

## STUDIES ON ISOLATED CELL COMPONENTS

### XVI. The Distribution of Acid Phenyl Phosphatase Activities in Rat Liver Brei Fractionated in the Zonal Ultracentrifuge

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#### ABSTRACT

The zonal ultracentrifuge has been used to separate the major components of rat liver brei (soluble phase, ribosomes, microsomes, mitochondria, membranous fragments, and nuclei) during one centrifugation, by using a 1200 ml sucrose gradient varying linearly with radius from 17 to 55 per cent (w/w) with a "cushion" of 66 per cent sucrose at the rotor edge at speeds up to 30,000 RPM. Liver brei was found to contain a family of phosphatases (phenol disodium phosphate substrate, sodium malonate buffers and Turgitol NPX, a non-ionic detergent). Activity maxima at pH 4.1 and 5.9 were observed in untreated brei prepared in 0.25 M sucrose. The addition of the non-ionic detergent Turgitol NPX selectively caused the release of considerable additional activity between these optima. The activity measured at pH 4.1 was primarily associated with the cytoplasmic granules, while the activities at pH 4.8, 5.4 and 5.9 were found in both soluble phase and particulate-mitochondria and membranous fractions. The activities present beyond the region of the gradient occupied by the soluble phase (sample layer) were all bound to particles sedimentable at 105,536 *g* (average) in the preparative ultracentrifuge. The data suggest that the different activities are not similarly distributed between soluble phase and particulate fractions. When the data are expressed in terms of specific activity, the area in the gradient between the microsomes and mitochondria now appears richest in all the acid phenyl phosphatase activities measured, while the soluble phase and larger particulate fractions appear relatively poor in activity. This part of the gradient is occupied by small, dense granules which may be the so called lysosomes. Pretreatment of the brei with Turgitol NPX prior to fractionation in the zonal ultracentrifuge resulted in the solubilization of acid phenyl phosphatase activities (almost all the activity was in the sample zone of the gradient) and the non-specific destruction of the formed elements of the brei. Essentially all of the activities present in the original brei measured under these conditions were recovered after zonal ultracentrifuge fractionations.

A major objective of research in cellular physiology has been to determine the intracellular molecular architecture (*e.g.* the distribution of enzymes and other molecular species) and to relate ultrastruc-

ture to function. This endeavor has involved the synthesis of two main lines of investigation (18, 30): (*a*) morphology and *in situ* localization by means of histochemistry (42), and (*b*) biochemical

analysis of isolated cellular components (3, 21, 23). For example, de Duve (19, 22) and his associates postulated the existence of a new family of subcellular particles in rat liver, called lysosomes, on the basis of the enzymatic analysis of brei fractionated by differential and density gradient centrifugation. The lysosomes are believed to be intermediate in size between microsomes and mitochondria and to contain a variety of acid hydrolases capable of degrading most of the major constituents of the cell. One of these hydrolases, "acid phosphatase," has been shown to be localized *in situ* in rat liver within granules having the morphological characteristics of the postulated lysosomes, by histochemical staining in both the light and electron microscopes (17, 24, 29, 39, 40).

Simple sedimentation methods yield cross-contaminated preparations. Extensive handling and recentrifugation is therefore required to obtain "purified" fractions (3, 21, 23). Use of high-speed density gradient centrifugation has greatly improved the obtainable resolution (11, 19, 20). This work, however, was restricted by the small volume contained in the SW-39 swinging-bucket rotor, limitations that have been largely overcome by the recent development of the zonal ultracentrifuge (5, 6). This device is capable of spinning a 1.6 liter gradient at speeds up to 30,000 RPM with a force in excess of 51,000 *g* at the rotor edge, and has achieved high-resolution separation of the subcellular components (soluble phase, ribosomes, microsomes, mitochondria, membranous fragments, and nuclei) from gram quantities of tissue during a single centrifugation.

The key enzyme to the development of the lysosome concept has been "acid phosphatase." This enzyme was usually measured with  $\beta$ -glycerol phosphate as substrate at pH 5.0 using either acetate (27) or acetate-borate-cacodylate buffers (16). However, considerable evidence now exists that "acid phosphatase" in rat liver is not a single enzyme but rather a family or system of enzymes with activity maxima covering a wide region of the acid range (1, 7, 9, 10, 26, 33, 37). Consequently, assays made at a single hydrogen ion concentration might easily lead to the development of an oversimplified picture.

The present investigation was undertaken to re-examine the distribution of "acid phosphatase" in rat liver brei with respect to (a) the heterogeneity of enzymes showing phosphomonoesterase activity over a wide range of hydrogen ion concentrations, and (b) the high resolution-mass isolation of sub-

cellular components that can be obtained with the zonal ultracentrifuge. The results of this study have been reported briefly elsewhere (47).

## MATERIALS AND METHODS

Adult male Sprague-Dawley rats were allowed to drink and feed freely until killing by a blow over the head and decapitation. The livers were perfused with cold Locke's solution (2) and homogenized in a manually operated Potter and Elvehjem grinder in the presence of cold 8.5 per cent sucrose. Approximately a dozen up and down strokes were required to achieve a complete disruption of the tissue. Large fragments of connective tissue were removed by filtration through several layers of cheesecloth.

The brei was fractionated in the zonal ultracentrifuge rotor B II using a 1200 ml sucrose gradient varying linearly with radius from 17 to 55 per cent (w/w) with a "cushion" of 66 per cent sucrose at the rotor edge at speeds of 10,000 to 30,000 RPM for 15 to 180 minutes (4, 6). All operations, including introduction of the sample layer in a short gradient (8.5 to 17 per cent sucrose) and the recovery of the gradient with its separated concentric zones of particles, were accomplished while the rotor was rotating at 5,000 RPM.

The presence of cellular components was determined by continuously analyzing the gradient for ultraviolet absorbance as it emerged from the rotor. The recordings were made directly at 260  $m\mu$  (without dilution) on the gradient as it passed through a quartz flow cell with a 0.2 cm light path. The data presented in this paper are based on the computed absorbance in a 1.0 cm light path.

