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PREPARATION OF MEMBRANE-BOUND AND OF SOLUBILIZED ($Na^+ + K^+$)-ATPase FROM RECTAL GLANDS OF *SQUALUS ACANTHIAS*

THE EFFECT OF PREPARATIVE PROCEDURES ON PURITY, SPECIFIC AND MOLAR ACTIVITY

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Summary

A simple method is described for the routine preparation of larger quantities of purified ($Na^+ + K^+$)-ATPase from the rectal glands from *Squalus acanthias* and for solubilization of the purified enzyme in a highly active form. Microsomes are prepared by homogenization of the glands in a Waring Blendor followed by differential centrifugation. They keep their activity for years when stored at −70°C. Based on an earlier method (Jørgensen, P.L. and Skou, J.C. (1971) Biochim. Biophys. Acta 233, 366—380), enzyme with a specific activity of 1500 μmol $P_i \cdot mg^{-1}$ protein $\cdot h^{-1}$ was prepared by treating the microsomes with low concentrations of deoxycholate followed by differential centrifugation, and with a yield of 70% of the activity in the deoxycholate-treated microsomes. The purified enzyme can be dissolved in deoxycholate in the presence of cholesterol, and after a single centrifugation to remove undissolved enzyme, the specific activity of the solubilized enzyme is increased to 2400—2600 μmol $P_i \cdot mg^{-1}$ protein $\cdot h^{-1}$. Precipitation of the solubilized enzyme leads to a decrease in specific activity to 1500 μmol $P_i \cdot mg^{-1}$ protein $\cdot h^{-1}$ and to a decrease in molar activity.

Introduction

The present paper describes a simple method for the preparation of larger quantities of purified ($Na^+ + K^+$)-ATPase from the rectal glands of *Squalus*

acanthias, and a procedure for solubilization of the enzyme in a purified active form.

Jørgensen [1] described a simplified version of the method used for purification of enzyme from the outer medulla of kidney. It gave a preparation with a specific activity of 1200—1500 μmol $P_i \cdot mg^{-1}$ protein $\cdot h^{-1}$. However the yield from kidney was low. Rectal glands from *Squalus acanthias* are a richer source, and Hokin et al. [2] described a procedure for preparation of larger quantities of enzyme from this tissue, involving solubilization, densitygradient centrifugation and $(NH_4)_2SO_4$ precipitation. The method gives a highly purified preparation from the points of view of protein purity and number of ^{32}P labelling sites $\cdot$ mg^{-1} protein. However, the specific and molar activity is about 2/3 of the activity of the enzyme kidney outer medulla purified to the same degree, and equal to the specific activity of the less-pure enzyme, which can be obtained by the simplified procedure [1]. The kidney enzyme was purified in a membranous form by using a low concentration of sodium dodecyl sulphate (SDS), which did not dissolve the enzyme-containing membranes, but removed and dissolved proteins which did not belong to the enzyme system. The membranes were then separated from the dissolved protein by centrifugation. As enzyme prepared from kidney outer medulla by solubilizing concentrations of detergent, deoxycholate, and $(NH_4)_2SO_4$ precipitation [3] gave the same lower specific and molar activity as obtained by Hoking et al. [2]. from rectal glands, solubilization and/or precipitation seem to decrease the activity.

In the present experiments, the enzyme was purified from rectal glands of *Squalus acanthias* in membranous form by the method described by Jørgensen and Skou [4], using deoxycholate as detergent. The purified membranes were dissolved in deoxycholate and then precipitated, to find the step at which the activity decreases. We tried to find a simple way for the routine preparation of purified enzyme in large quantities, to ensure that the preparative procedure did not lead to partial inactivation, and to see if the enzyme could be solubilized in a highly active form.

Methods

Preparation of microsomes. Rectal glands from *Squalus acanthias* were washed in sea water and stored on fishing-boats at 0°C in sea water to which was added 50 mg Polymyxin b, 0.5 g streptomycin and 600 mg penicillin. The glands were received up to 1 week after the sharks had been caught.

