Progesterone-Induced Lysis of Rat Kidney Lysosomes as Studied by Changes in Light-Absorbance

By P. BADENOCH-JONES and H. BAUM

Department of Biochemistry, Chelsea College, Manresa Road, London SW3 6LX, U.K.

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1. A rat kidney lysosomal fraction was prepared by the method of Maunsbach (1966) and characterized by its content of representative marker enzymes for lysosomes, mitochondria, peroxisomes and endoplasmic reticulum. 2. It was shown that both pH-dependent and progesterone-induced lysis lead to a decrease in the E_{520} of suspensions of this preparation. This decrease parallels quantitatively and temporally the release of soluble acid phosphatase. 3. It is suggested that E_{520} measurements are a valid method for the continuous measurement of changes in lysosomal integrity. 4. As an example, results are included which demonstrate the ability of Zn^{2+} to stabilize lysosomes against spontaneous and progesterone-induced lysis.

The effects of membrane-active agents on lysosomal integrity have been commonly measured by monitoring the release of soluble hydrolases from impure lysosome preparations. Typically, rat liver mitochondrial fractions have been used. Alternatively, lysosome lysis may be determined by measuring the change in E_{520} of suspensions of uncontaminated lysosomes. This method has been successfully used in a preliminary investigation of the lytic effects of streptolysins (Weissmann *et al.*, 1964) and pyrogenic steroids with the 5β -H configuration (Weissmann, 1965) on suspensions of granules from polymorphonuclear leucocytes.

Some workers have used preparations obtained from rat liver to measure, by absorbance changes, the effect on lysosomal integrity of pH and warming (e.g. Sawant *et al.*, 1964). These preparations, however, are likely to be grossly impure and contaminated with mitochondria (Beaufay, 1969).

Rat kidney lysosomes are quite easily separated from homogenates by virtue of their large size and high density, and so have been prepared in a relatively pure state (Maunsbach, 1964; Shibko & Tappel, 1965).

The purity of the preparation of Maunsbach (1966) was monitored by semi-quantitative electron microscopy, but apart from a demonstration that the preparation was enriched 6-10-fold over the homogenate in acid phosphatase activity, no enzymic analysis of the preparation was carried out. The preparation of Shibko & Tappel (1965) was used to measure, by determinations of E_{520} , changes in lysosomal integrity in response to warming and changes in pH. A detailed enzymic analysis of this preparation was published by Shibko & Tappel (1965) claiming that the preparation was enriched in lysosomal hydrolases by factors ranging from 60

for β -glucuronidase to 5.8 for cathepsins. These published results have been criticized by Beaufay (1969) on the grounds that there had been no attempt to achieve a quantitative recovery of enzyme activities during fractionation.

In the present work we have prepared a fraction enriched in rat kidney lysosomes by a method similar to that of Maunsbach (1966). We report its content of marker enzymes and the usefulness and validity of using changes of E_{520} for studying the lytic effects of progesterone on the lysosomes of this preparation.

Methods

Materials

Progesterone (preg-4-ene-3,20-dione) was obtained from Sigma (London) Chemical Co., London S.W.6, U.K. A fresh stock solution of this (25 mM) in ethanol was made up for each experiment. Polyethylene glycols, average mol. wt. 1000, 600, 400 and 200, were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K. as was *p*-nitrophenyl phosphate (disodium salt). The sucrose used was a general laboratory reagent (Fisons Ltd., Loughborough, Leicestershire, U.K.), and other chemicals used were of AnalaR grade.

Preparation of the lysosome fraction

From male Sprague-Dawley rats (800-1100 g body wt.; starved for 24 h) a 'primary granule fraction' was prepared by a method similar to that of Maunsbach (1966). Briefly, rats were killed by cervical dislocation, the kidneys removed, sliced and homogenized in ice-cold, unbuffered, 0.45 M-sucrose by four up-and-down strokes of a Potter-Elvehjem

homogenizer rotating at 1000 rev./min. The homogenate was filtered through a single layer of cheese-cloth and centrifuged at $150g_{\max}$ for 10min (twice) to sediment nuclei, intact cells and debris, in an 8×100 ml swing-out rotor.