Acid phosphatase activities were measured automatically with the Technicon Autoanalyzer (49) by a modified King-Armstrong procedure (31, 43). The module assembly used in the present study was similar to the one devised for alkaline phosphatase (36), and was identical with the one recommended by Technicon Instruments Corp. for the clinical assay of acid phosphatase in human serum. The concentration and flow rates of the reagents and the incubation temperature are shown in Table I. The substrate was disodium phenyl phosphate (Technicon Instruments Corp., Chauncey, New York). In this method, the free phenol produced is condensed rapidly with 4-amino-antipyrine followed by oxidation with  $K_3Fe(CN)_6$  under alkaline conditions. The reddish reaction product is measured at 505  $m\mu$ . Sodium malonate (Eastman Organic Chemicals, Rochester, New York) was used to buffer over the range pH 2.6 to 6.6. However, the substrate disodium phenyl phosphate is itself a powerful buffer (32) and contributes significantly to the buffer capacity of the system. The actual pH of the reaction mixture was determined after mixing the reagents in the same pro-

portions employed in the automated assay system and incubation at 37°C. To release particle-bound enzymes the non-ionic detergent 0.2 per cent Turgitol NPX<sup>1</sup> (Union Carbide Chemical Corp., New York) was included in the substrate. Assays in conjunction with zonal ultracentrifuge runs were made *in stream* on the gradient as it was recovered from the rotor and also on each of the 40-ml fractions obtained.

The protein content of the isolated fractions was estimated in the Autoanalyzer according to the

TABLE I  
Concentration and Flow Rates of Reagents Used  
in the Automated Assay of Acid Phosphatase

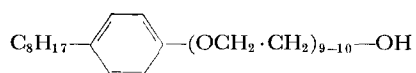
Reagent	Concentration	Flow rates (ml/ minute)*
Sample		0.3
Air		1.2
Sodium Malonate (Buffer)	0.2 M	0.8
Disodium phenylphosphate (Substrate)	12 gm/liter	0.8
NaOH	15 gm/liter	0.8
4-aminoantipyrine	15 gm/liter	0.8
Na <sub>2</sub> CO <sub>3</sub>	20 "	
NaHCO <sub>3</sub>	30 "	
K <sub>3</sub> Fe(CN) <sub>6</sub>	40 gm/liter	0.8
Na <sub>2</sub> CO <sub>3</sub>	30 "	
NaHCO <sub>3</sub>	30 "	

\* Based on specifications provided by Technicon Instruments Corp. on the flow rates through the manifold tubing on the proportioning pump of the Autoanalyzer. Tissue sample incubated with substrate and buffer for 12.5 minutes at 37°C.

adaptation of the Folin-phenol method (34) recommended by Technicon Instruments Corp. This was done on the material left after extraction of acid-soluble nucleotides with 70 per cent ethanol and 1 per cent perchloric acid, lipids with Bloor's solution, RNA with cold 0.5 N perchloric acid, and DNA with hot (70°C) 0.5 N perchloric acid.

Microscopic observations were made on the isolated fractions with a phase-contrast microscope using a dark-medium objective.

<sup>1</sup> This is similar in composition to Triton X100 used by de Duve (16). These products are described as mixtures of alkyl phenyl esters of polyethylene glycol and have the following general formula:—



## RESULTS

### *The Effect of pH on Acid Phenyl Phosphatase Activity in Rat Liver Brei*

According to MacDonald (35) the maximum rate of hydrolysis of phenyl phosphate by murine liver brei was obtained at pH 5.9. In this case, the substrate, pK<sub>2</sub> 5.72 (reference 32), was utilized to buffer the reaction as well. This particular hydrogen ion concentration is just at the limits of the effective buffer capacity range for sodium acetate which is most commonly employed in phosphatase assay systems. As a prelude, therefore, to a study of the distribution of acid phenyl phosphatase in rat liver components separated in the zonal ultracentrifuge, the effects of pH and the non-ionic detergent Turgitol NPX on enzymatic activity were examined.

It is known that "acid phosphatase" activity in rat liver brei is greatly affected by the anions present in the buffer mixture (26). This complication could be minimized by utilizing a single substance, in this case sodium malonate pK<sub>1</sub> 2.88 and pK<sub>2</sub> 5.68 (reference 53), to buffer over the entire pH range of known acid phenyl phosphatase activities in rat liver brei. Furthermore, it appears that malonate may function as a protective agent for the "enzyme" in murine liver brei (35).

The effects of pH and the non-ionic detergent Turgitol NPX on acid phenyl phosphatase activity in rat liver brei using sodium malonate buffers were examined in detail. The results of one of 10 similar experiments on 0.5 per cent rat liver brei can be seen in Fig. 1. In untreated brei, pH maxima were observed at pH 4.1 and 5.9. The addition of detergent to the system resulted in the release of considerable additional activity between these optima. A plot of the per cent change in activity induced by the detergent shows that most of the additional activity was between pH 4.4 and 5.2. This treatment also produced a considerable reduction in activity in the regions between pH 3.1 and 3.7 and pH 6.2 to 7.0.

The sodium malonate used in the assay system proved to be insufficient to overcome the strong buffering action of the disodium phenyl phosphate itself. As can be seen in Fig. 1, the addition of the substrate produces a variable shift of 0.4 to 1.0 pH units. The pH obtained by mixing buffer and substrate, however, was not affected by the presence of liver brei, the addition of Turgitol NPX, or incubation at 37°C for 12.5 minutes. The original

assay system developed by Kind and King (31) incorporated a 20/1 molar ratio of buffer (sodium citrate) to substrate, while the modification of this procedure recommended by Technicon Instruments Corp. utilized only a 3.6/1 ratio. We observed that in the Kind and King (31) procedure the addition of substrate shifted the pH from 4.9 to 5.05, while in the Technicon version the shift was from 4.8 to 5.15.

These data as well as earlier observations in the literature (1, 7, 9, 10, 26, 33, 37, 48) indicated that

gen ion concentrations on enzymatic activity was also determined. The activities were a direct linear function of brei concentration over a range from 0.2 to 1.6 per cent (w/v). This corresponded exactly to the linear range of the phenol assay in this system, 10 to 120  $\mu\text{gm/ml}$ .

