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ENZYMATIC ACTIVITIES OF THE MATRIX AND INNER MEMBRANE OF PIGEON-LIVER MITOCHONDRIA

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SUMMARY

Pigeon-liver mitochondria were fractionated by a modification of the method of SOTTOCASA *et al.*[5]. The various fractions obtained were examined by electron microscopy. Control experiments excluded the possibility that the activity of rotenone-insensitive NADH–cytochrome *c* reductase in the various submitochondrial fractions could be largely contributed by microsomal contamination. The submitochondrial localization of the following enzymes in pigeon liver was studied: pyruvate carboxylase, phosphopyruvate carboxylase, $NADP^+$–isocitrate dehydrogenase, glutamate dehydrogenase, aspartate aminotransferase, NAD^+–isocitrate dehydrogenase, the enzymes of β-oxidation, malonyl-CoA decarboxylase and the enzymes of fatty acid synthesis. The results indicated that a group of enzymes, consisting of pyruvate carboxylase, phosphopyruvate carboxylase, $NADP^+$–isocitrate dehydrogenase, aspartate aminotransferase, glutamate dehydrogenase and NAD^+–isocitrate dehydrogenase, is localized in a fraction practically free of both the inner and outer membranes. This fraction, in agreement with previous reports, is identified as the mitochondrial matrix. It is suggested that $NADP^+$–isocitrate dehydrogenase and aspartate aminotransferase be used as marker enzymes of the mitochondrial matrix. The enzymes of β-oxidation and those of fatty acid synthesis are localized in the inner membrane of pigeon-liver mitochondria 58.4% of the total activity of the mitochondrial malonyl-CoA decarboxylase was found in the inner membrane and 31.4% in the matrix.

INTRODUCTION

Recently different methods for the separation of the outer membrane from the inner membrane of mitochondria have been described[1–6]. By making use of these techniques the enzyme localization in mitochondria has been studied. There is, however, controversy about the localization of some enzymes. According to a group of authors, most of the enzymes of the citric acid cycle are localized in the mitochondrial matrix[5,7]. GREEN and co-workers[2–4], on the other hand, are of the opinion that these enzymes are localized in the outer membrane of mitochondria. These authors have suggested that also the enzymes involved in the mitochondrial metabolism of fatty acids are localized in the outer membrane[2–4]. In contrast with this

BEATTIE[8] has proposed that in rat-liver mitochondria the enzymes of β-oxidation are localized in the inner membrane–matrix fraction.

The main purpose of the present investigation was to prepare both an inner membrane and a matrix fraction of pigeon-liver mitochondria, essentially devoid of the outer membrane, and of characterizing the enzymatic composition of these two fractions.

Evidence has been obtained that a group of enzymes, consisting of $NADP^+$–isocitrate dehydrogenase, glutamate dehydrogenase, aspartate aminotransferase, NAD^+–isocitrate dehydrogenase, pyruvate carboxylase and phosphopyruvate carboxylase, is localized in the matrix of pigeon-liver mitochondria. The enzymes of fatty acid synthesis and those of β-oxidation are bound to the inner membrane.

Part of the present investigation has been communicated in a preliminary form[9,10].

EXPERIMENTAL

Preparation and fractionation of pigeon-liver mitochondria

Pigeon liver was gently homogenized in 0.25 M sucrose (1 g liver in 15 ml sucrose) using a Potter–Elvehjem homogenizer with a Teflon pestle. The homogenate was centrifuged 8 min at 800 $\times$ *g*. Mitochondria were sedimented from the supernatant at 4800 $\times$ *g* (10 min) and washed 3 times. The microsomal contamination of mitochondria and submitochondrial fractions was determined by assaying glucose-6-phosphatase (EC 3.1.3.9)[11] and NADPH–cytochrome *c* reductase (EC 1.6.2.3)[5] activity. Mitochondria were fractionated in two steps by a modification of the method of SOTTOCASA *et al.*[5]. At the end of the preparation, mitochondria were swollen in 10 mM Tris–phosphate buffer (pH 7.5) (20 min) and contracted (15 min) with 1/3 vol. of 1.8 M sucrose containing 2 mM ATP and 2 mM $MgSO_4$. The first step of the fractionation was performed by discontinuous density gradient centrifugation on 1.18 M sucrose at 60000 $\times$ *g* (3 h). The three fractions obtained are indicated as S_1 (soluble fraction), I_1 (interphase fraction) and P_1 (pellet). The P_1 pellet was suspended in 10 mM Tris–phosphate buffer (pH 7.5). After 15 min it was diluted with 1/3 vol. of 1.8 M sucrose containing 2 mM ATP and 2 mM $MgSO_4$. After another 10 min the suspension was sonicated 20 sec at 1.2 A (MSE sonicator) and the second step of fractionation was performed by discontinuous density gradient centrifugation as before. After centrifugation, three fractions were again obtained: S_2 (soluble fraction), I_2 (interphase fraction) and P_2 (pellet). This pellet was suspended in 0.25 M sucrose.

