ELECTROPHORETIC SEPARATION OF MULTIPLE
FORMS OF PARTICLE ASSOCIATED ACID PHOSPHATASE

John M. Allen* and Jon Gockermant

Department of Zoology, The University of Michigan, Ann Arbor, Mich.

A considerable body of evidence supports the contention that much, if not all, cellular acid phosphatase is contained within particles designated as lysosomes (deDuve, 1959; Novikoff, 1961). Release of the enzyme from these particles is effected by conditions that destroy the integrity of the lysosomal membrane. Often a discrepancy is found between the total activity of acid phosphatase and the activity of released, unsedimentable, enzyme. This discrepancy is assumed to be due to retention of soluble enzyme within collapsed lysosomal vesicles and to the secondary adsorption of enzyme to cellular structure. This could be explained alternately by the existence, within the lysosomal particle, of two categories of acid phosphatase: one present in readily releasable "soluble" form and the other present in a more firmly "bound" form. This alternate hypothesis is suggested by experiments of Barka (1961b), which showed the release of a chromatographically distinct acid phosphatase following the treatment of rat liver homogenates with Triton X-100. This paper deals with the electrophoretic demonstration of multiple acid phosphatases associated with particles and their differential release.

Materials and Methods

Quantitative determination of acid phosphatase activity was based on the estimation of alpha-naphthol liberated enzymatically from sodium alpha-naphthyl acid phosphate at pH 5.0. The liberated alpha-naphthol was postcoupled with the stabilized diazonium compound, Fast Red ITR (diazotized 4-diethylsulfonamido-2-aminoanisole), at pH 8.0 to yield a purple end product suitable for spectrophotometric measurement. This method is derived from that of Gomori (1953) for the estimation of esterase activity using naphtholic substrates. The reaction mixture for acid phosphatase determination contained the following components: 0.005 M sodium alpha-naphthyl acid phosphate (Dajac) and 0.05 M sodium acetate-acetic acid buffer at pH 5.0 (Gomori, 1955). Incubation was at 25°C. Following incubation, tubes were chilled in ice, and a volume of a coupling solution equal to that of the reaction mixture plus enzyme sample was added to each tube. This coupling solution was compounded as follows: (a) *Stock Michaelis veronal buffer*: 14.7 gm. sodium diethyl barbiturate and 9.7 gm. sodium acetate in a final volume of 250 ml. (b) *Working Michaelis veronal buffer*: 2 parts stock Michaelis veronal buffer, one part 0.2 N HCl, and 3 parts water (final pH 8.0). To 100 ml. of this working buffer were added 4 gm. of sodium lauryl sulfate (Fisher) and 200 mg. of Fast Red ITR (General Aniline and Film Corporation). After addition of the coupling solution, the tubes were incubated at 25°C. for 15 minutes to allow development and stabilization of the color. Optical density was measured with a Coleman Model 2A spectrophotometer at 545 millimicrons. Reaction rates were linear over a wide range of enzyme activity, and substrate concentration was not rate limiting. The con-

centration of sodium lauryl sulfate used produced complete solubilization of the azo-dye formed in the assay procedure. It also completely inhibited acid phosphatase activity and led to the resolubilization of protein precipitates, which formed when detergent-treated samples were diluted in the reaction mixture. This system of acid phosphatase assay appears to be completely compatible with the presence of high concentrations of Triton X-100 (and other detergents), which interfere with phosphate determination by the Fisk-SubbaRow procedure (Wattiaux & deDuve, 1956). Total nitrogen was determined by the method of Levy (1936).

Localization of acid phosphatase following electrophoresis was achieved with a coupling-type reaction. The reaction mixture contained 1 mg. per ml. of sodium alpha-naphthyl acid phosphate (Dajac) and 1 mg. per ml. of the diazonium salt of o-aminoazotoluene (Diazo Garnet GBC) (Dajac) in 0.05 M sodium acetate-acetic acid buffer at pH 5.0. Gels were incubated in this reaction mixture for 5 and 10 minutes at 25°C. The reaction was stopped by replacement of the reaction mixture by 7.5 per cent acetic acid. Developed gels could be stored in 7.5 per cent acetic acid at 4°C. for many days without deterioration.

In certain instances a Gomori-type reaction (Gomori, 1952) was utilized for the visualization of acid phosphatase after electrophoresis. The reaction mixture contained 0.05 M sodium acetate-acetic acid buffer at pH 5.0, 0.003 M lead nitrate, and 0.015 M sodium beta-glycerophosphate (Matheson, Coleman, and Bell). Following incubation at 25°C. for 5 and 10 minutes these gels were washed in repeated changes of distilled water over the course of one hour. The pattern was developed by immersion in 1.0 per cent ammonium sulfide for one minute.

After processing for enzyme visualization, the gels were photographed by transillumination under constant conditions of exposure and processing. All Figures have been enlarged twofold. In all Figures the cathode end of the gel is at the top.

Electrophoretic separation of acid phosphatase in acrylamide matrices was achieved by a modification of the methods proposed by Ornstein and Davis (1962) and Barka (1961a). Preparation of the acrylamide gels used in this study follows. *Lower gel solution*: 5 parts of 1.6 per cent N, N'-methylenebisacrylamide (American Cyanamide) in water, 10 parts of 0.25 per cent tetramethylethylene diamine (Rohm-Haas) in 0.04 M histidine-NaOH buffer (pH 7.5), 5 parts of 0.03 per cent potassium ferricyanide in water, and 5 parts of 0.56 per cent ammonium persulfate in water. Acrylamide (Eastman) was weighed into this mixture to yield a final acrylamide concentration of 10.0 per cent (wt./vol.) after volumetric dilution to 1.6 times the volume of the lower gel solution used. Gels were cast in soft glass tubes, 64 mm. in length and 5 mm. inside diameter, to yield a column of lower gel 43 mm. in length. Gels were layered with water as described by Ornstein and Davis (1962). After polymerization (about 40 minutes at 25–30°C.), 200 μl. of a sample gel were placed upon the lower gel column. This sample gel was compounded as follows: 1 part of 0.04 M histidine-NaOH buffer (pH 7.5), 2 parts of 10.0 per cent acrylamide and 2.5 per cent N,N', methylenebisacrylamide in water, and 1 part 0.004 per cent riboflavin in water. Two parts of this sample gel were mixed with one part of an appropriate tissue sample. This sample gel was polymerized by ultraviolet light emitted from a 15 watt fluorescent lamp tube. Polymerization required approximately 20 minutes. Spacer gels (Ornstein & Davis, 1962) were not employed. Stock solutions were stored at 4°C. and were stable for one week.

The bridge buffer for all separations was 0.01 M histidine-NaOH at pH 7.5. Electrophoresis was carried out for 90 minutes at 4°C. with an initial potential

of 100 volts across the gels supplied by a constant voltage power supply. Voltage drop across the gels was measured with an external volt meter with the electrodes placed in line with one of the gel tubes.

All work was carried out using liver tissue derived from 90 to 180-day-old albino male rats. Animals were starved for 12 to 14 hours prior to use but were provided with water *ad libitum* during this period. Tissue was homogenized in 0.25 M sucrose at 0° C. Homogenization was minimal and consisted of 5 up-down strokes using a smooth bore homogenizer and a Kel-F pestle rotated at approximately 1200 RPM. Homogenates were brought to a concentration of 20.0 per cent (wt./vol.) by addition of 0.25 M sucrose prior to centrifugation.

