribution of formazan particles with the distribution of cellular

organelles. In the present study the distribution of organelles was confirmed by phase optics and supravital staining although classical techniques could have been employed. Cytoplasmic morphology should be confirmed in all histochemical studies of oxidative enzymes since the nature of the formazan deposits makes the accurate identification of organelles practically impossible. The suggestion that the DPND technique is the best method for the demonstration of mitochondria (Scarpelli, Hess and Pearse, '58) may be seriously questioned in view of the distribution of this enzyme in the L cell (figs. 1, 2 and 8). If a histochemical procedure is to be employed for the demonstration of mitochondria, the cytochrome oxidase method of Burstone ('60) seems to be best suited (fig. 3).

Another complication introduced into previous histochemical studies of oxidative enzymes has been the failure to employ an intermediate electron acceptor. Since it is well established that electrons are transferred to tetrazolium salts at points in the electron transport chain beyond the level of the dehydrogenase (Nachlas, Margulies and Seligman, '60) it is obvious that dehydrogenase activity can be demonstrated only in those sites where appropriate endogenous transferring agents are present. This dependency on endogenous agents can be overcome quite easily by employing various exogenous compounds in the substrate medium. While exogenous diaphorase may be employed, commercially available preparations are not sufficiently purified and consequently other intermediate agents are recommended. Although a variety of redox dyes have been tested histochemically (Farber and Louviere, '56) the compound of choice is phenazine methosulfate which is readily available, inexpensive and easily employed. In the present study PMS was not essential for the demonstration of the DPN-linked dehyrogenases because of the wide-spread distribution of DPN diaphorase. Phenazine methosulfate was essential for the demonstration of the TPN-linked mitochondrial G6DH since TPN diaphorase was not present within the mitochondria and therefore not available to mediate the transfer of electrons to the tetrazolium salt. Since PMS increased the activity of the DPN-linked enzymes it was routinely employed for their demonstration also. This activation effect of PMS also was noted by Walker and Seligman ('61) who reported a 5-fold increase in SDH activity in fresh-frozen tissues. In the present study PMS was found to activate cells which were either fresh or fresh-frozen. Thus in addition to improving localization PMS also increases the reaction rate and markedly facilitates the specific demonstration of dehydrogenase activity.

Although it has been claimed that osmolar additives such as PVP or sucrose are essential for the accurate localization of oxidative enzyme activity (Scarpelli and Pearse, '58; Hess, Scarpelli and Pearse, '58), such a requirement was not evident in the present study. The addition of these substances to the incubating medium had no effect on the localization of the various enzymes and seemed to be merely an unnecessary complication of the procedure. It also had the disadvantage of decreasing the reaction rate and prolonging the incubation time. The concept that mitochondrial integrity is maintained by employing additives is probably not justifiable, especially in the case of tissues which have been frozen and subjected to cryostat sectioning. In view of the report of Ziegler and Linnane ('58) that certain substrates are not oxidized by intact mitochondria and that oxidative capacity is greatly augmented after freezing, it is probable that enzyme activity cannot be demonstrated by the usual histochemical procedure unless mitochondrial damage has occurred. The activation of certain of the enzymes after freezing, observed in the present study, is assumed to be due to increased accessibility of substrate to enzyme as the result of damage to the mitochondria.

It has been suggested that mitochondrial damage can lead to the production of diffusion artefact. Presumably this artefact occurs as the result of diffusion of enzyme, reduced coenzyme, or formazan. It is unlikely that any of these factors are a problem when PMS and Nitro-BT are employed. The varied and rather precise localization of the oxidative enzymes demonstrated in the present study would indicate that enzyme diffusion did not occur. Furthermore,

the addition of protective compounds to the media, which should minimize such diffusion, had no effect on enzyme distribution. Secondly it is also unlikely that diffusion of DPNH occurred under the conditions employed since differences in distribution were observed for the various DPN-linked dehydrogenases. In addition, in a starch gel electrophoretic study of LDH and DPND (Dewey and Conklin, '60) the same substrates were employed to localize these enzymes in the starch gel and therefore reduced DPN was produced as LDH was demonstrated. If the DPNH was able to diffuse from the regions of the LDH fractions to the areas where DPND was located it could serve as substrate for the latter. Since this never occurred it is probable that the DPNH is rapidly reoxidized in the presence of PMS and Nitro-BT. Although the starch matrix is not analogous to cytoplasm, it seems equally probable that in view of the redox potential and site of action of Nitro-BT (Nachlas, Margulies and Seligman, '60) and the activity of PMS that diffusion of DPNH does not occur. Finally, although some diffusion of the formazan can occur, it does not happen readily with Nitro-BT and is not a problem if the tissues are examined soon after completion of the procedure. In any event, such artefact is easily recognizable since the formazan tends to aggregate in large masses. Diffusion artefact never produces a reticular-like arrangement of formazan as observed after certain procedures (figs. 4–12). While the distribution of certain of the enzymes differs from that reported in previous histochemical investigations it is felt that, barring actual differences in the tissues investigated, the substrates utilized in this study are better suited for the precise localization of the various enzymes. It seems very likely that the active components of the substrate solutions are of more significance to studies of localization than chemical and physical protective agents.

