found for the acid phosphatase-containing granules. As will be shown in a subsequent paper (Appelmans, Wattiaux & Duve, 1955), these granules form a special group, entirely distinct from the oxidizing mitochondria and from the microsomes, and are probably represented by a relatively small number of individuals. Their cytological nature is unknown and any new information which could be obtained regarding their structure and enzymic equipment appeared therefore as particularly valuable. These facts have been commented upon also in a recent review (Duve & Berthet, 1954).

Experiments now in progress have thrown some doubt on the above interpretation, showing that the distribution of β-glucuronidase is not identical with that of acid phosphatase. The difference is slight, involving a shift of about 15% of β-glucuronidase from a lighter mitochondrial fraction to the microsomes, but it is reproducible and clearly beyond recognized experimental errors. Studies on tumours and precancerous tissues have also furnished indications that the two enzymes may behave in an independent manner. These investigations, which will be reported in later publications, must be pursued further before allowing definite conclusions. But they are mentioned in view of the important incidence they may have on the final interpretation of the results presented here.

SUMMARY

1. The β-glucuronidase and cathepsin of rat liver share a number of the properties previously described for acid phosphatase. Like this enzyme, they are enclosed within cytoplasmic granules and exert their full activity in an *in vitro* assay only if the granules have been subjected to a treatment which simultaneously releases the enzymes in soluble form.

2. The release of the three enzymes proceeds almost identically in washed particulate preparations activated either by the Waring Blendor, freezing and thawing or exposure to hypotonic media. Incubation at 0° in 0·15M-NaCl, or at 37° and pH 5 in 0·25M sucrose also causes a parallel liberation of β-glucuronidase and acid phosphatase. Cathepsin was not measured in these experiments.

3. Once released, acid phosphatase and β-glucuronidase are easily separable by means of ammonium sulphate fractionation. The partial purification of acid phosphatase is described.

4. In discussing the results obtained, it is concluded that the three enzymes behave essentially as though they were associated within the same granules. However, recent unpublished data suggest that this conclusion may have to be revised.

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Tissue Fractionation Studies

5. THE ASSOCIATION OF ACID PHOSPHATASE WITH A SPECIAL CLASS OF CYTOPLASMIC GRANULES IN RAT LIVER

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In a previous study of the intracellular distribution of acid phosphatase in rat liver, it was found that the bound form of the enzyme, although associated mainly with the mitochondria, is also recovered to some extent with the microsomes (Berthet & Duve, 1951). Similar observations were made by Palade (1951). Other mitochondrial enzymes, such as cytochrome oxidase, do not show this peculiarity, and it seemed of interest to ascertain the cause of the discrepancy. The investigations which were undertaken in order to clarify this point are reported in the present paper. They have led to the discovery that acid phosphatase is attached to a special type of cytoplasmic granules, differing both from the cytochrome oxidase-bearing mitochondria and from the glucose 6-phosphatase-containing micro-

somes. A new fractionation scheme has been developed as a result of these findings. Preliminary reports of these results have appeared elsewhere (Duve, Appelmans & Wattiaux, 1952; Duve, Gianetto, Appelmans & Wattiaux, 1953).

METHODS

Differential centrifuging

In most experiments, the starting material was a 0·25 M sucrose cytoplasmic extract, free of nuclei, prepared from the livers of rats fasted for 12 hr., by the technique described previously (Berthet & Duve, 1951; Appelmans & Duve, 1955).

The methods used for separating particulate fractions from this material were varied extensively and will be described in the text. To ensure reproducibility, the conditions of centrifuging will be defined in each case by the time-integral of the field prevailing in the middle of the fluid, expressed in the composite unit *g*-min., as well as by the sedimentation constant $S_{min.}$ of the lightest spherical particles to be sedimented completely, expressed in Svedberg units ($1S = 10^{-13}$ sec.). This nomenclature has been justified elsewhere (Duve & Berthet, 1953). The characteristics of the centrifuges used are given in Table 1. Attention is called to the last column of this table, showing the conditions required in each centrifuge to sediment completely the same group of particles, taking as standard of comparison the no. 40 rotor of the Spinco centrifuge. These values illustrate the statement that 'individual centrifuges... are less comparable, so far as complete sedimentation is concerned, when developing equal average fields than when turning at equal velocities' (Duve & Berthet, 1953).

