Mitochondrial–reticular cytostructure in liver cells

Joseph KATZ,*† Petronella A. WALS,* Sybil GOLDEN* and Luisa RAIJMAN‡

*Cedars–Sinai Medical Center, Los Angeles, CA 90048, U.S.A., and ‡Department of Biochemistry,
University of Southern California Medical School, Los Angeles, CA 90033, U.S.A.

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1. This study examines the structural relationship of mitochondria and the endoplasmic reticulum in liver. Livers of rat and Japanese quail were homogenized and fractionated in media of 0.25 M-sucrose, either 5 mM or 50 mM in sodium Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], pH 7.4 (2.2 mM or 22 mM in Na respectively), designated here as low- and high-salt media. Three particulate fractions were prepared by sequential centrifugation. A nuclear pellet sedimenting at 300 g was obtained as described by Shore & Tata [(1977) *J. Cell Biol.* **72**, 714–725], and from the resulting supernatant thereof a low-speed pellet (1100–1500 g) and a high-speed pellet (8000–10000 g) were prepared. In the low-salt medium the yields of mitochondrial matrix enzymes (citrate synthase, glutamate dehydrogenase, ornithine carbamoyltransferase) and their specific activities in the low-speed pellet were over twice those in the high-speed pellet. In the high-salt medium the yield of matrix enzymes was 4–5 times, and the specific activities were up to 3 times, higher in the low-speed pellet than in the high-speed pellet. Oxygen uptake and respiratory control ratio were also much higher in the low-speed pellets in both media. Some 50–65% of the microsomal marker enzyme glucose 6-phosphatase was in the supernatant from the high-speed pellet, and the rest sedimented with the mitochondria. 2. Repeated washing with the high-salt medium removes only a limited amount of reticulum. Washing with salt-free sucrose removes most of the reticulum, but a fraction remains strongly bound to mitochondria. 3. Homogenates from quail and rat liver were fractionated isopycnicly on Percoll gradients in either 0.25 M-sucrose or 0.25 M-sucrose/50 mM-sodium Hepes. Up to five particulate bands were separated and assayed. Mitochondria were present in two to three bands and were associated with endoplasmic reticulum. 4. As seen in the phase-contrast microscope the mitochondria prepared in the low-salt medium consist of separate organelles. In the high-salt medium the mitochondria appear as chains of from three to ten organelles not touching each other. On addition of univalent ions at concentrations above 20 mM, the mitochondria aggregate into chains, and at higher ionic strength larger multidimensional aggregates are formed. The dispersion and aggregation of mitochondria are reversible. Negatively stained electron micrographs reveal a branched mitochondrial structure, with mitochondria held together by strands of reticulum. 5. Our observations support the existence of a cytostructure of a mitochondrial network held together by lamellae of endoplasmic reticulum which are continuous with the mitochondrial outer membrane. The reticular network is stable at high ionic strength, but breaks up to single mitochondria at low ionic strength. The implication of our findings for cell structure, for the heterogeneity of mitochondria and for methods of preparation of mitochondrial and microsomal fractions is considered.

It is generally held that mitochondria are free organelles suspended in the cytosol; however,

Abbreviation used: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

† To whom correspondence should be addressed.

several investigators have observed a continuity between the mitochondrial outer membranes and reticular lamellae, and a few have proposed the existence of a stable mitochondrial–reticular complex (see the Discussion section), but this view has

received little attention. We present biochemical, light- and electron-microscopic studies establishing the firm attachment of mitochondria and reticulum, and evidence supporting the concept of a reticular-mitochondrial cytostructure in mammalian and avian liver. We show that the stability of this cytostructure depends on ionic strength.

In the conventional procedures for subcellular fractionation by differential centrifugation in 0.25 M-sucrose, the low-speed pellet (600–1000 g) is universally described as being mainly 'debris' and nuclei, and the sediment obtained from the supernatant at 8000–10000 g is taken to represent the mitochondrial fraction. The endoplasmic reticulum is said to be present as microsomal vesicles in the supernatant from this fraction. We show here that this scheme, described in numerous texts and manuals, is not altogether correct for liver. Depending on ionic strength, a variable amount of reticulum remains bound to mitochondria.