Although the enzymes studied were classifiable in order of relative activity, very little can be deduced as to the actual metabolic state of the cells since the activity of the same enzymes in uncultured fibroblasts has not been reported. The relative activities are in general agreement with reports of enzyme activity in various types of malignant cells (Monis, Nachlas and Seligman, '59; Stolk, '60; Rees and Huggins, '60). It is possible that the levels of oxidative enzyme activity of these fibroblasts will more closely resemble that of malignant cells rather than normal fibroblasts since, like other cultured cells, the L strain fibroblast has undergone changes as the result of continuous culture (Earle, Shelton and Schilling, '49).

The demonstration of differences in enzyme activity between the cells of the population suggests that biochemical analyses of total cell populations would be more meaningful if the metabolic state of individual cells was determined concomitantly by histochemical methods (Kahn, Conklin and Dewey, '62).

SUMMARY

The activity of certain Kreb cycle, glycolytic and pentose shunt enzymes was demonstrated histochemically in the L strain (929) fibroblast. The intracellular distribution of these enzymes was correlated with the cytoplasmic organelles demonstrable with phase optics and supravital staining. By this method of study it was possible to demonstrate that some of these enzymes were located exclusively in the mitochondria while others were located extramitochondrially as well.

The authors are deeply indebted to Mrs. Julia Ternak and Miss Denise Strelbitzky for their technical assistance.

LITERATURE CITED

Becker, N. H. 1961 The cytochemistry of anoxic and anoxic-ischemic encephalopathy in rats. II. Alterations in neuronal mitochondria identified by diphosphopyridine and triphosphopyridine nucleotide diaphorases. Am. J. Path., *38:* 587–597.

Brodie, A. F., and J. S. Gots 1951 Effects of an isolated dehydrogenase enzyme and flavoprotein on the reduction of triphenlytetrazolium chloride. Science, *114:* 40–41.

Burstone, M. S. 1960 Histochemical demonstration of cytochrome oxidase with new amine reagents. J. Histochem., *8:* 63–70.

Dewey, M. M., and J. L. Conklin 1960 Starch gel electrophoresis of lactic dehydrogenase from rat kidney. Proc. Soc. Exp. Biol. Med., *105:* 492–494.

Dewey, M. M., R. H. Kahn and J. L. Conklin 1962 A cytochemical and cytological analysis of the L-strain (929) fibroblast. (In preparation.)

Earle, W. R., E. Shelton and E. L. Schilling 1949 Production of malignancy *in vitro*. XI. Further results from reinjection of *in vitro* cell strains into strain C_3H mice. J. Nat. Cancer Inst., *10:* 1105–1113.

Farber, E., W. H. Sternberg and C. E. Dunlap 1956 Histochemical localization of specific oxidative enzymes. I. Tetrazolium stains for diphosphopyridine nucleotide diaphorase and triphosphopyridine nucleotide diaphorase. J. Histochem., *4:* 254–265.

Farber, E., and C. D. Louviere 1956 Histochemical localization of specific oxidative enzymes. IV. Soluble oxidation-reduction dyes as aids in the histochemical localization of oxidative enzymes with tetrazolium salts. Ibid., *4:* 347–356.

Farber, E., and E. Bueding 1956 Histochemical localization of specific oxidative enzymes. V. The dissociation of succinic dehydrogenase from carriers by lipase and the specific histochemical localization of the dehydrogenase with phenazine methosulfate and tetrazolium salts. Ibid., *4:* 357–362.

Hess, R., D. G. Scarpelli and A. G. E. Pearse 1958 Cytochemical localization of pyridine nucleotide-linked dehydrogenases. Nature, *181:* 1531–1532.

