Biochimica et Biophysica Acta, 330 (1973) 283–293

BBA 76510

THE SEPARATION AND ENZYMATIC CHARACTERIZATION OF INNER AND OUTER MEMBRANES OF RAT-HEART MITOCHONDRIA

H. R. SCHOLTE

Department of Biochemistry I, Medical Faculty, Erasmus University, Rotterdam (The Netherlands)*

(Received July 25th, 1973)

SUMMARY

1. The procedure of Sottocasa, G. L., Kuylenstierna, B., Ernster, L. and Bergstrand, A. [(1967) *J. Cell. Biol.* 32, 415–438] for the separation of liver mitochondrial membranes was applied to rat-heart mitochondria. It appeared that the procedure did not result in a separation of the outer and inner membranes. It was found that the addition of a proteinase was essential to obtain the separation, suggesting that peptide bonds are involved in the binding between the inner and outer membrane. An optimal yield of outer membranes was obtained at 30–60 μg trypsin (EC 3.4.21.4) per mg mitochondrial protein.

2. The contamination of the outer membrane fraction with inner membrane fragments was counteracted by the use of a high mitochondrial protein concentration.

3. The outer membranes were 6-fold purified in a yield of up to 34%. They contained monoamine oxidase (EC 1.4.3.4), rotenone-insensitive NADH-cytochrome *c* reductase, palmitoyl-CoA synthetase (EC 6.2.1.3) and lauroyl-CoA synthetase. Creatine kinase (EC 2.7.3.2) remained bound to the inner membranes, while adenylate kinase (EC 2.7.4.3) was solubilized.

INTRODUCTION

The inner and outer membranes of liver mitochondria can easily be separated by high-amplitude swelling in the presence of phosphate[1–3], or by the use of digitonin[4,5]. Both methods were applied to heart mitochondria. Whereat *et al.*[6] fractionated rabbit-heart mitochondria with the method of Sottocasa *et al.*[1], and obtained an outer membrane fraction which contained 45% of the monoamine oxidase activity, 6-fold enriched from the mitochondria.

Digitonin was used with less success. Oliveira *et al.*[7] obtained an outer membrane fraction from beef-heart mitochondria which contained 7% of the monoamine oxidase, 3-fold enriched. Fractionation of guinea-pig-heart mitochondria by digitonin in earlier work[8] resulted in an outer membrane fraction which contained 15% of rotenone-insensitive NADH-cytochrome *c* reductase, more than 15-fold enriched. Since, however, 11% of the microsomal marker enzyme NADPH-cytochrome *c* reductase was recovered in the outer membrane fraction, we concluded that a part

* Postal address: Box 1738, Rotterdam (The Netherlands).

of the outer membrane fraction was of microsomal origin. Electron microscopy of an ultrathin section of guinea-pig-heart mitochondria treated with digitonin revealed that the outer membranes were ruptured by the action of digitonin, but that the outer-membrane fragments remained in the vicinity of the inner membrane[9].

Addink *et al.*[10] showed with beef-heart mitochondria that monoamine oxidase is released from the inner membrane *plus* matrix fraction at much higher digitonin concentrations than used for the separation of rat-liver mitochondrial membranes.

Smoly *et al.*[11] and Hayashi and Capaldi[12] purified outer membranes from an aged and a freeze thawed mitochondrial fraction from beef heart, respectively. The former preparation was 5-fold enriched in monoamine oxidase activity and 15-fold in rotenone-insensitive NADH-cytochrome *c* reductase activity. In the latter preparation, however, the enrichment factors were 28 and 4.2, respectively.

The purpose of the present study was to achieve a rapid separation of inner and outer membranes from freshly isolated rat-heart mitochondria, in order to establish the localization of mitochondrial enzymes, also more labile ones, to be carried out on a small scale. Special attention was paid to microsomal contamination.

METHODS

The separation of rat-heart mitochondrial membranes

The mitochondria were isolated according to the method of Holton *et al.*[13] as described by Wit-Peeters[14], in 0.25 M sucrose, 10 mM Tricine–KOH and 1 mM EDTA (pH 7.4 at 0 °C). The final mitochondrial pellet was suspended in 2 ml medium per heart. A protein determination was done and the mitochondria were sedimented for 10 min at 25 000 × *g*.