Methods

Homogenization

Fed rats of the Sprague-Dawley strain (200–300 g body wt.) and female mature (egg-laying) Japanese quail (*Coturnix coturnix japonica*) were used. The animals were decapitated and bled, or in a few experiments they were anaesthetized and the livers perfused with 0.25 M-sucrose. Rat liver was homogenized with chilling in 10 vol. of medium by five to six strokes of a Teflon pestle in a Potter-Elvehjem glass homogenizer, at about 300 rev./min. Quail liver is more fragile and was homogenized manually. The medium was 0.25 M-sucrose alone or with 5 mM- or 50 mM-sodium Hepes buffer, pH 7.4, or with NaCl or KCl, as described in the text. The concentration of Na⁺ ions in the buffer at pH 7.4 is about 45% that of Hepes. The homogenate was adjusted in volume to 20 ml/g of liver and filtered through two layers of sheer nylon stocking.

Sedimentation

A nuclear pellet was separated essentially as described by Shore & Tata (1977a). Then 20 ml of 0.35 M-sucrose solution of the same buffer or salt content as the medium was placed in a 50 ml conical centrifuge tube and overlaid with 20 ml of the homogenate. The tubes were centrifuged in a refrigerated Sorvall 3-CB instrument, in a HG-4 swinging-bucket rotor, at 1100 rev./min (300 g_{max.}) for 10 min. A reddish sediment (unless livers were pre-perfused) was separated distinctly from the homogenate suspension, which remained largely in the upper layer. Then 35 ml of the supernatant was diluted to a sucrose concentration of 0.25 M and centrifuged sequentially to yield a low- and a high-speed pellet and a supernatant fraction. In our

earlier work reported in Tables 1–5, an older model refrigerated centrifuge (Sorvall 2B) was used, replaced more recently by Sorvall model RC-5B. A SS-34 conical rotor was used with both centrifuges. With the Sorvall 2B model a fraction sedimenting at 3000 rev./min (1100 g_{max.}) for 10 min was collected and the supernatant was centrifuged at 8200 rev./min (8000 g_{max.}) for 10 min to yield a second pellet. We observed that with the RC-5B centrifuge a substantially lower yield was obtained at 1100 g. The total run, including acceleration and deceleration, was 15.5 min for the 2B model and 12 min for the RC-5B. We found that similar yields of protein and enzymes to those at 1100 g and 8000 g were obtained when the RC-5B model was run at 3500 rev./min (1500 g) and 9200 rev./min (10000 g). We thus designate the pellets obtained at 1100 g and 8000 g in the 2B model and at 1500 g and 10000 g in the RC-5B model as low-speed and high-speed pellets respectively. For studies of oxygen uptake, the low- and high-speed pellets were resuspended in 0.35 M-mannitol/5 mM-potassium Hepes, pH 7.4. Oxygen uptake and the P/O ratio were determined as described by Rajjman & Bartulis (1979).

Isopycnic fractionation

This was done with Percoll (Pharmacia Fine Chemicals). For rat liver the gradient was that used by Reinhart *et al.* (1982), but in most experiments the Percoll was suspended in 0.25 M-sucrose or in 0.25 M-sucrose containing 5 mM- or 50 mM-sodium Hepes. In a 15 ml tube, 3 ml each of 52%, 42% and 31% Percoll and 2 ml of 19% Percoll were layered, followed by 2 ml of rat liver homogenate from about 0.2 g of liver, prepared in the same medium as the Percoll suspension. The tubes were centrifuged for 1 min at 20000 rev./min (50000 g_{max.}) in the SS-34 rotor of the Sorvall RC-5B instrument, with a total time of the run of about 9 min. A diffuse layer mainly of soluble protein was obtained at the top of the tube, and five particulate bands were well separated. A small dense pellet or erythrocytes was present at the bottom (Fig. 1). The appearance was much the same with pure sucrose or sucrose/5 mM-sodium Hepes, but differed from that in the presence of 50 mM-sodium Hepes. Band II was much heavier and Band V much smaller with the higher buffer concentration, and was sometimes nearly absent (see the Results section). The bands together with the intermediate zones were aspirated manually with a Pasteur pipette, the fractions were made up to 10 ml with 0.25 M-sucrose/50 mM-sodium Hepes, pH 7.4, and centrifuged for 10 min at 10000 g (except for the top fraction), and the pellets examined with the phase-contrast microscope and analysed for protein, glutamate dehydrogenase and glucose 6-phosphatase. This removed most of the

Fig. 1. Rat liver homogenates fractionated in Percoll. Left, Percoll in 0.25 M-sucrose; right, Percoll in 0.25 M-sucrose/50 mM-sodium Hepes. For details see the Methods section.