Enzymatic assays

The activity of succinate–cytochrome *c* reductase (EC 1.3.99.1), rotenone-sensitive (EC 1.6.2.1) and rotenone-insensitive (EC 1.6.2.2) NADH–cytochrome *c* reductase was determined as in ref. 5. Glutamate dehydrogenase (EC 1.4.1.2) activity was determined by measuring the rate of NADH oxidation by α-oxoglutarate *plus* NH_4^+; $NADP^+$–isocitrate dehydrogenase (EC 1.1.1.42) by the rate of $NADP^+$ reduction by DL-isocitrate; NAD^+–isocitrate dehydrogenase (EC 3.1.1.41) by measuring the rate of NAD^+ reduction by DL-isocitrate in 30 mM Tris–acetate buffer (pH 7.2) in the presence of 0.7 mM ADP[12]; aspartate aminotransferase (EC 2.6.1.1) by the rate of NADH oxidation in the presence of added malate dehydrogenase

(EC 1.1.1.37), α-oxoglutarate and aspartate[13]; phosphopyruvate carboxylase (EC 4.1.1.32) by the rate of phosphoenolpyruvate formation (this was determined enzymatically[14]); pyruvate carboxylase (EC 6.4.1.1) by the rate of oxaloacetate formation (this was determined enzymatically[15]). All these enzymes were assayed at 25°. Fatty acid synthesis activity was determined at 37.5° as in ref. 16; fatty acid oxidation was carried out at 37° under N_2 and measured spectrophotometrically by the formazan method of MII AND GREEN[17]. The activity of malonyl-CoA decarboxylase (EC 4.1.1.9) was measured by incubating in Warburg vessels, in a final volume of 3 ml, 33 μM [1,3-$^{14}C_2$]malonyl-CoA (specific activity, 4 mC/mmole), 30 mM phosphate buffer (pH 6.5), 1 mM $MnCl_2$ and 1–2 mg protein, at 30° for 15 min. At the end of the incubation time the reaction was stopped by adding 1 ml 4 M H_2SO_4. Labeled CO_2 development was favoured by shaking the Warburg vessels for a further 45 min at 37°. $^{14}CO_2$ was collected by hydroxide of hyamine in the center wall of the Warburg vessels and the radioactivity was measured in a Packard liquid-scintillation spectrometer.

Electron microscopy

Samples of intact mitochondria, interphase fraction I_1 and the second pellet P_2 were fixed under isotonic conditions with 0.078 M OsO_4 (in 0.02 M phosphate buffer (pH 7.3) containing 0.182 M sucrose) for 1.5 h at 0°. Aliquots of the pellets of each fraction were prefixed under isotonic conditions with 0.2 M glutaraldehyde (in 0.02 M phosphate buffer (pH 7.3) containing 0.06 M sucrose) for 1.5 h at 0°. The fixed pellets were embedded in Epon 812 by the procedure of LUFT[18]. The pellets were sectioned with a glass knife on LKB ultramicrotome. The sections were stained with 5 % uranyl acetate for 1 h at 45°. Other sections were stained with lead hydroxide according to KARNOVSKY (Method A)[19]. Some sections were stained with both techniques. They were then examined in a Hitachi, type HU-11, electron microscope.

RESULTS

Distribution of marker enzymes

Pigeon-liver mitochondria were fractionated by a modification[9] of the method of SOTTOCASA *et al.*[5]. When the original procedure consisting of combined swelling–shrinking and sonication followed by discontinuous density gradient centrifugation was used, the three fractions described by these authors were obtained. The distribution pattern of marker enzymes like succinate–cytochrome *c* reductase, rotenone-sensitive and rotenone-insensitive NADH–cytochrome *c* reductase was similar to that reported in ref. 5. However, the activity of glutamate dehydrogenase, $NADP^+$–isocitrate dehydrogenase, NAD^+–isocitrate dehydrogenase and phosphopyruvate carboxylase was distributed almost equally between the soluble fraction (matrix) and the pellet (inner membrane). About 10 % of these activities was found in the interphase fraction (outer membrane) (*cf.* ref. 20).

The modified procedure of fractionation consisted of two successive steps. In the first mitochondria were subjected to swelling–shrinking followed by discontinuous density gradient centrifugation. In this way three fractions were obtained (see Table I). Practically all the activity of succinate–cytochrome *c* reductase and rotenone-sensitive NADH–cytochrome *c* reductase was recovered in the pellet P_1.

The specific activity of rotenone-insensitive NADH–cytochrome *c* reductase was concentrated about 4-fold in the interphase fraction I_1, with a recovery of 38% of the total activity in this fraction (*cf.* ref. 21). The second step of fractionation

TABLE I

DISTRIBUTION AND SPECIFIC ACTIVITY OF SOME MARKER ENZYMES IN SUBMITOCHONDRIAL FRACTIONS OF PIGEON LIVER

M = mitochondria; S_1 = intermembrane material; I_1 = outer membrane; P_1 = mitochondria with residual outer membrane; S_2 = matrix; I_2 = outer membrane; P_2 = inner membrane. Step I: M = 100%; Step II: P_1 = 100%.