The supernatant suspension was removed and centrifuged at $10000g_{\max}$ for 3min in a 6×100 ml angle head of an MSE High-Speed 18 centrifuge. Acceleration time was 1.6min and deceleration time 2.2min with the brake on. The resultant pellet consisted of an upper light-brown layer and a small dark-brown 'button'. The supernatant and upper light-brown layer were removed by swirling; the lower dark-brown pellet was resuspended in unbuffered 0.5M-sucrose and washed and resedimented several times, the upper light-brown material being removed each time until a homogeneous dark-brown pellet remained. This dark-brown primary granule fraction was resuspended at about 3mg of protein/ml in 0.5M-sucrose as a stock suspension. Three to five rats, yielding 15–20g wet wt. of whole kidney, were used for each experiment; the final yield of the primary granule fraction was 0.2–0.5mg of protein/g wet wt. of kidney used.

Enzyme assays

The assays and conditions used were those used by Shibko & Tappel (1965), except for D-amino acid oxidase (EC 1.4.3.3) which was measured by the method of Robinson *et al.* (1962) with D-alanine as the substrate. Protein was measured by the method of Lowry *et al.* (1951) with bovine serum albumin as the standard.

Absorbance measurements

The E_{520} of the primary-granule-fraction suspension was measured continuously with a Hitachi-Perkin Elmer recording spectrophotometer. The glass cuvettes used had a 1cm light path; 2.5ml of suspending medium was used and $25\mu\text{l}$ of stock primary-granule-fraction suspension was added. All measurements were made at 30°C , and the initial absorbance in 0.25M-sucrose was set at about 0.4 E_{520} unit and designated 100% absorbance. Stirring of the suspensions was not necessary, since, under these conditions, no floating or settling was observed.

Release of soluble acid phosphatase

Soluble acid phosphatase was measured in the clear solution obtained after Millipore filtration of the primary-granule-fraction suspension through a $0.22\mu\text{m}$ filter (Swinnex-13 cellulose ester filter; Millipore Filter Corp., Bedford, Mass., U.S.A.); 0.1% (w/v) Triton X-100 was included in the acid phosphatase incubation medium. Measurement of

soluble acid phosphatase after Millipore filtration or centrifugation of the suspension at $10000g_{\max}$ for 10min gave identical results.

Results

Enzyme content

Marker-enzyme activities of the primary granule fraction are shown in Table 1. All the material isolated besides the primary granule fraction was pooled, and the enzyme activities of the primary granule fraction and the other pooled fractions were assayed and compared with the corresponding activities of the original homogenate. Percentage recoveries of the activities after fractionation are shown in Table 1. The three acid hydrolases assayed were all enriched, to a variable degree, over the homogenate. The activities of glucose 6-phosphatase and D-amino acid oxidase were low, indicating little contamination with endoplasmic reticulum or peroxisomes. The relatively high glutamate dehydrogenase activity indicates that the main contaminant of the fraction was mitochondrial.

pH-induced absorbance changes and release of soluble acid phosphatase

The primary granule fraction was suspended in 0.25M-sucrose containing 10mM-sodium acetate buffer, pH5.0, and the E_{520} was measured as a function of time. In a parallel experiment, the release of soluble acid phosphatase was measured in samples withdrawn at suitable intervals to determine whether there was a quantitative and a temporal relationship

Table 1. Marker-enzyme content of the rat kidney primary granule fraction

Results are means of four experiments. Relative specific activity = specific activity in the isolated fraction/specific activity in the original homogenate. For details see the text.