The glands were homogenized in a Waring Blendor in small portions for 60 s in 0.25 M sucrose, 5 mM EDTA, 30 mM histidine (adjusted to pH 6.8 with propanediol). The microsomes were isolated by the method of Jørgensen and Skou [5] and were resuspended in 0.25 M sucrose, 30 mM histidine-HCl (pH 6.8), 1 mM EDTA and 5—6 mg protein/ml. The yield was 8—10 g microsomal protein from 1 l glands. Stored at —70°C, the microsomes retained their activity for several years.

Enzyme preparation. The microsomal fraction was thawed, heated slowly to room temperature and diluted 1 : 1 (v/v) with 25 mM imidazole, 1 mM EDTA (adjusted to pH 7.7 at 20°C with propanediol) and with sodium deoxycholate

in concentrations which gave maximum activity after 30 min incubation at room temperature, pH 7.1 and 2.5 mg protein/ml [4]. The optimum concentration of deoxycholate was found to be 0.15—0.17% (w/v) (Fig. 1). The solution also contained 0.05% saponin (see below).

After incubation, the microsomal fraction was cooled to 0°C and centrifuged at this temperature at 5900 × *g* for 15 min, 31 000 × *g* for 30 min and, finally, 48 000 × *g* for 3 h (Table I) and the pellets were resuspended in 25% glycerol, 30 mM histidine, pH 6.8 (buffer 1) at 20°C. The resuspended pellets were combined and centrifuged at 220 000 × *g* for 1 h in a Beckman L75 ultracentrifuge at 12°C. The pellet was resuspended in buffer 1 at 2—4 mg protein/ml and stored at −20°C or, for prolonged storage, at −70°C.

Saponin was tested in a search for detergents with a broader optimum concentration range. The maximum activity obtained with saponin is higher and the inhibitor effect of increasing concentrations of saponin is lower than with deoxycholate (Fig. 1). Saponin in concentrations up to 0.5% (w/v) did not, however, remove inactive protein from the microsomal membrane to the same extent as deoxycholate, and was not an effective substitute for deoxycholate. 0.05% saponin was routinely included in the incubation medium together with deoxycholate because it gave a firmer precipitate and a certain degree of protection against the inhibitory effect of deoxycholate (Fig. 1).

Activity measurements. The ATPase activity was tested at 37°C in 30 mM histidine (pH 7.4), 3 mM ATP, 4 mM Mg^{2+}, 130 mM Na^+, 20 mM K^+, with and without 10^{-4} M ouabain. The test solution also contained 1 mM phospho*enol*-pyruvate and 3 μl/ml pyruvate kinase (dissolved in glycerol at 10 mg/ml, obtained from Boehringer).

Inorganic phosphate was determined by the method of Fiske and Subbarow [6].

The *p*-nitrophenylphosphatase activity was tested at 37°C in 30 mM histidine (pH 7.4), 20 mM Mg^{2+}, 10 mM *p*-nitrophenylphosphate and 150 mM K^+. The released *p*-nitrophenol was measured as the absorbance at 410 nm after the addition of 2 vols. 0.5 M Tris base. ϵ was assumed to be 18 100 $M^{-1} \cdot cm^{-1}$.

0.033% albumin [7] and 0.2 mM EGTA were added to the test solutions. This increased the activity of the ATPase (but not of the *p*-nitrophenylphosphatase) about 30% for the membrane-bound enzyme. In testing the solubilized enzyme, the albumin was increased to 0.1% (see below). Bovine serum albumin was obtained from Behring Werke AG [7].

^{32}P incorporation. The number of $Na^+ + Mg^+$-dependent phosphorylation sites was determined by labelling with ^{32}P from [^{32}P]ATP as described previously [8]. [^{32}P]ATP was obtained from the Radiochemical Centre (Amersham, U.K.) and was purified and converted to the Tris-salt by chromatography on a DEAE-Sephadex G-25 column (Pharmacia) [9].