Enzymes	*Total activity* (%)				*Specific activity* (*nmoles/min per mg protein*)			
	S_1	I_1	P_1	*Recovery*	*M*	S_1	I_1	P_1
Step I								
Succinate–cytochrome *c* reductase	0	0	98.1	98.1	121	0	0	163
Rotenone-sensitive NADH–cytochrome *c* reductase	1.9	0	100.2	102.1	206	46	0	284
Rotenone-insensitive NADH–cytochrome *c* reductase	3.4	38.1	46.8	88.3	152	63	580	98
Protein	8.2	10.0	72.8	91.0	—	—	—	—
	S_2	I_2	P_2	*Recovery*	P_1	S_2	I_2	P_2
Step II								
Succinate–cytochrome *c* reductase	4.5	6.6	86.3	97.4	163	25	110	289
Rotenone-sensitive NADH–cytochrome *c* reductase	11.1	6.4	86.5	104.0	284	107	185	504
Rotenone-insensitive NADH–cytochrome *c* reductase	18.1	46.5	20.3	84.9	98	60	465	41
Protein	29.5	9.8	48.7	88.0	—	—	—	—

TABLE II

SPECIFIC ACTIVITY OF GLUCOSE-6-PHOSPHATASE AND NADPH-CYTOCHROME *c* REDUCTASE IN MICROSOMES, MITOCHONDRIA AND SUBMITOCHONDRIAL FRACTIONS OF PIGEON LIVER

Fraction	*Glucose-6-phosphatase* (*nmoles P_i per min per mg protein*)	*Microsomal contamination* (%)	*NADPH–cytochrome c reductase* (*nmoles cytochrome c reduced per min per mg protein*)	*Microsomal contamination* (%)
Microsomes	190	—	87	—
Mitochondria	17.5	9.2	7.5	8.6
S_1	13.2	6.9	3.6	4.1
I_1	25.6	13.5	13.8	15.9
P_1	23.2	12.2	12.0	13.8
S_2	15.2	8.0	7.9	9.1
I_2	20.7	10.9	10.6	12.2
P_2	19.2	10.1	6.8	7.8

again gave three fractions. 86 % of the activity of succinate–cytochrome *c* reductase and rotenone-sensitive NADH–cytochrome *c* reductase of the first pellet P_1 was recovered in the second one (P_2). The activity of rotenone-insensitive NADH–cytochrome *c* reductase was concentrated in the interphase I_2.

Microsomal contamination of mitochondria and submitochondrial fractions

In Table II the figures for the microsomal contamination of intact mitochondria and their various fractions are given. As marker enzymes of microsomes, glucose-6-phosphatase and NADPH–cytochrome *c* reductase were used. On the basis of the specific activity of microsomal rotenone-insensitive NADH–cytochrome *c* reductase (= 262 nmoles cytochrome *c* reduced per min per mg protein), it was possible to calculate the microsomal contribution to the activity of this enzyme in the various submitochondrial fractions. These calculations showed that the activity of rotenone-insensitive NADH–cytochrome *c* reductase of I_1 and I_2 fractions was not appreciably influenced by that of microsomal enzyme. Moreover, it was calculated that only 11 % and 8 % of the total activity of rotenone-insensitive NADH–cytochrome *c* reductase by the outer membrane remained in the S_2 and P_2 fractions, respectively; therefore these two fractions were essentially free of the outer membrane.

