Heterogeneity of Acid Phosphatase in the Human Diploid Cell Strain WI-38.* (32530)

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Since the original description of lysosomes in rat liver(1), they have been observed in many vertebrate tissues(2), as well as in several cell cultures of human origin(3,4), and have been implicated in a variety of phenomena of cellular injury and necrosis(5-7).

Acid phosphatase is primarily a lysosomal enzyme. Typically, 70-80% of its activity is reported to be confined to these particles, with the remainder being distributed in other subcellular particles and in the supernatant(1,8).

Several reports have suggested that at least two different forms of acid phosphatase are present in a number of tissues(9-12). Nelson (13), Shibko and Tappel(14), and Yu *et al* (15) have presented evidence that, in rat tissues (liver and polymorphonuclear leukocytes), the acid phosphatase recovered from the lysosomal fraction may be a distinctly different enzyme from that found in the supernatant fraction. Neil and Horner(16) have reported similar data for guinea pig liver. However, this heterogeneity has not been established for other species or for cells grown *in vitro*.

During the course of our studies on the acid p-nitrophenyl-phosphatase activity of whole homogenates of the human diploid cell strain WI-38, we noted that the shape of the curves expressing enzyme activity as a function of hydrogen ion concentration in the presence and absence of magnesium ion suggested more than one form of acid phosphatase (orthophosphoric monoester phosphohydrolase EC 3.1 3.2) in these cell cultures. Since the acid phosphatase activity of human diploid cells increases during the degenerative phase of these cultures(17), we felt it would be of interest to investigate the possibility of multiple forms of this enzyme and their respective subcellular localizations in these cells.

Materials and methods. For these studies, starter cultures of human diploid cell strain WI-38(18) were obtained from Dr. L. Hayflick of the Wistar Institute. The methods of subcultivation and harvesting and preparation of the cells for enzyme assays have been described(17,19,20).

For preparation of the various subcellular fractions, the cells were suspended in 10^{-3} M phosphate buffer at pH 7.2 and stirred gently for 10 minutes. This treatment was followed by homogenization with a Dounce apparatus using, first, a loose-fitting plunger and then a tight-fitting plunger. Microscopic examination of random samples of the homogenate showed that this treatment resulted in virtually complete cell breakage. Immediately following the homogenization, 0.5 M sucrose was added to the mixture to give a final sucrose concentration of 0.25 M. The various fractions were then separated by centrifugation. Four subcellular fractions resulted from this procedure: 1) a nuclear fraction which may contain residual intact cells and which was sedimented at 500 $\times$ *g* for 10 minutes; 2) a lysosomal-mitochondrial fraction sedimenting at 12,500 $\times$ *g* (20 minutes); 3) a microsomal fraction which was sedimented at 105,000 $\times$ *g* (30 minutes); and 4) the supernatant. All preparative operations were carried out at or near 4°. Following separation, each fraction was treated with Triton X-100 (1% final concentration), and the acid phosphatase activity was then assayed.

Acid phosphatase was determined by an adaptation of the method of Bessey, Lowry and Brock(21), which depends on the enzymatic hydrolysis of p-nitrophenylphosphate. Aliquots of the appropriate homogenate fractions were incubated in 0.05 M citrate buffer

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at pH 5.0 which contained 6.1 mM substrate for 30 minutes at 37°. The reaction was stopped by the addition of alkali and the p-nitrophenol measured spectrophotometrically at 410 mμ.

For electrophoresis, 10 μl of the sample were applied to 1″ × 6-3/4″ cellulose acetate strips and placed in the chamber which contained sodium barbital-barbituric acid-tris buffer at pH 8.8 (Gelman HR buffer diluted to 2000 ml; Gelman Instrument Co., Ann Arbor, Mich.). A force of 300 volts (approximately 1 mA/strip) was applied for 60 minutes. Development and visualization of the strips were based on the enzymatic hydrolysis of p-nitrophenylphosphate. Following separation, a similar strip which had been soaked in p-nitrophenylphosphate (6.1 mM, pH 5.0) was layered over the strip containing the enzyme, and both were incubated for 45 minutes at 37° in a moist chamber. Following the incubation period, the paired strips were placed in a moist chamber which contained a reservoir of concentrated NH_3. The hydrolysis of the NH_3 vapor on the wet strips adjusted the pH so that direct visualization of the enzymatically-released p-nitrophenol was possible.