The high sucrose concentrations used in the gradients did not appear to affect the phosphatases adversely. Comparison of the pH activity curves in the presence of 8 and 55 per cent sucrose reveals an approximately 5 per cent reduction in the

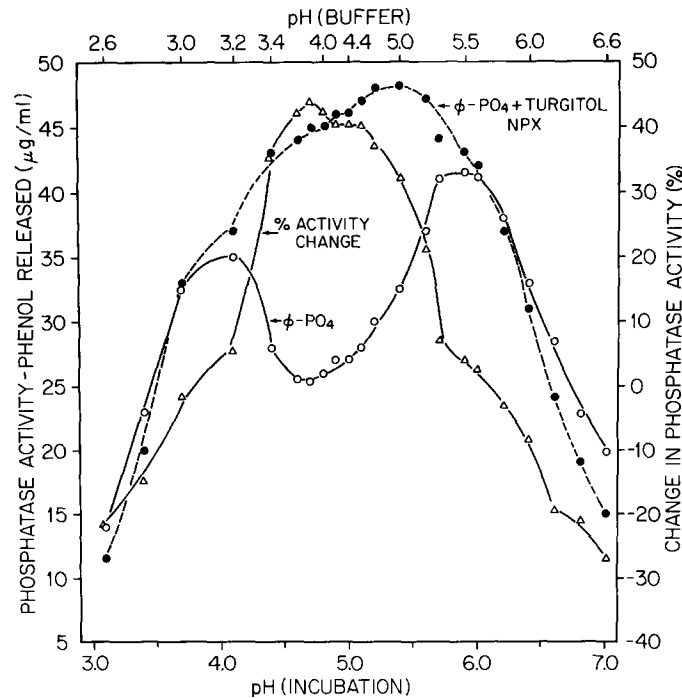


FIGURE 1 pH activity curves for acid phenyl phosphatase activities in rat liver brei in the presence and absence of Turgitol NPX. Buffer: sodium malonate.

more than one enzyme might be contributing to "acid phosphatase" activity in rat liver brei over a wide region of the acid range. It thus appeared necessary to study the distribution of phenyl phosphatase activities at more than one pH. Since it was not practical to run similar, complete pH-activity spectra on each of the some forty 40-ml fractions usually obtained from a typical zonal ultracentrifuge run, it was decided, instead, to monitor phenyl phosphatase activity at pH 4.1, 4.8, 5.4 and 5.9 to obtain some idea of the over-all distribution pattern.

The effect of brei concentration at these hydro-

amount of phenol released, in spite of the fact that the viscosity of 55 per cent sucrose is about 23 times that of 8 per cent sucrose at room temperature.

#### *The Distribution of "Acid Phosphatase" Activities in Zonal Ultracentrifuge Fractions of Rat Liver Brei*

Assays of phosphatase activities were made in liver breis subjected to a wide range of centrifugal fields, 10,000 to 30,000 RPM for 60 to 180 minutes. Under these conditions, separations of subcellular components are achieved by sedimentation to the

isopycnic position for the larger particles and by sedimentation rate for the smaller particles. The results of an experiment in which liver brei was subjected to 22,000 RPM for 120 minutes can be seen in Fig. 2. The phosphatase activities monitored at pH 4.8, 5.4 and 5.9 exhibited a similar distribution pattern. Large amounts of activity were associated with the soluble phase, mito-

of integration, all the activities present beyond the soluble phase peak (sample layer) in the gradient were considered to be particulate. The validity of this assumption will be demonstrated in the discussion of experimental data presented in Fig. 4. The data suggest that the phenyl phosphatase activities monitored at pH 4.1, 4.8, 5.4, and 5.9 have quite different distributions between "soluble" and

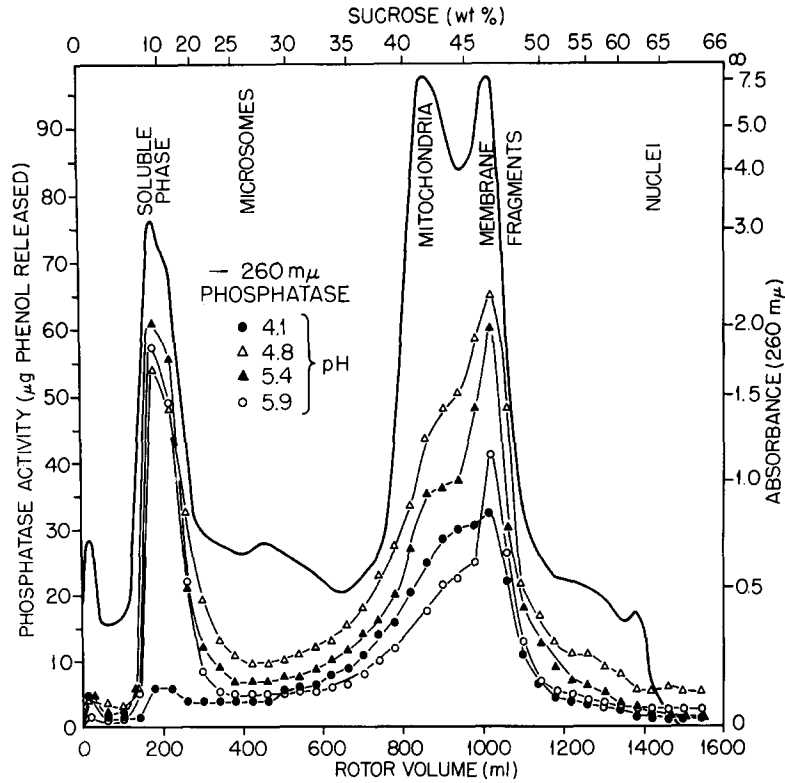


FIGURE 2 Distribution of acid phenyl phosphatase activities at pH 4.1, 4.8, 5.4, 5.9 in rat liver brei subjected to 22,000 RPM for 120 minutes in the zonal ultracentrifuge. Assays made with sodium malonate buffers and in the presence of Turgitol NPX.

chondria and membranous fragment fractions, while the microsomes and especially the nuclei appeared to contain low levels of activity. The activity measured at pH 4.1, however, exhibited a rather different distribution pattern, with just trace amounts observed in the soluble phase, while most of the activity was associated with the particulate, mitochondrial and membranous fractions.