SDS polyacrylamide gel electrophoresis was carried out according to Weber and Osborn [10] using 7.5% gels. Staining was according to Fairbanks et al. [11], and the gels were scanned in a Beckman Acta III with scanning equipment (aperture 0.1 mm).

Proteins were precipitated with 5% trichloroacetic acid, and estimated according to Lowry et al. [12], using bovine serum albumin as standard.

Density gradient centrifugation. 3 ml enzyme was put on a 28-ml gradient of

15--45% (w/v) sucrose, with 2 ml 55% sucrose in the bottom of the tube and centrifuged at 98 000 × *g* at 4°C in a Beckman Ti70 fixed angle rotor overnight. The gradient was pumped out and the samples around the activity peak collected and centrifuged 220 000 × *g* for 1 h. The sediment was resuspended in buffer 1 and stored at −20°C.

Solubilization of membrane-bound enzyme. Enzyme in buffer 1 was made 350 mM in NaCl and 2.8 mg/ml in protein. To this was added 1 vol. buffer 1 containing 0.4% deoxycholate, 0.2% saponin and 350 mM NaCl. After 10 min at 12°C, the solution was centrifuged at 220 000 × *g* at 12°C for 2 h. This gave a clear supernatant containing the solubilized enzyme. When cholesterol was included, buffer 1 with the detergents and NaCl was saturated with cholesterol at 50°C, cooled to room temperature and added to the enzyme suspension which was held at 0—2°C. The final temperature was 12°C.

Precipitation of enzyme. To the supernatant was added 1 vol. (30% (w/v) polyethyleneglycol (M_r = 6000) in buffer 1 (Ottolenghi, P., personal communication). The enzyme precipitated out of solution and was collected by centrifugation, washed twice and resuspended in buffer 1.

Results

Deoxycholate increases the specific activity of the microsomes from about 350 to 600—700 μmol $P_i \cdot mg^{-1}$ protein $\cdot h^{-1}$, Fig. 1. It can be further increased to about 1500 μmol $P_i \cdot mg^{-1}$ protein $\cdot h^{-1}$ by a few centrifugation steps (the 220 000 × *g* pellet), and with a yield of about 70% of the activity in the deoxycholate-treated microsomes, Table I.

To obtain this specific activity, it is necessary to collect the sediment after 31 000 × *g* before the following centrifugation, and to centrifuge the supernatant for 3 h at 48 000 × *g*. If the supernatant after the 31 000 × *g* centrifugation is centrifuged at a higher speed, e.g. 220 000 × *g* for 30 min, the distribution of protein and activity between sediment and supernatant is as above, but the specific activity of the resuspended sediment is 1/3 lower. This is not due to an activation during the 3 h run, but to a loss of total activity when sedimented at higher speed.

The 220 000 × *g* enzyme is not homogeneous. The pellet has a firm yellowish center and the rest is white and more loose. Be gentle stirring with the solution used for resuspension it is possible to separate the two parts; the white part has a specific activity of approximately 1650 μmoles $P_i \cdot mg^{-1}$ protein $\cdot h^{-1}$ for the ATPase and 230 μmol *p*-nitrophenol $\cdot mg^{-1}$ protein $\cdot h^{-1}$ for the *p*-nitrophenylphosphatase, while the specific activities of the yellowish part are 1200 and 175, respectively, Table I. The white loose part contains about 80% of the protein in the 220 000 × *g* pellet.

The 1500 μmol $P_i \cdot mg^{-1}$ protein $\cdot h^{-1}$ obtained with the 220 000 × *g* pellet enzyme is the same as was obtained by the more complicated and time-consuming solubilization procedure by Hokin et al. [2] on the same enzyme source, Table II. However, the 220 000 × *g* enzyme is less pure from the point of view of 96 000 and 56 000 dalton polypeptides, α and β chains in the following. The α + β chains comprise 72% against 92% of total protein, but the turnover is higher, 12 500 against 6300 based on ^{32}P labelling and 5500 against 3500 based on α-polypeptide content.