The fractionation of mitochondria was carried out according to the procedure of Sottocasa *et al.*[1], as described by Whereat *et al.*[6] with several modifications. The mitochondrial pellet was homogenized in a centrifuge tube with a loosely fitting pestle during 5 s in 3 ml containing 10 mM potassium phosphate, 10 mM Tris and trypsin (as a routine 30–60 μg trypsin per mg mitochondrial protein was used) (pH 7.5 at 0 °C with KOH). Then 1 ml 1.8 M sucrose, 2 mM $MgSO_4$, 2 mM ATP (disodium–dipotassium salt) was added. After 1 min at 0 °C with occasional homogenization by means of the pestle, the suspension was sonicated by 4 bursts of 5 s at 2.9 A (Branson Instruments, Danbury, Conn.). 6 min after the homogenization of the pellet in phosphate–Tris buffer with trypsin, an equivalent amount of soy-bean trypsin-inhibitor (3 times crystallized, Calbiochem) in 0.45 M sucrose was added.

A sample was taken to assess the recoveries, and 2.5 ml of the suspension was layered on top of a discontinuous sucrose gradient, consisting of 1 ml 1.2 M sucrose, 1 ml 1.0 M sucrose and 1 ml 0.6 M sucrose (Fig. 1). After centrifugation for 1 h at 249 000 × *g* a sharp white band was visible at the interface between 0.6 and 1.0 M sucrose. Around and at the interface between 1.0 and 1.2 M sucrose a diffuse brown band could be seen. On the bottom a firm brown pellet was formed. The fractions were obtained by slicing of the tube as indicated in Fig. 1. The particulate fractions were homogenized in 0.25 M sucrose, 10 mM Tricine–KOH and 1 mM EDTA (pH 7.4 at 0 °C). After a successful fractionation, Fraction 1 (the supernatant) yielded the enzymes from the intermembrane space, Fraction 2 (the sharp white band) the

outer membrane fragments, Fraction 3 (the diffuse brown band) mixed membranes, and Fraction 4 (the brown pellet) inner membranes *plus* matrix material.

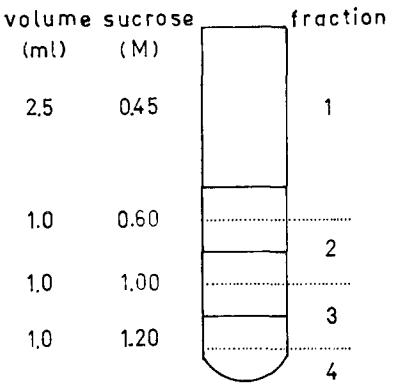

Fig. 1. Discontinuous sucrose gradient for the separation of the submitochondrial fractions. The tube was sliced after centrifugation as indicated by the dotted lines.

Assays

Malate dehydrogenase, EC 1.1.1.37, was expressed in units of μmoles NADH oxidized per min at 30 °C, and was determined as described in ref. 15; monoamine oxidase, μmoles tyramine deaminated per min at 37 °C and pH 7.95 (ref. 17); cytochrome *c* oxidase, EC 1.9.3.1, μatoms O consumed per min at 30 °C, ref. 5; kynurenine 3-hydroxylase, EC 1.14.1.2, μmoles NADPH oxidized per min at 37 °C, ref. 18; aspartate aminotransferase, EC 2.6.1.1, μmoles NADH oxidized per min at 30 °C, ref. 19; pyruvate kinase, EC. 2.7.1.40, μmoles NADH oxidized per min at 30 °C, ref. 20; creatine kinase, μmoles NADH oxidized per min at 30 °C, ref. 21; adenylate kinase, μmoles $NADP^+$ reduced per min at 30 °C, ref. 15; trypsin and nagarse, EC 3.4.21.14, μmoles benzoyl-L-arginine ethyl ester hydrolyzed per min at 25 °C, ref. 22; palmitoyl-CoA synthetase and lauroyl-CoA synthetase, μmoles acylcarnitine formed per min at 37 °C in the presence of 0.2 M KCl (ref. 23) in a volume of 0.25 ml, ref. 24; rotenone-intensitive NAD(P)H-cytochrome *c* reductases, μmoles cytochrome *c* reduced per min at 30 °C, ref. 1.