Differential centrifugation of these homogenates was carried out at 4°C. according to the following scheme: (a) *Nuclear fraction:* An initial sedimentation at 480 $\times$ g for 10 minutes was followed by two subsequent resuspensions and centrifugations at 480 $\times$ g for 10 minutes. The sediment from the final centrifugation was resuspended in 0.25 M sucrose to yield a final volume equal to that of the starting material. All subsequent sediments were resuspended in like fashion. (b) *Mitochondrial-lysosomal fraction*: The combined supernatant material from (a) was centrifuged at 20,000 $\times$ g for 30 minutes, and the sediment was resuspended and centrifuged at 20,000 $\times$ g for 30 minutes. The sediment was resuspended to initial volume as noted above. (c) *Microsomal fraction*: The combined supernatant material from (b) was centrifuged at $10^6 \times$ g for 60 minutes. After decantation of the supernatant fluid, the pellet surface was rinsed with a small volume of 0.25 M sucrose. The sediment was resuspended to initial volume as noted above. (d) *Supernatant fraction*: This consisted of the supernatant material and rinse from step (c). A Servall refrigerated centrifuge equipped with a SS-34 rotor was used in steps (a) and (b); a Spinco preparative centrifuge equipped with a #40 rotor was used for step (c). Gravity and time values refer to equilibrium fields exclusive of acceleration and deceleration.

Results

The electrophoretic system. Electrophoretic separation of maximally solubilized acid phosphatase (see below) by currently available methods was unsatisfactory. Starch gel matrices in both horizontal (Smithies, 1959; Allen *et al.*, 1963*b*) and vertical (Smithies, 1959) configurations showed several ill-defined regions of acid phosphatase activity but only after one to two hours of incubation at 25°C. The system of acrylamide electrophoresis proposed by Ornstein and Davis in 1962 (FIGURE 1, *a* and *b*) gave poor resolution at the normal acrylamide concentration (7.5 per cent) and showed only a single distinct component when 10.0 per cent acrylamide gels were used. The system of acrylamide electrophoresis developed by Barka (1961*a*; FIGURE 1, *c* and *d*) revealed two poorly defined groups of acid phosphatases in gels containing 7.5 per cent acrylamide, but in gels containing 10.0 per cent acrylamide the sites of acid phosphatase activity were more clearly demonstrable. The success of S. L. Allen *et al.* (1963*b*) in the electrophoretic separation of acid phosphatases of *Tetrahymena pyriformis* in starch gel matrices combined with a neutral range buffer prompted a search for a buffering system in the range between *p*H 6.5 and *p*H 8.5 that would be compatible with rat liver acid phosphatase. The combinations of parameters tested are summarized in TABLE 1. The most satisfactory system to emerge from these trials was an identical bed and bridge buffer composed of 0.01 M histidine-NaOH at *p*H 7.5 in combination with an acrylamide concentration of 10.0 per cent and a voltage gradient of 100 volts across the gels applied for 90
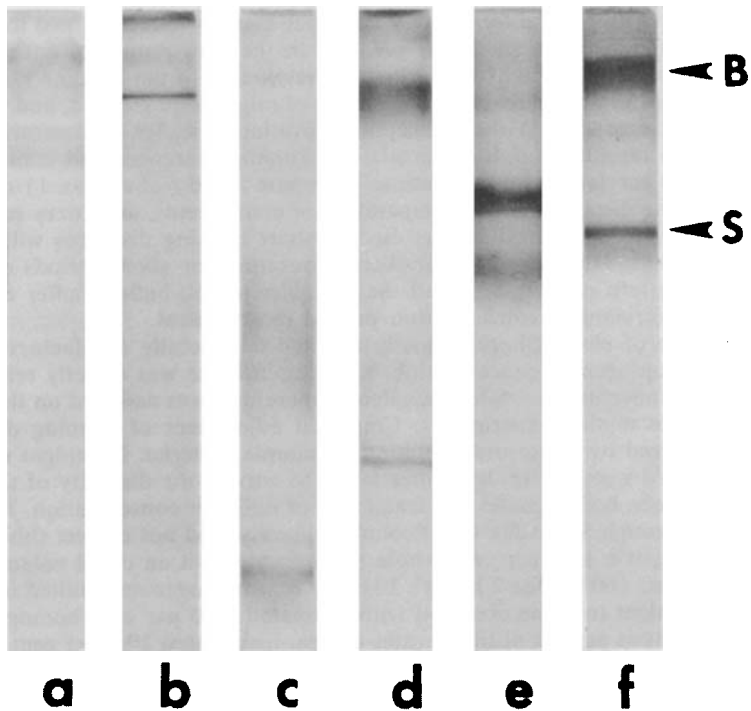
FIGURE 1.   Electrophoretic patterns of acid phosphatase activity derived from 10.0% whole homogenates of rat liver in 0.25 M sucrose and treated with 5.0% Triton X-100 to effect maximal solubilization of enzyme. (*a*) Ornstein and Davis method, 7.5% acrylamide, 10 minutes incubation at 25°C.; (*b*) as in *a* but with 10.0% acrylamide; (*c*) Barka method, 7.5% acrylamide, 10 minutes incubation 25°C.; (*d*) as in (*c*) but with 10.0% acrylamide; (*e*) electrophoresis as prescribed in "Materials and Methods" with 7.5% acrylamide, 5 minutes incubation 25°C.; (*f*) as in *e* but with 10.0% acrylamide. *B* designates firmly bound acid phosphatase released by Triton X-100, and *S* designates acid phosphatase solubilized or released by physical disruption. The pattern in *f* is identical in all respects to that shown in *e* of FIGURE 2, which was derived from a mitochondrial-lysosomal fraction after treatment with 5.0% Triton X-100.

TABLE 1

ELECTROPHORETIC VARIABLES*

Buffer	Buffer concentration		*p*H tested			Acrylamide concentration (in per cent)
Collidine	0.05M, 0.025M, 0.01M		6.5, 7.0, 7.5, 8.0			7.5, 8.75, 10.0
Histidine	0.05M, 0.025M, 0.01M		6.5	7.5	8.5	7.5, 8.75, 10.0
Boric Acid	0.05M	0.01M		7.5	8.5	7.5, 8.75, 10.0
Tris		0.01M		7.5	8.5	7.5, 8.75, 10.0
Tris-Borate	0.001 Tris–0.029 Boric Acid		7.5			10.0
Methylglycine		0.01M	7.5			7.5, 8.75, 10.0
Glycine	0.05M	0.01M			8.5, 9.5	7.5, 8.75, 10.0

* Measured with 10% rat liver homogenates in 0.25M sucrose and 100 volts across the gels for 90 minutes at 4°C.

minutes at 4°C. In general terms, with all buffer systems tested marked improvement in resolution was achieved at pH 7.5. In the acid range the pattern was squeezed, and the distance of migration was restricted. In the alkaline range the pattern separation was increased, the distance of migration extended, and pattern definition was degraded. Without exception elevation of buffer concentration was deleterious to resolution and produced short running distances and compressed patterns. Low acrylamide concentrations (compare e and f of FIGURE 1) resulted in long running distances, inferior separation of components, and fuzzy patterns. High acrylamide concentrations resulted in short running distances with compressed patterns. Higher voltage gradients operating for short periods of time resulted in pattern distortion. Of all the variables tested, buffer, buffer concentration, and acrylamide concentration proved most critical.

The system of electrophoresis finally adopted is generally satisfactory but is sensitive to homogenate concentration. Running distance was directly related to homogenate concentration. Site equivalence, therefore, was assigned on the basis of appropriate mixing experiments. Empirical adjustment of running distance may be achieved by appropriate dilution of sample material. Overnight dialysis against distilled water or bridge buffer failed to correct the disparity of running distance between homogenates (or fractions) of different concentration. Passage of samples through Sephadex G 50 columns likewise did not correct this difference. Mixture of a 10.0 per cent whole homogenate with an equal volume of a heat-inactivated (60°C. for 2 hours) 20.0 per cent homogenate resulted in separations equivalent to those observed with untreated 20.0 per cent homogenates. Overnight dialysis against distilled water of heat-inactivated 20.0 per cent homogenates did not impare their effectiveness in this respect. Addition of 1.0 per cent crystalline serum albumin or 0.1 M potassium chloride to 10.0 per cent homogenates did not increase running distance but, rather, further restricted migration.

