Tissue Fractionation Studies

2. THE NATURE OF THE LINKAGE BETWEEN ACID PHOSPHATASE AND MITOCHONDRIA IN RAT-LIVER TISSUE

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In the preceding paper (Berthet & Duve, 1951), the existence in rat-liver tissue of a mitochondria-linked form of acid phosphatase has been described and the cytological significance of this finding has been discussed. The nature of the linkage between enzyme and granule forms the object of the present communication.

Two facts relevant to this problem have already been reported, namely the great lability of the complex, which can be disrupted quantitatively by mechanical or physical means (Waring blender, freezing and thawing, hypotonic media), and its lack of enzymic activity towards added glycerophosphate at pH 5. Considerations based on these and other properties to be described in this paper, indicate that the enzyme is retained inside the mitochondrial structure by a membrane-like barrier, endowed with selective permeability and impermeable to glycerophosphate. A preliminary report of this work has already been published (Duve, Berthet, Berthet & Appelmans, 1951).

METHODS

The following general procedure has been followed in many of the experiments which will be reported. Mitochondria were separated from rat-liver tissue by the method previously described (Berthet & Duve, 1951), washed once and resuspended in a small quantity of cold 0·25 M-sucrose. Samples, of this suspension were suitably diluted with media of given composition and incubated. Except in the cases where the effect of temperature was studied, the incubation was carried out in the cold room, in an ice-water bath.

At various times after the beginning of the experiment, samples of the diluted suspension were centrifuged 10 min. at 20,000 *g*. Part of the supernatant was collected and kept for enzyme assay. A sample for the determination of the starting value was obtained in a similar way from a 0·25 M-sucrose dilution of the mitochondria. Another portion of the mitochondria was treated for 3 min. in the Waring blender, for the measurement of the total enzyme content.

When all the samples had been collected, their acid phosphatase activity was determined according to the technique described previously to provide an index of the amount of soluble enzyme present at the time the suspension was centrifuged.

In a number of experiments, additions to this procedure or deviations from it were introduced; these are mentioned in the text.

RESULTS

Effect of temperature. When mitochondria are kept in 0·25 M-sucrose at 0°, acid phosphatase is released very slowly, as shown by many of the graphs reproduced below. Raising the temperature produces a marked increase in the rate of liberation of the enzyme, and the activation which occurs under those conditions follows a characteristic course. The results which are represented graphically in Figs. 1–3 illustrate various aspects of this phenomenon.

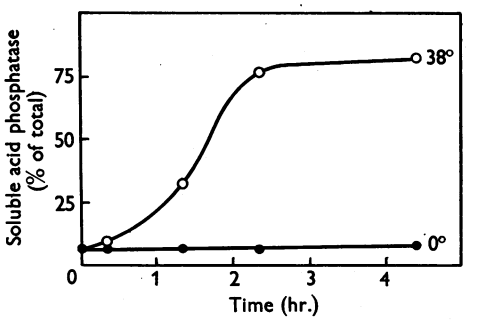

Fig. 1. Influence of temperature on release of acid phosphatase in 0·25 M-sucrose.

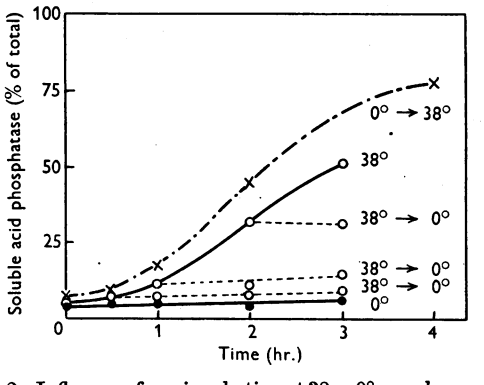

Fig. 2. Influence of pre-incubation at 38 or 0° on subsequent release of acid phosphatase at 0 and 38° respectively in 0·25 M-sucrose. ●——●. Fresh mitochondria, 0°; ○——○, fresh mitochondria, 38°; ○- - -○, brought back to 0° after preincubation at 38°; ×—·—×, 38°, after 3 hr. at 0°.

An experiment in which full activation was obtained after approximately 2·5 hr. incubation at 38° is shown in Fig. 1.