Where NADH oxidation was measured, 1.5 μM rotenone was present in order to inhibit the respiratory chain. Creatine kinase and pyruvate kinase were measured in the presence of 7.5 μg/ml oligomycin for the inhibition of the mitochondrial ATPase.

To abolish the latency of malate dehydrogenase, cytochrome *c* oxidase and aspartate aminotransferase, the fractions were pretreated with 0.025% Lubrol WX for 12 min at 0 °C.

Protein was determined according to Lowry *et al.*[25]. The amount of mitochondrial protein used in the fractionation is given after subtraction of the added amount of trypsin *plus* trypsin inhibitor. Although it is most likely that the trypsin-trypsin inhibitor complex is recovered in the supernatant (Fraction 1), no correction has been made for the amount of non-mitochondrial protein in Fraction 1.

RESULTS

Fraction of rat-heart mitochondria, the effect of proteinases

The modification of Whereat *et al.*[6] of the fractionation procedure of Sottocasa *et al.*[1] yielded an outer-membrane fraction, which contained only 14% of the mitochondrial monoamine oxidase, 1.7 times enriched. The contamination with inner-membrane fragments (cytochrome *c* oxidase) was considerable (8%). Variations of almost every step in the separation procedure were not able to improve the results. The difference between the results of Whereat *et al.*[6] and those mentioned here, could be attributed to a difference in the isolation procedure of the mitochondria. Whereat and coworkers isolated the mitochondria with the aid of the proteinase nagarse. In the present study the mitochondria were isolated without nagarse, since it destroys palmitoyl-CoA synthetase[26,27], one of the enzymes under study. As will be shown in the following experiments, it is likely that the nagarse which remained in the mitochondrial preparation was responsible for the separation of the mitochondrial membranes obtained by Whereat *et al.*[6]. This supposition is also supported by the low recovery (18.4%) of mitochondrial fatty acid synthesis in the subfractions[6], most likely due to proteolytic attack by the proteinase. Donaldson *et al.* [28] showed that lysosomal enzymes rapidly inactivated the fatty acid synthesizing systems from rat liver.

The addition of 0.1 mg nagarse in the swelling medium to 4.3 mg mitochondrial protein resulted in an outer membrane fraction which contained 42% of the monoamine oxidase activity, 5.6 times enriched. Preparation of the mitochondria with nagarse (stirring of an homogenate of two rat hearts for 30 min at 0 °C with 4 mg nagarse per 7 ml 0.25 M sucrose, 10 mM Tricine–KOH and 1 mM EDTA (pH 7.5 at 0 °C) and two washings of the mitochondrial pellet) and subsequent fraction of 17.8 mg of protein gave an outer-membrane preparation which contained 19% monoamine oxidase, 5.8 times enriched. Nagarse, however, was found in every fraction

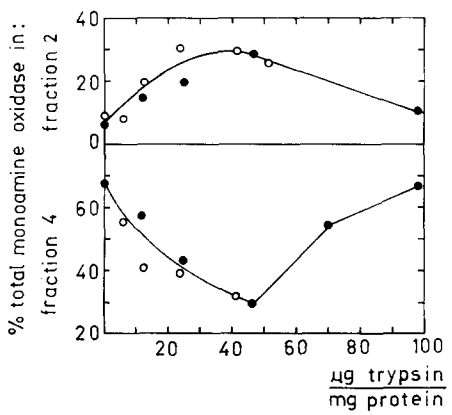

Fig. 2. The recovery of total mitochondrial monoamine oxidase activity in Fraction 2 and in Fraction 4, as a function of the ratio µg trypsin/mg mitochondrial protein. The trypsin inhibitor was added 30 min after the homogenization of the pellet in phosphate-Tris buffer with trypsin, see Methods. The points ● and ○ represent two different experiments in which, respectively, 2.1 and 3.9 mg of protein was used per fractionation. The average specific monoamine oxidase activities in the mitochondrial fractions were 7.6 and 5.3 munits/mg mitochondrial protein and the average recoveries were 76% and 81%.

and was replaced by trypsin, since the latter enzyme can be inhibited by several specific protein inhibitors.