Results and discussion. Table I summarizes the subcellular distribution of acid p-nitrophenylphosphatase activity in WI-38 cells. The results show that almost 50% of the specific activity (43% of the total activity) was localized in the lysosomal fraction.

TABLE I. Acid Phosphomonoesterase Activity—Subcellular Distribution.

Fraction	Activity, μmoles/min × 10^3	Specific activity, mμmoles/min/mg protein	% of total
Nuclear	.65	34.2	18.9
Lysosomal	1.65	90	49.7
Microsomal	.53	29	16
Supernatant	1.06	28	15.4
Whole homogenate	3.93	48.8	
% Recovery	99		

Following separation, each fraction was treated with Triton X-100 (1%) and the acid phosphatase activity assayed, as described in text.

These data also show that the lysosomal fraction is enriched in acid phosphatase activity as compared to the unfractionated, crude homogenate. Experiments involving treatment of the lysosomal fraction with Triton X-100 (1%) in 0.25 M sucrose with stirring and subsequent centrifugation at 105,000 × *g* resulted in almost 93% of the activity appearing in the supernatant. Similar treatment with 0.25 M sucrose alone resulted in only 26% of the activity appearing in the supernatant. Thus, we have isolated a subcellular fraction from cultured diploid human cells which meets many of the biochemical criteria for lysosomes: this fraction contains the greatest percentage of acid phosphatase activity; in terms of specific activity, this fraction is enriched over the whole crude homogenate; the activity is sedimentable and membrane-bound; and, once released by a detergent, the activity is no longer sedimentable.

Gordis and Nitowski(3) and Wattiaux(4) have reported similar data for cultures of Chang liver cells and HeLa cells, respectively. Thus, cell cultures, including diploid human cells, appear to contain lysosome-like granules similar to those described for tissues of other vertebrate species.

Our initial experiments on pH dependence and magnesium ion effect on acid phosphatase activity were carried out on whole homogenates. The data showed that, in the absence of magnesium ion, there was a gradual rise in enzyme activity from pH 3 to pH 5 and then a relatively rapid drop between pH 5 and pH 7.2. In the presence of magnesium ion, the shape of the curve was similar, but the activity optimum was shifted to pH 6.2. This shift in pH optimum suggested to us that two different forms of the p-nitrophenylphosphatase might be present in WI-38 cells. To elucidate this point a similar experiment was carried out on the lysosomal and supernatant fractions of WI-38 cells.

Fig. 1 shows a comparison of the effect of pH and magnesium ion on the acid phosphatases of the lysosomal and supernatant fractions. The lysosomal enzyme shows a broad activity optimum between pH 3.3 and 5.0 and then a gradual decline. Magnesium ion seems to have no effect on the activity. The supernatant enzyme activity shows a relatively sharp optimum at pH 5.5 with the activity falling off on either side of this pH.

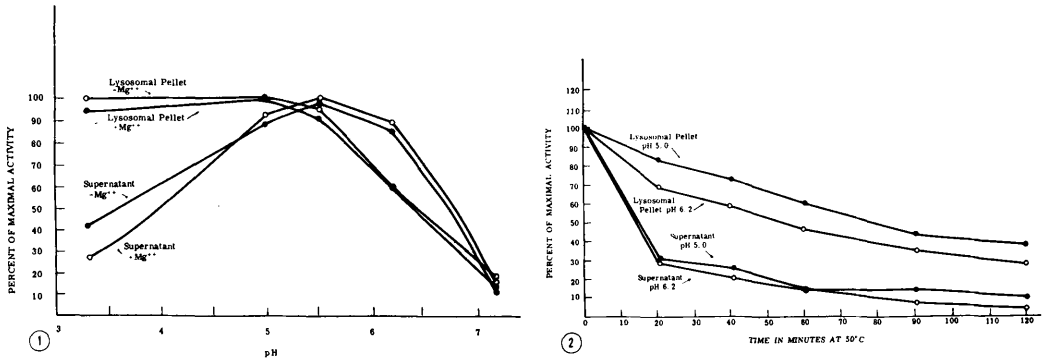

FIG. 1. Effect of pH and Mg^{++} on phosphomonoesterase activity in WI-38. Procedures for separation and method of measurement were as described in text. Mg^{++} was present at a final concentration of 1 mM.

FIG. 2. Thermal stability of acid phosphomonoesterases at 50°. The enzyme preparation was incubated at 50° and samples were taken at various times and assayed as described in text.

