PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 145, 157–160 (1974)

Acid Phosphatase Isoenzymes in Human Skin Fibroblasts (37768)

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Acid phosphatase, the first lysosomal enzyme described (1), has become a marker of lysosomal function in a variety of normal and pathologic states (2–5). Typically, 70–80% of acid phosphatase activity is confined to the lysosomes, with the remainder being distributed in other subcellular particles and in the supernatant (1, 6).

Acid phosphatase activity has been detected in multiple forms in a wide variety of tissues, including liver (7), kidney (8), leukocytes (9), red blood cells (10), and cultured human diploid cells (11). Its broad synthetic substrate specificity includes *p*-nitrophenylphosphate (11), phenolphthalein-di-phosphate (12), β-glycerophosphate (13), 4 -methyl-umbelliferyl-dihydrogen-phosphate (14), and α-naphthyl acid phosphate (15). The red cell enzyme is distinguished from the predominant acid phosphatase of other tissues by its lack of activity toward α-naphthyl acid phosphate (16, 17).

Despite the wealth of information characterizing the enzyme, its physiologic substrate and function remain unknown. Two pathologic states, however, are related to deficiencies of the enzyme; one involving lysosomal acid phosphatase deficiency (5), and the other characterized by total cellular acid phosphatase deficiency (18). In both instances, lipid has been found to accumulate in the liver and other tissues.

To gain a better understanding of this enzyme, studies have been conducted on cultivated diploid cells from fetal human lung (11). This communication extends these studies to cultivated human skin fibroblasts and describes the localization and characteristics of isoenzyme forms in various subcellular fractions.

Materials and Methods. Human skin biopsies were obtained from 20 control patients and cultivated in Eagles Minimal Essential Medium (GIBCO) supplemented with 15% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 mg/ml fungizone as previously described (19). All assays were performed on cells harvested at confluency between the tenth and sixteenth passages. When confluent, fibroblasts were harvested using 0.25% trypsin in Hanks' Balanced Salt Solution. The cells were washed three times in isotonic saline and then resuspended in distilled water, 0.25 *M* sucrose, or a 0.2% Triton X-100 solution in distilled water. Cells to be assayed for total cellular enzyme activity were disrupted by freeze-thawing five times in dry ice. Cells to be subjected to differential centrifugation were resuspended in 0.25 *M* sucrose and disrupted by nitrogen cavitation at a pressure of 600 psi for 15 min (20). The suspension was then centrifuged in a Spinco Model L ultracentrifuge according to the method of Cristofalo (11) for preparation of subcellular fractions. Subcellular fractions, including the nuclear pellet, the mitochondrial–lysosomal fraction, and the microsomal fraction were resuspended in distilled water or in distilled water containing 0.2% Triton X-100 and subjected to freeze-thawing five times.

Whole blood was collected in heparinized syringes and allowed to sediment at 37° for 1–2 hr. The leukocyte-rich plasma was then expressed, and the white blood cells were collected by centrifugation at 2000*g* for 10 min. The cells were washed three times in 0.25 isotonic saline. The pellet was resus-

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pended in 0.2% Triton X-100, and the cells were disrupted by freeze-thawing five times in dry ice. The red blood cells were washed three times in normal saline and then resuspended in distilled water to a final dilution of 1:20. To insure complete cell lysis, the suspension was freeze-thawed three times.

p-Nitrophenylphosphatase activity was determined by an adaptation of the method of Bessey *et al.* (21) in an incubation mixture containing 20 μl of enzyme preparation, 200 μl of 0.05 *M* *p*-nitrophenylphosphate in 0.1 *M* acetate buffer at pH 4.8, and 180 μl of distilled water. The mixture was incubated at 37° for 30 min and the reaction stopped by the addition of 3 ml of 0.25 *N* NaOH. Optical density at 410 μm was determined in a Coleman spectrophotometer.