Fig. 2 shows the relation between the amount of trypsin used, and the percentage of monoamine oxidase recovered in the outer-membrane Fraction 2, and the inner-membrane Fraction 4. There appeared to be an optimal trypsin protein ratio of 30–60 μg trypsin per mg mitochondrial protein, which yielded the highest monoamine oxidase in Fraction 2, and the lowest in Fraction 4. This ratio was used in the following experiments. Fortunately the trypsin treatment used had no effect upon the activities of monoamine oxidase and the other enzymes under study. The fact that a proteinase is essential to get the separation suggests that peptide bonds are involved in the binding between the inner and outer membrane. The finding that there is an optimum in the trypsin *vs* outer membrane release curve (Fig. 2) may indicate that another (pro-)enzyme, which must be activated by trypsin, is involved in the breakage of the binding between the membranes. This enzyme present in the mitochondrial fraction, must be stimulated at a low trypsin concentration, and destroyed at a high trypsin concentration, in order to explain the curve of Fig. 2.

When trypsin inhibitor was added before the trypsin, only 6% of monoamine oxidase activity was recovered in Fraction 2. Already after 6 min trypsinization 35% of the marker appeared in Fraction 2. Longer incubation time did not result in a considerable increase of this activity. A 6 min treatment was therefore used in the following experiments.

The effects of sonication time, high amplitude swelling and protein concentration on the separation

Fig. 3 shows that both sonication and high amplitude swelling were necessary to obtain the separation. Increasing the sonication time gave rise to a far better separation. But to protect the matrix enzymes from a complete escape from the inner-membrane *plus* matrix Fraction 4 to the soluble Fraction 1, 4 bursts of 5 s were routinely used, since it was found previously that the release of matrix enzymes is a function of the sonication time used[29].

The partition of mitochondria at low protein concentration (1.8 mg per fractionation, see Methods) gave the following results (not shown). Monoamine oxidase is 4.3-fold enriched and 31% recovered in Fraction 2. Adenylate kinase, which is

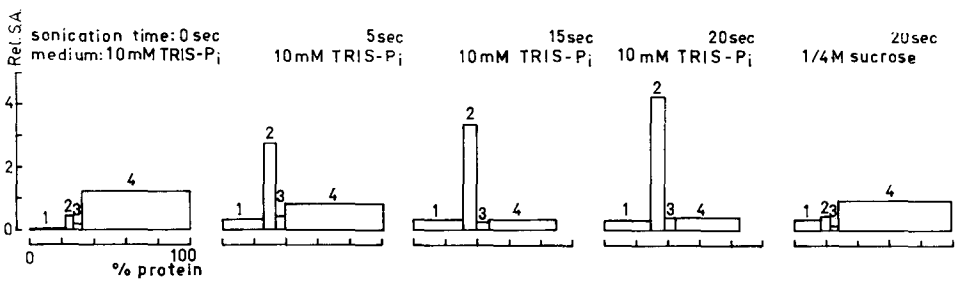

Fig. 3. The partition of monoamine oxidase as a function of the sonication time. In the last graph the swelling medium 10 mM Tris–10 mM phosphate was replaced by 0.25 M sucrose. Per partition 0.10 mg trypsin was used/1.78 mg mitochondrial protein. The average specific monoamine oxidase activity of the mitochondrial fractions was 5.39 munits/mg mitochondrial protein and the average recovery was 74%.

located in the intermembrane space, escapes and is 81% recovered in Fraction 1. Creatine kinase, which is located at the outer surface of the inner membranes, is also solubilized by 78%, most probably by the high amplitude swelling treatment with phosphate[9]. Unfortunately the outer membranes were very impure, as judged by the high contamination (19%) with the inner-membrane marker enzyme cytochrome *c* oxidase. About one half of the matrix enzymes aspartate aminotransferase and malate dehydrogenase are solubilized as a consequence of the swelling and the sonication. The cytosolic marker enzyme pyruvate kinase contaminates the mitochondria to a very low extent (about 0.3% of the total cellular activity is recovered in the mitochondrial fraction) and has a tendency to bind to the inner membrane fragments (37% is recovered in Fraction 4).