Thus, the optimum activity of the supernatant enzyme is on the alkaline side. The more striking difference between the two, however, is the very low activity of the supernatant enzyme between pH 3 and 4. Magnesium ions may have had a slightly inhibitory effect on this enzyme at pH 3.5, and this inhibition presumably accounts for the shift in pH optimum in whole homogenates. These differences in pH optima are similar to those reported by Yu *et al*(15) for leukocytes; Nelson(13) for rat liver; Neil and Horner (16) for guinea pig livers, and Moore and Angelletti(12) for unfractionated rat liver. However, Shibko and Tappel(14) reported pH curves for acid phosphatase from the lysosomal and supernatant fraction of rat liver which were similar to each other.

Table II shows the effects of several known acid phosphatase inhibitors on the activity of acid phosphatase from the lysosomal and supernatant fractions of WI-38 cells. Here, where possible, the inhibitors were used both at pH 5.0 and 6.2 to control pH effects on the enzyme activity with and without inhibitor. Both fluoride and tartrate showed a much higher percent inhibition of the acid phosphatase activity from the lysosomal fraction than from the supernatant fraction. Alloxan, on the other hand, showed the reverse effect, inhibiting the supernatant enzyme by 62% and the lysosomal enzyme by only 13.5%. These results are similar to those reported previously by a number of workers(13,14,16) and confirm the differences observed for p-nitrophenylphosphatase of different subcellular fractions. Shibko and Tappel(14) have shown that, for rat liver, the differences in sensitivity to these inhibitors were evident only when p-nitrophenylphosphate is used as substrate. Our data thus far do not include substrates other than p-nitrophenylphosphate; however, the observed difference in reactivity with this substrate strongly suggests that the respective reactions involve different molecular forms of the enzyme.

TABLE II. Fluoride, Tartrate and Alloxan Inhibition of Acid Phosphomonoesterase of WI-38.

Fraction	pH	% Inhibition NaF	% Inhibition L(+) tartrate	% Inhibition Alloxan
Lysosomes	5.0	84.3	68.6	13.5
Supernatant	5.0	31	21.7	61.8
Lysosomes	6.2	68.1	46.6	—
Supernatant	6.2	10	9.2	—

Fluoride and tartrate were present at 20 mM; alloxan at 0.1 M. The assay procedure was carried out as described in text.

The rates of thermal inactivation of the acid phosphatases of these two fractions were investigated at several temperatures and at both pH 5.0 and 6.2. Results obtained at 50° are shown in Fig. 2. The lysosomal enzyme at pH 5.0 shows a gradual decline in activity to 40% of its original value after 2 hours. Similar data were obtained for this fraction measured at pH 6.2. The acid phosphatase from the supernatant fraction, at both pH 5.0 and 6.2, shows a rapid inactivation at

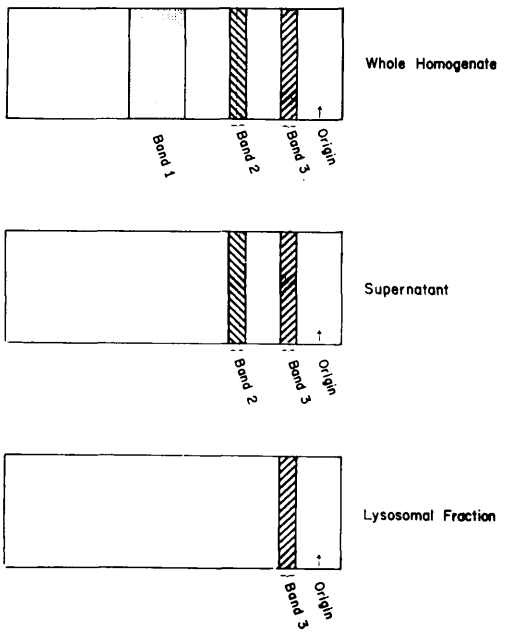

FIG. 3. Acid phosphatase isoenzymes in different subcellular fractions of WI-38 cells. Electrophoresis was carried out as described in text.

50° to about 30% of the original activity, and then a gradual decline which parallels the lysosomal enzymes. Heat inactivation curves of similar form have been shown for rat liver homogenates by Nelson(13). The biphasic nature of this inactivation curve suggested to us that the supernatant enzyme might be completely inactivated in 20-30 minutes at 50°, and that the remaining activity represented contamination of this fraction with lysosomal enzyme. Subsequent experiments using selective inhibition with 20 mM sodium fluoride have shown that, in fact, the supernatant enzyme is highly heat-sensitive and the residual activity after 30 minutes at 50° is due to contamination of the supernatant with the lysosomal enzyme.