Separation of fractions was performed by means of a pipette with a narrow drawn-out tip bent to an angle of 90°, operated with a rubber bulb fitted with valves, allowing controlled sucking. In low-speed centrifuging, this step becomes the most variable factor, as part of the sediment is loosely packed and easily removed with the supernatant fluid. The sediments were resuspended by means of a micro-homogenizer of the Potter & Elvehjem (1936) type.

Enzyme assays

Acid phosphatase. This was measured as before at 37° in the presence of 0·05 M β-glycerophosphate and 0·05 M acetate, adjusted to pH 5 with HCl. For measurements of free activity, the incubations were carried out for 10 min. in the presence of 0·25 M sucrose, under which conditions the enzyme enclosed within intact granules is usually unreactive (Appelmans & Duve, 1955). Total activity was estimated on preparations previously treated 3 min. in the Waring Blendor, usually after a 1:10 dilution with distilled water, or frozen and thawed at least 10 times. Bound activity is obtained as the difference between total and free activity.

Glucose 6-phosphatase. This was determined by the amount of inorganic phosphate formed at 37° in the presence of 0·008 M glucose 6-phosphate and 0·05 M cacodylate buffer, pH 6·5. The substrate was prepared by an enzymic method (Hers, Beaufays & Duve, 1953).

Cytochrome oxidase. This was assayed by the optical test of Cooperstein & Lazarow (1951), which was run in the Beckman Model DU spectrophotometer, using cells of 1 cm. light path. A suitable dilution of the enzyme was first prepared in 0·005 M phosphate buffer, pH 7·4, and kept for a few minutes at 0°. This precaution was necessary to obtain maximal initial rates. The reaction was started by adding 0·1 ml. of this dilution to 3 ml. of a solution containing $1·7 \times 10^{-5}$ M reduced cytochrome *c* in 0·03 M potassium phosphate buffer, pH 7·4. The decrease in optical density at 550 mμ. was measured at regular intervals for 3–6 min., following which the blank was read after addition of a small amount of solid potassium ferricyanide to the mixture.

Under these conditions the oxidation of cytochrome *c* is practically of first order with respect to the concentration of reduced pigment. One unit of activity is defined as the amount of enzyme causing the decadic logarithm of the concentration of reduced cytochrome *c* to decrease by one unit/min./100 ml. of incubation mixture. The calculations are performed as described by Cooperstein & Lazarow (1951). The cytochrome *c* was prepared from ox or horse heart by the method of Keilin & Hartree (1945). Before assay, enough $Na_2S_2O_4$ was added to reduce approximately 90% of the cytochrome. The rate of autoxidation was negligible.

The medium used in the measurements of O_2 consumption contained 0·008 M sodium glutamate, 0·008 M sodium fumarate, 0·001 M potassium adenosine triphosphate, 0·001 M-$MgCl_2$, 0·02 M potassium phosphate buffer, pH 7·4, and 0·125 M sucrose. Vessels, containing 2·5 ml. of this mixture and 0·2 ml. of enzyme, were incubated at 30° in a Warburg apparatus, with air as gas phase and 20% (w/v) KOH in the centre well. Readings were taken every 5 min., after an equilibration period of 10 min. The O_2 uptake proceeded linearly during at least 30 min. and the slope of the line was used to calculate the q_{O_2} (μl. O_2/min./g. liver or mg. N).

Nitrogen was estimated by a micro-Kjeldahl method.