Percoll, which was found to interfere in the assay of protein.

The above gradient was not satisfactory for quail liver. The gradient was prepared by layering 3 ml each of 42%, 31% and 19% Percoll, followed by 2 ml of 9.5% Percoll and topped with 2 ml of liver homogenate. The tubes were centrifuged as described for rat liver. There was an upper diffuse zone, followed by four bands and a small pellet of erythrocytes. The densities were determined with coloured density marker beads, purchased from Pharmacia Fine Chemicals.

Assays

For protein and enzyme assays, pellets were suspended in the homogenization medium, and homogenates and suspensions were sonicated (with chilling; three times for 20 s each), subdivided and frozen. Protein was determined as described by Bradford (1976), by using a Bio-Rad (Richmond, CA, U.S.A.) kit, citrate synthase (EC 4.1.3.7) as

described by Srere (1969), glutamate dehydrogenase (EC 1.4.1.3) as described by Schmidt (1974), and glucose 6-phosphatase (EC 3.1.3.9) as described by Baginski *et al.* (1974). Ornithine carbamoyltransferase (EC 2.1.3.3) was measured in 1 ml incubation volume, containing 200 mM-Tris/HCl, pH 8.5, 10 mM-ornithine, 10 mM-carbamoyl phosphate, and enough protein to form no more than 0.1 μ mol of citrulline in 10 min at 37°C. The reaction was stopped with 0.5 ml of 5% HClO₄, and citrulline was measured in the deproteinized supernatants as described by Ceriotti & Spandrio (1963).

Microscopy

All fractions were routinely examined by a visual phase-contrast microscope. For examination by electron microscopy, suspensions were stained without fixing by sodium phosphotungstate or uranyl acetate. Small drops of suspensions were allowed to settle on Formvar/carbon-coated grids, and excess liquid was removed with filter paper. For negative staining the grids were treated with a 0.1% solution of sodium phosphotungstate, excess fluids were removed, and, to remove the interfering sucrose, inverted successively on four to five drops of water. For positive staining with uranyl acetate, the grids were treated with a 1% solution and the excess was washed away. The grids were then dried in air and examined in a Philips 301 electron microscope operating at 80 kV.

Results

Distribution of enzymes

In Table 1 we report the distribution of protein and of three mitochondrial-matrix enzymes (citrate synthase, glutamate dehydrogenase, ornithine carbamoyltransferase) and the reticular marker enzyme glucose 6-phosphatase in the nuclear, low-speed and high-speed pellets, and the supernatant. One half of each liver was homogenized in 0.25 M-sucrose containing 5 mM-sodium Hepes buffer, pH 7.4, and the other half in 0.25 M sucrose with 50 mM of this buffer. Examination of the fractions with a phase-contrast microscope revealed that most of the 300 g pellet consisted of nuclei (Fig. 2). It also contained erythrocytes, plasma membranes and a small amount of partially broken cells or tissue. The latter, presumably the 'debris' mentioned in the literature, account at most for a few per cent of the total homogenate protein. The nuclear fraction contained a few mitochondria in the 5 mM-sodium Hepes medium, but considerably more in the preparation with the 50 mM buffer. The low-speed fraction consisted of mitochondria, with a few nuclei and erythrocytes. Only mitochondria could be discerned in the high-speed pellet. The appearance of

Table 1. *Distribution of enzymes of rat liver fractions*

One half of each liver was homogenized in 0.25 M-sucrose/5 mM-sodium Hepes, pH 7.4, and the other half in 0.25 M-sucrose/50 mM-sodium Hepes, pH 7.4. For details of the fractionation, see the Methods section. Results are means of three closely agreeing experiments. The units are: for protein, mg/g of liver; for enzyme-activity units, $\mu\text{mol/min per g of liver}$; for enzyme distribution, the percentage of the total activity recovered in the fraction; for specific activity (sp. act.), nmol/min per mg of protein. The ratios of enzyme specific activities in the homogenate were set to 1.00 (actual values are given in parentheses).