The possibility that the observed differences in activity results from changes in the environment of these enzymes following differential centrifugation seems unlikely, since determinations of activity differences *vs* pH in the presence of the selective inhibitors allow simultaneous resolution of the two enzyme forms in crude homogenates.

The heterogeneity of acid phosphatase activity in WI-38 cells is clear from these data. However, the question of subcellular localization is still doubtful since the possibility remains that the enzyme detected in the supernatant results from leakage of enzymes from the lysosomes during preparation of the fractions. To elucidate this point, an experiment was designed in which the lysosomal fraction was prepared and resuspended in either 0.25 M sucrose or H_2O. After 15 minutes of incubation at 20°, the suspension was recentrifuged at 12,500 × *g* and the lysosomal pellet re-isolated. Lysosomes show the usual properties of osmotic swelling and lysing; thus, treatment with water should result in leakage of acid phosphatase from these bodies. One could then test the activity in the extract and pellet with inhibitors and compare these results with results found on the supernatant fraction. The data from this experiment are shown in Table III. Treatment of the lysosomal fraction with water resulted in the release of about one-third of the activity into the extract. Similar incubation with 0.25 M sucrose resulted in less than 20% of the activity appearing in the extract. In both cases, the enzyme that leaks out of the lysosome is highly sensitive to fluoride inhibition, as is

TABLE III. Effect of H_2O Treatment on Lysosomal Acid Phosphomonoesterase Activity in WI-38.

	Treatment							
	H_2O				Sucrose			
Fraction	Activity, μmoles/min × 10^3		% Inhibition, NaF (20 mM)		Activity, μmoles/min × 10^3		% Inhibition, NaF (20 mM)	
pH→	5.0	6.2	5.0	6.2	5.0	6.2	5.0	6.2
Pellet	.72	.39	85	76	1.06	.50	85	85
Extract	.34	.17	84	69	.23	.13	73	67
Supernatant	.98	.83	27	17	—	—	—	—

The procedure for this experiment is described in text.

typical of the lysosomal fractions, while the supernatant enzyme, as previously shown, exhibits a much lower level of sensitivity to fluoride.

This experiment indicates that the acid phosphatase activity in the supernatant fraction is not the result of leakage from the lysosomes. An alternative but less likely possibility is that the enzyme which is found in the supernatant is less tightly bound to the lysosomes and leaks rapidly and completely from these granules during the initial stages of preparation.

Electrophoresis of the different fractions of WI-38 homogenates on cellulose acetate showed that at least 3 bands were present in the whole homogenate (Fig. 3); a diffuse, faintly developed band which migrates most rapidly (Band 1) followed by two less rapidly migrating fractions (Bands 2 and 3). Only band 3 was present in the lysosomal fraction, whereas both band 2 and band 3 were present in the supernatant fraction. Thus, one acid phosphatase isozyme is present in the supernatant fraction which is absent in the lysosomal pellet (Band 2). The presence of a band in the supernatant identical to the band in the lysosomal pellet may result from contamination of the supernatant fraction by the lysosomal fraction.

The data presented above show that the acid p-nitrophenylphosphatases of strain WI-38 diploid human cells are distinctly different enzymes which probably have different subcellular localizations in the intact cell.

None of the fractions studied can, in any way, be considered pure, and molecular heterogeneity within each fraction has not been thoroughly investigated. We have shown some leakage of lysosomal acid phosphatase in preparations suspended in 0.25 M sucrose (Table III); the thermal inactivation studies (Fig. 2) and the electrophoresis patterns (Fig. 3) indicate cross-contamination of the supernatant and lysosomal phosphatases. However, the demonstrated presence of cross-contamination reinforces the argument for different enzymes at different locations since, in a purified preparation, the differences observed in pH optima, thermal stability and sensitivity to inhibitors would be enhanced.

Summary. A subcellular fraction has been prepared from human diploid cell strain WI-38 which has the characteristics of lysosomes: this fraction contains the greatest percentage of acid phosphatase activity and in terms of specific activity is enriched over the whole homogenate. The activity is sedimentable and membrane-bound and, once released by a detergent, is no longer sedimentable. In addition, we have shown that two forms of acid phosphatase exist in WI-38 cells based on differences in subcellular localization, pH optimum, sensitivity to inhibitors, thermal stability and electrophoretic mobility.

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