Kahn, R. H., J. L. Conklin and M. M. Dewey 1962 Cytological and cytochemical characterization of cells grown *in vitro*. J. Nat. Cancer Inst., (in press).

Monis, B., M. M. Nachlas and A. M. Seligman 1959 Histochemical study of three dehydrogenase systems in human tumors. Cancer, *12:* 1238–1247.

Morgan, J. F., H. J. Morton and R. C. Parker 1950 Nutrition of animal cells in tissue culture. I. Initial studies on a synthetic medium. Proc. Soc. Exp. Biol. Med., *73:* 1–8.

Nachlas, M. M., K. C. Tsou, E. De Souza, C. S. Cheng and A. M. Seligman 1957 Cytochemical demonstration of succinic dehydrogenase by the use of a new p-nitrophenyl substituted ditetrazole. J. Histochem., *5:* 420–436.

Nachlas, M. M., D. G. Walker and A. M. Seligman 1958 A histochemical method for the demonstration of diphosphopyridine nucleotide diaphorase. J. Biophys. Biochem. Cytol., *4:* 29–38.

——— 1958 The histochemical localization of triphosphopyridine nucleotide diaphorase. Ibid., *4:* 467–474.

Nachlas, M. M., S. I. Margulies and A. M. Seligman 1960 Sites of electron transfer to tetrazolium salts in the succinoxidase system. J. Biol. Chem., *235:* 2739–2743.

Nachlas, M. M., S. I. Margulies, J. D. Goldberg and A. M. Seligman 1960 The determination of lactic dehydrogenase with a tetrazolium salt. Anal. Biochem., *1:* 317–326.

Novikoff, A. B., W. Y. Shin and J. Drucker 1961 Mitochondrial localization of oxidative enenzymes: Staining results with two tetrazolium salts. J. Biophys. Biochem. Cytol., *9:* 47–61.

Pearse, A. G. E. 1957 Intracellular localization of dehydrogenase systems using monotetrazolium salts and metal chelation of their formazans. J. Histochem., *5:* 515–527.

——— 1960 Histochemistry; Theoretical and Applied. Little, Brown and Co., Boston.

Rees, E. D., and C. Huggins 1960 Steroid influences on respiration, glycolysis, and levels of pyridine nucleotide-linked dehydrogenases of experimental mammary cancers. Cancer Res., *20:* 963–971.

Scarpelli, D. G., and A. G. E. Pearse 1958 Physical and chemical protection of cell consituents and the precise localization of enzymes. J. Histochem., *6:* 369–376.

Scarpelli, D. G., R. Hess and A. G. E. Pearse 1958 The cytochemical localization of oxidative enzymes. I. Diphosphopyridine nucleotide diaphorase and triphosphopyridine nucleotide diaphorase. J. Biophys. Biochem. Cytol., *4:* 747–752.

Schneider, W. C., and G. H. Hogeboom 1956 Biochemistry of cellular particles. Ann. Rev. Biochem., 25: 201–224.

Stolk, A. 1960 Histochemical analysis of three dehydrogenase systems in the renal adenocarcinoma of *Aplocheilus lineatus*. Naturwissenschaften, *47:* 188.

Thorne, C. J. R. 1960 Characterization of two malic dehydrogenases from rat liver. Biochem. Biophys. Acta, *42:* 175–176.

Walker, D. G., and A. M. Seligman 1961 Formalin fixation in the cytochemical demonstration of succinic dehydrogenase of mitochondria. J. Biophys. Biochem. Cytol., *9:* 415–427.

Ziegler, D. M., and A. W. Linnane 1958 Studies on the electron transport system. XIII. Mitochondrial structure and dehydrogenase activity in isolated mitochondria. Biochem. Biophys. Acta, *30:* 53–63.

PLATE 1

EXPLANATION OF FIGURES

All figures are of the L strain fibroblast. × 900.

1 The L strain fibroblast as viewed with phase optics. Note the juxtanuclear zone (J), granular zone (G), and the peripheral nongranular zone (N).