Enzyme	Homogenate specific activity (nmol/min per mg of protein)	Relative specific activity	Recovery (%) (total for all isolated fractions)
Arylsulphatase	1.5	7.8	95.0
β -Galactosidase	4.2	4.2	95.9
Acid phosphatase	48.0	8.2	100.3
Glutamate dehydrogenase	15.0	0.45	106.6
Glucose 6-phosphatase	85.0	0.16	96.4
D-Amino acid oxidase	12.0	0.09	91.2
Protein			96.2

Fig. 1. Correlation between decreases in particulate acid phosphatase activity and in E_{520} of the primary granule fraction during incubation at pH 5.0

Correlation between decreases in particulate acid phosphatase activity (●), and in E_{520} (continuous line) of the primary granule fraction during incubation at 30°C in 0.25M-sucrose containing 10mM-acetate buffer, pH 5.0. The method of calculating particulate acid phosphatase activity is given in the text.

between these two parameters, i.e. absorbance decrease and enzyme release.

The results are shown in Fig. 1. The absorbance is plotted as a percentage of the initial absorbance of the suspension. The total acid phosphatase activity minus the soluble activity at zero time is designated 100% particulate activity, and in Fig. 1 particulate activity is plotted as a percentage of this initial particulate activity. When plotted in this way for comparison, there is a good correlation between these two parameters.

Permeability of primary granule fraction to different compounds

Lysosomes have been shown to act as osmometers and are osmotically supported by a suspending medium containing a solute to which the lysosomal membrane is not permeable. Relative permeabilities of the membrane to different solutes have been measured (Lloyd, 1969, 1971; Lee, 1970, 1971), by suspending the lysosomes in iso-osmotic solutions of different solutes and measuring the release of soluble enzymes, as an index of lysis.

Analogous experiments were carried out in the present study. The primary granule fraction was suspended in 0.25M unbuffered solutions of different solutes (adjusted to pH 7.0 with 0.1M-NaOH) and the decrease in E_{520} /min measured as a percentage of the initial absorbance. The results are shown in Table 2, where the initial absorbance decrease shows an inverse dependence on the molecular weight of the supporting solute (quantitative correlations of absorbance changes in polyethylene glycols are

Table 2. Initial rate of decrease in E_{520} of the primary granule fraction suspended in 0.25M-solutions of solutes of different molecular weights

At the high rates of lysis in glycerol and water the rate of absorbance decrease was measured over a short period (15s) and this rate was then expressed in terms of absorbance decrease/min. With the other suspending media the absorbance decrease/min was calculated from the measured (nearly linear) decrease over the first 2min after addition of the primary granule fraction. The molecular weights quoted for the polyethylene glycols are weight-average molecular weights.

Solute	Mol. wt.	Initial rate of absorbance decrease (% of the initial absorbance/min)
Raffinose	504	0
Sucrose	342	0
Glucose	180	3
Glycerol	92	50
Water	18	118
Polyethylene glycol	1000	0
	600	0.4
	400	6
	200	27

Fig. 2. Decrease in E_{520} and in particulate acid phosphatase activity in suspensions of the primary granule fraction after addition of progesterone

Decrease in E_{520} (continuous line) and in particulate acid phosphatase activity (●) in suspensions of the primary granule fraction after addition of progesterone (final concn. 0.25 mM) (B). The progesterone was added as 25 μ l of a stock solution in ethanol. A corresponding volume of ethanol was added to the control (A). The absorbance decreases on addition of Triton X-100, to a final concentration of 0.1% (v/v) (D), and on suspension of the fraction in water (C), are also shown.

complicated because of the wide distribution of molecular weight species in a sample of a given average molecular weight). With the primary granule fraction suspended in water there is a very rapid absorbance decrease to a final absorbance of 20–40% of the initial absorbance of the suspension in 0.25M-sucrose. When absorbance decreases were induced by suspension of the sample in solutions of low-molecular-weight solutes, the final absorbance was approximately equal to that of the water-lysed sample.

Effect of progesterone on absorbance

The time-course of absorbance decrease on addition of progesterone (final concn. 0.25mM) to a suspension of the primary granule fraction in unbuffered 0.25M-sucrose is shown in Fig. 2.