In one of the attempts to improve fractionation, the protein concentration was highly (11-fold) increased (Table I, Fig 4). It turned out that this was very important to get a purer outer-membrane fraction. Monoamine oxidase, rotenone-insensitive NADH-cytochrome *c* reductase, palmitoyl-CoA synthetase and lauroyl-CoA synthetase are enriched in Fraction 2. The fact that these four activities are purified in Fraction 2 to the same extent, implies a low contamination of the outer-membrane fraction with microsomes. The localization of palmitoyl-CoA synthetase in the outer membranes is in complete agreement with the work of De Jong and Hülsmann[26] and Pande and Blanchaer[27] who were able to localize the enzyme on the outer surface of cardiac outer-mitochondrial membranes. In the mixed membrane Fraction 3 the

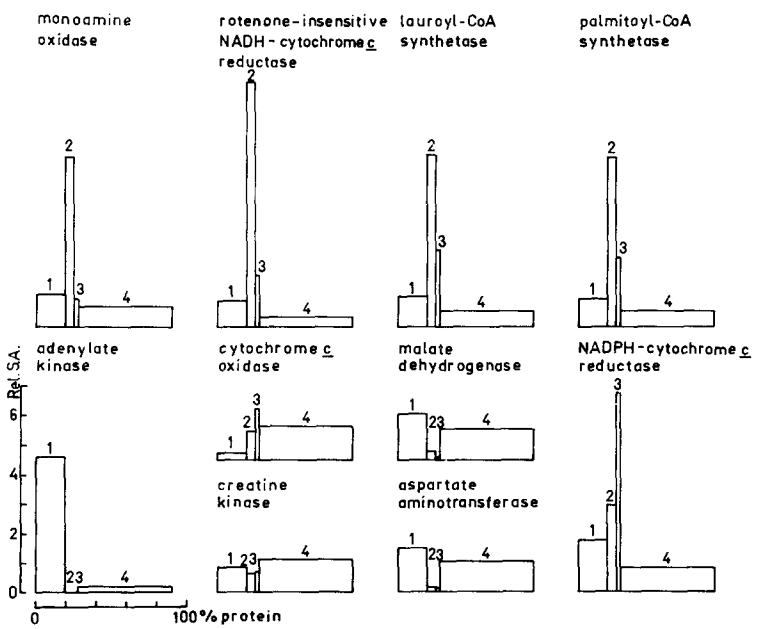

Fig. 4. The partition of monoamine oxidase, rotenone insensitive NADH-cytochrome *c* reductase, lauroyl-CoA synthetase, palmitoyl-CoA synthetase, adenylate kinase, cytochrome *c* oxidase, creatine kinase, malate dehydrogenase, aspartate aminotransferase and NADPH-cytochrome *c* reductase. 0.70 mg trypsin was used per 20.5 mg mitochondrial protein. The absolute and percentage values are given in Table I.

TABLE I

THE FRACTIONATION OF RAT-HEART MITOCHONDRIA OF A HIGH PROTEIN CONCENTRATION

0.70 mg trypsin was used per 20.5 mg mitochondrial protein. The fractionation was carried out according to Methods. The corresponding "De Duve-Plots" are given in Fig. 4.

	*Absolute values mitochondria**	*Percentage values*					
		*Mitochondria**	*1*	*2*	*3*	*4*	*Recovery*
Protein (mg)	19.8	100	19.5	5.6	2.5	62.2	89.9
Monoamine oxidase (munits)	42.7	100	21.3	31.8	2.4	43.0	98.5
Rotenone-insensitive NADH-cytochrome *c* reductase (units)	1.04	100	17.2	45.9	4.4	20.1	87.6
Lauroyl-CoA synthetase (munits)	259	100	20.0	32.3	6.5	33.6	92.4
Palmitoyl-CoA synthetase (munits)	285	100	19.0	32.0	5.8	33.0	89.8
Adenylate kinase (units)	1.20	100	90.1	0.0	0.0	12.5	102.6
Creatine kinase (units)	12.7	100	16.8	3.6	1.8	68.9	91.1
Cytochrome *c* oxidase (units)	80.7	100	4.7	5.5	4.3	69.8	84.3
Aspartate aminotransferase (units)	45.2	100	29.3	0.9	0.4	64.0	94.6
Malate dehydrogenase (units)	65.1	100	30.4	1.6	0.3	64.5	96.8
NADPH-cytochrome *c* reductase (munits)	78.6	100	34.8	16.8	17.0	52.0	120.6