The results of a similar experiment, in which, in addition, the effects of pre-incubation at 38 and 0° on the subsequent rate of release at 0 and 38° respectively were investigated, are given in Fig. 2.

Fig. 3 shows in diagrammatic form the amounts of soluble acid phosphatase present in a 0·25M-sucrose suspension of mitochondria before and after standing 2·5 hr. at various temperatures.

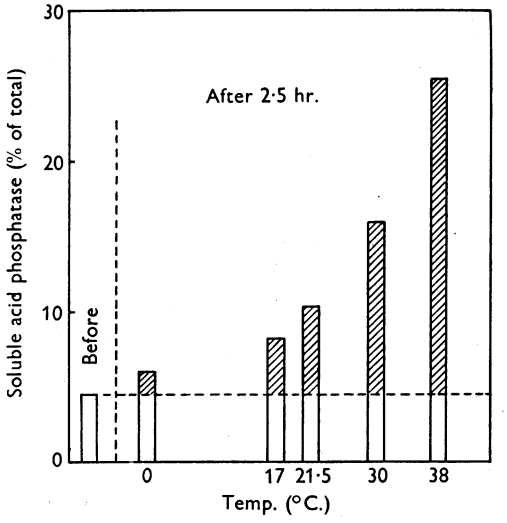

Fig. 3. Percentage of acid phosphatase released after 2·5 hr. standing at various temperatures in 0·25M-sucrose.

The main conclusion which may be drawn from these experiments is that chemical reactions are involved in the activation. This is supported by the magnitude of the temperature effect and by the general aspect of the results of Fig. 3. Although the particular shape of the activation curve does not allow a more detailed analysis of these results, it may further be deduced from Fig. 2 that liberation of the enzyme must be intimately linked with a temperature-sensitive reaction or reactions, since a previous incubation at 38° does not increase the rate of release at 0°. On the other hand, some change must occur even at 0°, in view of the increased rate of thermal activation which takes place after 3 hr. standing at this temperature.

Another significant point is the characteristic S-shape of the activation curve shown in Fig. 1. The slow initial rate of release of the enzyme and its progressive increase with time, are reminiscent of similar features which have already been described (Berthet & Duve, 1951), for the activation which occurs when mitochondria are incubated at 38° in a mixture containing equal volumes of 0·25M-sucrose and of the substrate used for the enzyme assays. The reason for this similarity will be given below. The abrupt flattening of the curve at a point where only 80% of the enzyme has dissolved is explained by the fact, discussed in the last section, that a fraction of the enzyme always remains attached to the particles, even in fully active preparations.

Effect of distilled water. Dilution of 0·25M-sucrose suspensions of mitochondria with 15–25 vol. of distilled water causes a very rapid activation, even at 0°. The results of seven experiments of this

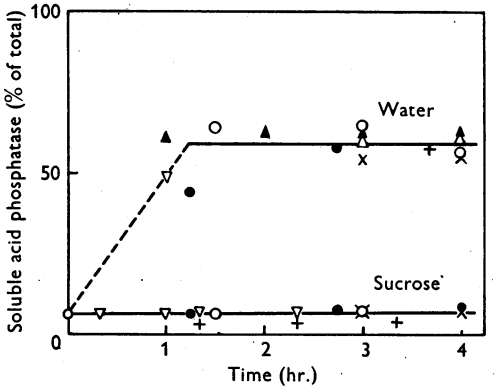

Fig. 4. Effect of distilled water on release of acid phosphatase. The signs represent independent experiments.

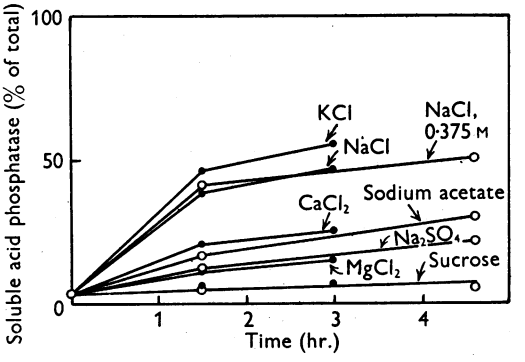

Fig. 5. Effect of ionic composition of suspending medium on release of acid phosphatase. All concentrations, 0·25M, except NaCl where indicated in graph.

type have been condensed in Fig. 4, which shows that a plateau corresponding to the release in soluble form of approximately 60% of the total enzyme content, is reached at the end of 1 hr. Here again, as will be shown below, the preparation attains maximum activity, even though a considerable part of the enzyme remains attached to the particles. Moreover, other observations suggest that the actual activation phenomenon occurs very

rapidly and is not preceded by a lag period as observed for thermal activation.