There was a rapid decrease in absorbance, which resulted in a final absorbance similar to that of the water-lysed sample. Addition of the same concentration of progesterone to a water-lysed sample did not cause any change in absorbance. Soluble acid phosphatase was measured at timed intervals and the decrease in particulate activity, calculated as above, is included in Fig. 2.

Addition of Triton X-100 to a final concentration of 0.1% (v/v) to the primary granule fraction in 0.25M-sucrose led to a rapid and almost complete absorbance decrease, whereas as stated above the water-lysed preparation still showed some 20–40% of the initial absorbance in 0.25M-sucrose. It is probable that the residual E_{520} observed in pH- and progesterone-lysed lysosomes, and in lysosomes suspended in water or solutions of solutes of low molecular weight, is caused by debris from disrupted lysosomes. Suspension of the sample in water results in a residual E_{520} and residual particulate acid phosphatase activity. In the presence of detergent (Triton X-100) the fall of E_{520} almost to zero is probably due to complete disruption and solubilization of the lysosomes. As progesterone- and pH-induced lysis results in a residual E_{520} , this suggests that with these treatments the lysosomes are disrupted by an osmosis-mediated rather than a detergent-like action. This is in agreement with studies by us (Badenoch-Jones & Baum, 1973) on the mechanism of progesterone-induced lysis of rat liver lysosomes, where evidence was presented suggesting that progesterone increases the permeability of the lysosomal membrane to the supporting solute (sucrose), which equilibrates across the membrane and results in osmotic lysis of the lysosomes.

In the present work it was also found that addition of high concentrations of phosphatidylcholine (final concn. 70 $\mu\text{g}/\text{ml}$) to the primary granule fraction in 0.25M-sucrose induced an absorbance decrease resembling that induced by Triton X-100, whereas lower concentrations of lysophosphatidylcholine

(0.5–7 $\mu\text{g}/\text{ml}$) caused an absorbance decrease resembling that induced by progesterone, with the final absorbance corresponding to the value attained by a water-lysed preparation. This suggests that, at high concentration, lysophosphatidylcholine causes complete disruption of the primary granule fraction by a detergent-like action, whereas at lower concentrations lysis is induced by an osmosis-mediated effect.

Progesterone-induced changes in absorbance of mitochondrial suspensions

The main contaminant of the primary granule fraction appears (on the basis of marker-enzyme activity) to be mitochondria. Mitochondria have a high capacity for swelling and contraction, accompanied by absorbance changes (Packer *et al.*, 1968). Thus it was decided to measure any absorbance changes that might be induced by progesterone in mitochondrial suspensions.

Absorbance measurements were carried out on a suspension of rat kidney mitochondria. The upper, light-brown pellet of the $10000g_{\text{max}}$, $\times 3$ min centrifugation of the homogenate was used. [This fraction was studied by semi-quantitative electron microscopy by Maunsbach (1966) and consisted almost entirely of mitochondria.] The same conditions were used as for measurements on the primary granule fraction. The absorbance change on addition of progesterone to a final concentration of 0.25mM to such a mitochondrial suspension in 0.25M-sucrose is shown in Fig. 3. The absorbance initially decreased on addition of

Fig. 3. Change in E_{520} induced by the addition of progesterone to a suspension of rat kidney mitochondria

Change in E_{520} induced by the addition of progesterone (final concn. 0.25mM) to a suspension, in 0.25M-sucrose, of rat kidney mitochondria (B). A corresponding volume of the ethanol solvent (25 μl) was added to the control (A). For comparison, the absorbance decrease induced in a suspension of the primary granule fraction under the same conditions is included (C).