Fraction	Homogenate		Nuclear pellet		Low-speed pellet		High-speed pellet		Supernatant		Recovery (%)	
	5	50	5	50	5	50	5	50	5	50	5	50
Protein	[Sodium Hepes] (mM) ...											
	(mg/g)	184	196									
	(%)	100	100	12	15	22	22	13	10	50	51	98
Citrate synthase	(units/g)	13	14									
	(%)	100	100	7.6	16	61	61	25	13	7.7	4.1	100
	(sp. act.)	73	70	45	77	224	192	127	83	9.6	6.5	—
Glutamate dehydrogenase	(units/g)	126	134									
	(%)	100	100	7.8	16	65	63	21	12	5.7	3.7	100
	(sp. act.)	560	545	386	880	1840	1760	820	590	55	33	—
Ornithine carbamoyltransferase	(units/g)	396	362									
	(%)	100	100	6.7	16	69	68	27	13	N.D.	N.D.	103
	(sp. act.)	1920	1850	1040	2070	5940	5670	3560	2260	—	—	97
Glucose 6-phosphatase	(units/g)	23	26									
	(%)	100	100	7.2	10	12	20	24	24	52	42	95
	(sp. act.)	129	120	75	86	86	117	220	283	—	—	—
Glucose 6-phosphatase	sp. act. ratio	(1.76)	(1.71)	0.94	0.69	0.22	0.34	0.98	1.99	8.4	9.5	—
citrate synthase		1.00	1.00									
Glucose 6-phosphatase	sp. act. ratio	(0.197)	(0.192)	1.10	0.53	0.26	0.32	1.25	2.23	11.1	13.9	—
glutamate dehydrogenase		1.00	1.00									
Glucose 6-phosphatase	sp. act. ratio	(0.07)	(0.065)	1.07	0.64	0.22	0.32	0.92	1.93	—	—	—
ornithine carbamoyl-transferase		1.00	1.00

Fig. 2. Phase-contrast microscopy of nuclear pellet, prepared as described by Shore & Tata (1977a). The nuclear pellet is from rat liver homogenized in 0.25 M-sucrose/5 mM-sodium Hepes. Magnification $\times 400$.

the mitochondria differed markedly in the two buffers, as discussed below.

The recovery of protein and enzyme activity in the four fractions was nearly quantitative, ranging from 95 to 103% of that in the homogenate (Table 1). The relative content of the three matrix enzymes was very similar, but their distribution among the fractions differed for the two buffer concentrations. In the low-salt medium about 7% was in the nuclear pellet, 65% in the low-speed pellet, 20–25% in the high-speed pellet, and 6 or 7% in the supernatant. In the high-salt buffer there was more protein and twice as much mitochondrial-matrix enzymes in the nuclear pellet (consistent with the microscopic findings), but only one-half of the matrix enzymes in the high-speed pellet. Thus in the low-salt medium the mitochondrial enzyme activity in the low-speed pellet was 2.5–3 times that in the high-speed pellet, and in the high-salt medium, 5-fold. The specific activities of the matrix enzymes were similar for the low-speed fractions from the two media, but they were 1.8 to nearly 3 times higher than in the high-speed pellet. Thus the low-speed pellet contained most of the mitochondria; moreover, there was much more non-mitochondrial material in the high-speed pellet. Only 40–50% of the activity of the

glucose 6-phosphatase, a classical microsomal enzyme, was in the supernatants. About one-quarter of the total glucose 6-phosphatase activity was in the high-speed fraction. There was substantially more of the enzyme in the pellets when the medium contained 50 mM buffer, and the specific activity in the high-speed pellet was at least twice as high as in the original homogenate.

As an index of the association of reticulum to mitochondria, the ratios of activity of glucose 6-phosphatase to the mitochondrial-matrix enzymes were computed and were normalized with those in the homogenate set to unity (Table 1). The lowest ratio (0.2–0.25) was in the low-speed pellet prepared with the 5 mM buffer. In the high-speed pellet the ratio with low salt was close to 1, about the same as in the homogenate, but with 50 mM buffer the ratio was about 2, indicating a marked enrichment with reticulum. The distribution of NADPH-cytochrome *c* reductase, another microsomal marker enzyme, followed closely that of glucose 6-phosphatase (results not shown).