Relation between electrophoretic pattern and the physical state of the mitochondrial-lysosomal fraction. Supernatant material ($10^6 \times$ g, 60 minutes, 4°C.), derived from untreated mitochondrial-lysosomal fractions, when subjected to electrophoresis, showed minimal acid phosphatase activity in the gel. This activity was represented as a single component of low intensity (FIGURE 2,a). Similar fractions subjected, prior to centrifugation and electrophoresis, to 6 minutes in a blendor (Eberbach microattachment, 2 cm. blade, 15,000 RPM, 0°C.), to 30 minutes sonication (MSE Ultrasonic Disintegrator, ⅜" probe, 18,000 to 20,000 CPS, 0°C.), or to 12 cycles of freeze-thawing showed marked increase of enzyme activity at this site above appropriate control levels but showed no new sites (FIGURE 2, b and c). In all cases one or two minor satellite bands (barely visible in photographs) were present just ahead of the main acid phosphatase component. Material subjected to sonication for 60 minutes showed, rather than the presence of these minor satellites, distinct division of the main component into a doublet.

Enzymatic digestion of mitochondrial-lysosomal fractions (FIGURE 2, d), prior to centrifugation and electrophoresis, with pronase (Calbiochem, 1 mg. per ml. fraction, 2 hours at 25°C.) or phospholipase c (Worthington, 1 mg. per ml. fraction, 2 hours at 25°C.) elevated activity of the above-noted acid phosphatase site over control levels (2 hours incubation in 0.25 M sucrose). Neither enzyme was as effective in this respect as the physical treatments.

Triton X-100 treatment of mitochondrial-lysosomal fractions (Rohm-Haas, 5.0 per cent final concentration, 30 minutes, 0°C.) prior to centrifugation and electrophoresis, resulted in a marked increase in the acid phosphatase activity of the component released by physical treatment and enzymatic digestion. Most
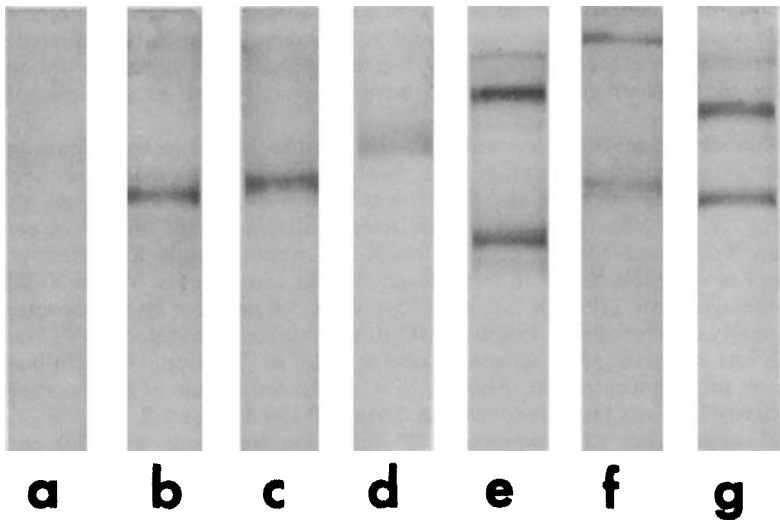
FIGURE 2.   Influence of the physical state of the mitochondrial-lysosomal fraction upon electrophoretic pattern. In all cases the sample consisted of supernatant material derived from a mitochondrial-lysosomal fraction after specified treatment. All gels were incubated for 5 minutes at 25°C. Differences in migration seen in this Figure are due to variations in the properties of the sample analyzed. In all cases site equivalence was assigned on the basis of appropriate mixing experiments, e.g., compare *f* and *g*. (*a*) control, sample (equivalent in all cases to 66.6 μl. of the appropriate tissue preparation) liberated 25.1 mμM alpha–naphthol in 5 minutes at 25°C.; (*b*) blendor, 6 minutes sample liberated 66 mμM alpha–naphthol in 5 minutes at 25°C.; (*c*) sonicated 30 minutes, sample liberated 92.4 mμM alpha-naphthol in 5 minutes at 25°C.; (*d*) pronase, 1 mg. per ml., 2 hours at 25°C., sample liberated 46.2 mμM alpha–naphthol in 5 minutes at 25°C.; (*e*) 5.0% Triton X-100, 30 minutes, sample liberated 220 mμM alpha–naphthol in 5 minutes at 25°C.; (*f*) digitonin, 2 hours, sample liberated 130 mμM alpha-naphthol in 5 minutes at 25°C.; (*g*) 1:1 mixture of sample *e* with sample *f*. Pattern indicates that slow migrating acid phosphatase in *f* is identical to slow migrating component "B" in *e*.

significantly, however, this treatment also resulted in the appearance of an acid phosphatase site of high activity and intermediate mobility (FIGURE 2, *e*). This Triton X-100 released acid phosphatase site appeared as a doublet (poorly re-produced in photographs), with the faster moving component showing slightly less activity. Minor components of acid phosphatase activity were also present following treatment of mitochondrial-lysosomal fractions with Triton X-100. One of these was a doublet of modest activity migrating more slowly than the major Triton X-100 released doublet (FIGURE 2, *e*). The other minor component, a single site of low activity, migrated slightly faster than the major Triton X-100 released doublet (FIGURE 2, *e*). Ignoring the presence of doublet sites and satel-lite sites, a total of 4 components of acid phosphatase activity were present in these electrophoretic preparations. Further discussion will be limited to the rap-idly migrating acid phosphatase released by physical treatment (designated henceforth as component "S;" FIGURE 1, *f*) and the major intermediate com-ponent released by Triton X-100 (designated henceforth as component "B;" FIGURE 1, *f*).

Effective electrophoresis of compõnents "S" and "B" was a function of con-centration of Triton X-100 (FIGURE 3, *a-d*). One-tenth per cent Triton X-100

was minimally effective in the release of these components. One per cent Triton X-100 resulted in moderate release, and 5.0 per cent Triton X-100 effected maximal release of these components. Further elevation of the Triton X-100 concentration to 10.0 per cent resulted in no additional release of acid phosphatase activity.

Other detergents were less effective than Triton X-100 in the release of acid phosphatase activity (FIGURE 3, d-g). Within the Triton series, 5.0 per cent Triton X-45 was as effective as 5.0 per cent Triton X-100 in the release of component "S" but was ineffectual in the release of component "B." Five per cent Triton X-205 and 5.0 per cent Triton X-305 were patently less effective than 5.0 per cent Triton X-100 in the release of both components. Triton X-305 was less effective than Triton X-205. Brij 35 (Atlas, 5.0 per cent final concentration) was nearly as effective as Triton X-100 in the release of component "S" but was much less effective in the release of component "B." Digitonin (Nutritional, 1.0 per cent final concentration, 2 hours, 25°C.) effected release of both components of activity but was less effective than Triton X-100 (FIGURE 2, f).