Effect of salts. The action of salts is characteristic. In the absence of sucrose, they are unable to prevent a progressive liberation of the enzyme, even when present in hypertonic concentration. This effect is particularly rapid with potassium chloride and sodium chloride; it is much slower with sodium acetate, calcium chloride, magnesium chloride and sodium sulphate (Fig. 5).

In order to avoid any error in the estimation of soluble enzyme in the experiments described in Fig. 5, parallel tests were made in the presence of equivalent concentrations of the various salts used, with a dialysed preparation of acid phosphatase, extracted from mitochondria with distilled water. Inhibitions of 5% with potassium chloride, 20% with calcium chloride and 38% with sodium sulphate were observed, and the results obtained were corrected accordingly.

In the presence of 0·25M-sucrose, the effect of salts is entirely different. Except for a slight and practically instantaneous increase in soluble acid phosphatase, the significance of which will be discussed below, they exert little or no influence on the rate of release of the enzyme. A typical experiment, illustrating the difference between the actions of 0·44M-sodium chloride in the presence and in the absence of 0·25M-sucrose is represented in Fig. 6.

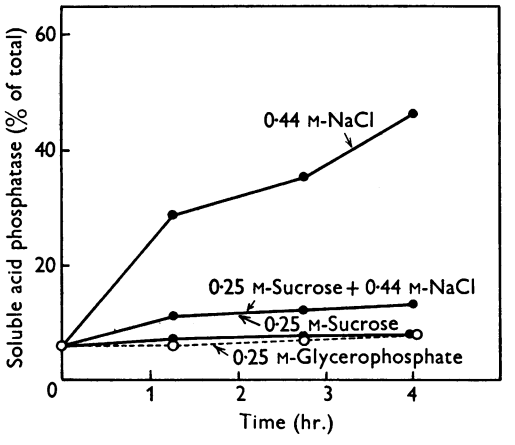

Fig. 6. Inhibition by 0·25M-sucrose of activating effect of 0·44M-NaCl. Protection by 0·25M-glycerophosphate.

Additional information concerning the mechanism by which salts effect liberation is furnished in Table 1. In this experiment, mitochondria were suspended in 0·25M-sucrose containing 0·5M-sodium chloride and centrifuged. The supernatant contained 12·5% of the total activity. Equal quantities of the sedimented granules were resuspended in 0·25M-sucrose + 0·5M-sodium chloride and in 0·25M-sucrose respectively, and assayed for free enzyme activity. Whereas the activity of the salt containing suspension was only 23% of the total, the granules resuspended in sucrose alone possessed 89% of the total activity and had therefore become fully active.

Table 1. *Disruption of salt-enriched mitochondria in* 0·25M-*sucrose*

Preparation	Acid phosphatase activity (mg. P/g./10 min.)	Acid phosphatase activity (% of total)
Total activity of original mitochondria (blender)	0·71	100
Supernatant of washing with 0·25M-sucrose + 0·5M-NaCl	0·09	12·5
Precipitate, resuspended in 0·25M-sucrose + 0·5M-NaCl	0·12	23
Precipitate, resuspended in 0·25M-sucrose	0·63	89

These results are easily explained if it be admitted that mitochondria behave as osmotic systems, impermeable to sucrose but permeable to water and various salts, and that the release of the enzyme is the consequence of an osmotic disruption of the granules. When suspended in water, their breakdown is due to the penetration of water, a phenomenon which is very rapid. In salt solutions the same process occurs, but only when enough salt has diffused into the granules to decrease the effective osmotic pressure of the surrounding medium below the critical value. The rate of diffusion of the salt thus becomes the limiting factor. In the presence of 0·25M-sucrose, osmotic disruption is prevented, but not the penetration of salt. Consequently, such salt-enriched mitochondria will disintegrate if they are resuspended in pure sucrose, because water enters the granules more rapidly than the salt can leave them (Table 1).