A comparison of the relative amounts of the acid phenyl phosphatase activities in the "soluble" and "particulate" phases obtained in this run (Fig. 2) can be seen in Table II. For the purpose

"particulate" phases in rat liver brei fractionated in the zonal ultracentrifuge.

A rather different distribution pattern of acid phenyl phosphatase in liver brei emerges when the data from this run are expressed in terms of specific activity (microgram phenol released/milligram protein) as can be seen in Fig. 3. The region in the gradient between the microsomes and the mitochondria appears to have the highest concentration of phosphatase activity in terms of total protein. This part of the gradient, as revealed by phase microscopy, contains large numbers of small, dense-

TABLE II  
*Comparison of Soluble Phase and Particulate Acid Phenyl Phosphatase Activities in Rat Liver  
 Brei Fractionated in the Zonal Ultracentrifuge*

Activity is given in  $\mu\text{g}$  phenol released/ml. Percentages in parentheses are expressed in terms of per cent of total activity in the gradient.

	pH			
	4.1	4.8	5.4	5.9
Soluble phase activity	24 (6.8)	156 (19.3)	155 (24.5)	139 (31.2)
Particulate activity	331 (93.2)	652 (80.7)	478 (75.5)	307 (68.8)
Total activity	355	808	633	446
Sol. act./part. act.	0.072	0.239	0.324	0.453

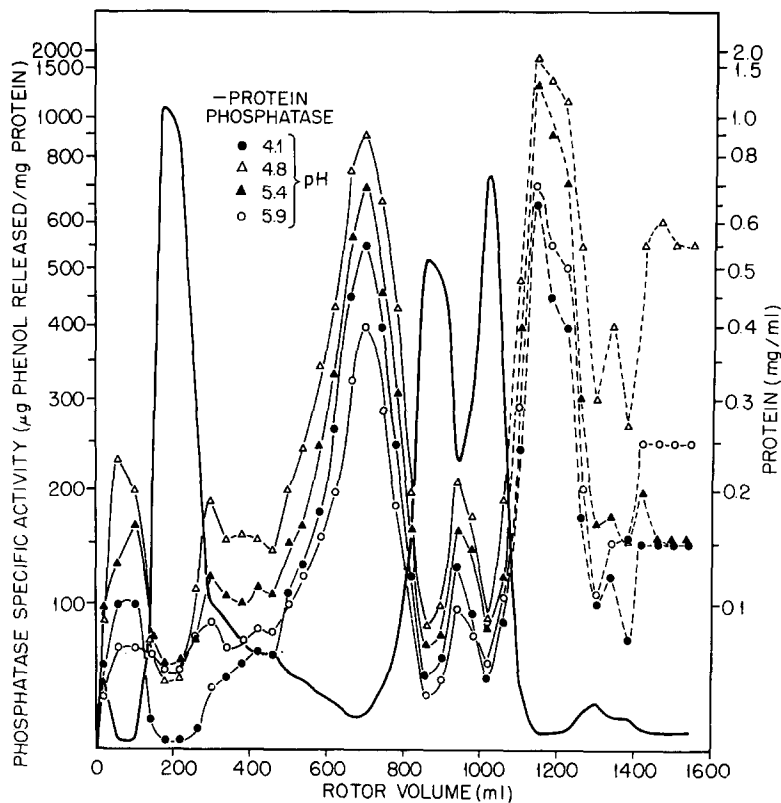


FIGURE 3 Specific activities of acid phenyl phosphatase at pH 4.1, 4.8, 5.4, 5.9 in rat liver brei subjected to 22,000 RPM for 120 minutes in the zonal ultracentrifuge. Protein assays made on material remaining after extraction of acid soluble nucleotides, lipids, RNA, and DNA.

appearing granules. The validity of the data on specific activity in the gradient beyond the membranous fragments fraction is questionable now because the measurements of protein and acid phosphatase are just at the lower limit of resolution of the assay systems.

The significance of the specific activity data

would be jeopardized, however, if the activities found in the intermediate zone were the result of leakage of enzymes out of the larger particles as they were sedimented through the sucrose gradient. Two lines of evidence mitigate this possibility.

First, in low-speed runs (10,000 RPM for 60 minutes) a much larger portion of the activities is

found in the intermediate area. The ultraviolet absorbance in this area is about the same in both high- and low-speed runs. These observations suggest that at least part of the activities are associated with small but dense granules which sediment more slowly than mitochondria and membranous components.

Second, all the activities beyond the soluble phase region were attached to particles (Fig. 4).

taken from aliquots of fractions beyond the soluble phase peak were found to be almost completely devoid of acid phenyl phosphatase activities. Absorbance at  $260\text{ m}\mu$  and distribution of subcellular components in the gradient are shown in Fig. 4 for the purpose of orientation.

Non-ionic detergents such as Turgitol NPX or Triton X-100 (19) are included in the assay systems for "acid phosphatase" to release enzymes