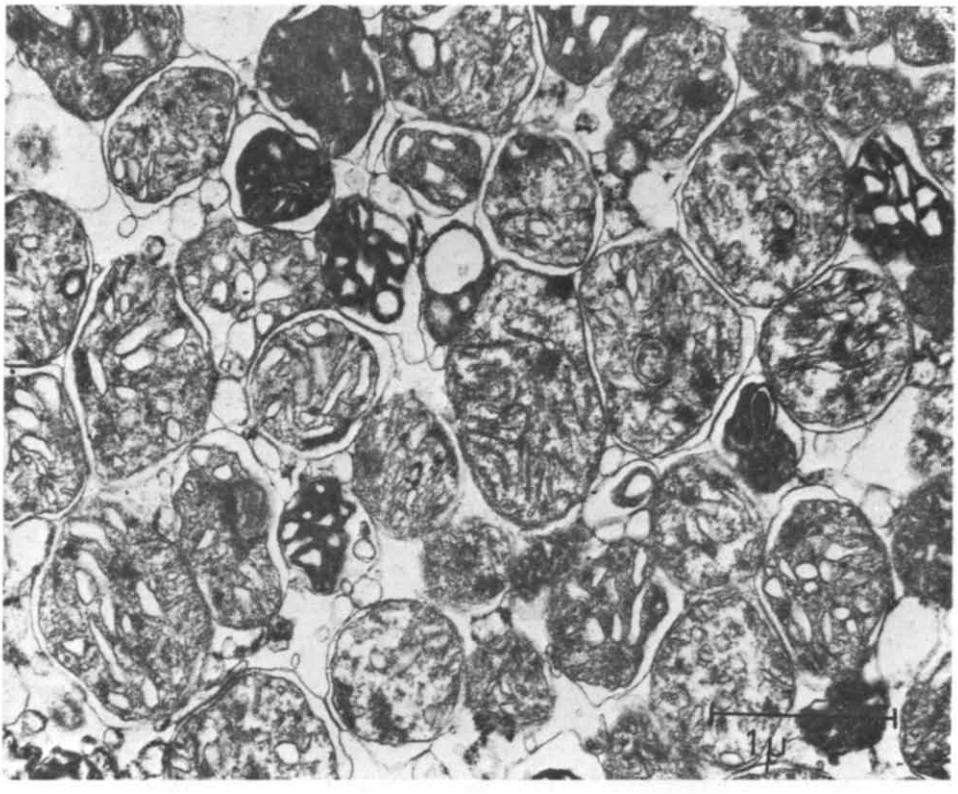

Fig. 1. Electron micrograph of thin sections of glutaraldehyde–OsO_4-fixed isolated pigeon-liver mitochondria stained with uranyl acetate–lead hydroxide. × 24600. Scale line = 1 μ.

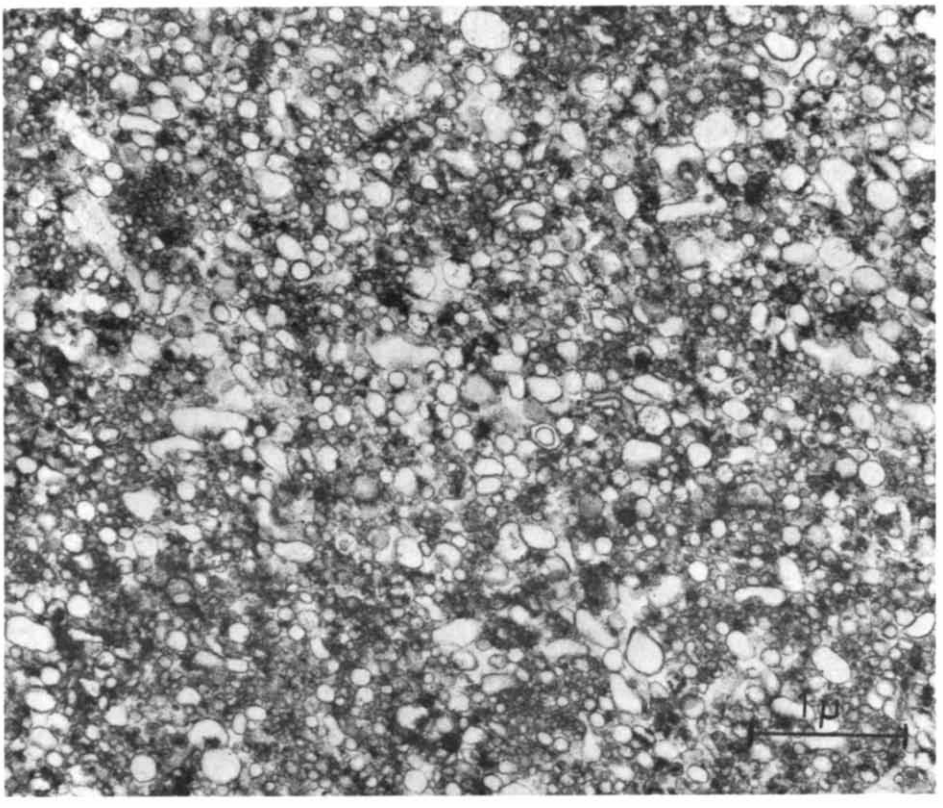

Fig. 2. Electron micrograph of thin sections of glutaraldehyde–OsO_4-fixed fraction I_1 of pigeon-liver mitochondria stained with uranyl acetate–lead hydroxide. This fraction consists of the outer membrane of mitochondria. × 20400. Scale line = 1 μ.

Electron microscopy observations

Fig. 1 shows a thin section of the freshly isolated pigeon-liver mitochondria used for the fractionation. The structural integrity of mitochondria as well as a differentiation between the outer and inner mitochondrial membranes can be seen. Fig. 2 is a micrograph of the interphase fraction I_1 obtained after the first step of fractionation. It consists principally of small vesicles, bordered by a single membrane, characteristic of the outer membrane[5,6]. Fig. 3 represents an electron micrograph of the pellet P_2 obtained after the second step of fractionation of pigeon-liver mitochondria. It consists of inner membrane fragments of various sizes which have no attached outer membrane (*cf.* ref. 22).