In addition to these main effects, there are other secondary actions of salts which will be discussed in the last section.

Effect of glycerophosphate and of the enzyme substrate. In several experiments, a solution of 0·25M-sodium glycerophosphate adjusted to pH 6·1 with hydrochloric acid was used as suspending medium. In all these experiments, the curve of release of the enzyme was found to coincide perfectly with that observed in 0·25M-sucrose. An example is given in Fig. 6. In terms of the osmotic interpretation put forward above, this implies that mitochondria are as impermeable to glycerophosphate as to sucrose, a fact sufficient to account for the lack of detectable activity of the bound enzyme.

A similar test was carried out using as suspending medium equal vol. of 0·25M-sucrose and of the enzyme substrate. This duplicated the conditions under which the assays on intact mitochondria, described in the preceding paper (Berthet & Duve,

1951), were performed. A very slow liberation occurs in the medium, so that it is almost as effective as 0·25M-sucrose in protecting the granules against osmotic disruption. The fact that little activation takes place during the first 10 min. of the enzyme assays—an important factor in the discussion of the results reported in the preceding paper—is thus entirely explained, since the initially slow thermal activation becomes dominant in the absence of osmotic factors.

The protection afforded by this medium is not surprising when its composition, which is given in Table 2, is considered. The sucrose and glycerophosphate concentrations alone, make the medium

Table 2. *Composition of incubation medium for standard enzyme assay*

Component	Molarity
Sucrose	0·125
Glycerophosphate (8% doubly ionized)	0·05
Boric acid	0·08
Cacodylic acid (8% ionized)	0·08
Acetic acid (70% ionized)	0·08
Na^+	0·30
Cl^-	0·185
Total	0·900

iso-osmotic with 0·175M-sucrose. If the mitochondria are only slightly permeable to boric or cacodylic acid, the effective osmotic pressure should reach a value equivalent to that of 0·25M-sucrose. It is quite possible that the medium is entirely adequate osmotically, and that the small activating effect it produces is due to its low pH (pH 5).

As pointed out previously (Berthet & Duve, 1951) the use of the *ABC* buffer mixture was fortuitous, since acetate alone would have been just as effective as a buffer. However, it appears that the other components of the buffer served an unexpected purpose. The use of this buffer and the adoption of short incubation periods, made it possible to demonstrate the lack of activity of the bound enzyme, a property which can become completely obscured when other assay conditions are used, as in the experiments of Palade (1951).

Effect of other substances and of detergents. Fig. 7 illustrates the behaviour of the acid phosphatase-bearing mitochondria in 0·25M-glucose and 0·25M-glycerol. Glucose is not as effective as sucrose in protecting the granules and probably enters them at a slow but significant rate. With glycerol, activation is very rapid. As in previous similar cases, the amount of soluble enzyme present reaches a plateau far below the total content of the granules.

The action of two detergents, saponin and sodium deoxycholate, was also investigated. In these, as in some of the experiments with salts, the results are complicated by inhibiting effects. These were measured separately on a preparation of soluble acid phosphatase. In Fig. 8 are represented the quantities of soluble enzyme present in 0·25M-sucrose suspensions of mitochondria kept for 1 hr. at 0° with various concentrations of the two detergents, corrected for inhibition effects. It is of interest to note that approximately one-tenth of the concentration of both saponin and deoxycholate necessary to produce full activation of the mitochondria is sufficient to cause complete haemolysis of human erythrocytes.

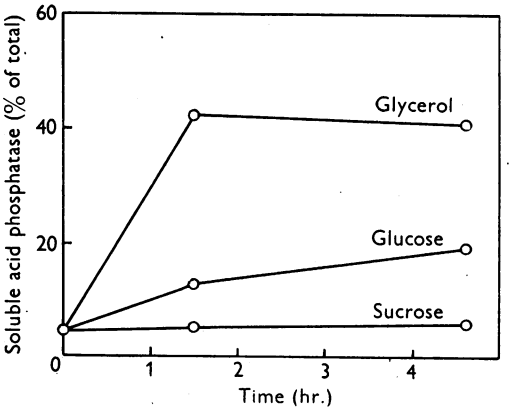

Fig. 7. Effect of 0·25M-glucose and glycerol on release of acid phosphatase.