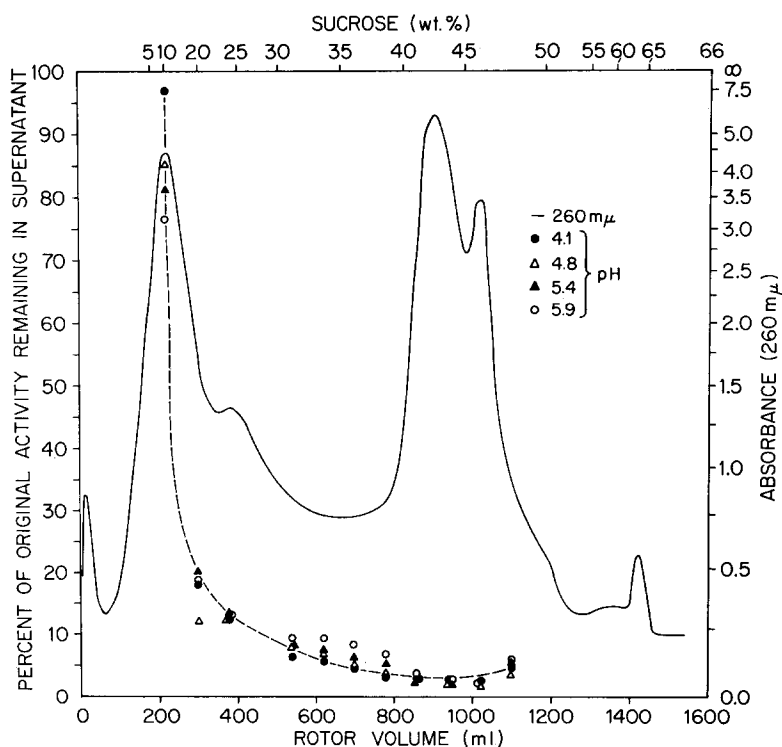


FIGURE 4 Binding of acid phenyl phosphatase activities at pH 4.1, 4.8, 5.4, 5.9 in rat liver brei to particles. Brei initially was subjected to 20,000 RPM for 60 minutes in the zonal ultracentrifuge. Aliquots of fractions centrifuged for 90 minutes at  $144,880\text{ }g_{\text{max}}$ , and supernatants assayed for activity in the presence of sodium malonate buffers and Turgitol NPX.

These data were obtained from liver brei that had been initially subjected to 20,000 RPM for 60 minutes in the zonal ultracentrifuge. The over-all distribution pattern of acid phenyl phosphatase activities was similar to that seen in Fig. 2. Ten-ml aliquots of the isolated fractions were diluted by the addition of 2 ml of 8.5 percent sucrose (to make it possible to sediment particles that had reached their isopycnic position in the gradient), and then centrifuged for 90 minutes at 40,000 RPM (maximum  $144,880\text{ }g$ ) in the Spinco Model L preparative ultracentrifuge. The supernatants

confined within granules and apparently unreactive toward external substrates. It appeared desirable to test this hypothesis by determining whether any acid phenyl phosphatase-containing granules in rat liver brei survive exposure to Turgitol NPX under conditions where the ratio of detergent to liver material is similar to that employed in the assay system. Accordingly, fresh 20 per cent (w/v) rat liver brei prepared in 8.5 per cent sucrose was mixed with an equal volume of ice-cold 20 per cent Turgitol NPX (2 gm detergent in 10 ml of 8.5 per cent sucrose), and a 12.5 ml aliquot was

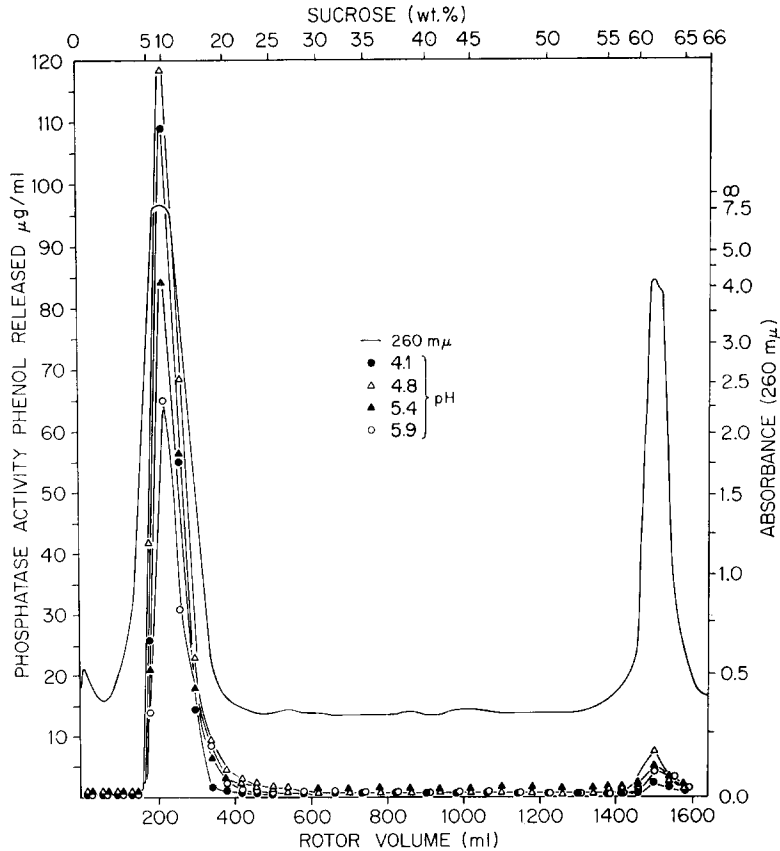


FIGURE 5 Distribution of acid phenyl phosphatase activities at pH 4.1, 4.8, 5.4, and 5.9 in rat liver brei pretreated with the nonionic detergent Turgitol NPX before being subjected to 20,000 RPM for 60 minutes in the zonal ultracentrifuge.

immediately subjected to 20,000 RPM for 60 minutes in the zonal ultracentrifuge. The results of this experiment appear in Fig. 5. Absorbance readings at 260 m $\mu$  revealed the absence of structural elements, microsomes, mitochondria, etc., invariably observed in intact-untreated brei. Most of the acid phenyl phosphatase activities at the hydrogen ion concentrations measured were present in the soluble phase-sample layer in the gradient. Little activity was observed in the material that was sedimented out into the sucrose "cushion." Microscopic observation of this region

of the gradient revealed the presence of large amounts of membranous material, small granules the size of nucleoli, and a few whole cells (Fig. 6). Integration of the phosphatase activities distributed in the gradient showed that a significant fraction was present outside of the soluble phase; *i.e.*, sample layer (Table III). However, these calculations were weighted by the traces of activity which were spread out in the approximately 1000 ml of the gradient between the sample layer and the 66 per cent sucrose "cushion." These traces of activity represented a deflection of about

FIGURE 6 Phase-contrast micrographs of material sedimented out into the 66 per cent sucrose "cushion" after exposure of rat liver brei to Turgitol NPX prior to fractionation at 20,000 RPM for 60 minutes in the zonal ultracentrifuge: *a*, membranous material and small granules; *b*, one of the few whole cells observed.  $\times 1900$ .