Enzyme distribution

In Table III it is shown that after the first step of fractionation practically all the activity of pyruvate carboxylase, phosphopyruvate carboxylase, glutamate dehydrogenase, aspartate aminotransferase, $NADP^+$–isocitrate dehydrogenase and NAD^+–isocitrate dehydrogenase was recovered in the pellet P_1. After the second step of fractionation about 70% of both pyruvate carboxylase and phosphopyruvate carboxylase, 61% of glutamate dehydrogenase, about 80% of aspartate amino-

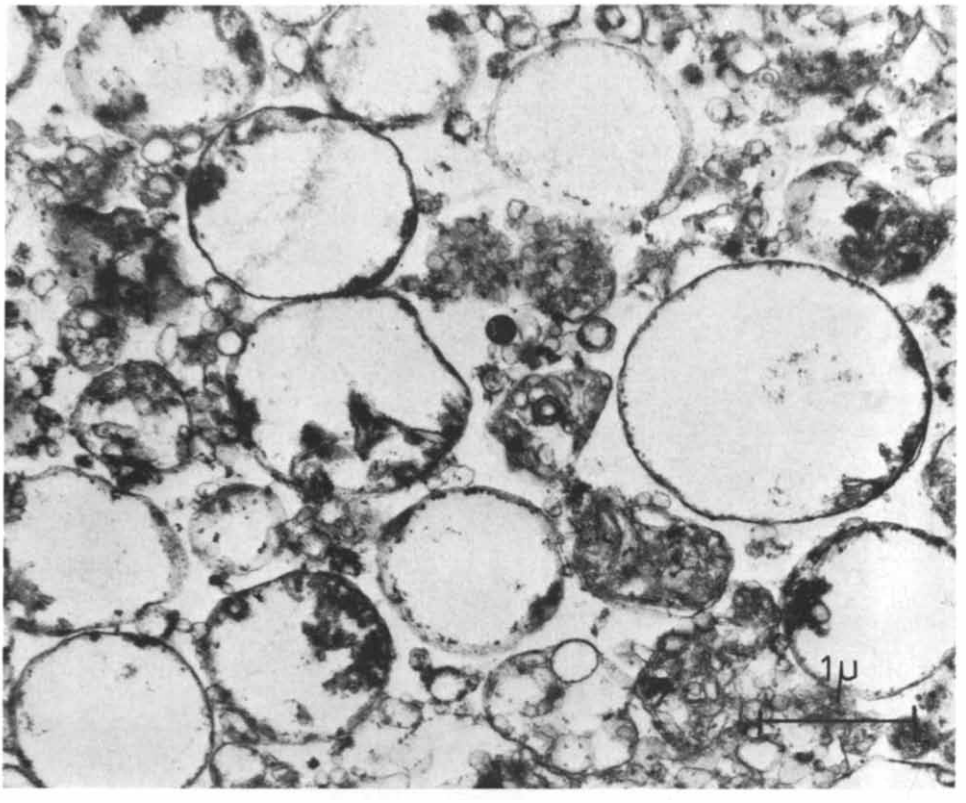

Fig. 3. Electron micrograph of thin sections of glutaraldehyde–OsO_4-fixed fraction P_2 of pigeon-liver mitochondria stained with uranyl acetate–lead hydroxide. This fraction appears to consist primarily of disrupted inner membrane. × 21 000. Scale line = 1 μ.

transferase, 91 % of $NADP^+$–isocitrate dehydrogenase and 76 % of NAD^+–isocitrate dehydrogenase were found in the soluble fraction S_2.

Subcellular distribution of malonyl-CoA decarboxylase

The subcellular distribution of malonyl-CoA decarboxylase in the pigeon liver was also studied. The results of Table IV show that in pigeon liver, like in rat liver[23,24], malonyl-CoA decarboxylase is principally a mitochondrial enzyme. Moreover, this table shows that, by disrupting pigeon-liver mitochondria, the specific activity of malonyl-CoA decarboxylase increases 2.5 times with respect to intact mitochondria[23].

Submitochondrial distribution of lipid metabolism enzymes

In Table V results on the submitochondrial distribution of the enzymes of β-oxidation, malonyl-CoA decarboxylase and the enzymes of fatty acid synthesis are reported. After the first step of fractionation, the activity of these enzymes was concentrated in the P_1 fraction. After the second step of fractionation, the enzymes of β-oxidation were found exclusively in the inner membrane. The low recovery of this enzymatic activity, as compared to the higher recovery of the other enzymes, may be due to inactivation of the enzyme complex during the fractionation procedure.

TABLE III

LOCALIZATION AND SPECIFIC ACTIVITY OF SEVERAL ENZYMES IN THE MATRIX SPACE OF PIGEON-LIVER MITOCHONDRIA

See legend of Table I for abbreviations. Step I: M = 100%; Step II: P_1 = 100%.