β-Glycerophosphatase activity was determined (13) in an incubation mixture containing 100 μl of enzyme preparation and 900 μl of 0.02 *M* β-glycerophosphate in 0.05 *M* acetate buffer, pH 5.0. The mixture was incubated for 60 min at 37° and the reaction terminated by the addition of 1 ml of 10% trichloroacetic acid. After 10 min the mixture was centrifuged for 10 min at 3000 rpm. Inorganic phosphate was determined in 1 ml of the resultant supernatant solution by the addition of 0.2 ml of 1/25% ammonium molybdate in 2.5 *N* sulfuric acid and 0.1 ml of Fiske Subbarow reducing reagent (Sigma 661-8). Optical density at 660 μm was determined immediately in a Coleman spectrophotometer.

Red cell acid phosphatase activity was determined utilizing 4-methylumbelliferyl-dihydrogen-phosphate as substrate according to a method modified from Swallow *et al.* (22). The reaction was terminated by the addition of 3 ml of 0.2 *M* glycine buffer, pH 10.7. Fluorescence was then measured immediately using an Aminco-Bowman fluorescence spectrophotometer (excitation 360 μm, emission 450 μm).

Protein was determined by the method of Lowry *et al.* (23).

Polyacrylamide gel electrophoresis was performed according to the method of Li *et al.* (9). Fifty to 100 micrograms of protein was applied to each gel. Acid phosphatase activity was detected by the method of Barka using sodium α-naphthyl acid phosphate and Fast Garnet GBC (15). The gels were washed in 0.1 *M* acetate buffer, pH 5.0, after electrophoresis followed by staining for 60–180 min at room temperature. After staining the gels were washed three times in distilled water and then immersed in a 7% acetic acid solution.

Starch gel electrophoresis was carried out according to the method of Swallow *et al.* (23) on a 13% starch gel. Fifty microliters of sample (300–400 μg protein) was applied to each sample slot. Acid phosphatase isoenzymes were located after electrophoresis using 4 -methylumbelliferyl-dihydrogen-phosphate (British Drug House) as substrate according to Swallow *et al.* (22) and α-naphthyl acid phosphate (Sigma) as substrate according to Swallow and Harris (16).

Results. Acid phosphatase activity was detected in cultivated human fibroblasts using *p*-nitrophenylphosphate, β-glycerophosphate, and 4-methylumbelliferyl-dihydrogen-phosphate as substrates (Table I).

Four to five isoenzymes of acid phosphatase activity were detected on polyacrylamide gel electrophoresis in total cell lysates of cultivated human fibroblasts (Fig. 1). All isoenzymes were likewise detected in the lysosomal subcellular fraction. A single faint

TABLE I. Acid Phosphatase Activity in Cultivated Human Fibroblasts.

Substrate	Number of samples	Specific activity (mean ± standard deviation)
p-Nitrophenylphosphate	11	1.04 ± 0.10[a]
4-Methylumbelliferyl-dihydrogen-phosphate	5	157.40 ± 52.11[b]
β-Glycerophosphate	5	9.76 ± 3.30[c]

[a] Micromoles of substrate cleaved/mg protein.

[b] Nanomoles substrate cleaved/mg protein.

[c] Micrograms inorganic phosphate/mg protein.

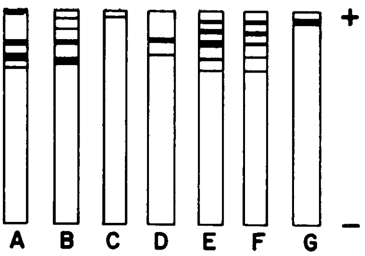

FIG. 1. Isoenzymes of acid phosphatase activity in cultivated human fibroblasts on polyacrylamide gel electrophoresis: Activity in total cell lysate (A), lysosomal fraction (B), microsomal fraction (C), and supernatant (D); activity after treatment with Triton X-100 in total cell lysate (E), lysosomal fraction (F), and microsomal fraction (G).

slow-moving isoenzyme was found in the microsomal fraction. Acid phosphatase activity in the soluble (100,000g) cellular supernatant fraction was confined to one or two isoenzymes of intermediate mobility. Treatment of subcellular fractions with Triton X-100 greatly enhanced the activity of the slow-moving (anionic) isoenzymes. In total cellular and lysosomal preparations, two anionic isoenzymes were clearly solubilized by treatment with the nonionic detergent; in the microsomal subcellular fraction, the single anionic component present was also solubilized by this treatment.