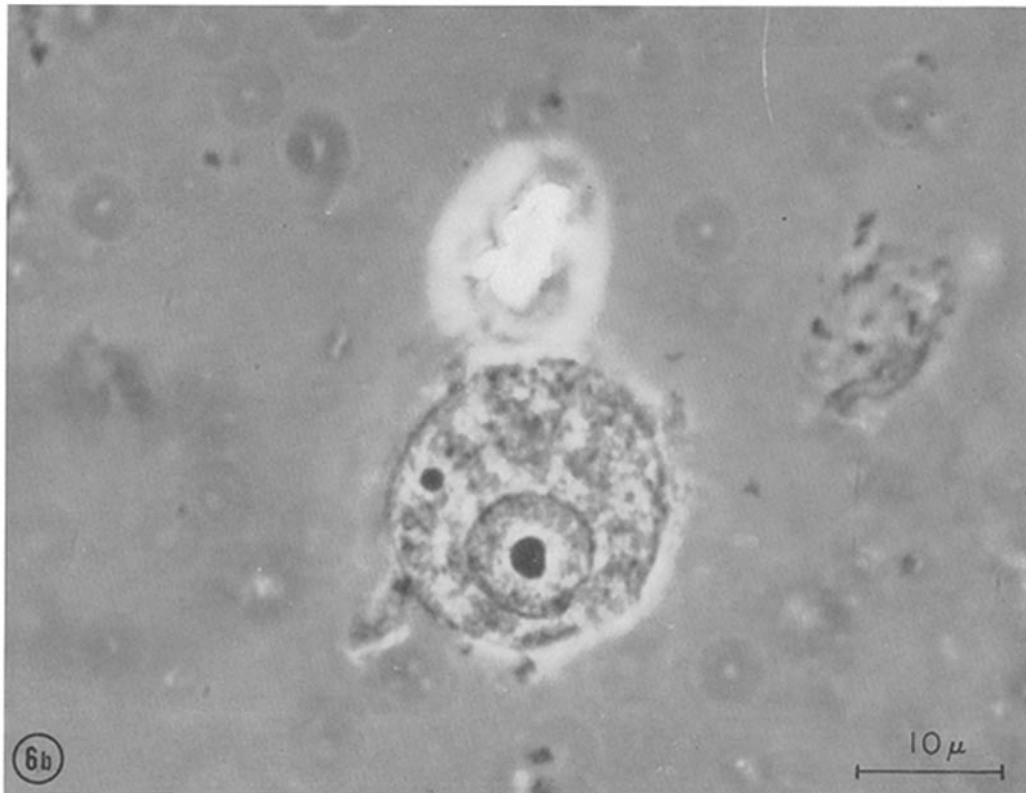
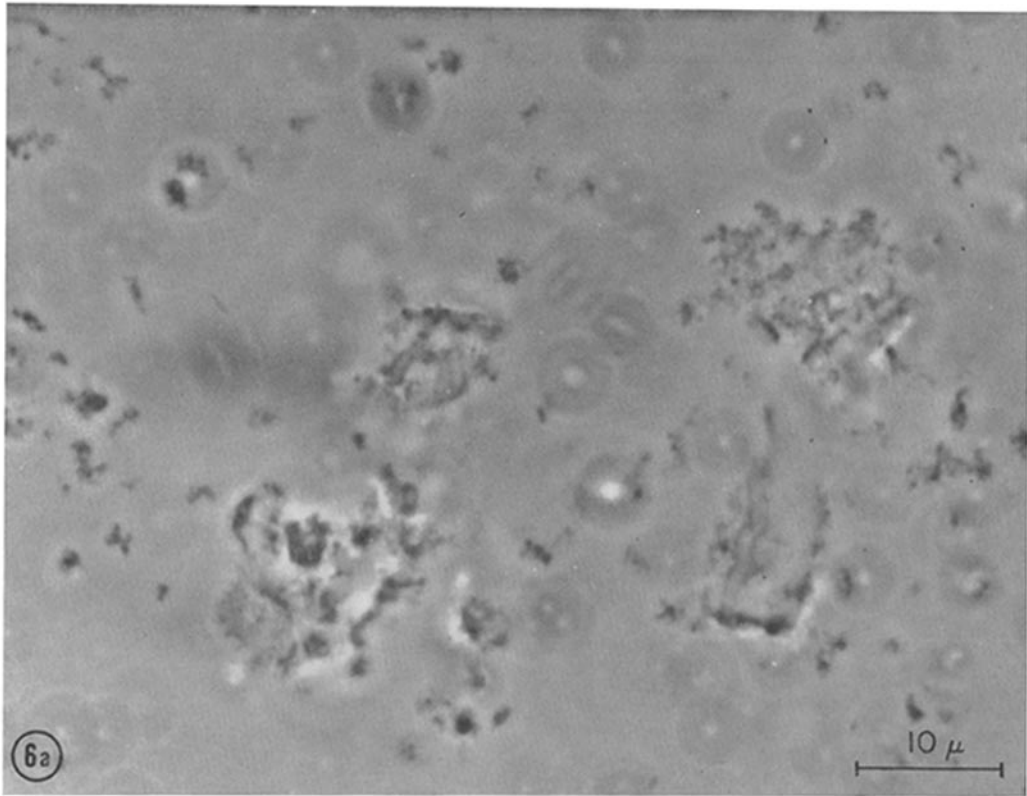


TABLE III

*Effect of Exposure of Rat Liver Brei to Turgitol NPX Prior to Zonal Ultracentrifuge Fractionation on the Distribution of Acid Phenyl Phosphatase Activities*

Data expressed in terms of per cent of total phosphatase activity observed in gradient (0 to 66 per cent sucrose).

Sucrose	pH			
	4.1	4.8	5.4	5.9
<i>Per cent</i>				
1 to 20 (sample layer-soluble activity)	93.2	89.8	79.8	84.5
21 to 54 (bulk of gradient)	4.0	5.1	13.6	7.6
55 to 66 (material in "cush-ion")	2.8	5.1	6.6	7.9

TABLE IV

*Per Cent Recovery of Acid Phenyl Phosphatase Activities in Rat Liver Brei Fractionated in the Zonal Ultracentrifuge*

Centrifuge operated 60 minutes at speed indicated.