The appearance of component "B" following treatment with 5.0 per cent Triton X-100 was not due to a modification of the electrophoretic properties of component "S." Supernatants derived from blendor-treated, mitochondrial-lysosomal fractions, when treated with 5.0 per cent Triton X-100, showed electrophoretic patterns identical to untreated supernatants; i.e., only component "S" appeared. However, resuspended sediments derived from these centrifugations, when treated with 5.0 per cent Triton X-100, showed both components "S" and
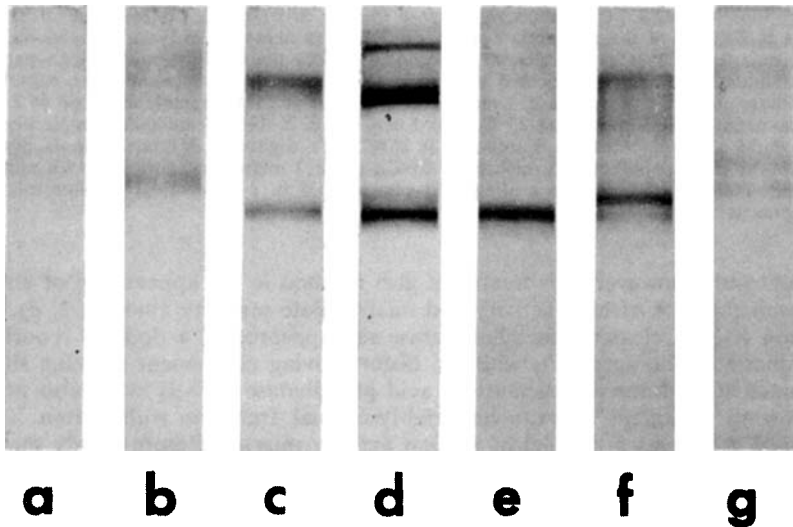

a   b   c   d   e   f   g

FIGURE 3. Effect of detergent type and concentration upon electrophoretic separation of acid phosphatase. Mitochondrial-lysosomal fractions were treated as specified below, centrifuged at $10^6 \times g$ for 60 minutes, and the supernatant used for analysis. All gells were incubated for 5 minutes at 25°C. (a) control, incubated at 0°C. for 30 minutes prior to centrifugation; (b) 0.1% Triton X-100, incubated at 0° C. for 30 minutes prior to centrifugation; (c) 1.0% Triton X-100, incubated at 0°C. for 30 minutes prior to centrifugation; (d) 5.0% Triton X-100, incubated at 0°C. for 30 minutes prior to centrifugation; (e) 5.0% Triton X-45, incubated at 0°C. for 30 minutes prior to centrifugation; (f) 5.0% Triton X-205, incubated at 0°C. for 30 minutes prior to centrifugation; (g) 5.0% Triton X-305, incubated at 0°C. for 30 minutes prior to centrifugation.

"B." If such sediments were subjected to several cycles of washing (0.25 M sucrose) and centrifugation prior to detergent treatment, the amount of component "S" progressively decreased while the amount of component "B" was unchanged.

Mitochondrial-lysosomal fractions were made 20.0 per cent (v/v) in n-butanol. After standing for one hour (0°C.) these preparations were centrifuged at 37,500 × g for 60 minutes (4°C.), and the aqueous layer was removed for electrophoresis. Appreciable amounts of component "S" were found to be present. Interpretation of the effectiveness of this treatment in the release of component "B" was complicated by the severe distortion of electrophoretic patterns of butanol extracts. Mixing experiments (butanol extracts mixed 1:1 with blendor-treated samples prepared as above) consistently showed low enzyme activity in the region of the gel occupied by component "B." Since this material was not present in blendor-treated samples it must be concluded that minimal release of component "B" was effected by butanol extraction. Similar treatment of mitochondrial-lysosomal fractions with carbon tetrachloride released only component "S."

Relation between total acid phosphatase activity and the physical state of the mitochondrial-lysosomal fraction. The influence of treatments employed to release acid phosphatase prior to electrophoretic separation upon the total activity of the enzyme in mitochondrial-lysosomal fractions was assayed by quantitative means (FIGURE 4). Treatment with 5.0 per cent Triton X-100 always resulted in an increase in total activity at least 15 per cent higher than that obtained by any other procedure. Addition of 5.0 per cent Triton X-100 to samples previously treated by other means resulted in total activity values comparable to those obtained with this detergent alone.

Within the Triton series, no other member was as effective as Triton X-100 in terms of total measurable acid phosphatase activity (FIGURE 4). Triton X-45 appeared as effective as the other treatments employed, but Triton X-205 and Triton X-305 were much less effective. One-tenth per cent and 1.0 per cent Triton X-100 treatment produced total activity values equivalent to those obtained following 5.0 per cent Triton X-100 treatment.
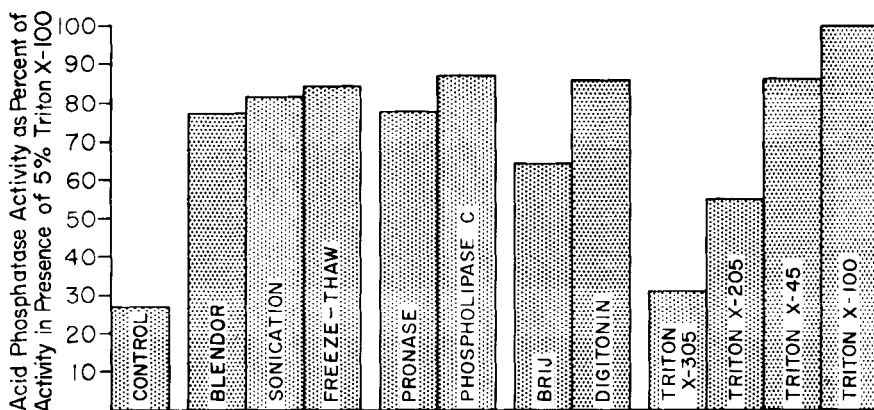

FIGURE 4. Influence of various treatments upon the total acid phosphatase activity of the mitochondrial-lysosomal fraction. Conditions of treatment are specified in the body of the text.
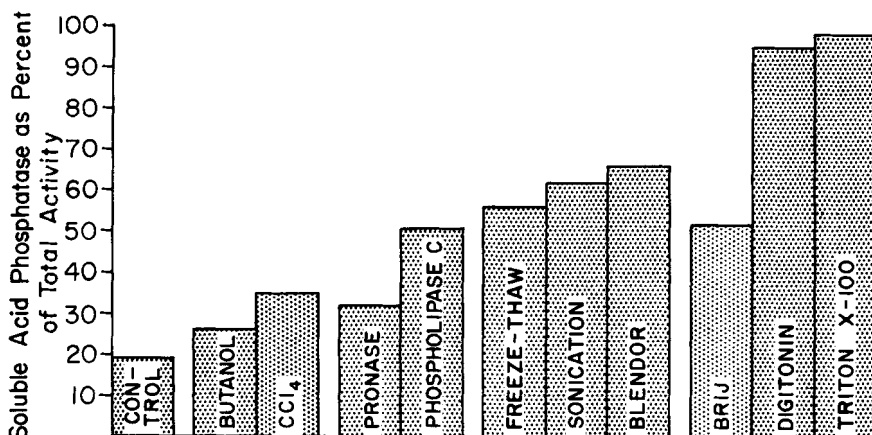
FIGURE 5. Unsedimentable (soluble) acid phosphatase activity of the mitochondrial-lyso-somal fraction after various treatments. Conditions of treatment and manipulation are speci-fied in the body of the text.

Relation between unsedimentable or soluble acid phosphatase activity and the physical state of the mitochondrial-lysosomal fraction. The influence of treatments employed to release acid phosphatase in electrophoretic experiments upon unsedimentable acid phosphatase activity in mitochondrial-lysosomal fractions was assayed by quantitative means (FIGURE 5). In these experiments unsedimentable activity was defined as that which remained in the supernatant phase following centrifugation of previously treated mitochondrial-lysosomal fractions at $10^6 \times g$ for 60 minutes. In the case of samples subjected to sonication or 5.0 per cent Triton X-100 the centrifugation time was extended to six hours to insure sedimentation of particulate material.

Five per cent Triton X-100 and digitonin treatment resulted in the appearance of 98.0 per cent and 95.0 per cent respectively of the total acid phosphatase activity in the unsedimentable phase. This behavior was in marked contrast to other treatments which released 65.0 per cent or less of the total activity. One-tenth per cent Triton X-100 released 73.0 per cent of the total activity of the mitochondrial-lysosomal fraction into the unsedimentable phase.

Butanol and carbon tetrachloride extracts of mitochondrial-lysosomal fractions showed a small increase in activity of the aqueous phase (roughly equivalent to unsedimentable activity) above that which appeared in the unsedimentable phase of control preparations (FIGURE 5). In these experiments, the per cent of the activity solubilized was based upon the activity of the whole, untreated mitochondrial-lysosomal fractions. Thus no measure of possible inactivation of acid phosphatase by butanol or carbon tetrachloride is available.