RESULTS

Sedimentation experiments

In order to investigate the cause of the atypical distribution found for bound acid phosphatase in previous experiments, the sedimentation behaviour

Table 1. *Characteristics of centrifuges*

Centrifuge	Fluid volume (ml.)	$r_{max.}$ (cm.)	$r_{min.}$ (cm.)	Equivalent relative values*	
				g (av.)†	rev./min.
Corda, type 2047	50	15	8	2·16	1·1
M.S.E., superspeed attachment	7·5	7	3	1·26	1·28
Spinco, model L rotor no. 20	100	12·8	7·5	1·6	1·01
Spinco, model L rotor no. 40	10	8·1	4·8	1	1

* Necessary for the complete sedimentation of the same group of particles (same value of $S_{min.}$).
† Field in the middle of centrifuged fluid, i.e. at 0·5 ($r_{max.} + r_{min.}$) cm. from axis.

of this enzyme was compared with that of the respiratory activity, which has been shown by numerous workers to belong exclusively to mitochondria. At first, the rate of oxygen consumption in the presence of suitable substrates was used to measure respiratory activity. In later investigations, this test was replaced by the simpler cytochrome oxidase assays.

Fig. 1 shows the results of an experiment in which equal amounts of unwashed mitochondria were subjected to increasing centrifugal forces, in the M.S.E. superspeed attachment. The sediments were collected and analysed for nitrogen, free and total acid phosphatase, and respiratory activity. The results obtained, expressed as percentage of the corresponding values for the original suspension, are plotted against the time-integral of the average field applied.

It is seen that the respiratory activity of the sediments increases almost linearly with the centrifugal force and reaches the value of the original suspension at about 50000 *g*-min. ($S_{min.} = 14000\ S$). The sedimentation of bound acid phosphatase is only about half complete at this point, and continues to rise with increasing centrifugal force, but less steeply. Only an insignificant increase of the nitrogen content of the sediments is associated with this second part of the curve.

Similar experiments, in which cytochrome oxidase was measured instead of oxygen consumption, gave essentially the same results. In some cases, anomalous results were obtained. In Fig. 2 are shown sedimentation curves determined on two cytoplasmic extracts prepared from the same liver in 0·25 M and 0·88 M sucrose, respectively. The abnormal shape of the phosphatase curve probably reflects irregular decantation. This step is not easily performed in a reproducible manner when the tubes contain loosely packed precipitates and incompletely sedimented material. The data, however, serve to show that the sucrose concentration of the suspending medium does not materially affect the dissociation between the two activities; this dissociation was observed even in 1·4 M sucrose.

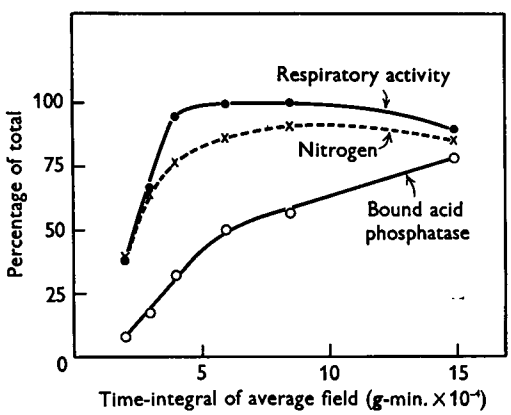

Fig. 1. Sedimentation curves of nitrogen, respiratory activity and bound acid phosphatase of a mitochondrial suspension. Original preparation: unwashed mitochondria, isolated in the M.S.E. superspeed attachment by 200000 *g*-min. ($S_{min.} = 3600\ S$) and diluted 1:10 in 0·25 M sucrose. From this suspension, 7·5 ml. portions were centrifuged at increasing average field-time values (see abscissa) in the M.S.E. centrifuge. The sediments were collected and analysed. The results are expressed as percentage of the total amounts present in the same volume of the original suspension.

Separation experiments

In a number of experiments, it could be shown that the partial dissociation of acid phosphatase from respiration, brought about by a single sedimentation, is further increased by the use of procedures known to favour the separation of particles of differing sedimentation constant.