Speed	pH			
	4.1	4.8	5.4	5.9
<i>RPM</i>				
10,000	94.6	92.4	98.8	114.3
	97.7	100.8	99.1	97.2
	90.5	98.0	88.6	87.4
	95.8	109.6	105.4	103.6
	96.7	95.4	98.1	104.8
	87.5	96.5	95.8	94.3
15,000	68.8	71.6	75.6	81.3
	103.2	98.5	133.6	
20,000	96.2	90.2		92.5
	86.9	101.2	93.2	91.6
Average recovery	91.8	95.4	98.7	96.3

0.005 OD from the base-line, and the reliability of these data is questionable since small shifts in the base-line could introduce large errors into the final results in assays extending over the greater part of an hour.

The percentage of phosphatase activity of the original brei recovered after zonal ultracentrifuge fractionation was determined for a number of runs. As can be seen in Table IV, essentially all of the original activities at pH 4.1, 4.8, 5.4 and 5.9 were recovered. This is extremely good recovery, considering the large number of individual assays made over a period of several hours. It is also clear that the fractionation has not uncovered the effects of any major activators or inhibitors.

## DISCUSSION

The observations described above tend to emphasize the fact, already well established in the literature (1, 7, 9, 10, 26, 37, 45, 48, 54), that "acid phosphatase" in the livers of rats and other mammals is not a single enzyme but rather constitutes a heterogeneous family of enzymes. This condition is not surprising when one considers the ubiquitous role of reactions involving the hydrolysis or transfer of phosphate esters in cellular metabolism. The common denominator is the ability of these enzymes to hydrolyze the universal substrates, usually alcoholic or phenolic phosphate esters, below pH 7.0 in the standard assay procedures.

Once the heterogeneity of the "acid phosphatase" is recognized, other factors become apparent. The results obtained by the assay for "acid phosphatase" depend upon the assay conditions. Previous workers have observed that the nature of the substrate, pH, anionic species in the buffer mixture, inorganic ions, and organic compounds exert a profound influence on the level of "acid phosphatase" activity (1, 25-27, 35, 38, 52). It thus becomes essential to specify rigorously and in detail all the conditions of assay to avoid spurious comparisons. However, even when these requirements are met, difficulties still remain because more than one enzyme may be contributing to the total activity measured (7, 9, 10, 26, 37).

At the present time it is impossible to estimate with any certainty the exact number and nature of the enzymes contributing to the hydrolysis of phenyl phosphate by rat liver preparations in the acid range. However, at least two enzymes or groups of enzymes appear to be involved: the first, an apparently non-specific phosphatase, present in the soluble phase and lysosomal fractions, which is able to act on phosphoproteins and a variety of other phosphate esters including phenyl phosphate but not on  $\beta$ -glycerophosphate (46); the second, "glucose-6-phosphatase," which appears to act

on phenyl phosphate at approximately 20 per cent of its maximum rate of activity on glucose-6-phosphate and is apparently unreactive towards  $\beta$ -glycerophosphate (12). It is to be expected that at least some of the enzymes which act on  $\beta$ -glycerophosphate should also act on phenyl phosphate, but the relationships remain to be worked out. These questions should be clarified as more of the enzymes capable of hydrolyzing or transferring phosphate esters are isolated in a pure form and their chemical and physical properties ascertained.

It is thus evident that the term "acid phosphatase" is of dubious significance unless all these considerations are kept in mind.

A rat liver homogenate is a heterogeneous and a multicomponent system, and a number of factors must be considered in order to understand the pH-activity data and the zonal centrifuge fractionation data.

1. The pH and the anionic species in the buffer mixture are known to affect the rate of substrate hydrolysis by the enzymes involved (13, 26).
2. The pH and anions of the buffer may act indirectly by affecting the exchange of substrate and reaction products across the semi-permeable membranes of the cytoplasmic granules, or by influencing the stability of these granules under the conditions of assay. For example, it has been reported that pH influences the rate of spontaneous release of "acid phosphatase" activity in granules isolated from rat liver brei (15). These factors may account for the observations in the present study that the detergent failed to release appreciable amounts of additional activity at pH 4.1, yet this activity appeared to be associated with the granular components in zonal ultracentrifuge fractionations. However, the enzyme(s) contributing to this activity may be bound to the surface of the granules. Additional work is necessary to resolve these questions.
3. The pH and particularly the detergent may adversely affect the stability of the enzymes themselves. Glucose-6-phosphatase, for example, appears to be readily inactivated by surface active agents such as deoxycholate (14). This enzyme should contribute at least in part to the peak of phenyl phosphate hydrolysis observed at pH 5.9 in intact brei. According to de Duve (15), the lysosomes appeared to be maximally stable at this pH,

yet in the present study the addition of the detergent Turgitol NPX apparently failed to release additional phenyl phosphatase activity. Furthermore, there does not appear to be a peak of activity associated with the microsomal fraction which apparently contains all the glucose-6-phosphatase in rat liver (22). These results suggest that the activity of this enzyme is abolished either partially or completely by the detergent under the conditions of assay, while additional phenyl phosphatase activity is simultaneously released from the lysosomes and possibly other cytoplasmic granules by the detergent. This conclusion should remain tentative, however, until the effects of Turgitol NPX and malonate buffers on the stability of glucose-phosphatase have been definitely established.

de Duve (19) has proposed that "acid phosphatase" in normal, living hepatic cells is restricted to the interior of a special class of lytic granules known as lysosomes, and is entirely without action on external substrates. In this way "acid phosphatase" and the other acid hydrolases would be prevented from digesting the major biochemical constituents of the cell. According to this view, the "acid phosphatase" activity that is always present in the soluble phase of breis is the result of the rupture of a small part of the lysosomes during homogenization. If all the enzymes contributing to the hydrolysis of phenyl phosphate in the acid range had been confined within a single species of cytoplasmic granules, then two types of results should have been obtained in the present study:

First, one would have expected the addition of detergent to whole brei to result in a uniform activity increase over the entire pH range investigated. This was clearly not the case. Inspection of the pH activity data reveals activity maxima at pH 4.1 and 5.9 in untreated rat liver brei, while the addition of detergent results in the preferential release of additional activities between these optima, and a slight repression at the extremes of the pH spectrum.

Second, one would have expected a constant ratio of "soluble" versus "particulate" phase activities measured at various hydrogen ion concentrations. The data show that the activities measured at pH 4.1, 4.8, 5.4 and 5.9 have quite different distributions.