In Table 2, similar experiments with quail liver are presented. The distribution patterns of citrate synthase and glutamate dehydrogenase at high and low buffer concentrations resembled that for rat liver, and the yield of glucose 6-phosphatase in the mitochondrial fraction was even higher than with rat liver.

Oxygen uptake

The oxygen uptake and respiratory control ratios of the mitochondrial fractions prepared in the two sucrose media were compared (Table 3). The oxygen uptake of the low-speed pellet in the presence of ADP was the same for both media, about 150 nmol of O_2 /min per mg of protein (Table 3). In this fraction, the oxygen uptake before ADP addition (State 2) and upon its exhaustion (State 4) were similar, and the respiratory control ratios were 5–6. The oxygen utilization in the high-speed fractions was less. In these fractions the approach to State 4 was slow, especially in that prepared in the high-salt medium; the respiratory control ratio (Table 3) could not be determined reliably, and there appeared to be some ATP hydrolysis. The oxygen uptake was less than that expected from dilution by the microsomal content, and it appears that oxidation and probably oxidative phosphorylation are decreased in the mitochondria in the high-speed pellet, especially in that from the high-salt medium.

The oxygen uptake of the low-speed pellet, the major mitochondrial fraction, is about twice the maximal values reported for mitochondria of normal liver by Yamazaki (1975), and 3 times that reported by Reinhart *et al.* (1982), and the respiratory control ratios are comparable with values in the literature. When the pellets were washed one or two times there

Table 2. *Distribution of enzymes in fractions from livers of Japanese quail*
 Fractions were prepared as described in the legend to Table 1. Results are means of two closely agreeing experiments. For definition of units, see Table 1.

Fraction	Homogenate		Nuclear pellet		Low-speed pellet		High-speed pellet		Supernatant		Recovery (%)	
	5	50	5	50	5	50	5	50	5	50	5	50
Protein	183	191	7.6	16	21	20	16	12	57	49	102	97
Citrate synthase	100	100	4.0	23	64	59	30	12	7.8	5.5	106	100
	24	24	15	34	74	71	47	26	3.5	3.0	-	-
Glutamate dehydrogenase	49	49	4.1	25	67	60	22	8.9	5.7	3.5	99	97
	100	100	98	372	820	760	370	203	27	18	-	-
Glucose 6-phosphatase	16.3	16.7	4.2	15	20	27	26	27	51	33	101	102
	90	88	53	79	83	119	153	198	79	48	-	-
Glucose 6-phosphatase citrate synthase	(3.66)	(3.64)	1.03	0.64	0.30	0.53	0.88	2.10	-	-	-	-
Glucose 6-phosphatase glutamate dehydrogenase	1.00	1.00	1.10	0.62	0.30	0.46	1.20	2.90	-	-	-	-

Table 3. *Enzyme activity and oxygen uptake of mitochondrial fractions from rat liver*

Enzyme units are $\mu\text{mol/min}$ per g of liver. Specific activity (sp. act.) is expressed as nmol/min per mg of protein. The enzyme ratio in the homogenate was set to 1.00. Results are the means of three closely agreeing experiments.

Fraction		Low-speed pellet		High-speed pellet	
		5	50	5	50
Protein	[Sodium Hepes] (mM) ... (mg/g)	20	20	14	11
Glutamate dehydrogenase	(units/g)	65	66	24	13
	(sp. act.)	1700	1720	753	674
Glucose 6-phosphatase	sp. act. ratio	0.21	0.31	1.20	1.90
glutamate dehydrogenase					
O ₂ uptake	(nmol/min per mg)				
State 2		22	27	15	14
State 3		144	157	72	47
Respiratory control ratio	(State 3/State 2)	5.7	5.6	3.9	2.9
P/O ratio*		1.6	1.7		

* With succinate.

was a small increase in oxygen uptake, but there was a decrease in uptake and respiratory control after the third wash. It appears that mitochondria were damaged by handling.