Enzymes	*Total activity* (%)				*Specific activity* (*nmoles/min per mg protein*)			
	S_1	I_1	P_1	*Recovery*	*M*	S_1	I_1	P_1
Step I								
Pyruvate carboxylase	3.6	2.6	77.1	83.3	34	15	9	36
Phosphopyruvate carboxylase	2.5	1.3	81.6	85.4	147	44	21	165
Glutamate dehydrogenase	0	0	99.9	99.9	670	0	0	920
Aspartate aminotransferase	3.0	1.0	98.3	102.3	265	98	25	358
$NADP^+$–isocitrate dehydrogenase	4.8	2.5	91.8	99.1	206	120	51	260
NAD^+–isocitrate dehydrogenase	6.5	2.1	92.6	101.2	165	131	35	210
	S_2	I_2	P_2	*Recovery*	P_1	S_2	I_2	P_2
Step II								
Pyruvate carboxylase	70.0	2.7	16.3	89.0	36	85	10	12
Phosphopyruvate carboxylase	69.5	4.0	16.3	89.8	165	388	68	55
Glutamate dehydrogenase	61.3	6.4	28.1	95.8	920	1910	610	530
Aspartate aminotransferase	79.6	2.8	26.5	108.9	358	965	104	195
$NADP^+$–isocitrate dehydrogenase	91.0	4.3	19.4	114.7	260	802	125	104
NAD^+–isocitrate dehydrogenase	75.9	4.7	19.5	100.1	210	540	101	84

TABLE IV

DISTRIBUTION AND SPECIFIC ACTIVITY OF MALONYL-CoA DECARBOXYLASE IN SUBCELLULAR FRACTIONS OF PIGEON LIVER

Fraction	*Units/g liver**	*Specific activity***
Intact mitochondria	—	0.92
Disrupted mitochondria	88.0	2.20
Microsomes	20.8	0.40
Supernatant 105000 × *g*	62.7	0.95

* Expressed as nmoles [1,3-$^{14}C_2$]malonyl-CoA decarboxylated per min per g liver.
** Expressed as nmoles [1,3-$^{14}C_2$]malonyl-CoA decarboxylated per min per mg protein.

The enzymes of fatty acid synthesis were almost exclusively recovered in the inner membrane fraction. Malonyl-CoA decarboxylase was distributed among the inner membrane and the soluble fractions. 58.4% of its activity remained bound to the inner mitochondrial membrane, while 31.4% was found in the soluble fraction.

Comparison between the ratio of mitochondrial protein to soluble protein and the purification of the enzymes recovered in the soluble fraction S_2

In Table VI the specific activities and the purification of the enzymes recovered in the soluble fraction S_2 are compared with the ratio of mitochondrial protein to

TABLE V

DISTRIBUTION AND SPECIFIC ACTIVITY OF β-OXIDATION ENZYMES, FATTY ACID SYNTHESIS ENZYMES AND MALONYL-CoA DECARBOXYLASE IN SUBMITOCHONDRIAL FRACTIONS OF PIGEON LIVER

See legend of Table I for abbreviations. Step I: M = 100%; Step II: P_1 = 100%.

Enzymes	*Total activity (%)*				*Specific activity (nmoles/min per mg protein)*			
	S_1	I_1	P_1	*Recovery*	*M*	S_1	I_1	P_1
Step I								
Fatty acid oxidizing enzymes*	0	0	82.8	82.8	2.9	0	0	3.3
Fatty acid synthesizing enzymes**	2.4	1.7	90.9	95.0	0.24	0.07	0.04	0.3
Malonyl-CoA decarboxylase***	6.0	3.6	78.7	88.3	1.74	1.27	0.63	1.88
	S_2	I_2	P_2	*Recovery*	P_1	S_2	I_2	P_2
Step II								
Fatty acid oxidizing enzymes*	0	0	56.1	56.1	3.3	0	0	3.8
Fatty acid synthesizing enzymes**	12.8	6.5	58.4	77.7	0.30	0.13	0.20	0.36
Malonyl-CoA decarboxylase***	31.4	9.8	58.4	99.6	1.88	2.0	1.91	2.25

* The specific activity is expressed as nmoles formazan formed per min per mg protein.

** The specific activity is expressed as nmoles [1,3-$^{14}C_2$]acetyl-CoA incorporated into fatty acids per min per mg protein.

*** The specific activity is expressed as nmoles [1,3-$^{14}C_2$]malonyl-CoA decarboxylated per min per mg protein.

TABLE VI

COMPARISON BETWEEN THE RATIO OF MITOCHONDRIAL PROTEIN TO SOLUBLE PROTEIN AND THE PURIFICATION OF THE ENZYMES OF THE SOLUBLE FRACTION S_2

Enzymes	*Specific activity of M*	*Specific activity of S_2*	*Purification*
$NADP^+$–isocitrate dehydrogenase	206	802	3.9
Aspartate aminotransferase	265	965	3.6
NAD^+–isocitrate dehydrogenase	165	540	3.3
Glutamate dehydrogenase	670	1910	2.8
Phosphopyruvate carboxylase	147	388	2.6
Pyruvate carboxylase	34	85	2.5