* Trypsin-treated mitochondria (see Methods).

microsomal marker enzyme NADPH-cytochrome *c* reductase is highly enriched. The escape of matrix enzymes is lowered by the use of a higher mitochondrial protein concentration. Furthermore in this experiment creatine kinase remains bound to the inner-membrane *plus* matrix Fraction 4. Only 17% is solubilized as a consequence of the phosphate treatment. In another partition (not shown) carried out at about the same protein concentration, 8% release was found. 90% of adenylate kinase activity is recovered in Fraction 1. The remaining activity in Fraction 4 is most probably due to nucleosidemonophosphate kinase (EC 2.7.4.4) which in liver mitochondria is located in the matrix[30].

Negative staining and electron microscopy of Fraction 2 showed the virtual absence of inner-membrane subunits, which were, however, abundantly present in Fraction 4. Fraction 2 contained some outer-membrane ghosts, but most outer membranes were fragmented into smaller pieces.

Kynurenine 3-hydroxylase, an outer membrane enzyme in liver[31], is absent in rat-heart mitochondria, confirming the results with beef-heart mitochondria[12].

DISCUSSION

The problem of the separation of heart mitochondrial inner and outer membranes was first tackled by Green and co-workers[32–35]. They designed several procedures involving ageing of beef-heart mitochondria for 24 h, followed by sonication *plus* or *minus* phospholipase, or a treatment with cholate. Unfortunately all these procedures gave rise to damage of the inner membranes, which were partly fractionated into light vesicles sedimenting in the fraction which was supposed to contain outer membranes. Moreover, a part of the matrix enzymes escaped and was believed to be loosely associated with the outer membranes. Consequently many enzymes originating from the inner membrane *plus* matrix compartment were believed to be located in the outer membranes.

Later on Smoly *et al.*[11] demonstrated that the outer membrane fraction obtained by the procedure of Green *et al.*[34] was indeed enriched in outer-membrane marker enzymes: monoamine oxidase was 5 times enriched and rotenone-intensitive NADH-cytochrome *c* reductase was even 15 times enriched. This discrepancy must be due to the fact that monoamine oxidase is only located in the outer-membranes, while rotenone-insensitive NADH-cytochrome *c* reductase is located in the outer membranes as well as in the sarcoplasmic reticulum. It can also be concluded that the microsomes are copurified with the outer membranes. This contamination of the outer-membrane preparation was ruled out by the fact that the membrane preparation had a very low activity in glucose-6-phosphatase (EC 3.1.3.9), which was believed to be a microsomal marker enzyme in beef heart. In heart, however, the activity of glusose-6-phosphatase is much lower than in liver, and is most probably due to non-specific phosphatases like acid phosphatase (EC 3.1.3.2) or alkaline phosphatase (EC 3.1.3.1)[36,37]. In organs which are not able to carry out gluconeogenesis, like heart, NADPH-cytochrome *c* reductase is a suitable marker enzyme for the microsomes.

Recently Hayashi and Capaldi[12] isolated a beef-heart outer-mitochondrial membrane fraction with inverse properties compared with the preparation of Smoly *et al.*[11]. This preparation, obtained by freezing and thawing in a hypotonic medium followed by sucrose gradient centrifugation, was 28 times enriched in monoamine

oxidase, but only 4.2 times in rotenone-intensitive NADH-cytochrome *c* reductase. Also in this communication glucose-6-phosphatase was used as a microsomal marker enzyme.

It is clear that in an outer-membrane preparation the outer-membrane enzymes must be purified to the same extent compared with pure mitochondria. A difference in the purification of monoamine oxidase and rotenone-insensitive NADH-cytochrome *c* reductase may be due to (a) contamination of the mitochondria with microsomes, (b) purification of microsomes with the outer membranes and (c) a differential release of enzymes from the outer-membrane preparation.