Starch gel electrophoresis of cultivated human fibroblasts, leukocytes, and red blood cells revealed the presence of an isoenzyme in fibroblasts and leukocytes which stained with 4-methylumbelliferyl-dihydrogen-phosphate but not with α-naphthyl acid phosphate (Fig. 2 a and b). Comparison of these isoenzyme patterns with those of the red cell acid phosphatases of the same subject indicated that his isoenzyme in leukocytes and fibroblasts corresponded to an isoenzyme in red cells.

Discussion. The present investigations in cultivated human fibroblasts demonstrate multiple molecular forms of acid phosphatase activity in this tissue. A single anionic isoenzyme is present in the microsomal fraction (that subcellular fraction containing endoplasmic reticulum). This isoenzyme is apparently tightly membrane bound, being solubilized only by treatment with Triton X-100. Multiple additional isoenzymes of acid phosphatase activity are detectable in the lysosomal subcellular fraction in addition to the anionic form corresponding to the microsomal isoenzyme. The cationic isoenzymes are readily solubilized by physical disruption of this subcellular fraction.

Lysosomal acid hydrolases have been found to occur in multiple molecular forms in rat kidney; these isoenzymes differ in electrophoretic mobility on polyacrylamide gel electrophoresis (8, 24). The acidic forms of these hydrolases are readily solubilized by physical disruption, while the basic (cationic) forms are more firmly bound to the insoluble lysosomal residue (24). In contrast to the work of Goldstone and Koenig (8) in rat kidney, then, the isoenzyme of acid phosphatase present in the endoplasmic reticulum of cultivated human fibroblasts is anionic (acidic) rather than cationic (basic). Additional isoenzymes found in the lysosomal fraction migrate toward the negative electrode, and hence are less acidic than the microsomal isoenzyme. The significance of this finding in terms of the modification of lysosomal hydrolases during intracellular

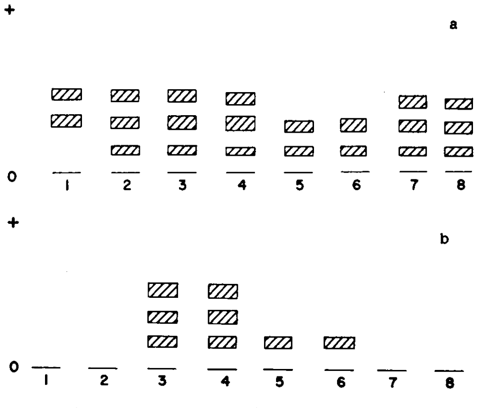

FIG. 2. Isoenzymes of acid phosphatase activity in red blood cells, leukocytes and cultivated human fibroblasts on starch gel electrophoresis. (a) 4-Methylumbelliferyl-dihydrogen-phosphate as substrate: red blood cells (1, 2, 7, 8); leukocytes (3, 4); fibroblasts (5, 6). (b) α-Naphthyl acid phosphate; note the presence of the second anodal band in fibroblasts using 4-methylumbelliferyl-dihydrogen-phosphate which is not detectable with α-naphthyl acid phosphate as substrate, indicating "red cell" acid phosphatase activity.

transport is not entirely clear. Goldstone and Koenig (25) have postulated that the basic, less-soluble isoenzymes are those synthesized first in endoplasmic reticulum. Late modification of this molecular form by addition of sialic acid is then thought to result in the presence of multiple, more soluble anionic forms in the lysosomal fraction. If this process is a general one, then the presence of multiple cationic isoenzymes of acid phosphatase in the lysosomal subcellular fraction of cultivated human fibroblasts is difficult to explain. It is possible that basic groups on the protein moiety of this glycoprotein enzyme modify the electrophoretic mobility independently of acidic sugars or that the enzyme is desialated during preparation. Alternatively, molecular aggregation during the processing of subcellular fractions may account for some of these findings. These possibilities are currently under investigation.