Quantitative and electrophoretic determination of the distribution of acid phosphatase among cellular fractions. The results of quantitative differential fractionation studies are summarized in FIGURE 6. The distribution of total activities was found to be essentially the same with either of 5.0 per cent Triton X-100 or blendor treatment (6 minutes) of the fractions. Approximately 50.0 per cent of the total activity of the whole homogenate was recovered in the mitochondrial-lysosomal fraction with the remainder distributed more-or-less equally among other fractions. Thus, a unimodal distribution of total acid phosphatase activity is suggested.

Results of an electrophoretic analysis of fractions derived from differential centrifugation are shown in FIGURE 7. In these studies the fractions were treated with the blendor for 6 minutes (FIGURE 7, top row) or with 5.0 per cent Triton X-100 (FIGURE 7, bottom row). The treated samples were not centrifuged prior to electrophoresis. Samples subjected to the blendor showed only component "S" (FIGURE 7, top row). This component was concentrated in the mitochondrial-lysosomal fraction. Samples treated with Triton X-100 showed both components "S" and "B" (FIGURE 7, bottom row). Both components were concentrated in the mitochondrial-lysosomal fraction.

Differential extraction of component "B" and reconstitution of the total electrophoretic pattern. Twenty per cent aqueous homogenates (wt./vol.) of rat liver were sonicated for 30 minutes. The resulting sonicate was added to 10 volumes of acetone at −70°C. The precipitate was collected by filtration under suction, washed repeatedly with cold acetone (−70°C.), and dried under vacuum. The resulting acetone powder was suspended in 10 volumes of ice-cold distilled water (wt./vol.). This slurry was centrifuged at $10^6 \times$ g for 60 minutes. The precipitate was resuspended in a volume of ice-cold distilled water equal to that used previously and was recentrifuged. This procedure was repeated twice more. Following the fourth wash and centrifugation, the precipitate was resuspended in a volume of distilled water equal to that used for the first wash. An aliquot of this suspension (A) was treated with Triton X-100 (5.0 per cent). This extract was then examined by electrophoresis. Such preparations showed only the presence of component "B" (FIGURE 8, *a*). Supernatants obtained from blendor-treated whole homogenates (B) were treated with Triton X-100 (5.0 per cent) prior to electrophoresis; these showed only component "S" (FIGURE 8, *b*). A 1:1 mixture of A and B resulted in an electrophoretic pattern typical
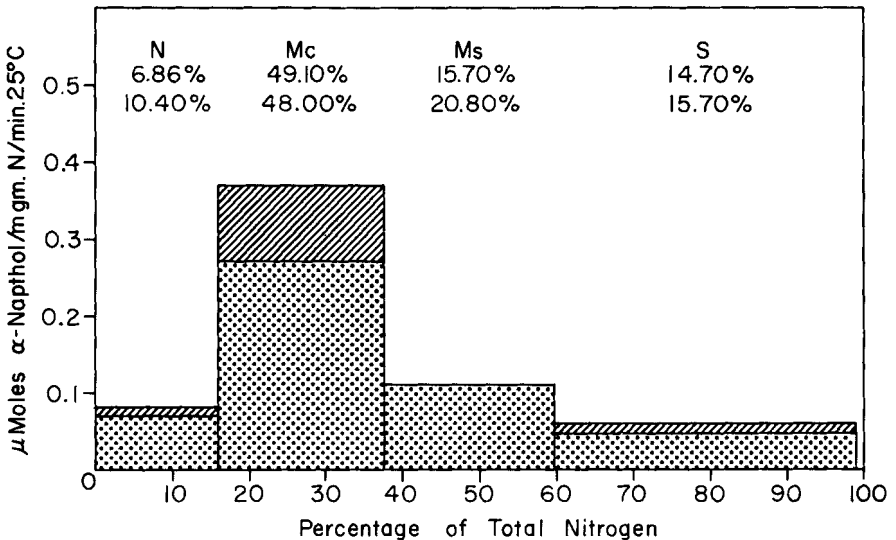

FIGURE 6. Distribution of total acid phosphatase among cell fractions following blendor treatment for 6 minutes (*stippled*) or treatment with 5.0% Triton X–100 for 30 minutes (*cross hatched*). Values above each bar refer to per cent of total activity in the various fractions following Triton X–100 treatment (top row) and blendor treatment (bottom row). *N*, Nuclear fraction; *Mc*, mitochondrial fraction; *Ms*, microsomal fraction; *S*, soluble fraction.
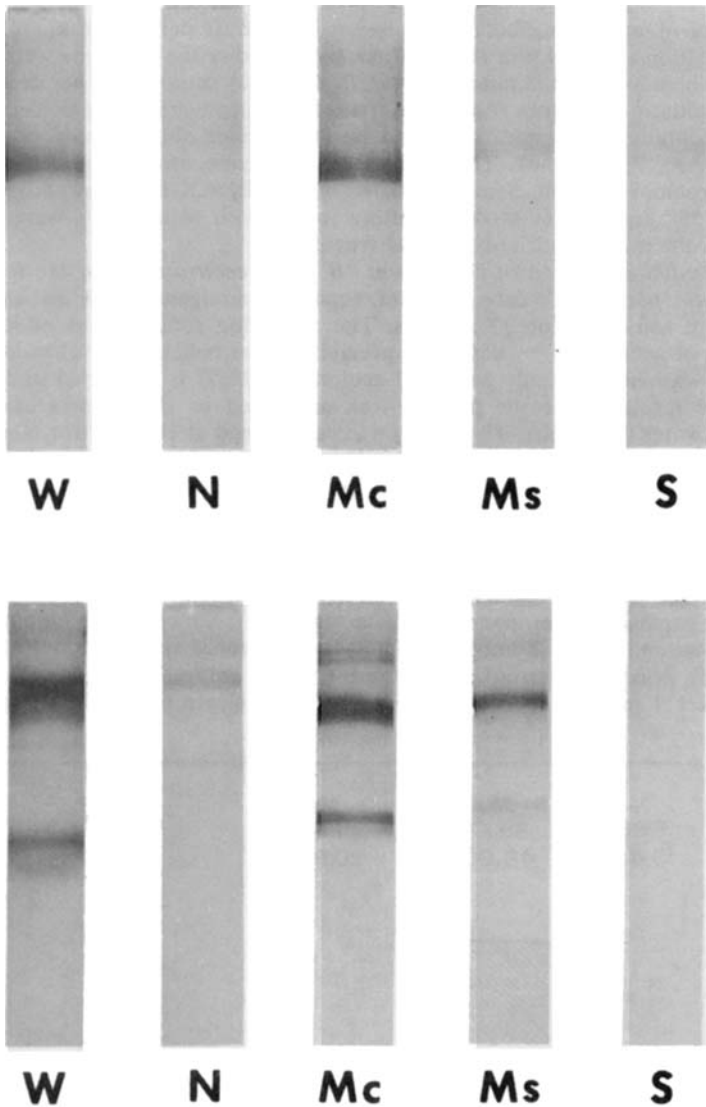
FIGURE 7. Distribution of electrophoretically mobile acid phosphatase among cell fractions following blendor treatment for 6 minutes (top) or treatment with 5.0% Triton X–100 for 30 minutes (bottom). Fraction samples identical to those used in FIGURE 6. All gels incubated 5 min. 25°C. *W*, whole homogenate, diluted to 10.0%; *N*, nuclear fraction, undiluted; *Mc*, mitochondrial fraction, undiluted; *Ms*, microsomal fraction, undiluted; *S*, soluble fraction, undiluted. Samples used for electrophoresis contained the following acid phosphatase activities in 200 μl. of a mixture of one part sample and two parts sample gel stock (equivalent to 66.6 μl. of the appropriate tissue preparation): Blendor treated (top): *W*, 127 mμM alpha-naphthol in 5 minutes 25°C.; *N*, 26.4 mμM alpha-naphthol in 5 minutes at 25°C.; *Mc*, 122.1 mμM alpha-naphthol in 5 minutes at 25°C.; *Ms*, 52.8 mμM alpha-naphthol in 5 minutes at 25°C.; *S*, 13.2 mμM alpha-naphthol in 5 minutes at 25°C. Triton X–100 treated (bottom): *W*, 168.3 mμM alpha-naphthol in 5 minutes at 25°C.; *N*, 23.1 mμM alpha-naphthol in 5 minutes at 25°C.; *Mc*, 165 mμM alpha-naphthol in 5 minutes at 25°C.; *Ms*, 52.8 mμM alpha-naphthol in 5 minutes at 25°C.; *S*, 16.5 mμM alpha-naphthol in 5 minutes at 25°C.
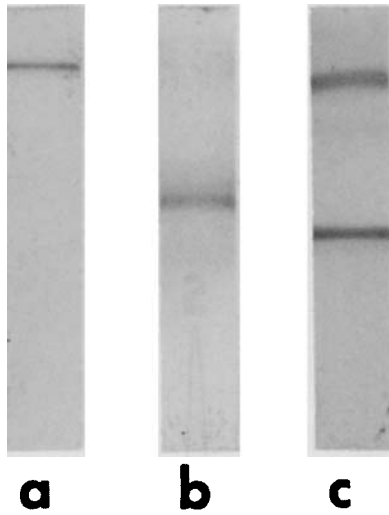
a   b   c