One series of experiments, aimed at isolating a head fraction in as great a state of purity as possible, was performed as follows. Starting from a 1:10 cytoplasmic extract

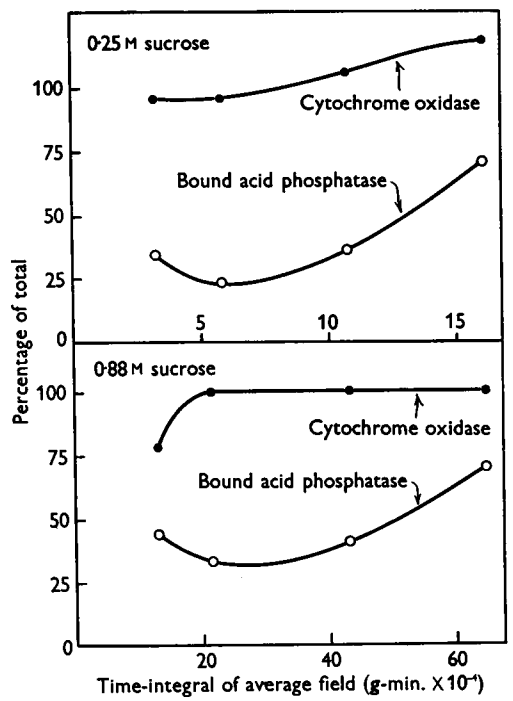

Fig. 2. Influence of sucrose concentration on dissociation between bound acid phosphatase and cytochrome oxidase. Sediments isolated from 10 ml. portions of 1:14 cytoplasmic extracts, in no. 40 rotor of Spinco centrifuge. Upper half: extract prepared in 0·25 M sucrose; lower half: extract prepared in 0·88 M sucrose from the same liver. For significance of coordinates, see Fig. 1.

in 0·25 M sucrose, the heavier granules, termed 'head fraction', were separated in the Corda centrifuge by 35 000 *g*-min. ($S_{min.} = 35\,000\ S$) and washed twice under the same conditions. A fairly large amount of loosely packed particles were removed, together with the first supernatant, which was spun in the M.S.E. superspeed attachment at 120 000 *g*-min. ($S_{min.} = 6000\ S$). The resulting sediment was resuspended in the first washing of the head fraction and recentrifuged at the same speed. The second washing of the head fraction served to wash the new sediment in the same manner, yielding the lighter 'main fraction'. Three experiments of this type were performed. In a fourth experiment, a 'head fraction' was isolated as before, but a combined 'head + main fraction' was prepared directly in the M.S.E. centrifuge from a separate portion of the cytoplasmic extract.

The two fractions were subjected to various analyses, the results of which are summarized in Table 2. To facilitate comparison between experiments, 'main' values were calculated in the fourth experiment by substracting the 'head' values from those obtained on the combined 'head + main' fraction.

The heavier 'head' fraction, isolated by this procedure, contained one-third to two-fifths of the total mitochondrial nitrogen and of the respiratory activity. The remainder was recovered in the 'main' fraction. In general appearance, the two fractions were almost undistinguishable, except for a slightly pinker tinge in the 'main' fraction, indicating a greater contamination by microsomes, also reflected in the glucose 6-phosphatase assays. Microscopic examination showed a greater proportion of smaller granules in the 'main' fraction but no other obvious difference.

In contrast, striking differences were found in the acid phosphatase content of the two fractions. The specific activity of this enzyme in total homogenates usually lies between 50 and 70 μg. P/10 min./mg. N. It is consistently lower in the 'head' fraction and much higher in the 'main' fraction. This difference becomes still more apparent if the bound activities are considered. In Expt. 3, particularly, bound phosphatase was almost absent from the 'head' fraction and 30 times more abundant in the 'main' fraction. Here again, the unequal distribution of acid phosphatase is not reflected in the partition of nitrogen, since the specific q_{O_2} of the two fractions is practically the same.

Table 3 summarizes the results of an experiment in which the layering technique, first introduced by Hogeboom, Schneider & Palade (1948), was used. This experiment was carried out in the no. 20 rotor of the Spinco centrifuge, accommodating tubes of 100 ml. capacity. Ten ml. of a 1:6 cytoplasmic extract in 0·25 M sucrose were first pipetted into the tube, following which 90 ml. of 0·3 M sucrose were slowly introduced through this layer by means of a thin capillary, the tip of which extended to the bottom of the tube. As a result, the extract was layered over the sucrose, with a fairly sharp interface. After centrifuging at 65 000 *g*-min. ($S_{min.} = 14\,000\ S$), the supernatant was decanted and centrifuged at 225 000 *g*-min. ($S_{min.} = 4000\ S$). The two sediments and the final supernatant were assayed for cytochrome oxidase and bound acid phosphatase.