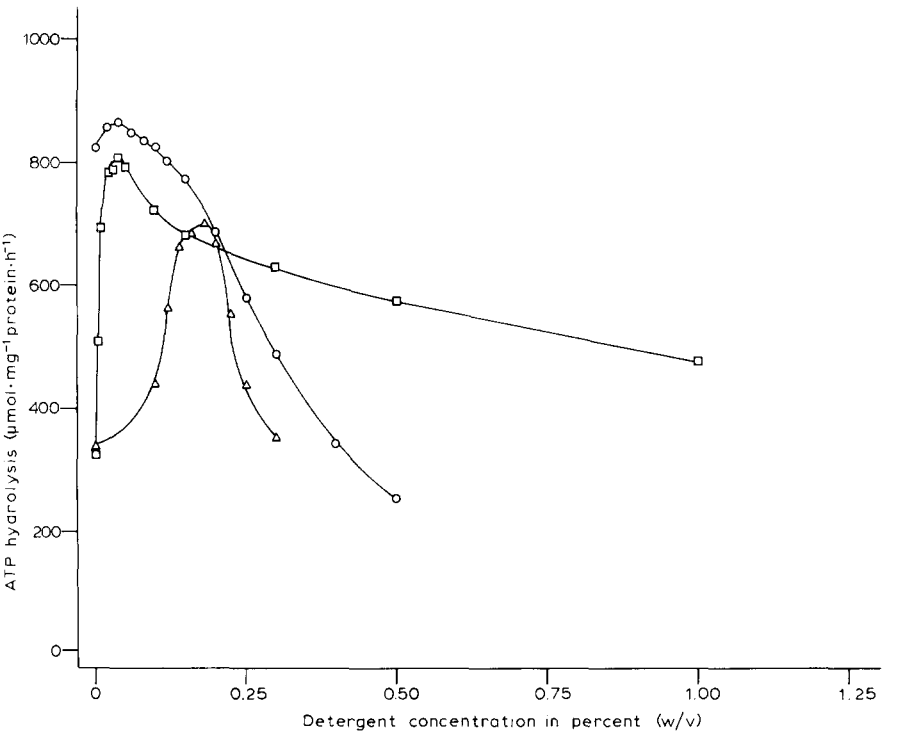

Fig. 1. The effect of detergents on the activity of $(Na^+ + K^+)$-ATPase in microsomes from the rectal gland of *Squalus acanthias*. The microsomes were incubated with varying concentrations of deoxycholate (△——△) and saponin (□——□) and with saponin 0.05% and varying concentrations of deoxycholate (○——○).

TABLE I

SPECIFIC ACTIVITY AND PROTEIN RECOVERY UPON PURIFICATION OF $(Na^+ + K^+)$-ATPase

Values for specific activity and protein concentration are given as the mean ± S.E., n is the number of experiments. pNPPase, *p*-nitrophenylphosphatase; DOC, deoxycholate.

	Specific activity (μmol $P_i \cdot mg^{-1}$ protein $\cdot h^{-1}$)			Recovery in % of total in microsomes		
	ATPase	pNPPase	Ratio	Protein	ATPase	pNPPase
Microsomes	285 ± 9	60 ± 1	4.8	100	—	—
DOC treated microsomes	752 ± 14	112 ± 1	6.7	100	100	100
Pellet 6100 × *g* (n = 4)	680 ± 21	93 ± 3	7.3	6	6	6
Pellet 31 000 × g (n = 4)	1354 ± 54	183 ± 5	7.4	18 ± 0.5	32 ± 0.4	32 ± 0.6
Pellet 48 000 × *g* (n = 4)	1326 ± 38	177 ± 3	7.5	33 ± 0.6	59 ± 1	58 ± 1
Pellet 220 000 × *g* (n = 4)	1517 ± 24	201 ± 1	7.5	36 ± 0.2	73 ± 3	71 ± 2
White pellet (n = 3)	1656 ± 14	232 ± 6	7.1			
Yellow pellet (n = 3)	1217 ± 73	175 ± 12	7.0			
Gradient enzyme (n = 4)	2020 ± 68	299 ± 6	6.8	12 ± 0.7	32 ± 0.9	32 ± 2.4
Solubilized enzyme * (n = 8)	2419 ± 53	353 ± 7	6.9	13	38	39
PEG precipitated (n = 3)	1524 ± 55	274 ± 1	5.6	13	26	32