These findings establish that, for mammalian and avian liver, the low-speed mitochondrial fraction is much superior to the fraction collected at higher centrifugal speed with respect to oxygen uptake and specific activity of matrix enzymes and is less contaminated with endoplasmic reticulum.

Effect of cations

Several published procedures for the preparation of mitochondria (e.g. Johnson & Lardy, 1967) recommend low ionic strength for obtaining good yields, but to our knowledge the effect of ionic strength on the preparation of cell organelles has never been studied systematically. Surveys of recent literature show that although some workers use 0.25 M-sucrose without any addition, the majority include buffers, frequently 10 mM-Tris or -triethanolamine. Occasionally, higher concentrations and bivalent cations are used (see, e.g., Lewis & Tata, 1973). The results in Tables 1 and 2 show a marked effect of buffer concentration on the distribution and association of mitochondria and reticulum. The effect of cations was explored further (Table 4). For a 1:20 (w/v) liver homogenate in pure sucrose, the cation concentration is (assuming a 20% extracellular space) about 1–1.5 mM-Na⁺ and 5–6 mM-K⁺. Thus the addition of 2.2 mM-Na⁺ in the 5 mM-sodium Hepes buffer had little effect on the cation concentration and on the distribution pattern compared with that prepared in unbuffered sucrose. However, when livers were pre-perfused and homogenized with 0.25 M-sucrose, the effect of low salt became evident. Without salt, the nuclear pellet was

decreased in size and in protein content (Table 4). Under the microscope it appeared to represent a nearly pure preparation of nuclei, with few mitochondria. This correlated with the rather low citrate synthase activity in this fraction and a negligible yield of glucose 6-phosphatase. The addition of 10 mM-NaCl to the homogenization medium markedly increased the mitochondrial content of the nuclear fraction. The protein yield was increased 3-fold, that of citrate synthase 4-fold, and that of glucose 6-phosphatase by up to 10-fold. There was little difference between 5 mM- or 50 mM-sodium Hepes buffer if the Na⁺ concentration in the former was increased to 20 mM with NaCl.

The increase in salt concentration shifted markedly the sedimentation of mitochondria toward a lower centrifugal force. As shown in Table 4, the yield of citrate synthase in the high-speed pellet was 31% when livers were pre-perfused and homogenized in sucrose, declining to 20–25% with low-salt media and to only some 10% in the presence of 50 mM-sodium Hepes; the reverse distribution pattern was observed for glucose 6-phosphatase. It is commonly believed that during homogenization the endoplasmic reticulum is converted into vesicles which are usually sedimented at 100 000 g. However, in a large series of experiments, when unbuffered sucrose was used, only 50–68% (mean 61%) of the glucose 6-phosphatase was present in the post-mitochondrial supernatant. The yield was lowered with increasing ionic strength, ranging from 31 to 54% (mean 48%) in the presence of 50 mM-sodium Hepes buffer (22 mM-Na⁺). About 40% of the glucose 6-phosphatase sediments with mitochondria. In Table 4, we have made a rough estimate of the mass of endoplasmic protein in the sediments. This was calculated from the specific activity of the

Table 4. *Effect of Na⁺ and K⁺ on the distribution of citrate synthase and reticulum*

The homogenates were prepared in 0.25M-sucrose supplemented with NaCl, KCl or sodium Hepes at the concentrations listed in the Table, and the homogenate fractions were prepared as described in the Methods section. The distribution of citrate synthase is reported as percentage of total activity recovered in the fractions obtained from 1g of liver, and specific activity (sp. act.) as nmol/min per mg of protein. Values for total and microsomal protein are the protein content of each fraction obtained from 1g of liver. The reticular protein content was calculated from the yield of glucose 6-phosphatase in the fractions and the specific activity of the glucose 6-phosphatase in the 100000g (1h) pellet obtained from the supernatant. It was assumed that endoplasmic reticulum (microsomes) represents 75% of the protein in this fraction. The activity of citrate synthase was $12.5 \pm 1.2 \mu\text{mol/min per g of liver}$ (range 9.7–15.6), and the specific activity of glucose 6-phosphatase in the 100000g (microsomal) pellet averaged $0.30 \mu\text{mol/min per g of protein}$.