As is shown in Table 3, the layering procedure which should, on theoretical grounds, allow a more efficient separation of particles of different sedimentation constant, does in fact emphasize the dissociation between cytochrome oxidase and bound acid phosphatase. So does repeated washing, as comes out clearly in the balance presented in Table 4.

The starting material, in this experiment, was a 1:6 cytoplasmic extract in 0·25 M sucrose. A heavier sediment was separated in the no. 40 rotor of the Spinco centrifuge by 54 000 *g*-min. ($S_{min.} = 10\,500\ S$) and washed twice at

Table 2. *Subfractionation of mitochondria*

The terms 'Head' and 'Main' refer to the two subfractions; for details of experimental procedure, see text.

	Expt. 1		Expt. 2		Expt. 3		Expt. 4	
	'Head'	'Main'	'Head'	'Main'	'Head'	'Main'	'Head'	'Main'*
Nitrogen (mg./g. liver)	2·08	5·16	2·40	5·00	2·40	4·60	2·94	3·76
Acid phosphatase activity (μg. P/10 min./g. liver)								
Total	88	1030	109	620	46	780	103	997
Free	34	304	23	170	26	175	19	53
Bound	54	726	86	450	20	605	84	944
(μg. P/10 min./mg. N)								
Total	42	200	46	124	19	170	35	265
Free	16	59	10	34	11	39	6	14
Bound	26	141	36	90	8	131	29	251
Respiration								
(μl. O_2/min./g. liver)	—	—	—	—	10·4	21·2	15·7	20·7
(μl. O_2/min./mg. N)	—	—	—	—	4·33	4·6	5·35	5·5
Glucose 6-phosphatase								
(μg. P/10 min./g. liver)	41·2	505·8	—	—	—	—	—	—

* 'Main' values calculated by substracting 'Head' from 'Head + Main' values.

33 000 g-min. ($S_{min.}$ = 17 300 S). The three supernatants were combined and centrifuged at 160 000 g-min. ($S_{min.}$ = 3500 S) to separate a lighter sediment. In a parallel experiment, a heavy fraction was isolated in the same rotor by layering 3 ml. of the extract over 6·5 ml. of 0·3 M sucrose and centrifuging at 74 000 g-min. ($S_{mm.}$ = 7700 S).

Despite a fairly large loss of bound phosphatase, owing to injuries suffered by the granules in the course of the experiment, the results of the assays demonstrate the efficiency of washing in separating the two activities. As can be seen in Table 4, each washing removes more bound phosphatase than cytochrome oxidase, yielding a final sediment with a phosphatase:oxidase ratio of 0·12 and a supernatant, from which a lighter sediment is isolated, in which this ratio is 15 times larger. The layering technique gives results comparable to those obtained with one centrifuging and one washing.

Quantitative fractionation

The importance of the dissociation which could be brought about experimentally between two enzymes, showing roughly similar distributions by the conventional fractionation procedure, made it imperative to devise a scheme which would make differences of this kind immediately apparent. On the basis of the results obtained, the following procedure was decided upon.

The nuclei and cytoplasmic extract were separated in the usual manner. The Spinco no. 40 rotor was used for all the subsequent steps of the fractionation. A heavy mitochondrial fraction was first isolated from the cytoplasmic extract by centrifuging at 33 000 g-min. ($S_{min.}$ = 17 300 S) and washed twice under the same conditions. The combined supernatants were centrifuged at 250 000 g-min. ($S_{min.}$ = 2300 S) to separate a light mitochondrial fraction, which was also washed twice. The microsomes were sedimented from the resulting supernatants by 3 000 000 g-min. ($S_{min.}$ = 190 S), and washed, leaving the final supernatant.