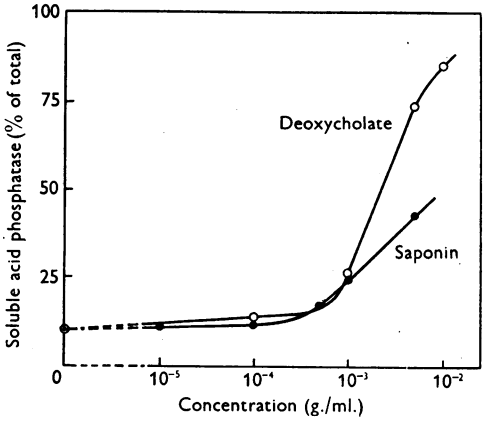

Fig. 8. Acid phosphatase released after 1 hr. standing in the presence of various concentrations of saponin or sodium deoxycholate.

Effect of pH. This was only studied in media which were made isotonic partly with sucrose, partly with salts, so that the results are complicated by osmotic effects. In an experiment covering the pH range from 3·7 to 8·5, maximum stability was observed between pH 5·5 and 6·5, with a slight and progressive increase in the rate of activation at values below or above this limit.

Retention of active enzyme by particulate material. It will have been noticed that in all the experiments described above, in which the activation has reached a limiting value, the amount of soluble enzyme present in the mitochondria suspension is always less than the total acid phosphatase content. This is true whatever the activating agent used, whether it be an increase in temperature (Fig. 1), distilled water (Fig. 4) or 0·25 M-glycerol (Fig. 7). It is also true in the case of mitochondria treated in the Waring blender or disrupted by freezing and thawing. Only in one instance, with mitochondria which had aged 13 days at 0° (Berthet & Duve, 1951), was almost the entire enzyme content of the granules in the soluble form.

Another aspect of this phenomenon, illustrated by the data in Table 3, is that the amount of enzyme retained by the particulate material varies with the nature of the activating agent, and is relatively constant with each type of activating agent.

Finally, there are two experimental observations which have a bearing on this problem, namely that the free activity of intact mitochondria is always larger than their non-sedimentable activity (Berthet & Duve, 1951), and the peculiar liberation produced by salts in the presence of 0·25 M-sucrose (Fig. 6).

A point of special importance in understanding these phenomena appeared to be the actual activity of the enzyme retained by the particulate material in all these experiments. In order to investigate this matter, tests were first performed on the resuspended precipitates, but these did not furnish unequivocal results, because it is impossible to resuspend a precipitate of mitochondria without causing some mechanical damage. Moreover, this damage is greater when the mitochondria have been treated with a salt-containing solution of sucrose, even if the same medium is used to resuspend them. Salts, in addition to the effects already described, cause a loosening of the mitochondrial structure. This effect is generally not sufficient to produce an actual liberation of bound acid phosphatase, but renders the granules more sensitive towards other activating agents. It is perhaps related to the fact, demonstrated by spectrophotometric determina-

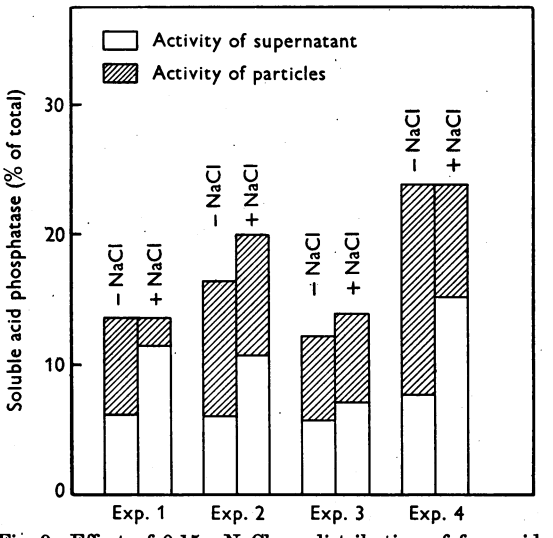

Fig. 9. Effect of 0·15 M-NaCl on distribution of free acid phosphatase activity between supernatant and particles in intact mitochondria.