2 Supravitally stained with Janus green B. The stain is localized in the granules (mitochondria) of the granular zone.

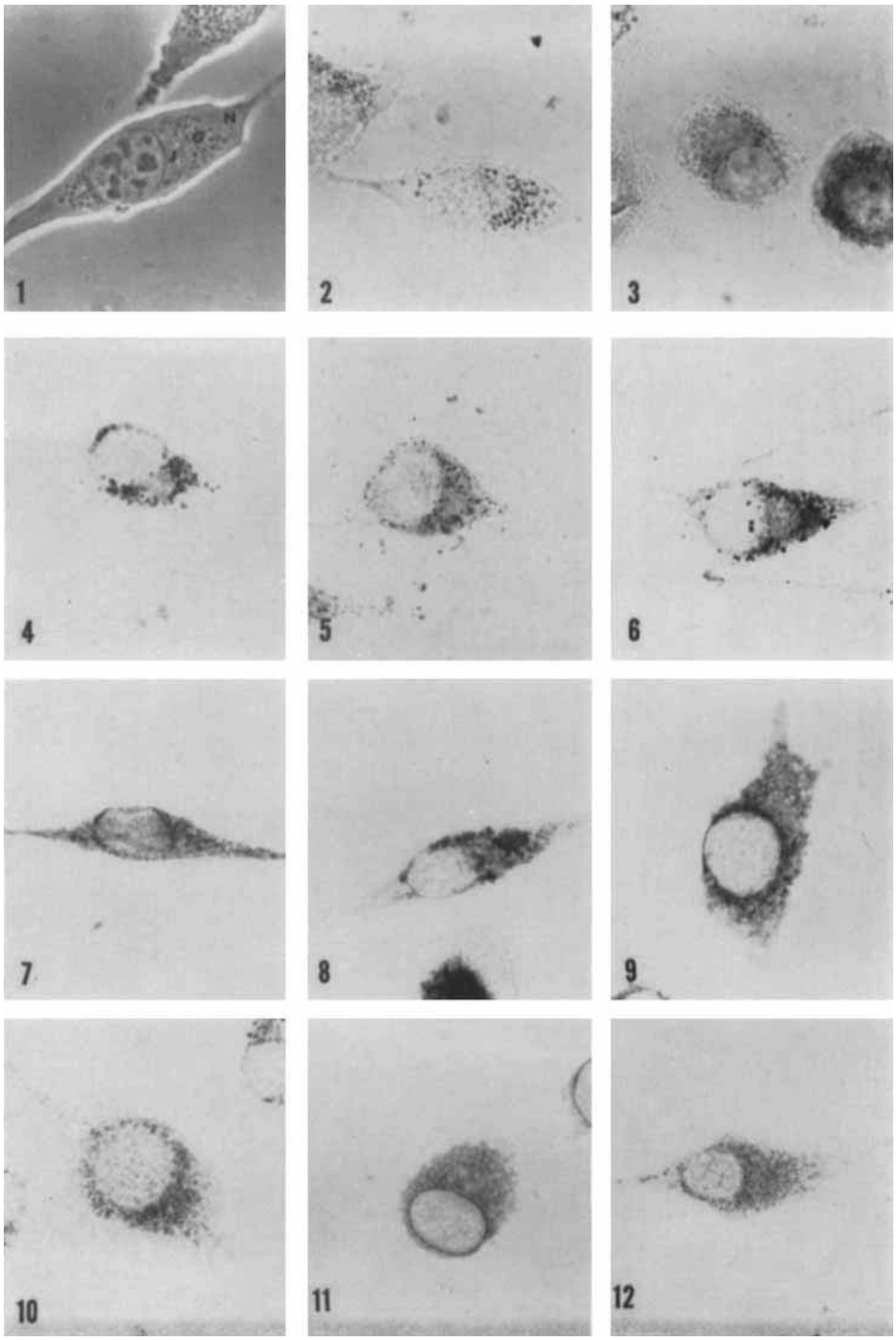
3–4 Cytochrome oxidase and succinic dehydrogenase activity, respectively, restricted to the mitochondria of the granular zone.

5–6 Isocitric and malic dehydrogenase activity, respectively. Note that while activity is greatest in the mitochondria of the granular zone, there is a reticular distribution of activity in the juxtanuclear and nongranular zones as well.

7–10 β-hydoxybutyric dehydrogenase, DPN diaphorase, lactic and α-glycerophosphate dehydrogenase activity, respectively. Note the presence of both reticular and granular formazan deposits throughout the cytoplasm.

11 TPN diaphorase activity. Observe that while activity is present in all three zones of the cytoplasm, it is nongranular in nature. The vacuoles present in the region of the granular zone indicates the absence of mitochondrial activity.

12 Glucose-6-phosphate dehydrogenase activity. This enzyme also has a dual localization and is present throughout the cytoplasm.


1
2
3
4
5
6
7
8
9
10
11
12