The results of a representative experiment are given in Table 5. It will be noted that the oxidase and phosphatase are recovered mostly in the two mitochondrial fractions but are distributed very unequally between them. On the other hand, glucose 6-phosphatase is present in low quantities in these fractions, even in the lighter one, and comes

Table 3. *Subfractionation by layering procedure*

For details of experimental procedure, see text.

Fraction	Percentage of activity of extract: Cytochrome oxidase	Total acid phosphatase	Bound acid phosphatase*	Ratio, bound phosphatase: cytochrome oxidase
Cytoplasmic extract	100	100	100 (73)	1
Heavy sediment	88	30	34 (25)	0·39
Light sediment	14	41	51 (37)	3·6
Final supernatant	1·5	26	16 (12)	10·7
Recovery	103·5	97	101 (74)	0·98

* Figures in brackets show bound activity as percentage of total activity of extract.

Table 4. *Efficiency of washing and of layering procedure*

For details of experimental procedures, see text.

Fraction	Percentage of activity of extract: Cytochrome oxidase	Total acid phosphatase	Bound acid phosphatase*	Ratio, bound phosphatase: cytochrome oxidase
Cytoplasmic extract	100	100	100 (81)	1
Heavy fraction (H.F.)	88	45	48 (39)	0·55
Supernatant	22	56	49·5 (40)	2·25
H.F. washed once	91	27	25 (20)	0·27
First washing	6	14	13·5 (11)	2·25
H.F. washed twice	73	14	8·5 (7)	0·12
Second washing	5	8	8·5 (7)	1·7
Light fraction, unwashed	26	46	48 (39)	1·85
Final supernatant	3	24	8·5 (7)	2·83
Recovery	102	84	65 (53)	0·64
Layering experiment				
Sediment	82	29	26 (21)	0·32
Supernatant	31	56	42 (34)	1·35
Recovery	113	85	68 (55)	0·60

* Figures in brackets show bound activity as percentage of total activity of extract.

Table 5. *Quantitative fractionation*

For details of experimental procedure, see text.

Fraction	Nitrogen (mg./g. liver)	Percentage of activity of homogenate: Cytochrome oxidase	Total acid phosphatase	Bound acid phosphatase*	Glucose 6-phosphatase
Homogenate†	34·2	100	100	100 (78·5)	100
Nuclei, washed twice	5·3	16·5	7·3	6·2 (4·9)	10·7
Heavy mitochondrial fraction, washed twice	3·9	60·5	20·6	17·6 (13·8)	1
Light mitochondrial fraction, washed once	3·3	20	43·9	47·2 (37)	6·4
Microsomes, unwashed	9·8	2	17·6	11 (8·6)	79·3
Final supernatant	11·3	0	9·2	0	2·3
Recovery	33·6	99	98·6	82 (64·3)	99·7

* Figures in brackets show bound activity as percentage of total activity of homogenate.
† Sum of nuclei + cytoplasmic extract.

Table 6. *Separation of the 'fluffy layer'*

Mitochondria, together with the 'fluffy layer', were spun down in the no. 40 rotor of the Spinco centrifuge by 160 000 *g*-min. ($S_{min.} = 3500\ S$) and washed once at 100 000 *g*-min. ($S_{min.} = 5700\ S$). The top layer of this sediment was removed by means of a glass rod, resuspended and centrifuged at 350 000 *g*-min. ($S_{min.} = 1600\ S$), yielding a well packed sediment, which could easily be separated mechanically into two fractions, containing the pink 'fluffy layer' and the underlying yellow mitochondrial precipitate, respectively. Enzyme activity expressed as μg. P/10 min./g. liver.

Fraction	Acid phosphatase: Activity	%	Glucose 6-phosphatase: Activity	%
Yellow sediment	110	7·5	238	7
Pink 'fluffy layer'	21	1·5	336	10

* Percentage of total activity of extract.

down predominantly with the microsomes. A comparison of these observations with those obtained by the classical technique (Berthet & Duve, 1951) indicates that the main result achieved by the new procedure is a subfractionation of the mitochondria into a heavier fraction, rich in oxidase and poor in phosphatase, and a lighter one, showing an inverse relationship with respect to its content of the two enzymes.