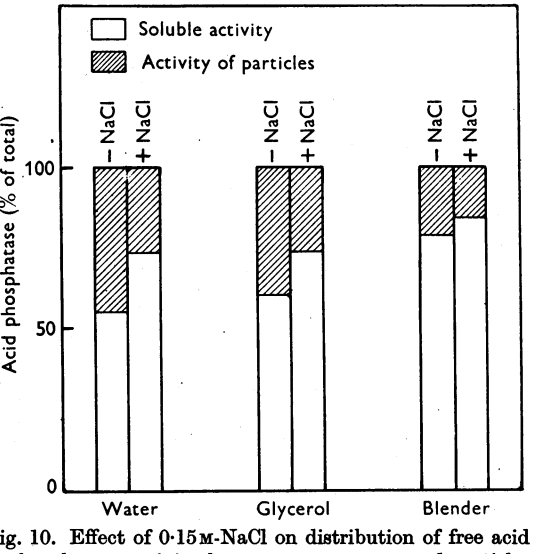

Fig. 10. Effect of 0·15 M-NaCl on distribution of free acid phosphatase activity between supernatant and particles in disintegrated mitochondria.

Table 3. *Retention of acid phosphatase by particulate material after full activation*

Activating agent	No. of exp.	Acid phosphatase retained by particulate material, % of total: Unwashed precipitate	Precipitate washed once	Precipitate washed twice
Waring blender	5	20±2	—	—
Distilled water	6	40·6±1·4	—	—
Freezing and thawing	1	32	19	19
Incubation at 38° in 0·25 M-sucrose	1	20	—	—
0·25 M-glycerol	2	59	—	—
		39		
Ageing at 0° (13 days)	1	15	4	—

Table 4. *Adsorption of acid phosphatase by intact mitochondria*

Suspension medium	Acid phosphatase (μg. P/10 min.) Supernatant of mitochondria	Soluble enzyme added	Supernatant of mitochondria + added enzyme	Difference (adsorbed enzyme)
0·25 M-sucrose	5·8	66	54·1	17·7
0·25 M-sucrose + 0·15 M-NaCl	13·1	66	73·7	5·4

tions, that nucleic acids are detached, apparently from the surface of the mitochondria, under the influence of strong salt solutions, even in 0·25 M-sucrose.

In order to circumvent these difficulties, tests were made on the original suspensions and on the supernatants, the differences between the two values being taken as representing the activities of the particles. Special precautions were taken to prevent activation during the tests, which were carried out at either 38 or 0°, under the conditions described in the preceding paper for intact mitochondria (Berthet & Duve, 1951). Fig. 9 shows diagrammatically the results of four experiments of this type, with intact mitochondria suspended in 0·25 M-sucrose or in 0·25 M-sucrose + 0·15 M-sodium chloride. It is clear that the main effect of the salt is to detach from the granules a fraction of enzyme which is already fully active when bound. In two experiments, a slight activation is also observed, a consequence of the labilizing effect of salts, described above.

In Fig. 10 are the results of a similar experiment on mitochondria which had been activated by distilled water, 0·25 M-glycerol and treatment in the Waring blender respectively. They show unambiguously that the portion of enzyme retained by the particles is fully active in all cases and that it is partly brought into solution, as in the experiments on intact mitochondria, by the addition of 0·15 M-sodium chloride.

Table 5. *Adsorption of acid phosphatase by microsomes*

Fraction	Acid phosphatase activity (μg. P/10 min.)
Washed microsomes	4
Microsomes + acid phosphatase	101·2
Supernatant of first centrifugation	89·2
Precipitate of first centrifugation	11·2
Supernatant of second centrifugation	2·7
Precipitate of second centrifugation	9·0
Supernatant of third centrifugation	0·6
Precipitate of third centrifugation	6·2

From these results it may be concluded that the linkage by which a portion of acid phosphatase remains attached both to intact and disintegrated mitochondria, does not modify the activity of the enzyme, and must therefore be different from that in the native complex which is entirely inactive.