Fraction	*Protein (mg)*	*Ratio of mitochondrial protein to soluble protein*
M	18	—
S_2	3.9	4.6 (3.7)*

* The value in parentheses is that corrected by assuming a 100% protein recovery in the two steps of fractionation of mitochondria.

soluble protein. It can be seen that the activity of $NADP^+$–isocitrate dehydrogenase and aspartate aminotransferase in the S_2 fraction is 3.9 and 3.6 times higher, respectively, than that of unfractionated mitochondria. These values are very near to that of the ratio of mitochondrial protein to soluble protein, equal to 4.6. This ratio was

obtained when no correction for the loss of protein during the fractionation was applied. By assuming a 100 % protein recovery in the two steps of fractionation, the value of the ratio of mitochondrial protein to soluble protein proved to be 3.7. The concentration factor of pyruvate carboxylase, phosphopyruvate carboxylase, glutamate dehydrogenase and NAD^+–isocitrate dehydrogenase in the soluble fraction with respect to unfractionated mitochondria approached that of $NADP^+$–isocitrate dehydrogenase and aspartate aminotransferase.

These data allow the identification of a group of enzymes, consisting of $NADP^+$–isocitrate dehydrogenase, aspartate aminotransferase, pyruvate carboxylase, phosphopyruvate carboxylase, glutamate dehydrogenase and NAD^+–isocitrate dehydrogenase, that can be assigned to the matrix. This indicates an enzymatic individuality of this space.

DISCUSSION

Recently much attention has been paid to the study of the synthetic enzymes of mitochondria. The activity of these enzymes appears to be closely connected to that of the tricarboxylic acid cycle, oxidative phosphorylation and nicotinamide adenine nucleotide transhydrogenase. In this context the topography of the mitochondrial enzymes is highly relevant. The main purpose of the present investigation was to study the localization of the mitochondrial enzymes involved in glutamate metabolism[25], gluconeogenesis[15], isocitrate metabolism and fatty acid metabolism[16] in pigeon liver. The mitochondria of this tissue are particularly suitable for these studies, since they have an extremely high content of phosphopyruvate carboxylase[26] and a relatively high content of pyruvate carboxylase[27], enzymes of fatty acid synthesis, glutamate dehydrogenase and $NADP^+$–isocitrate dehydrogenases.

The fractionation of pigeon-liver mitochondria by the method of SOTTOCASA *et al.*[5] gave a clear-cut separation of the inner membrane from the outer membrane of mitochondria. However, when the localization of the enzymes involved in glutamate metabolism, gluconeogenesis and the citric acid cycle was studied by this method, equivocal results were obtained. In fact glutamate dehydrogenase, $NADP^+$–isocitrate dehydrogenase, NAD^+–isocitrate dehydrogenase and phosphopyruvate carboxylase distributed almost equally between the matrix and the inner membrane of mitochondria (see also ref. 20).

The distribution pattern of marker enzymes and the electron microscopy observations show that in the first step of the modified procedure of fractionation of the mitochondria a soluble fraction S_1, probably containing the material between the two membranes, and an interphase fraction I_1, containing almost exclusively the outer membrane with practically no contamination by inner membrane and matrix, can be obtained. The pellet P_1 of the first step of fractionation consists essentially of the inner membrane with the matrix enclosed *plus* residual outer membrane. In the second step of fractionation the residual outer membrane moves to the interphase I_2 and a clear-cut separation of the matrix (soluble fraction S_2) from the inner membrane (pellet P_2) appears to be achieved.

It can clearly be seen that, by using the modified procedure of fractionation, the mitochondrial enzymes involved in gluconeogenesis, pyruvate carboxylase and phosphopyruvate carboxylase, and those of glutamate metabolism, glutamate dehy-

drogenase, aspartate aminotransferase and both $NADP^+$– and NAD^+–isocitrate dehydrogenases, are localized in the matrix (*cf.* refs. 5 and 7). The concentration factor of these enzymes agrees well with the value of the ratio of mitochondrial protein to matrix protein (Table VI). It must be emphasized that the concentration factor of $NADP^+$–isocitrate dehydrogenase and aspartate aminotransferase is practically equal to the value of this ratio. Therefore, it seems warranted to use these two enzymes as markers of the mitochondrial matrix.

The enzymes involved in the synthesis and oxidation of fatty acids in mitochondria are recovered in the inner membrane. Malonyl-CoA decarboxylase appears to have a dual localization: 58.4 % of its activity is found in the inner membrane and 31.4 % in the matrix.