Conflicting reports appeared on the localization of mitochondrial creatine kinase and adenylate kinase. (a) Pette[38] and Klingenberg and Pfaff[39] located the enzymes in the intermembrane space of rat-heart mitochondria, since they were able to release the enzymes from the mitochondria with 0.1 M phosphate. (b) Addink *et al.*[10] found that 21% of the creatine kinase activity and 19% of the adenylate kinase activity was released from beef-heart mitochondria by a digitonin concentration which is able to detach 100% of the monoamine oxidase activity. Addink *et al.*[10] concluded that both enzymes have a bimodal distribution in beef-heart mitochondria: in the intermembrane space, and in the inner membrane *plus* matrix space (c) Farrell *et al.*[40] reported the association of creatine kinase with the outer of beef-heart mitochondria. This conclusion, however, is not based on experimental evidence. (d) Scholte *et al.*[9] found that treatment of rat-heart mitochondria with digitonin resulted in a release of 89% of the adenylate kinase, while only 3% of the creatine kinase activity was solubilized. Since creatine kinase becomes partially latent in submitochondrial particles, it was concluded that mitochondrial creatine kinase is bound to the outer surface of the inner mitochondrial membrane, while adenylate kinase is present in a soluble form in the intermembrane space. The 11% of the adenylate kinase activity, which was found in the matrix space, was attributed to nucleosidemonophosphate kinase (EC 2.7.4.3) activity.

The partitions described in this paper support the conclusions of Scholte and coworkers[9] on the binding of creatine kinase to the inner mitochondrial membrane, and the localization of adenylate kinase in the intermembrane space. The discrepancy between these results with those of Addink *et al.*[10], may be explained by the escape of the bulk of the adenylate kinase activity from the beef-heart mitochondria during isolation, which leads to a relative higher contribution to mitochondrial adenylate kinase activity by the matrix nucleosidemonophosphate kinase.

Part of this paper has been presented elsewhere[41].

ACKNOWLEDGEMENTS

Professor Dr W. C. Hülsmann is acknowledged for this stimulating interest and encouragement. Miss J. D. van Esch and Mr M. J. Edixhoven have contributed to this paper by their skilful technical assistance. I am indebted to Drs R. Stoeckart and Mr H. G. Jansen from the Institute of Anatomy of our University, for the electronmicroscopical examination of the membrane fractions.