Fig. 4. Protective effect of ZnSO_4 against lysis of the primary granule fraction induced by the addition of progesterone

The protective effect of ZnSO_4 against lysis of the primary granule fraction (suspended in 0.25M-sucrose) induced by the addition of progesterone (final concn. 0.25 mM) (●), or by suspension of the fraction in 0.25M-sucrose buffered at pH 5.9 with 10mM-acetate buffer, pH 5.9 (○). The rate of lysis (as judged by the decrease of E_{520}) in the absence of ZnSO_4 was designated 100%, in each case, or 0% protection. The measured rate of lysis in the presence of ZnSO_4 was hence expressed as a percentage protection by ZnSO_4 where 100% protection indicates no lysis.

progesterone, then gradually increased again almost to its initial value. The basis of this apparent, low-amplitude, reversible swelling is obscure and requires further investigation. It might reflect a process dependent on the coupled oxidation of endogenous substrate, since energy-dependent oscillations of mitochondrial volume (albeit under conditions quite different from those described here) have frequently been observed (Packer *et al.*, 1968). Whatever the basis for this effect there was a distinct and reproducible difference in progesterone-induced absorbance changes of the lysosomal and mitochondrial suspensions. The contaminating mitochondria in the primary granule fraction therefore appear not to interfere significantly with measurement of lysosome lysis by absorbance measurements.

Stabilizing effect of Zn^{2+} on pH-induced and progesterone-induced lysis

Low concentrations of Zn^{2+} have been reported to stabilize rat liver lysosomes against spontaneous thermal and pH-induced lysis (Chrapil *et al.*, 1972). We suspended the primary granule fraction in 0.25M-sucrose containing 0–10mM- ZnSO_4 (ZnSO_4 did not appreciably alter the pH of the medium) and measured the rate of absorbance decrease either on addition

of 0.25mM-progesterone or on addition of sodium acetate buffer (final concn. 10mM, pH 5.9). The results are shown in Fig. 4. Complete protection was afforded against spontaneous absorbance decrease at pH 5.9 with 2mM- ZnSO_4 , and there was a partial protection against progesterone-induced lysis, which increased with increasing zinc concentration.

Discussion

To use a lysosome suspension for absorbance studies it is important to know the purity of the isolated preparation and the functional significance of such a non-specific parameter as absorbance change.

The primary granule fraction isolated in this work was purified several times over the homogenate in terms of its acid hydrolase content. The lysosomes present in such a primary granule fraction have been shown to be protein resorption droplets from the proximal convoluted tubule, and are formed by fusion of a lysosome and a protein-containing phagosome and hence are classified as phago-lysosomes (Straus, 1964). Therefore the degree of purification of hydrolases may not be very high even for a pure preparation. This contrasts with the situation in rat liver, where the lysosomes constitute only a small percentage of total cellular protein and are expected to be purified some 120-fold in an uncontaminated preparation (Beaufay, 1969).

All particles present in a suspension might be expected to scatter light to varying degrees and so affect the E_{520} . It is thus important to ensure that the mitochondrial content of the preparation is low because, as noted above, these particles have a high capacity for swelling and contracting with an associated absorbance change. In the preparation isolated here the main contaminant appears to be mitochondria, by virtue of the glutamate dehydrogenase content of the preparation. The low activity of glucose 6-phosphatase and D-amino acid oxidase in the isolated preparation is indicative of a low contamination with endoplasmic reticulum and peroxisomes. Moreover, at least insofar as progesterone-induced lysis is concerned, the extent of contamination with mitochondria appears not to be sufficient to interfere significantly with lysosome absorbance measurements. Indeed, the correlation between the final absorbance in water-lysed preparations and in those lysed in the presence of progesterone suggests that a study of absorbance changes is of general validity for this preparation.

This conclusion is reinforced by the evidence of a good quantitative correlation between absorbance decrease and induced lysosome lysis, as judged by release of soluble acid phosphatase. Therefore E_{520} changes of suspensions of this preparation might provide a simple parameter for measuring the effects

of membrane-active agents on lysosomal stability and permeability, provided that aggregation of the particles can be avoided and that the agents used are completely soluble.

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