Pretreatment of liver ...	Citrate synthase									
	Perfused with 0.25 M-sucrose					None				
	0.25M-sucrose		0.25M-sucrose/ 10mM-NaCl			0.25M-sucrose		0.25M-sucrose/ 50mM-KCl		
Medium ...	(%)	(sp. act.)	(%)	(sp. act.)	(%)	(%)	(sp. act.)	(%)	(sp. act.)	(%)
Homogenate	63	67	67	67	55	60	60	60	63	63
Nuclear pellet	3.8	14	14	82	6.5	26	85	7.7	44	22
Low-speed pellet	58	172	61	216	72	156	173	67	198	68
High-speed pellet	31	147	24	154	22	93	52	24	113	11
Supernatant	5.6	5	4.5	7	6.5	6	6	6.3	7	4.8
										6
Pretreatment of liver ...	Total and reticular protein (mg/g)									
	Perfused with 0.25 M-sucrose					None				
	0.25M-sucrose		0.25M-sucrose/ 10mM-NaCl			0.25M-sucrose		0.25M-sucrose/ 50mM-KCl		
Medium ...	Total	Microsomal	Total	Microsomal	Total	Total	Microsomal	Total	Microsomal	Total
Homogenate	202	53	208	54	202	203	53	204	45	193
Nuclear pellet	11	0.8	20	3.6	16	38	9.8	21	4.3	27
Low-speed pellet	44	7.8	38	7.2	50	45	12	42	5.5	39
High-speed pellet	35	13	37	17	24	25	10	26	8.5	21
Supernatant	117	34	109	28	125	105	21	105	29	106
										22

glucose 6-phosphatase in the fraction sedimenting at 10000g for 1 h, assuming 75% purity for the microsomal pellet and assuming uniform enzyme distribution in the reticulum. These values indicate that a substantial part of the protein in the high-speed pellet is non-mitochondrial.

Effect of washing

To assess the stability of the association between mitochondria and reticulum, the low- and high-speed pellets were washed three times with 20 vol. of the respective media (Table 5). About one-third of the citrate synthase was removed from the low-speed pellet by 0.25 M-sucrose, and most of the glucose 6-phosphatase. The citrate synthase was recovered by centrifugation at 8000g, but not the glucose 6-phosphatase. On the other hand, washing of the pellet with sucrose containing 50 mM buffer removed little citrate synthase and only about one-third of the glucose 6-phosphatase. Three washes increased the specific activities of citrate synthase and glutamate dehydrogenase by 20–25%. The relative ratio of glucose 6-phosphatase to mitochondrial-matrix enzymes served as a sensitive index of the association of reticulum to mitochondria. In the absence of salts, the ratio in the low-speed pellet decreased after three washings from about 0.2 to 0.05; in the presence of 50 mM-Hepes, three washings decreased the ratio in the low-speed pellet from about 0.4 to 0.25. In the high-speed pellet the glucose 6-phosphatase/citrate synthase ratio decreased after three washings with salt-free sucrose from 0.7 to 0.26; with 50 mM-Hepes this ratio declined from 2 or over to about 1.1; thus, even after three washings in this medium, the ratio of glucose 6-phosphatase to matrix enzymes in this fraction exceeded that of the total cell homogenate. Thus at high ionic strength there is only a limited removal of glucose 6-phosphatase, but at low ionic strength most of it is removed. Complete removal is very difficult, if not impossible. Similar observations were reported by Brunner & Bygrave (1969), who washed mitochondria ten times with 0.25 M-sucrose/10 mM-Tris/1 mM-EDTA, pH 7.4. Four washes removed 50–60% of the glucose 6-phosphatase from the mitochondrial pellet, but there was virtually no further loss in the subsequent six treatments.

We found that three extractions with salt-free sucrose also removed 70–80% of the NADPH-cytochrome *c* reductase and of cytochrome *P*-450, but only about one-half of the cytochrome *b₅*, and of UDP-glucuronyltransferase (results not shown).