Swallow *et al.* (22) have recently reported the existence of red cell acid phosphatase in liver, kidney, spleen, leukocytes, cultured fibroblasts, and other tissues, in contrast to previous work (26). In the present investigations an isoenzyme was detected on starch gel electrophoresis of human leukocytes and fibroblasts which did not cleave α-naphthyl acid phosphate but was active against 4 -methylumbelliferyl-dihydrogen-phosphate, and which corresponded to an acid phosphatase isoenzyme in red cell hemolysates. This confirms the presence of "red cell" acid phosphatase in cultivated human fibroblasts.

Summary. Five isoenzymes of acid phosphatase activity were detected by polyacrylamide gel electrophoresis in cultivated human fibroblasts. All of the isoenzymes were present in the lysosomal subcellular fraction, while a single anionic isoenzyme was detected in the microsomal subcellular fraction. The anionic isoenzymes tended to be firmly membrane bound and were solubilized by treatment with Triton X-100. Red cell acid phosphatase isoenzymes were studied using starch gel electrophoresis. An isoenzyme formerly considered to be unique to the red blood cell was detected in leukocytes and in cultivated human fibroblasts.

1. De Duve, C., Granelto, R., Appelmans, F., and Wattiaux, R., Nature, (London) **172**, 1143 (1953).
2. Essner, E., and Novikoff, A. J., Ultrastruct Res. **3**, 374 (1960).
3. Allison, A. C., Proc. Roy. Soc. Med. **59**, 868 (1966).
4. Comfort, A., Lancet **2**, 1325 (1966).
5. Nadler, H. L., and Egan, T. J., N. Engl. J. Med. **282**, 302 (1970).
6. De Duve, C., and Berthet, J., Int. Rev. Cytol. **3**, 225 (1955).
7. Nelson, B. D., Proc. Soc. Exp. Biol. Med. **121**, 998 (1966).
8. Goldstone, A., and Koenig, H., Life Sci. **9**, 1341 (1970).
9. Li, C. Y., Yam, L. T., and Lam, K. W., Histochem. Cytochem. **18**, 473 (1970).
10. Hopkinson, D. A., Spencer, N., and Harris, H., Nature (London) **199**, 969 (1963).
11. Cristofalo, V. J., Kabakjian, J. R., and Kritchersky, D., Proc. Soc. Exp. Biol. Med. **126**, 649 (1967).
12. Huggins, C., and Talalay, P., J. Biol. Chem. **159**, 399 (1945).
13. Hirschhorn, R., Hirschhorn, K., and Wessmann, G., Blood **30**, 84 (1967).
14. White, I. N. H., and Butterworth, P. J., Biochem. Biophys. Acta **193**, 1229 (1971).
15. Barka, T., J. Histochem. Cytochem. **9**, 542 (1961).
16. Swallow, D. M., and Harris, H., Ann. Hum. Genet. London **36**, 141 (1972).
17. Sorenson, S. A., Clin. Genet. **1**, 294 (1970).
18. Nadler, H. L., *in* "Lysosomes and Storage Diseases" (H. G. Hers and F. Van Hoof, eds.), Academic Press, pp. 475–484, (1973).
19. Nadler, H. L., Inouye, T., and Justice, P., Nature (London) **213**, 1261 (1962).
20. Nadler, H. L., Dowben, R. M., and Hsia, D. Y. Y., Blood **34**, 52 (1969).
21. Bessey, D. A., Lowry, D. H., and Brock, M., J. Biol. Chem. **164**, 321 (1964).
22. Swallow, D. M., Povey, S., and Harris, H., Ann. Hum. Genetics **37**, 31 (1973).
23. Lowry, O. H. Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem. **193**, 265 (1951).
24. Goldstone, A., Konecny, P., and Koenig, H., Fed. Eur. Biochem. Soc. Lett. **13**, 68 (1971).
25. Goldstone, A., and Koenig, H., Biochem. J. **132**, 267 (1973).
26. Hopkinson, D. A., Spencer, N., and Harris, H., Hum. Genet. **16**, 141 (1964).

Received Sept. 4, 1973. P.S.E.B.M., 1974, Vol. 145.