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A rapid and simple procedure to deplete rat-liver mitochondria of lysosomal activity

Lysosomal activities that contaminate rat-liver mitochondria prepared by the conventional methods often hamper the exact localization of lysosomal and mitochondrial isoenzymes[1–5]. It has been shown[6–8] that lysosomes present in subcellular

enzyme preparations are responsible for the inactivation of both mitochondrial and soluble enzymes, and TAPPEL *et al.*[9] and MELLORS *et al.*[10] have demonstrated that added lysosomes rupture mitochondrial membranes and uncouple oxidative phosphorylation.

Thus many procedures have been worked out for the removal of the lysosomes. An elegant and profoundly studied method is that of WATTIAUX and co-workers[11,13], based upon the findings that pretreatment of the rat with Triton WR-1339 causes a decrease in the density of the lysosomes, which allows the separation of these particles from the mitochondria on a sucrose gradient. This method, however, suffers from several disadvantages. The whole procedure is time consuming and the mitochondrial membranes may be damaged by the solubilizing effect of the detergent. In particles of livers of Triton-treated rats cytochrome *c* oxidase (EC 1.9.3.1) is recovered in part in the supernatant[11], which is never the case in partitions of normal rat livers[13]. It is also known that centrifugation on sucrose gradients affects the mitochondrial phosphorylating machinery.

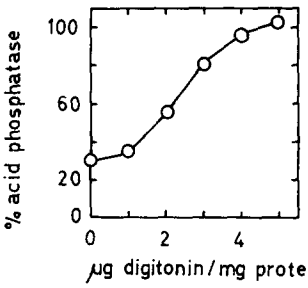
BAUDHUIN[14] found that low concentrations of digitonin abolish the latency of acid phosphatase (EC 3.1.3.2) in a rat-liver mitochondrial preparation. SCHOLTE[6] showed that digitonin in a concentration that releases the outer membranes from rat-liver mitochondria[15] also solubilizes most of the acid phosphatase from the contaminating lysosomes. These findings suggested that low concentrations of digitonin probably could be used to deplete rat-liver mitochondria of lysosomes, without affecting the mitochondrial integrity.

Treatment for 20 min at 0° of a suspension of rat-liver mitochondria (50 mg/ml) with 5 μg digitonin per mg protein abolishes the latency of acid phosphatase (Fig. 1).

To prepare purified mitochondria we adopted the procedure of MYERS AND SLATER[16], but carried it out rapidly (with higher centrifugal forces and shorter centrifugation times), and at pH 7.5, in order to minimize lysosomal attack on the mitochondria. Rat liver was rinsed with ice-cold 0.25 M sucrose–1 mM Tris–HCl (pH 7.5), dried between filter-paper, weighed, thoroughly minced and washed, and homogenized in an electrically-driven Potter–Elvehjem homogenizer with a teflon pestle. The 15 % homogenate was centrifuged for 1 min at 3200 $\times$ g_{av}, and the supernatant centrifuged for 2 min at 17000 $\times$ g_{av}. All centrifugation times are excluding the acceleration and deceleration times. The precipitate was suspended by homogenization in 1 ml 0.25 M sucrose–1 mM Tris–HCl (pH 7.5) per g original liver, and 1 ml 0.25 M sucrose–1 mM Tris–HCl (pH 7.5) containing 1.75 mg digitonin per g original liver was added. The digitonin was recrystallized from ethanol. After 2 min standing with shaking in an ice-water bath, the mixture was diluted with 3 vol. 0.25 M sucrose–1 mM Tris–HCl (pH 7.5) and centrifuged for 4.5 min at 19000 $\times$ g_{av}. The fluffy layer on the pellet was removed by washing and the pellet was resuspended in 0.25 M sucrose and centrifuged for 5.5 min at 19000 $\times$ g_{av}. This washing procedure was repeated and the final pellet resuspended in 0.25 M sucrose.

The effect of using different amounts of digitonin in this procedure on the activity of acid phosphatase, adenylate kinase (EC 2.7.4.3) and glutamate dehydrogenase (EC 1.4.1.3) in the final mitochondrial preparation is given in Fig. 2. Less than 10 % of the acid phosphatase activity remained after treatment with 1.5 mg digitonin per g liver. The glutamate dehydrogenase activity was unaffected, indicating that the inner mitochondrial membrane does not become leaky under the conditions used.

At concentrations of digitonin up to 2 mg/g liver, the activity of the inter-membrane enzyme adenylate kinase[17] is little affected indicating that the outer membrane must have remained intact. The decrease in adenylate kinase with 3 mg digitonin per g liver probably reflects the beginning of rupture of the outer mitochondrial membrane. The residual acid phosphatase activity shows no latency, but cannot be decreased by washing with sucrose.



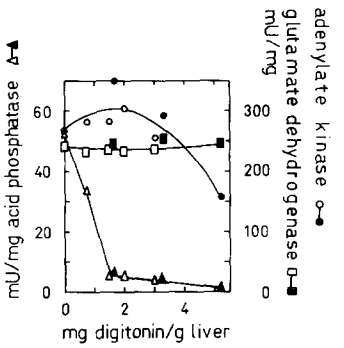


Fig. 1. Abolition of the latency of acid phosphatase in a rat-liver mitochondrial preparation by low concentrations of digitonin. The mitochondria were incubated as described in the text. The activity of acid phosphatase was measured as described in ref. 13. Total acid phosphatase activity of the mitochondrial preparation was (100 % value) measured after sonication.