It is probable that several different phenomena are responsible for this second type of linkage. There is no doubt that part of the enzyme is retained by adsorption, and that salts have an eluting effect on this adsorption complex. The data of Table 4 refer to an experiment, in which adsorption of dissolved acid phosphatase by intact mitochondria, and its partial elution by sodium chloride, could actually be observed. As shown by Table 5 and by observations described in the preceding paper (Berthet & Duve, 1951), the ability to adsorb the enzyme is not restricted to mitochondria, but is shared by microsomes and by nuclei. It may therefore be considered a non-specific phenomenon. A peculiar fact is that 0·25 M-sodium-glycerophosphate, adjusted to pH 6·1 with hydrochloric acid, causes no elution, although it contains a fair amount of sodium chloride (Fig. 6).

Mitochondria which have been disrupted osmotically (water, glycerol), may in addition to the adsorbed fraction, retain another fraction of enzyme trapped inside the collapsed granule. That some such phenomenon may take place is suggested by the results of Table 6, which show that an additional quantity of enzyme can be extracted with water from a precipitate of mitochondria disrupted with distilled water and centrifuged down in the presence of sodium chloride.

Table 6. *Extraction of acid phosphatase with distilled water from mitochondria disrupted in distilled water and washed with salt*

Fraction	Soluble acid phosphatase (% of total)
Original suspension	4
A. In supernatant, after 4 hr. in distilled water	62
B. In supernatant of A, with previous addition of NaCl to 0·15 M concentration	80
C. In supernatant of precipitate from B, resuspended in distilled water	16

Finally, one cannot exclude the possibility that a small portion of the enzyme is firmly attached to sub-microscopic granules forming part of the mitochondrial inclusions.

DISCUSSION

In view of the combined evidence presented in this and the preceding paper, it may be safely concluded that the linkage between acid phosphatase and its particulate support cannot be a simple molecular association, either chemical or physical; obviously structural factors are involved. The information, obtained by methods which are entirely self-sufficient, requires no predefined morphological picture for its interpretation.

The evidence presented indicates that the enzyme acid phosphatase is associated, in a manner which suppresses its activity under the usual assay conditions, with a group of fairly large cytoplasmic entities, of which two-thirds are sedimented in a 0·25 M-sucrose homogenate of liver tissue, by centrifuging for 10 min. at 8500 *g*. The enzyme can then be extracted in fully active form from these entities by a number of methods.

Of the various agents which are found to bring about partial or total release of the enzyme, there are several which may be termed unspecific, in that they do not permit any definite conclusion to be drawn as to the structure of the carrier granule. For instance, the fact that exposure to the Waring blender or to repeated freezing and thawing causes liberation of acid phosphatase, simply shows that the binding of the enzyme requires a certain structural integrity of the carrier granule, but does not help to characterize further the nature of its structure.

There is, however, a group of facts which do not admit of several interpretations. The enzyme is only retained by its carrier if a certain kind of substance, which may be ionic (glycerophosphate) or non-ionic (sucrose) in nature, is present in sufficient concentration in the suspending medium. A progressive release occurs when these substances are replaced, in equimolar concentration, by a variety of others, which may also be ionic (sodium chloride, potassium chloride) or not (glycerol). The rate at which this phenomenon takes place depends on the nature of the substance present. The addition of a protecting substance in adequate concentration is sufficient to suppress completely the liberation which occurs in the presence of non-protecting substances. These facts can only be understood if it be admitted that the enzyme-bearing granules behave as osmotic systems.

Consequently, any representation of these granules must at least include the essential features of an osmotic system: a limiting barrier capable of depressing to a greater or lesser extent the diffusion of solutes present within or outside the granule; an interior medium containing a number of osmotically active components, to which the barrier is not permeable. The simplest model obeying this description is that of a sac, consisting of fluid enclosed in a semi-permeable membrane.

All the facts reported in this work can be fitted into this picture, simply by assuming that acid phosphatase is present in a diffusible form inside the sac. Mechanical agents, such as the Waring blender and other homogenizing devices, may cause injuries to the membrane. Media which are hypotonic with respect to osmotically effective substances, to which the membrane is impermeable, will induce swelling of the granules, with the consequent distention or disruption of the membrane. Detergents are known to dissociate lipoprotein complexes and to disintegrate biological membranes. Repeated freezing and thawing probably acts in several ways, by splitting or denaturing architectural components, creating local increases in osmotic pressure and causing tearing effects due to ice crystals. Finally, the thermal activation may be interpreted as a consequence of autolytic changes affecting the membrane, and the particular S-shape of the curve might indicate either that these changes are autocatalytic or that the 'survival-time' of the granules is dependent on a statistically distributed property. In all cases, therefore, the release of acid phosphatase may be related to damages suffered by a membrane. In addition, the impermeability of the membrane to glycerophosphate, which is demonstrated by the protection afforded by this substance, is sufficient to explain the lack of detectable enzymic activity when intact granules are incubated under mild conditions with glycerophosphate.