We have previously found that the enzymes responsible for fatty acid synthesis in rat-liver mitochondria are localized in the inner membrane[16]. The same distribution is found in pigeon-liver mitochondria (Table V). BEATTIE[8], by fractionating rat-liver mitochondria by the digitonin method, has demonstrated that the enzymes of β-oxidation are localized in the inner membrane–matrix fraction of mitochondria. The data of Table V indicate that in pigeon-liver mitochondria, like in rat-liver mitochondria (unpublished results), the enzymes responsible for the oxidation of fatty acids are localized in the inner membrane. The finding that both the enzymes of fatty acid synthesis and those of β-oxidation are present in the same fraction appears to have some relevance with respect to the problem of mechanism by which fatty acids are synthesized in the mitochondria. QUAGLIARIELLO *et al.*[16] have demonstrated that fatty acid synthesis in rat-liver mitochondria occurs only by chain elongation *via* acetyl-CoA. The present data would be in agreement with the proposal that in mitochondria chain elongation of fatty acids, except the last reducing step that is NADPH-dependent, is essentially carried out by the reverse mechanism of fatty acid oxidation[c8].

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REFERENCES

1 D. F. PARSONS, G. R. WILLIAMS AND B. CHANCE, *Ann. N. Y. Acad. Sci.*, 137 (1966) 643.
2 E. BACHMANN, D. W. ALLMANN AND D. E. GREEN, *Arch. Biochem. Biophys.*, 115 (1966) 153.
3 D. W. ALLMANN, E. BACHMANN AND D. E. GREEN, *Arch. Biochem. Biophys.*, 115 (1966) 165.
4 D. E. GREEN, E. BACHMANN, D. W. ALLMANN AND J. F. PERDUE, *Arch. Biochem. Biophys.*, 115 (1966) 172.
5 G. L. SOTTOCASA, B. KUYLENSTIERNA, L. ERNSTER AND A. BERGSTRAND, *J. Cell Biol.*, 32 (1967) 415.
6 C. SCHNAITMAN, V. G. ERWIN AND J. W. GREENWALT, *J. Cell Biol.*, 32 (1967) 719.
7 C. SCHNAITMAN AND J. W. GREENWALT, *J. Cell Biol.*, 38 (1968) 158.
8 D. BEATTIE, *Biochem. Biophys. Res. Commun.*, 30 (1968) 57.
9 S. PAPA, C. LANDRISCINA, N. E. LOFRUMENTO AND E. QUAGLIARIELLO, *Abstr. 5th Meeting Federation European Biochem. Socs., Praha, 1968*, p. 178.
10 C. LANDRISCINA, S. PAPA, N. E. LOFRUMENTO, P. CORATELLI AND E. QUAGLIARIELLO, *Boll. Soc. Ital. Biol. Sper.*, 44 (1968) 2127.
11 A. E. HARPER, in H. U. BERGMEYER, *Methods of Enzymatic Analysis*, Academic Press, New York, 1963, p. 788.
12 H. F. CHEN AND G. W. E. PLAUT, *Biochemistry*, 2 (1963) 752.

13 H. U. BERGMEYER AND E. BERNT, in H. U. BERGMEYER, *Methods of Enzymatic Analysis*, Academic Press, New York, 1963, p. 837.
14 R. CZOK AND L. ECKERT, in H. U. BERGMEYER, *Methods of Enzymatic Analysis*, Academic Press, New York, 1963, p. 224.
15 S. PAPA, N. E. LOFRUMENTO AND E. QUAGLIARIELLO, *Biochim. Biophys. Acta*, 110 (1965) 442.
16 E. QUAGLIARIELLO, C. LANDRISCINA AND P. CORATELLI, *Biochim. Biophys. Acta*, 164 (1968) 12.
17 S. MII AND D. E. GREEN, *Biochim. Biophys. Acta*, 13 (1954) 425.
18 J. H. LUFT, *J. Biophys. Biochem. Cytol.*, 9 (1961) 409.
19 M. J. KARNOVSKY, *J. Biophys. Biochem. Cytol.*, 11 (1961) 729.
20 K. R. NORUM, M. FARSTAD AND J. BREMER, *Biochem. Biophys. Res. Commun.*, 24 (1966) 797.
21 D. S. BEATTIE, *Biochem. Biophys. Res. Commun.*, 31 (1968) 901.
22 D. F. PARSONS, G. R. WILLIAMS, W. THOMPSON, D. WILSON AND B. CHANCE, in E. QUAGLIARIELLO, S. PAPA, E. C. SLATER AND J. M. TAGER, *Mitochondrial Structure and Compartmentation*, Adriatica Editrice, Bari, 1967, p. 29.
23 C. LANDRISCINA AND E. QUAGLIARIELLO, *Abstr. Intern. Congr. Olive Oil Dietetics, Bari, 1968*, p. 3.
24 H. R. SCHOLTE, *Biochim. Biophys. Acta*, 170 (1969) 137.
25 S. PAPA, J. M. TAGER, A. FRANCAVILLA AND E. QUAGLIARIELLO, *Biochim. Biophys. Acta*, 172 (1969) 20.
26 W. GEVERS, *Biochem. J.*, 103 (1967) 141.
27 D. B. KEECH AND M. F. UTTER, *J. Biol. Chem.*, 238 (1963) 2609.
28 W. SEUBERT, I. LAMBERTS, R. KRAMER AND B. OHLY, *Biochim. Biophys. Acta*, 164 (1968) 498.