FIGURE 8. Physical separation of component S and component B. (*a*) washed acetone powder derived from sonicated whole homogenate and treated with 5.0% Triton X–100. Incubated for 5 minutes at 25°C. The gel shows only the presence of component B. (*b*) Supernatant of whole homogenate that had been treated with the blendor. Incubated for 5 minutes at 25°C., the gel shows only the presence of component S. (*c*) 1:1 mixture of samples from *a* and *b*. Incubated 10 minutes at 25°C. The gel shows both components S and B. Compare this to FIGURE 2, *e*.

of those obtained with whole homogenates treated with 5.0 per cent Triton X-100 (FIGURE 8, *c*; compare with *f* of FIGURE 1).

The relative amounts of components "S" and "B" in electrophoretic material. Ten per cent homogenates of rat liver (0.25 M sucrose) were treated with 5.0 per cent Triton X-100 and subjected to electrophoresis as usual. After cytochemical localization of acid phosphatase (5 minutes at 25° C.) these preparations were analyzed spectrophotometrically with an appropriate modification of the Beckman DU spectrophotometer[3] (Johnson & Starkweather, 1962; see FIGURE 9). Preliminary measurements indicated that 41.0 per cent of the total acid phosphatase activity in these gels was accounted for by component "B" and 39.0 per cent was accounted for by component "S."

Control procedures in electrophoretic analysis. Alkaline phosphatase activity was examined in mitochondrial-lysosomal fractions treated with 5.0 per cent Triton X-100. After electrophoresis these gels were incubated in 0.1 M Tris-HCl buffer at *p*H 9.5 containing 1 mg. per ml. of sodium alpha-naphthyl acid phosphate and 1 mg. per ml. of Fast Red TR (diazotized 4-chloro-o-toluidine). Just perceptible amounts of reaction product were present in the region of the gel occupied by component "S" after 60 minutes of incubation at 25°C. No reaction was visible in the area occupied by component "B" after this time.

Similar gels were also incubated for 5 and 10 minutes at 25°C. in Gomori type substrates for the visualization of acid phosphatase activity. These gels showed patterns of activity identical to those observed with the coupling-type reaction.

Other gels, when incubated in reaction mixtures containing Diazo Garnet GBC but no substrate, were unreactive after 1 hour at 25°C.
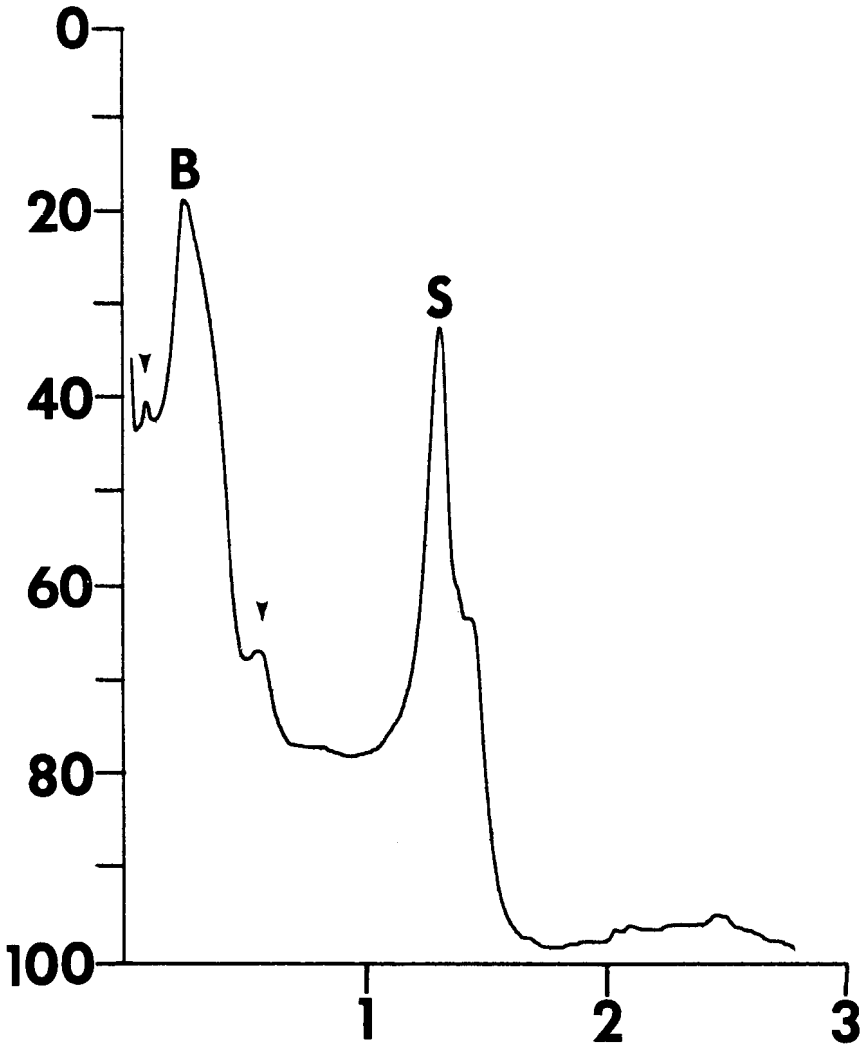
FIGURE 9. Densitometric tracing on an acid phosphatase pattern derived from a 10.0% whole homogenate of rat liver that had been treated with 5.0% Triton X-100. Gel was incubated for 5 minutes at 25°C. Measured at 540mμ in 7.5% acetic acid. Ordinate, % Transmission; abscissa, distance from origin in centimeters. Origin (cathode) lies at the ordinate line. *S*, component S; and *B*, component B. *Arrows* indicate minor components. Compare this tracing with FIGURE 1, *f*. Shoulder on anode face of peak *S* is due to the presence of minor satellite bands. (Scanning mechanism available from John D. Johnson, Ann Arbor, Mich.)

Discussion

The experiments reported in this paper suggest that two major groups of acid phosphatases are present in rat liver cells. These enzyme categories are resident within a particulate component and their release is accomplished by physical disruption of the restrictive element. One category of acid phosphatase (Component "S") appears to be present in a soluble form and is released by physical

treatment of the particle. The other category of enzyme (Component "B") appears to be firmly bound to the structure of the particle and is effectively solubilized only by detergent treatment.