* Test time 30 s.

TABLE II

MOLAR AND SPECIFIC ACTIVITY OF DIFFERENT PREPARATIONS OF $(Na^+ + K^+)$-ATPase

Different levels of purification are shown together with data on the enzyme prepared from shark tissue [2] and dog kidney [3]. Values are the mean ± S.E., n is the number of experiments. PEG, polyethyleneglycol.

	Specific activity (μmol · mg^{-1} protein · h^{-1})		Phosphorylation (nmol ^{32}P/mg)
	ATPase	pNPPase	
Microsomes	288 ± 9 (n = 8)	60 ± 2 (n = 8)	0.50 ± 0.02 (n = 8)
220 000 × g pellet enzyme	1517 ± 24 (n = 4)	201 ± 1 (n = 4)	2.02 ± 0.06 (n = 4)
Gradient enzyme	2020 ± 68 (n = 4)	299 ± 6 (n = 4)	2.75 ± 0.17 (n = 4)
Solubilized enzyme	2419 ± 53 (n = 8)	353 ± 7 (n = 8)	3.60 ± 0.10 (n = 5)
PEG-precipitated enzyme	1524 ± 55 (n = 3)	274 ± 1 (n = 3)	3.10 ± 0.04 (n = 3)
$(NH_4)_2SO_4$ precipitated shark enzyme [2]	1510		4.08
$(NH_4)_2SO_4$ precipitated dog kidney enzyme [3]	1500		3—4 *

Amount of α-chains		Molar activity per ^{32}P-site · min^{-1}		Molar activity per α-chain · min^{-1}		α + β chains in percent of total
In percent of total	Per ^{32}P-site (molar ratio) ***	ATPase	pNPPase	ATPase	pNNPase	
		9 700 ± 523	1990 ± 77			
47 ± 1.4 (n = 5)	2.5	12 494 ± 405	1665 ± 59	5499	756	72 (n = 5)
59 ± 1.0 (n = 5)	2.2	12 313 ± 405	1828 ± 76	5572	825	88 (n = 5)
60 ± 1.2 (n = 3)	1.75	11 070 ± 207	1702 ± 36	6399	987	92 (n = 3)
60 ± 1.2 (n = 3)	2.00	8 191 ± 335	1473 ± 73	4063	730	91 (n = 3)
72	1.8	6 300		3500		92
70		6 500 **		3700		100

* Ouabain binding sites.

** Molar activity per ouabain binding site.

*** Based on a molecular weight of 96 000.

Solubilization

The amount of protein which goes into solution at a given deoxycholate concentration increases with the concentration of NaCl or KCl in the solution and at a given salt concentration with the deoxycholate concentration [13,14]. The specific activity of the solubilized enzyme decreases with the amount of protein which goes into solution. With 1.4 mg protein/ml of the 220 000 × g pellet

TABLE III

THE EFFECT OF CHOLESTEROL ON SOLUBILIZED ENZYME

The effect of cholesterol in saturating concentrations on the specific activity of the deoxycholate solubilized $(Na^+ + K^+)$-ATPase. The test time is 30 s, and values are given ± S.E.