This picture is complicated to a certain extent by side phenomena, most of which are due to non-specific secondary adsorption of the enzyme on particulate material.

It may be concluded that a sac-like representation of the acid phosphatase-bearing granules is both necessary and sufficient to account for their experimentally determined behaviour, at least as far as the release or the retention of acid phosphatase is concerned. This does not mean, of course, that the content of the sac is not itself structurally organized. On the contrary, it is very probable that an internal structure exists, but its study is beyond the scope of the methods used in this work.

There is one alternative interpretation which, though not very probable, should be mentioned, namely that the membrane does not surround the whole granule, but only that part of it containing acid phosphatase.

A second point, which has already been discussed (Berthet & Duve, 1951) concerns the nature of the granules to which these conclusions apply. We do not know what these granules are, and it is only because they appear largely in a fraction whose content has been identified by several workers with the cell mitochondria that we have used this name to

characterize them. At least, if they are mitochondria, then in the cytoplasm of liver cells a significant amount of smaller mitochondria must exist which do not sediment completely in 10 min. at 8500 *g*, since the acid phosphatase-bearing particles which are recovered in the microsome fraction show all the properties of the larger ones, and it is reasonable to assume that both groups are part of a homogeneous population of granules of varying size.

There is very good agreement between the conclusions reached in this work and the results which have been obtained by other methods. Both Claude (1946) and Hogeboom, Schneider & Pallade (1948) state that large granules or mitochondria isolated by differential centrifugation swell and disrupt in hypotonic media, and consider this phenomenon to be osmotic in nature. It is also mentioned by Hogeboom *et al.* (1948) that mitochondria prepared in saline are less stable than in sucrose. The existence of a structurally defined membrane around the mitochondria has been further demonstrated by electron microscope studies (Claude & Fullam, 1945; Dalton, Kahler, Kelly, Lloyd & Striebich, 1949). The observations of the latter authors are of special interest, describing various stages in the disruption of mitochondria, from incipient leakages of the mitochondrial contents up to completely empty 'ghosts', occurring in isotonic saline.

The membrane theory has been disputed by Huennekens & Green (1950) on the basis of results showing that some pyridino-proteins of the 'cyclophorase-complex' can be selectively dissociated into apoenzyme and coenzyme. However, their data do not disprove the existence of a membrane, but show simply that the coenzymes cannot be considered as simply in solution in the intramitochondrial fluid. In addition, their results, like those of Harman (1950), which have also been quoted against the membrane hypothesis, are obtained on material separated by low-speed centrifugation from homogenates prepared in 0·9% potassium chloride by means of the Waring blender. Both these factors have been shown to cause considerable damage to the mitochondrial structure.

SUMMARY

1. The influence of various physical and chemical agents on the ability of cytoplasmic granules to retain acid phosphatase has been investigated.

2. The best protection of the complex is observed when the granules are kept at 0° in a medium containing 0·25 M-sucrose or glycerophosphate.

3. When these substances are absent or replaced by a variety of others, a progressive release of the enzyme occurs, at a rate which is dependent on the nature of the substance added. The phenomenon is very rapid in distilled water, 0·25 M-glycerol, and 0·15 or 0·44 M-sodium chloride or potassium chloride. It is completely inhibited when 0·25 M-sucrose is present as well in the suspending medium.

4. The rate of release in 0·25 M-sucrose is increased by an increase in temperature, and by addition of small amounts of saponin or deoxycholate.

5. Acid phosphatase is easily adsorbed by various structural entities. In contrast to the native bound form of the enzyme, the adsorbed form is fully active enzymically, and is partly eluted by salts.

6. From a consideration of all the experimental data, it is concluded that the acid phosphatase-bearing granules are osmotic systems and must therefore possess a semi-permeable membrane and an osmotically active internal medium.

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