Minimal acid phosphatase activity was demonstrable by quantitative or electrophoretic means when native mitochondrial-lysosomal material was analyzed. Disruption of these particles by physical means released 55.0 to 65.0 per cent of the total activity of the fraction into the unsedimentable (or soluble) phase, permitting the electrophoretic separation of one component of acid phosphatase activity (S). It is clear that such treatment leaves an appreciable fraction of the total activity of acid phosphatase associated with sedimentable structure. Much of the activity of this sedimentable enzyme is measurable by routine quantitative means but is not demonstrable by electrophoresis. Treatment of the mitochondrial-lysosomal fraction with 5.0 per cent Triton X-100 placed virtually all quantitatively determined acid phosphatase activity in the soluble or unsedimentable phase. This treatment simultaneously made possible the electrophoretic separation of two categories of the enzyme. One of these was identical to that released by physical disruption of the particles (component "S"); the other, component "B," represented a unique enzyme released only by appropriate detergent treatment.

This interpretation assumes both components of acid phosphatase activity to be resident within the same population of particles. In the present work no attempt was made to separate lysosomal particles from mitochondrial particles. The studies of deDuve (1959), however, render unlikely the possibility of an association of acid phosphatase with mitochondria. Likewise, microscopic cytochemical studies indicate that hepatic acid phosphatase is associated only with lysosomelike structures (Novikoff, 1960; Essner & Novikoff, 1961, 1962; Holt, 1959; Holt & Hicks, 1961). The fractionation data presented here are consistent with the assumption that both categories of acid phosphatase are resident within a single population of particles. Neither quantitative nor electrophoretic determinations gave evidence of disparate behavior on the part of these enzymes in terms of sedimentation characteristics. There is, however, no assurance that, within this population, the two components reside in the same particle. The question of heterogeneity of the lysosomal population is at present open. deDuve (1959) has pointed out that the distributions of lysosomal enzymes, "whether determined on the basis of sedimentation rate or of density, are not identical." Electron microscopic cytochemical localization of acid phosphatase (Holt & Hicks, 1961) indicates that not all dense hepatic bodies (lysosomes) contain demonstrable acid phosphatase. The presence of acid phosphatase in lipofucsin bodies and in the "large heterogeneous bodies" of Kupffer cells (Essner & Novikoff, 1961) likewise suggests a source of heterogeneity within the acid phosphatase containing particles derived by centrifugation. It is, however, most unlikely that appreciable contribution to the electrophoretic pattern could have been made by enzyme derived from either source. Kupffer cells or other components of the reticulo-endothelial system contribute only about 2.0 per cent of the total acid phosphatase activity of whole rat liver (Wattiaux et al., 1956). Lipofucsin bodies are rare in the hepatic cells of young adult rats.

The relative amounts of component "S" and component "B" in whole liver tissue can only be estimated. Twenty per cent homogenates of whole liver tissue, when sonicated as usual, showed 70.5 per cent of the total activity in the supernatant phase and 29.5 per cent of the total activity in the sediment. Assuming minimal adsorption of soluble enzyme (component "S") to this sediment, the activity recovered in the supernatant phase in such an experiment should be a

rough measure of the relative amount of component "S" in the whole tissue. Preliminary quantitation of electrophoretic material, however, indicated that the activity (based on planimetric integration of densitometric tracings) was nearly evenly divided between component "S" (39.0 per cent) and "B" (41.0 per cent). Neither method of estimation is satisfactory. Quantitative assay of homogenates and fractions measures acid phosphatase activity other than that assignable to component "S" or "B." Electrophoretic analysis cannot account for immobile enzyme or for cathodally migrating enzyme. In spite of such problems of interpretation, it may be concluded that an appreciable fraction of the total acid phosphatase of rat liver is represented by component "B." Available data indicate this fraction to be 30.0 to 40.0 per cent of the total.

The findings of the present study are at variance with the interpretation generally given to the nature of lysosomal acid phosphatase (deDuve, 1959). The enzyme is assumed to be contained within the lysosome in such a condition that it is released in a soluble form following rupture of the particle membrane. Total acid phosphatase activity, however, is consistently greater than that of unsedimentable (soluble) enzyme (Berthet et al., 1951). The disparity between total activity and unsedimentable activity is accounted for on the basis of secondary adsorption of enzyme (Berthet et al., 1951; Appelmans & deDuve, 1955; deDuve, 1959). The quantitative data of the present study are completely compatible with this interpretation. The electrophoretic data are, however, at odds. They indicate, rather, that an appreciable portion of the acid phosphatase activity that is recovered in sediments after particle disruption is distinct from that released into the unsedimentable phase. The former activity (component "B") can be effectively solubilized only by high concentrations of Triton X-100. It, therefore, differs from the more readily solubilized enzyme (component "S") both in terms of electrophoretic mobility and in the nature of its physical associations with lysosomal structure.

The nature of the binding of component "B" is open to question. That the binding of this component is remarkably firm is attested to by the failure of strenuous physical treatment to dislodge it. Extensive extraction with acetone and carbon tetrachloride likewise effected no perceptible solubilization of this component. It was readily extracted by high concentrations of Triton X-100 and to a lesser extent by digitonin. Minimal solubilization was achieved with butanol. These treatments have in common the ability to disperse lipoprotein complexes (Morton, 1955). Within the lysosome such complexes would be expected to be found in the lysosomal membrane or in the lysosomal matrix (Koenig, 1962). At present no decision between these alternatives can be made. It is however, suggestive that many electron micrographs of acid phosphatase localization show the enzyme to be situated around the periphery of the particle in close association with the lysosomal membrane (Essner & Novikoff, 1961, 1962). Preparative procedures employed in such studies might well remove readily soluble enzyme while leaving firmly bound enzyme in situ.

Solubilization of a chromatographically distinct acid phosphatase by 0.1 per cent Triton X-100 has been reported by Barka (1961b). The Triton X-100 solubilized enzyme was concentrated in sediments derived from distilled water homogenates of rat liver. Such results are compatible with those presented here, although the extent of solubilization by Triton X-100 was only slightly greater than reported for distilled water alone. The modest release of the chromatographically distinct acid phosphatase was doubtless due to the low concentration of detergent employed by Barka (1961b). Solubilization of acid phosphatase is minimal at a 0.1 per cent concentration of Triton X-100. The common use of

low concentrations of Triton X-100 for the solubilization of acid phosphatase is due to historical accident (Glassman, 1950), to the fact that low concentrations are as effective in terms of total activity values as higher ones (Walker & Levvy, 1953; Wattiaux & deDuve, 1956), and to problems of quantitative assay in the presence of elevated levels of detergent (Wattiaux & deDuve, 1956). The methods of quantitative assay and electrophoresis employed by us are compatible with concentrations of Triton X-100 as high as 10.0 per cent. There was no evidence that high levels of this detergent were deleterious to acid phosphatase activity.

The degree of heterogeneity of acid phosphatase of mammalian tissues is a moot question. Barka (1961a) has demonstrated 3 electrophoretically distinct acid phosphatases in crude aqueous homogenates of rat liver and 4 chromatographically distinct (Barka, 1961b) components in the same material after treatment with 0.1 per cent Triton X-100. Three electrophoretically distinct forms of the enzyme have been reported in rat brain homogenates in 0.3 per cent Triton X-100 (Anderson et al., 1962). Two major and two minor groups of acid phosphatase have been reported here.