Fig. 2. The effect of incubation of rat-liver mitochondria with increasing amounts of digitonin on the activity of glutamate dehydrogenase, adenylate kinase and acid phosphatase. The mitochondria were prepared as indicated in the text. The assays were carried out as described in Table I. The open and blocked symbols are from two different experiments. △, acid phosphatase; □, glutamate dehydrogenase; ○, adenylate kinase. mU/mg, 1 nmole substrate/min per mg protein.

TABLE I

THE ACTIVITIES OF MARKER ENZYMES IN 'NORMAL' MITOCHONDRIA (M + L) COMPARED WITH MITOCHONDRIA DEPLETED OF LYSOSOMAL CONTAMINATION (M)

The enzymes were assayed as described in ref. 6, after sonication of the fractions for 1 min per ml enzyme suspension, at 21 kHz with an amplitude from peak to peak of 2 μm. Catalase (EC 1.11.1.6) was measured as described by BEERS *et al.*[18], and calculated from the decrease in H_2O_2 absorption at 240 nm ($A_{240\ nm} = 0.036\ mM^{-1}\ cm^{-1}$). The enzyme activities (except catalase) are expressed in munits (1 nmole/min) per mg protein.

	Number of experiments	*M + L*	*M*
Protein (mg/g liver)	5	12.8 ± 1.5*	14.2 ± 2.3
Acid phosphatase	5	52.4 ± 3.3	4.7 ± 0.3
Adenylate kinase	4	206 ± 15	240 ± 35
Propionyl-CoA carboxylase (EC 6.4.1.3)	3	25.6 ± 2.6	25.3 ± 1.9
Glutamate dehydrogenase	1	266	292
Rotenone-insensitive NADPH cytochrome *c* reductase	2	3.70, 3.15	3.72, 4.40
Glucose-6-phosphatase (EC 3.1.3.9)	2	24.4, 25.3	16.8, 9.5
Catalase (units/mg protein)	2	141, 92	74, 105

* Standard error of mean.

In Tables I and II the properties of the mitochondria freed from lysosomal activity (M) are compared with mitochondria prepared in the same way, but without the digitonin (M+L). The mitochondrial activities of the preparations are comparable.

TABLE II

COMPARISON OF THE OXIDATIVE PHOSPHORYLATION AND NADH OXIDATION IN M + L AND M

The ATPase activities were measured according to PULLMAN[19], and the P:O ratios and the respiratory-control indices (R.C.I.) according to ESTABROOK[20].

	M + L	*M*
ATPase (munits/mg protein):		
without additions	90.3, 65.9	76.8, 47.1
plus 0.1 mM 2,4-dinitrophenol	217, 365	266, 302
Succinate (60 mM) oxidation:		
P:O	1.43	1.48
R.C.I.	12.3	13.9
L-Glutamate (30 mM) oxidation:		
P:O	2.13	2.21
R.C.I.	5.8	13.4
NADH oxidation (munits/mg protein):		
intact mitochondria	0	0
sonicated mitochondria	24	24
sonicated mitochondria + 2.5 μM rotenone	0	0

Not shown in Table II is that the P:O ratios and the respiratory-control indices of the digitonin-treated mitochondria remain constant for a longer period than normal preparations. The microsomal marker enzymes, glucose-6-phosphatase and rotenone-insensitive NADPH cytochrome *c* reductase, and the peroxysomal marker enzyme catalase are not considerably lowered in the M preparation, but the acid phosphatase activity is lowered from 52.4 to 4.7 munits/mg protein (Table I). This remaining activity is very low compared with that found by LEIGHTON *et al.*[12] for their preparation (16.7 munits/mg).

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The action of cations and anions on the respiration and ATPase activity of submitochondrial particles

Recently the effect that salts have on the oxidative phosphorylation process of submitochondrial particles has been studied in several laboratories. Although SMITH AND BEYER[1] reported that K^+ did not affect the process, the data of PAPA *et al.*[2] indicate that salts at higher concentrations inhibit the phosphorylation reaction. On the other hand, CHRISTIANSEN *et al.*[3], using low concentrations of salts in their incubation media, found that different salts diminish the P:O ratios of submitochondrial particles either by increasing the respiratory rate or by decreasing the phosphorylation rate or both. As these results are in apparent contradiction with those obtained in our laboratory in which K^+ was found to increase the State 3/State 4 ratio of K^+-depleted mitochondria[4], the effect that various salts have on the respiration and ATPase activity of submitochondrial particles was examined.

Bovine heart mitochondria were prepared according to the method of LÖW AND VALLIN[5] and were stored for at least 24 h in 0.25 M sucrose and 1.0 mM EDTA (pH 7.3) at —4°. EDTA sonic particles were prepared according to the method of LEE *et al.*[6]. Respiration was measured polarographically and ATPase was measured as indicated in Fig. 1. Inorganic phosphate was determined in the 6% trichloroacetic acid supernatant by the method of SUMNER[7].

In submitochondrial particles oxidizing NADH, the tested salts, KCl, KNO_3, potassium formate and potassium acetate, stimulated O_2 uptake to a similar extent (Fig. 1). On the other hand, the salts tested inhibited ATPase to a different degree;