REFERENCES

1 Sottocasa, G. L., Kuylenstierna, B., Ernster, L. and Bergstrand, A. (1967) *J. Cell Biol.* 32, 415–438

2 Parsons, D. F., Williams, G. R. and Chance, B. (1966) *Ann. N.Y. Acad. Sci.* 137, 643–666
3 Parsons, D. F., Williams, G. R., Thompson, W., Wilson, D. and Chance, B. (1967) in *Mitochondrial Structure and Compartmentation* (Quagliariello, E., Papa, S., Slater, E. C. and Tager J. M., eds), pp. 29–70, Adriatica Editrice, Bari
4 Lévy, M., Toury, R. and André, J. (1967) *Biochim. Biophys. Acta* 135, 599–613
5 Schnaitman, C., Erwin, V. G. and Greenawalt, J. W. (1967) *J. Cell Biol.* 32, 719–735
6 Whereat, A. F., Orishimo, M. W. and Nelson, J. (1969) *J. Biol. Chem.* 244, 6498–6506
7 Oliveira, M. M., Weglicki, W. B., Nason, A. and Nair, P. P. (1969) *Biochim. Biophys. Acta* 180, 98–113
8 Scholte, H. R. (1970) *Het Metabolisme van Malonyl-CoA in Mitochondriën*, Ph.D. Thesis, pp. 1–87, Mondeel Offsetdrukkerij, Amsterdam
9 Scholte, H. R., Weijers, P. J. and Wit-Peeters, E. M. (1973) *Biochim. Biophys. Acta* 291, 764–773
10 Addink, A. D. F., Boer, P., Wakabayashi, T. and Green, D. E. (1972) *Eur. J. Biochem.* 29, 47–59
11 Smoly, J. M., Wakabayashi, T., Addink, A. D. F. and Green, D. E. (1971) *Arch. Biochem. Biophys.* 143, 6–21
12 Hayashi, H. and Capaldi, R. A. (1972) *Biochim. Biophys. Acta* 282, 166–173
13 Holton, F. A., Hülsmann, W. C., Myers, D. K. and Slater, E. C. (1957) *Biochem. J.* 67, 579–594
14 Wit-Peeters, E. M. (1969) *Biochim. Biophys. Acta* 176, 453–462
15 Sottocasa, G. L., Kuylenstierna, B., Ernster, L. and Bergstrand, A. (1967) in *Methods in Enzymology* (Estabrook, R. W. and Pullman, M. E., eds), Vol. 10, pp. 448–463, Academic Press, New York
16 McCaman, R. E., McCaman, M. W., Hunt, J. M. and Smith, M. S. (1965) *J. Neurochem.* 12, 15–23
17 Aas, M. (1971) *Biochim. Biophys. Acta* 231, 32–47
18 Saito, Y., Hayaishi, O. and Rothberg, S. (1957) *J. Biol. Chem.* 229, 921–934
19 Karmen, A., Wroblewski, F. and La Due, J. A. (1955) *J. Clin. Invest.* 34, 126–131
20 Bücher, Th. and Pfleiderer, G. (1955) in *Methods in Enzymology* (Colowick, S. P. and Kaplan N. O., eds), Vol. 1, pp. 435–440, Academic Press, New York
21 Bücher, Th., Luh, W. and Pette, D. (1964) in *Handbuch der Physiol. Pathol. Chem. Anal.* (Lang, K. and Lehnartz, E., eds), 10th Edn, Vol. 6A, pp. 292–339, Springer-Verlag, Berlin
22 Schwert, G. W. and Tanaka, Y. (1955) *Biochim. Biophys. Acta* 16, 570–575
23 Aas, M. (1970) *Biochim. Biophys. Acta* 202, 250–258
24 Van Tol, A. and Hülsmann, W. C. (1969) *Biochim. Biophys. Acta* 189, 342–353
25 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* 193, 265–275
26 De Jong, J. W. and Hülsmann, W. C. (1970) *Biochim. Biophys. Acta* 197, 127–135
27 Pande, S. V. and Blanchaer, M. C. (1970) *Biochim. Biophys. Acta* 202, 43–48
28 Donaldson, W. E., Wit-Peeters, E. M. and Scholte, H. R. (1970) *Biochim. Biophys. Acta* 202, 35–42
29 Wit-Peeters, E. M., Scholte, H. R., Van den Akker, F. and De Nie, I. (1971) *Biochim. Biophys. Acta* 231, 23–31
30 Heldt, H. W. and Schwalbach, K. (1967) *Eur. J. Biochem.* 1, 199–206
31 Okamoto, H., Yamamoto, S., Nozaki, M. and Hayaishi, O. (1967) *Biochem. Biophys. Res. Commun.* 26, 309–314
32 Bachmann, E., Allmann, D. W. and Green, D. E. (1966) *Arch. Biochem. Biophys.* 115, 153–164
33 Allmann, D. W., Bachmann, E. and Green, D. E. (1966) *Arch. Biochem. Biophys.* 115, 165–171
34 Green, D. E., Bachmann, E., Allmann, D. W. and Perdue, J. F. (1966) *Arch. Biochem. Biophys.* 115, 172–180
35 Bachmann, E., Lenaz, G., Perdue, J. F. and Orme-Johnson, N. (1967) *Arch. Biochem. Biophys.* 121, 73–87
36 Hulsmans, H. A. M. (1960) *Verdeling van Enzymactiviteiten bij Fractionering van Hartspierweefsel*, M.D. Thesis, pp. 1–100, Poortpers, Amsterdam
37 Grinna, L. S. and Barber, A. A. (1972) *Biochim. Biophys. Acta* 288, 347–353
38 Pette, D. (1966) in *Regulation of Metabolic Processes in Mitochondria* (Tager, J. M., Papa, S. Quagliariello, E. and Slater, E. C., eds), BBA Library, Vol. 7, pp. 28–49, Elsevier, Amsterdam

39 Klingenberg, M. and Pfaff, E. (1966) in *Regulation of Metabolic Processes in Mitochondria* (Tager, J. M., Papa, S., Quagliariello, E. and Slater, E. C., eds), BBA Library, Vol. 7, pp. 180–201, Elsevier, Amsterdam
40 Farrell, Jr, E. C., Baba, N., Brierley, G. P. and Grümer, H.-D. (1972) *Lab. Invest.* 27, 209–213
41 Scholte, H. R. (1972) *Abstr. Commun. Meet. FEBS Amsterdam*, Vol. 8, p. 149, North Holland, Amsterdam