	ATPase activity (μmol · mg^{-1} protein · h^{-1})	pNPPase activity in (μmol · mg^{-1} protein · h^{-1})
Without cholesterol	1801 ± 89 (n = 3)	255 ± 23 (n = 3)
With cholesterol	2419 ± 53 (n = 8)	353 ± 7 (n = 8)

TABLE IV

THE EFFECT OF TEST TIME ON ATPase AND *p*-NITROPHENYLPHOSPHATASE ACTIVITIES

Activity given as μmol inorganic phosphate or *p*-nitrophenol released from ATP and *p*-nitrophenylphosphate respectively, as a function of test time. (S.E., $n = 4$).

Test time	ATPase activity ($\mu mol \cdot mg^{-1}$ protein)	pNPPase activity ($\mu mol \cdot mg^{-1}$ protein)
30 s	20.7 ± 0.3	2.93 ± 0.04
1 min	37.6 ± 0.6	5.41 ± 0.07
2 min	68.9 ± 1.3	10.07 ± 0.15
5 min	150.2 ± 2.0	21.81 ± 0.31
10 min	272.4 ± 4.6	39.26 ± 0.87

enzyme, the highest activity is obtained using 0.2% deoxycholate, 0.1% saponin and 350 mM NaCl. About 35% of the protein is solubilized, and by this is meant that it does not sediment after 2 h at 220 000 × *g* and that the supernatant is clear. On a Sepharose 6B-CL (Pharmacia) column equilibrated with 0.15% deoxycholate, 0.05% cholate and 350 mM NaCl it elutes with a K_{av} of 0.38 but with loss of activity (see Ref. 7).

The specific activity both of the ATPase and the *p*-nitrophenylphosphatase increases by the addition of cholesterol from 1800 to 2400 $\mu mol\ P_i \cdot mg^{-1}$ protein $\cdot h^{-1}$ for the ATPase and from 255 to 353 μmol *p*-nitrophenol $\cdot mg^{-1}$ protein $\cdot h^{-1}$ for the *p*-nitrophenylphosphatase (test time 30 s, see below). Phosphatidylserine and phosphatidylethanolamine added to the solubilizing medium did not have an effect like cholesterol, on the contrary, they decreased the activity.

The solubilized enzyme is relatively stable when stored at −20°C; after 5—10 days storage the activity was 5—10% lower. In the test medium at 37°C the activity is not linear as a function of time as it is for the membrane-bound enzyme. This is partly due to the detergent added to the test medium with the enzyme. Albumin in the test solution tended to stabilize the activity (see Ref. 15). Optimum effect was obtained with 0.1% albumin and with a dilution of the enzyme which gave 0.02% deoxycholate and 0.01% saponin or less in the test solution.

Even under these conditions the activity was not linear with time, Table IV. The values given in Table I—III for the solubilized enzyme are based on measurements of hydrolytic activity after 30 s.

Solubilization of the enzyme leads to an increase in specific activity, both for the ATPase and the *p*-nitrophenylphosphatase and to purification from the point of view of protein and site numbers. The molar activity based on ^{32}P labelling is slightly decreased to about 11 000/min for the ATPase based on 30 s incubation. Based on initial values, the specific activity is about 2600 $\mu mol\ P_i \cdot mg^{-1}$ protein $\cdot h^{-1}$ and the molar activity is equal to that of the 220 000 × *g* pellet enzyme and the gradient enzyme.

Solubilization with deoxycholate in the presence of cholesterol has thus no deleterious effect on the molar activity.

Polyethyleneglycol precipitation

When the deoxycholate solubilized enzyme is precipitated with polyethyleneglycol, it becomes stable in the test solution as it was before solubilization. It has the same protein composition as the solubilized enzyme. The specific activity decreases and becomes identical to the specific activity of the Lubrol (a nonionic polyoxyethylene ether) solubilized ammonium sulphate precipitated enzyme from the same source [2] and of the deoxycholate solubilized ammonium sulphate precipitated enzyme from outer medulla of kidney [3]. The molar activity decreases and become closer to the molar activity of the two ammonium sulphate precipitated enzymes, Table III.