The 'fluffy layer'

The peculiar sedimentation properties of acid phosphatase suggested the possibility that this enzyme might be associated with the pinkish, poorly sedimented 'fluffy layer', which is found on top of mitochondrial precipitates. In the fractionation scheme described above, this layer forms a conspicuous part of the first sediment obtained in the isolation of the light mitochondrial fraction. However, it is usually removed to a large extent in the course of washing and comes down mainly with the microsomes. Therefore the possibility considered seemed rather unlikely. Further evidence against it is furnished by the results of Table 6, showing that the 'fluffy layer' is distinctly poorer in acid phosphatase, but richer in glucose 6-phosphatase, than the lighter portion of the yellow mitochondrial sediment.

DISCUSSION

The results reported are in complete agreement with those of Novikoff, Podber, Ryan & Noe (1953), who have described a partial dissociation of acid phosphatase from succinoxidase, together with certain other data indicative of biochemical heterogeneity in particulate fractions. In assessing the significance of such results, the possibility of artifacts must however be examined. Cytochrome oxidase and the related succinoxidase are strongly attached to the insoluble framework of the mitochondria. Acid phosphatase, on the other hand, is easily released in soluble form from injured granules, and may subsequently undergo an artificial redistribution amongst particulate elements, owing to adsorption phenomena. In the present work, intact granules have been estimated selectively as the difference between total and free activity. The fact that the enzyme recovered in the lighter fractions is present mainly in bound form shows clearly that the separation of acid phosphatase from cytochrome oxidase reflects a true difference in the sedimentation characteristics of the granules in which the two enzymes are concentrated.

The observed heterogeneity could be a property either of the granules themselves or of the isolated fractions. In the first interpretation, which is apparently favoured by Novikoff *et al.* (1953), both enzymes are taken to be present in all the granules, but in a ratio which varies with one of the factors, size or density, which determine the sedimentation

behaviour of the particles. In the other, the fractions are considered as mixtures of two distinct classes of granules, having slightly different sedimentation properties and characterized each by one of the two enzymes studied.

In judging the relative merits of these two hypotheses, it must be kept in mind that fractions isolated by differential centrifuging are always mixtures of granules of differing sedimentation constant. It is theoretically possible to increase the resolution, either by the use of a layering procedure or by repeated washings; but such attempts soon become defeated by the progressive agglutination which occurs in the preparation. As was shown in a previous study (Hers, Berthet, Berthet & Duve, 1951), it is already very difficult to free mitochondria completely from microsomes and one must expect these complications to be even greater with particles of closer sedimentation behaviour.

Examined in this light, the results obtained in the layering and washing experiments stand out as particularly significant. In every instance, these procedures were found to enhance the dissociation between the two enzymes. Under the best conditions, one-third of the respiratory activity was isolated practically free of acid phosphatase, and more than 70% of the cytochrome oxidase was separated in association with less than 10% bound phosphatase. Conversely, fractions containing 10–20% of the original bound phosphatase of the extract and only traces of cytochrome oxidase have also been obtained. As was pointed out above, the nitrogen content of the isolated fractions appeared to correlate essentially with their respiratory activity, irrespectively of their content in acid phosphatase.

Such results suggest very strongly that the classical mitochondrial fraction is made up mostly of granules possessing oxidative activity but devoid of acid phosphatase. In view of the numerous morphological observations made by other authors, these granules can only be the true mitochondria. The results of the sedimentation experiments are in agreement with this conclusion, indicating that the cytochrome oxidase-containing granules behave as a fairly homogeneous population, with a sedimentation constant of approximately 14 000 Svedberg units (in 0·25 M sucrose at 0°); using the limit values assigned by Holter, Ottesen & Weber (1953) to the density of mitochondria (1·10–1·20), this figure would correspond to diameters ranging between 0·55 and 0·94 μ.