It should be remembered that very little is

known about the identity of the phosphatases active in the various fractions. This is a crucial factor in interpreting results, in view of the lack of specificity inherent in the use of alcoholic or phenolic substrates. Recent work has suggested that at least a portion of the "acid phosphatase" (38, 41, 48) and "acid ribonuclease" (44) enzymes found in the soluble phase fractions of liver homogenates may be quite different from those associated with the cytoplasmic granules. The observations reported in the present study suggest that this may also be true for the enzymes that hydrolyze phenyl phosphate in the acid range. A large number of compounds are known to selectively inhibit the enzymes contributing to "acid phosphatase" activity (1, 25, 26, 28, 35, 38, 41, 45, 48). Future studies in which these inhibitors are employed in enzymatic assays performed on fractions isolated by improved procedures should help to clarify this question.

Several aspects of the structure-linked latency and release of "acid phosphatase" activity in rat liver brei first reported by de Duve (16, 19, 22) and his colleagues were confirmed in the present study. Treatment of the brei with a nonionic detergent, Turgitol NPX, results in the almost complete solubilization of acid phenyl phosphatase activities. This was shown in the experiment where the liver brei was pretreated with the detergent prior to fractionation in the zonal ultracentrifuge. Furthermore, it was evident that the structural elements normally found in liver brei, microsomes, mitochondria, etc., failed to survive exposure to Turgitol NPX. The significance of the trace amounts of activity apparently trailing out into the bulk of the gradient between the sample layer and the 66 per cent sucrose "cushion" is difficult to assess in the light of the unreliability of data depending upon extremely small deflections from the base-line in the colorimeter. These experiments demonstrate that alkyl phenyl esters of polyethylene glycol detergents solubilize the majority of cell structures and do not rupture lysosomes specifically under the conditions of our experiments.

The data obtained from fractionations in the zonal ultracentrifuge suggest that the various acid phosphatase activities are not equally distributed in the subcellular components of rat liver brei. The activity at pH 4.1 appears to be primarily associated with the cytoplasmic particles, while the activities at pH 4.8, 5.4 and 5.9 are associated

with both the soluble phase and particulate fractions. However, the distribution patterns that one sees depend upon how the data are presented. When the data from a zonal ultracentrifuge fractionation are recalculated and plotted in terms of specific activity, an entirely different picture emerges. This is similar to the method, adopted by de Duve and his colleagues (19, 22), of presenting the data in terms of specific activity within the various fractions that had been obtained by means of differential centrifugation. The region between the microsomes and mitochondria now appears to be richest in phenyl phosphatase activity at all the hydrogen ion concentrations monitored, while the soluble phase and larger particles appear to be relatively poor in activity, suggesting that these regions of the gradient are heavily contaminated with other material.

It is thus evident that specific activity measurements in subcellular fractions isolated from tissues may be misleading, since they reflect the amount of contamination present and do not necessarily indicate the degree to which an enzyme or activity may be concentrated within a given species of particles.

To establish the significance of the observed phenyl phosphatase distribution patterns with respect to the lysosome concept, it is necessary to know exactly how the mitochondria are distributed in the gradient. In the present study mitochondria were identified on morphological criteria in the phase contrast microscope. As a result of the centrifugal forces employed, the mitochondria were sedimented to form a sharp peak at their isopycnic position in the gradient, 43 per cent sucrose. These observations have been confirmed in experiments reported elsewhere (50, 51) using cytochrome-*c*-oxidase as the biochemical marker for the mitochondria. Furthermore, the lower speed zonal centrifuge fractionations carried out in the present study indicate that at least part of the acid phenyl phosphatase activities may be associated with particles which sediment slower than the mitochondrial and membranous components. These results suggest that the region in the gradient between the microsomes and the mitochondria might contain part of the lysosomal complement defined by de Duve as small dense granules free of cytochrome oxidase activity but containing a very high concentration of "acid phosphatase." This conclusion should remain tentative until the development of automated

assay procedures makes it feasible to determine the distribution patterns of the other acid hydrolases also thought to be confined within the lysosomes (19). In addition, new low-speed rotors now under development in this laboratory may make it possible to achieve a better separation of cytoplasmic granules such as lysosomes and mitochondria based on differences in sedimentation rate (8).

de Duve and his associates (11, 19) have recently reported data obtained by density gradient centrifugation which indicate a considerable heterogeneity in "acid phosphatase"- and "acid hydrolase"-containing granules (lysosomes) in rat liver brei. These observations have been confirmed in the present study by the apparently bimodal distribution of acid phenyl phosphatase-containing granules in the sucrose density gradient following fractionation in the zonal ultracentrifuge, which suggests that at least two types of acid phenyl phosphatase-containing particles may be present.

It is evident from the recovery data that the fractionation failed to uncover the effects of any major inhibitors or activators in liver.

It must be recognized that subcellular particles are distributed in the density gradient of the zonal ultracentrifuge on the basis of purely physical characteristics, sedimentation rate and density. Thus several populations of particles may be found in a particular zone by virtue of their physical characteristics under the conditions of centrifugation. For example, on the basis of enzymatic and morphological criteria the mitochondria appear to be restricted to a narrow zone around 43 per cent sucrose (50, 51). A very high level of acid phenyl phosphatase activities is also found in

this zone, part of which, at least, may be associated with granules other than the mitochondria. Preliminary observations in the electron microscope indicate that this zone contains a variety of particles in addition to the mitochondria, including smaller electron-opaque granules and large pieces of the endoplasmic reticulum some of which appear to be attached to the mitochondria. Although the zonal ultracentrifuge has many advantages over earlier centrifugal fractionation procedures, considerable additional work must be done before truly homogeneous populations of subcellular components can be obtained with these systems.

The experiments just described illustrate the kind of work in tissue fractionation that can be accomplished using zonal ultracentrifuge rotors. Furthermore, it is evident that the components of cells can now be visualized in a new way—as a continuous spectrum of the various classes of subcellular particles. Devices of this type have the further advantage of being preparative as well as analytical; subcellular components are readily separated in mass and are available for subsequent chemical and morphological analysis, and the distribution of classes of particles in the gradient might also be used as a diagnostic tool for various types of physiological manipulations.

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