The ATPase activity decreases more than the *p*-nitrophenylphosphatase activity, i.e. the ATPase/*p*-nitrophenylphosphatase ratio decreases from about 7.5 to 5.6.

Discussion

The rectal glands keep their enzymatic activity when stored on ice for at least a week on the fishing-boats, which makes it easy to arrange the delivery. Microsomes can be prepared by a simple procedure which does not involve dissection of the tissue, and they keep their activity for years stored at −70°C. The enzyme with a specific activity of 1500 μmol $P_i \cdot mg^{-1}$ protein $\cdot h^{-1}$ can be prepared by a short incubation with deoxycholate followed by a few differential centrifugations which can be accomplished in less than one day, and with a yield of 70% of the activity in the deoxycholate-treated microsomes. This specific activity is comparable to the one obtained by the simplified procedure for preparation of enzyme from outer medulla from kidney [1], but the preparation of microsomes in large quantities is simpler from rectal glands.

Solubilization with deoxycholate in the presence of NaCl and cholesterol followed by a single centrifugation leads to a purification which, from the point of view of $\alpha + \beta$ chains, are comparable to the one obtained by the Hokin procedure [2]. However, the molar activity based on ^{32}P labelling as well as α-chain content is about twice as high. Based on 30-s test-values, the specific activity is equal to the highest specific activity which has been obtained in enzyme prepared in membranous form from outer medulla of kidney [1]. Based on initial activity, it is even higher. The purified enzyme can thus be dissolved in a form which has the same or higher specific activity as can be obtained in membranous form and without a decrease in molar activity. When the enzyme is precipitated, the specific activity decreases to the value obtained by the procedure of Hokin et al. [2], and the molar activity decreases. As saponin in low concentrations does not, or only to a slight extent, increase the activity of the precipitated enzyme, it seems unlikely that the decrease in activity is due to vesicle formation upon precipitation. This suggests that it is due to the aggregation of the enzyme molecules. It seems likely that the molar activity of the ATPase of the microsomal enzyme, which has not been in contact with detergents, is a minimum number. The lower molar activity of the precipitated enzyme then suggests that the precipitation leads to partial inactivation.

The molar activity of the ATPase increases and the *p*-nitrophenylphosphatase decreases when the microsomes are treated with a low concentration of

deoxycholate, Table III. As *p*-nitrophenylphosphate can penetrate plasma membranes [16], while ATP cannot, the change in molar activity can be explained from an opening of vesicles by deoxycholate, provided that there is a mixture of opened, of right-side-out and of right-side-in closed vesicles in the microsomal preparation. It is, however, not possible to exclude an activating effect of the detergent. However, if this is the case, the detergent is bound firmly enought to withstand both the washing during the differential and the density gradient centrifugation, Table II.

It has been proposed that the enzyme molecule contains 2α and 2β chains (see Ref. 17) and this seems to find support from determinations of the molecular weight of the enzyme system [15,19]. It has also been proposed that only 1 out of 2α-chains are labelled with ^{32}P from [^{32}P]ATP (see Ref. 17).

What does it then mean that the ratio between the number of α-chains to ^{32}P labelling sites is more than 2 : 1 in the membrane-bound enzyme, Table III? Is there a poly-peptide with the same molecular weight as the α-chain in the preparation which does not belong to the system? Or are there silent α-chain molecules, and does the decrease in the ratio and the increase in the molar activity per α-chain seen when the enzyme is solubilized mean that they become active? And why is it that the ratio is less than 2 : 1 in the solubilized and in the solubilized precipitated enzyme prepared by Hokin et al. [2]? Is it due to uncertainties in the determination of the amount of α-chains for due to an overestimation of the molecular weight of the α-chain? Or does it mean that more than 1 out of 2 of the α-chains are labelled? And, if so, does it mean that, under certain conditions, all α-chains could be labelled?

Another problem, to which we shall return, is whether the kinetic properties of enzyme with high and low molar activity are different.

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