Consequently, the main information provided by the present study is that typical mitochondria are probably free of acid phosphatase, but that, when isolated by differential centrifuging, they are contaminated by granules of a second type which are very rich in this enzyme. The cytological identification of this second group of granules raises a difficult problem. In the first place, judging from the sedimentation data, they appear to form a much more heterogeneous population, with sedimentation constants of about 1000–10 000 Svedberg units, making it almost impossible to isolate them in good yield without significant contamination by mitochondria or microsomes. Moreover, the present indications are that they must be very few in proportion. In several experiments, fractions showing an almost tenfold increase in specific phosphatase activity over that of the original homogenate have been isolated. These fractions still contained significant amounts of cytochrome oxidase and glucose 6-phosphatase and it could be estimated that the pure granules must have a specific activity of at least 1500 μg. P/10 min./mg. N and be associated with 4% or less of the cell's nitrogen. Fractions showing specific activities of this order of magnitude have actually been separated in preliminary trials performed by means of density-gradient centrifuging, and it is hoped that improved methods will make it possible to prepare samples of granules pure enough for morphological examination.

In the meantime, some indirect information is provided by the results recorded in this and previous papers. From the sedimentation data, it can be calculated that the diameters of the granules must range mostly between 0·25 and 0·8 μ. if their density is low (1·10) or between 0·13 and 0·4 μ. if their density is high (1·30). Despite the uncertainty of these calculations, they tend to show that the acid phosphatase-containing granules are comparable in size to small mitochondria rather than to microsomes. This conclusion is further supported by the fact that they can be separated to a large extent from glucose 6-phosphatase, and by the experiments in which they were shown to be associated with the mitochondrial sediment rather than with the superimposed 'fluffy layer'. A confirmation of this finding is to be found in the work of Laird, Nygaard, Ris & Barton (1953), who made an extensive study of the 'fluffy layer' and found no evidence for a concentration of acid phosphatase in this fraction. Finally, it must be recalled that studies of the release of acid phosphatase have led to the conclusion that the granules behave as typical osmotic systems, surrounded by a semi-permeable membrane.

Whether they represent a specialized form of mitochondria or an entirely distinct cytological entity remains to be established. The most comparable bodies are the 'droplets' recently isolated from kidney tissue by Straus (1954), who found them to contain little succinoxidase but considerable amounts of acid phosphatase. These 'droplets' have been studied histologically by Oliver (1948);

they increase greatly in size after the intraperitoneal injection of egg white and are believed by this author to be concerned with the tubular reabsorption of proteins. They are, however, of much larger size than was estimated for the liver granules (0·5–1·5 μ. in untreated animals, 1–5 μ. in animals injected with egg white) and their specific acid phosphatase activity (185–270 μg. P/10 min./mg. N) is distinctly lower than that found in the best liver fractions and much lower, therefore, than the values estimated for the pure liver granules.

In addition to their theoretical interest, the results obtained entail a conclusion of great practical importance, showing that two enzymes may be both recovered to a large extent in the mitochondrial fraction and still be entirely unrelated cytologically. This view is supported by a number of recent reports, describing evidence of biochemical heterogeneity in isolated mitochondria (Novikoff *et al.* 1953; Laird *et al.* 1953; Holter *et al.* 1953; Kuff & Schneider, 1954; Paigen, 1954). As will be shown in a subsequent publication, the use of the new fractionation method outlined in the present paper provides a partial solution to the problems raised by these findings.

SUMMARY

1. A comparative study has been made of the sedimentation properties of bound acid phosphatase and of cytochrome oxidase or the related respiratory activity, in rat-liver fractions. Acid phosphatase has been found to sediment more slowly than the oxidase.

2. The partial dissociation between the two activities, achieved by a single centrifuging, is further enhanced by the use of a layering procedure or by repeated washings.

3. It is concluded from the results obtained that the classical mitochondrial fraction is made up essentially of typical mitochondria, possessing oxidative properties but devoid of acid phosphatase. The latter enzyme is believed to be associated with a special class of granules, very few in proportion, entirely distinct from microsomes and comparable in size to small mitochondria. In the classical fractionation procedures, these granules are unequally distributed between the mitochondria and the microsomes, occurring mostly in the former fraction.

4. A new scheme of fractionation is described, which will make it possible to distinguish between true mitochondrial enzymes and contaminants of the acid phosphatase type.

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