The reality of this heterogeneity is an important question. There has been a tendency to maximize the number of enzymatic sites demonstrable electrophoretically and to assume that the patterns seen represent minimal resolutions of enzyme heterogeneity. In the case of the lactate dehydrogenase isozymes, certain conditions of electrophoresis lead to the formation of multiple sites (Markert, 1963). Such site multiplication is presumably due to minor molecular change such as combination of the isozymes with different amounts of cofactor (Fritz & Jacobson, 1963). Increased heterogeneity within groups of esterase isozymes has been induced by treatment with iodoacetamide (Allen, personal communication). The satellite bands observed here (particularly associated with component "S") are probably due to minor structural changes (FIGURE 2, e). The cleavage of component "S" into a clear doublet by prolonged sonication supports this contention. The significance of the double configuration of component "B" is not clear. There is little possibility that the two major electrophoretic components of acid phosphatase reported here are artifacts of preparation. The fact that these components could by physically separated and independently demonstrated electrophoretically is evidence in point. S. L. Allen et al. (1963a) have succeeded in demonstrating multiplicity in the acid phosphatases of Tetrahymena. Studies of substrate specificities, inhibitor sensitivities, growth conditions, and cellular localization (Allen et al., 1963a), physicochemical properties, and genetic control (Allen et al., 1963b) indicate that these acid phosphatases represent a family of enzymes and that much, if not all, of the observed heterogeneity is biologically meaningful. On the basis of the limited data available, molecular multiplicity may be assumed to be characteristic of the acid phosphatases.

Summary

The acid phosphatases of rat liver mitochondrial-lysosomal fractions have been examined by quantitative and electrophoretic means. Disruption of mitochondrial-lysosomal material by freeze-thawing, sonication, or by blendor treatment released approximately 55.0 to 65.0 per cent of the total acid phosphatase activity of the fraction into the unsedimentable phase. Electrophoretic preparations of this material showed a single acid phosphatase-active site. Treatment of mitochondrial-lysosomal fractions with 5.0 per cent Triton X-100 released 98.0 per cent of the total acid phosphatase activity of the fraction into the unsedi-

mentable phase. Electrophoretic preparations of this material showed two major sites of acid phosphatase activity. One of these was identical to that resolved following physical disruption. The other site was characteristically seen only after treatment with Triton X-100. This acid phosphatase was also released by treatment with digitonin but to a lesser extent. Quantitative and electrophoretic examination indicated that both components of acid phosphatase were concentrated in the mitochondrial-lysosomal fraction. Physical separation of the acid phosphatase released by detergent treatment from the acid phosphatase released by physical disruption was achieved. It was concluded that two categories of acid phosphatase may reside in lysosomal particles. These phosphatases differ in the nature of their binding to lysosomal structure as well as in their electrophoretic properties.

References

ALLEN, S. L. Personal Communication.

ALLEN, S. L., M. S. MISCH & B. M. MORRISON. 1963a. Genetic control of an acid phosphatase in Tetrahymena: Formation of hybrid enzyme. Genetics 48: 1635–1658.

ALLEN, S. L., M. S. MISCH & B. M. MORRISON. 1963b. Variations in the electrophoretically separated acid phosphatases of Tetrahymena. J. Histochem. Cytochem. 11: 706–719.

ANDERSON, J. P., S. K. SONG & N. CHRISTOFF. 1962. The cytochemistry of acid phosphatase in neural tissue. In Proc.. IV Intern. Congr. Neuropathology 1: 75–79.

APPLEMANS, F. & C. DEDUVE. 1955. Tissue-fractionation studies. 3. Further observations on the binding of acid phosphatase by rat-liver particles. Biochem. J. 59: 426–433.

BARKA, T. 1961a. Studies of acid phosphatase. I. Electrophoretic separation of acid phosphatases of rat liver on polyacrylamide gels. J. Histochem. Cytochem. 9: 542–547.

BARKA, T. 1961b. Studies of acid phosphatase. II. Chromatographic separation of acid phosphatases of rat liver. J. Histochem. Cytochem. 9: 564–571.

BERTHET, J., L. BERTHET, F. APPLEMANS & C. DEDUVE. 1951. Tissue fractionation studies. 2. The nature of the linkage between acid phosphatase and mitochondria in rat-liver tissue. Biochem. J. 50: 182–189.

DEDUVE, C. 1959. Lysosomes, a new group of cytoplasmic particles. In Subcellular Particles: 128–159. T. Hayashi, Ed. Ronald Press. New York, N. Y.

ESSNER, E. & A. B. NOVIKOFF. 1961. Localization of acid phosphatase activity in hepatic lysosomes by means of electron microscopy. J. Biophys. Biochem. Cytol. 9: 773–784.

ESSNER, E. & A. B. NOVIKOFF. 1962. Cytological studies on two functional hepatomas. J. Cell Biol. 15: 289–312.

FRITZ, P. F. & K. B. JACOBSON. 1963. Lactic dehydrogenase: subfractionation of isozymes. Science 140: 64, 65.

GLASSMAN, H. 1950. Hemolytic activity of some nonionic surface-active agents. Science 111: 688, 689.

GOMORI, G. 1952. Microscopic Histochemistry. Univ. Chicago Press. Chicago, Ill.

GOMORI, G. 1953. Human esterases. J. Lab. Clin. Med. 42: 445–453.

GOMORI, G. 1955. Preparation of buffers for use in enzyme studies. In Methods in Enzymology, Vol. I. : 138–146. S. Colowick and N. Kaplan, Eds. Academic Press. New York, N. Y.

HOLT, S. J. 1959. Factors governing the validity of staining methods for enzymes, and their bearing upon the Gomori acid phosphatase technique. Exptl. Cell Res. Suppl. 7: 1–27.

HOLT, S. J. & R. M. HICKS. 1961. The localization of acid phosphatase in rat liver cells as revealed by combined cytochemical staining and electron microscopy. J. Biophys. Biochem. Cytol. 11: 47–66.

JOHNSON, J. D. & W. STARKWEATHER. 1962. A simple, improved microdensitometric recording method for slide mounted electrophoretic systems. Proc. 10th Ann. Detroit Conf. Assoc. Anal. Chemists, Paper #15.

KOENIG, H. 1962. Histological distribution of brain gangliosides: lysosomes as glycolipoprotein granules. Nature 195: 782–784.

LEVY, M. 1936. Studies on enzymatic histochemistry. XVII. A micro Kjeldahl estimation. Compt. Rend. Trav. Lab. Carlsberg 21: 101–110.

MARKERT, C. L. 1963. Lactate dehydrogenase isozymes: dissociation and recombination of subunits. Science 140: 1329–1330.

MORTON, R. K. 1955. Methods of extraction of enzymes from animal tissues. In Methods in Enzymology, Vol. I. : 25–51. S. Colowick and N. Kaplan, Eds. Academic Press. New York, N. Y.

NOVIKOFF, A. B.   1960.   The liver cell. Some new approaches to its study. Am. J. Med. 29: 102–131.

NOVIKOFF, A. B.   1961.   Lysosomes and related particles. *In* The Cell, Vol. II. : 423–488. J. Brachet and A. Mirsky, Eds. Academic Press. New York, N. Y.

NOVIKOFF, A. B. & E. ESSNER.   1962.   Pathological changes in cytoplasmic organelles. Fed. Proc. 21: 1130–1142.

ORNSTEIN, L. & B. DAVIS.   1962.   Disk Electrophoresis. Parts I and II. Distillation Products Industries. Rochester, N. Y.

SMITHIES, O.   1959.   Zone electrophoresis in starch gels and its application to studies of serum proteins. *In* Advances in Protein Chemistry, Vol. 14. : 65–113. C. Anfinsen, M. Anson, K. Bailey, and J. Edsall, Eds. Academic Press. New York, N. Y.

WALKER, P. & G. LEVVY.   1953.   The preparation and properties of β-glucuronidase. 6. Activity in rat liver preparations. Biochem. J. 54: 56–65.

WATTIAUX, R., P. BAUDHUIN, A. M. BERLEUR & C. DEDUVE.   1956.   Tissue fractionation studies. 8. Cellular localization of bound enzymes. Biochem. J. 63: 608–612.

WATTIAUX, R. & C. DEDUVE.   1956.   Tissue fractionation studies. 7. Release of bound hydrolases by means of Triton X–100. Biochem. J. 63: 606–612.