To examine the sedimentation pattern of washed mitochondria which were low in reticulum, rat liver was homogenized in 0.25 M-sucrose and the nuclear fraction removed. The mitochondria were sedimented at 10000g and washed with 3 × 10 vol. of 0.25 M-sucrose. The relative specific-activity ratio of

glucose 6-phosphatase to glutamate dehydrogenase decreased to 0.045 (initial homogenate set at 1.00). The mitochondria were taken up in either salt-free sucrose or sucrose containing 5 mM- or 50 mM-sodium Hepes and sedimented at 2000g and 10000g to obtain a low- and a high-speed fraction. With sucrose containing no or low salt, there was no sediment at 10000g, and 98% of the protein and the enzymes were recovered in the low-speed pellet. With 50 M-Hepes buffer there was a small 10000g pellet. Some 92% of the glutamate dehydrogenase and 89% of the glucose 6-phosphatase were recovered in the low-speed pellet, and 6% and 10% respectively in the high-speed pellet. It appears that, when the amount of bound reticulum is very low, the mitochondria are recovered almost completely in the low-speed pellet in both low- and high-salt sucrose.

Isopycnic separation

The rate of sedimentation at a fixed centrifugal force depends on the difference in density of medium and organelles, and on viscosity, but to a major extent it is determined by particle size. For a sphere the rate is proportional to volume; for non-spherical particles the rate will depend greatly on shape. On the other hand, in isopycnic centrifugation the position of a particle at equilibrium will depend solely on its density. It was thus decided to examine the association of reticulum to mitochondria in Percoll media. The preparation of mitochondria in this medium has been described by Reinhart *et al.* (1982). The gradient is self-forming, is established rapidly, and equilibrium occurs within minutes at lower centrifugal speeds than with sucrose. Nearly all of the soluble protein, and 40–60% of endoplasmic reticulum, as estimated from the glucose 6-phosphatase activity, remained at the top of the tube (Table 6). Four or five well-defined bands (Fig. 1) accounted for some 40% of protein in the rat liver homogenate, and about 35–50% of the glucose 6-phosphatase, but the recovery of mitochondria as measured by the yields of glutamate dehydrogenase was nearly quantitative (Table 6). Visually the distribution pattern appeared quite different in low- and high-salt media (Fig. 1). Band I was very prominent in 0.25 M-sucrose or 0.25 M-sucrose/5 mM-sodium Hepes, but slight with 50 mM-sodium Hepes. Band V was prominent in low-salt media, but was slight or absent with 5 mM-Hepes. The situation was reversed for Band II.

Microscopic examination showed fat droplets on the surface. Band I in low-salt sucrose consisted predominantly of nuclei, present as large aggregates; plasma-membrane sheets and unidentified small organelles were also present. In 50 mM-sodium Hepes there were only a few unidentified organelles in Band I, and the nuclei were present in Band II, which also contained many organelles. Bands IV

Table 5. *Effect of washing on mitochondrial fractions*

One half of rat liver was homogenized in 0.25M-sucrose and one half in 0.25M-sucrose/50mM-sodium Hepes, pH7.4. Low- and high-speed pellets were prepared as described in the Methods section, and washed three times by suspending in 20 vol. of the respective media and centrifuging at the original speed. Results are expressed as in Tables 1-3. Total protein in the homogenate averaged 195mg/g of liver. Microsomal protein was calculated as described in Table 4. The activities of citrate synthase and glucose 6-phosphatase in the homogenate were respectively 14.5 and 18.6 μ mol/min per g of liver.

Pellet	Medium	No. of washes	Protein (mg/g)		Citrate synthase		Glucose 6-phosphatase		Glucose 6-phosphatase ratio	
			Total	Microsomal	(%)	(sp. act.)	(%)	(sp. act.)	citrate synthase	
Low-speed	0.25mM-sucrose	0	41	5.7	60	210	11	57	0.18	
		1	32	3.3	50	215	6	43	0.13	
		2	27	1.8	44	227	3.4	27	0.080	
		3	23	1.0	41	225	2.0	18	0.048	
	0.25mM-sucrose/50mM-sodium Hepes	0	40	11	64	209	24	102	0.37	
		1	39	9.0	63	228	20	98	0.31	
		2	35	7.8	62	251	17	91	0.27	
		3	32	7.2	60	259	16	90	0.27	
High-speed	0.25mM-sucrose	0	27	10	30	154	20	162	0.69	
		1	19	6.3	30	220	8.4	105	0.32	
		2	17	5.5	27	224	7.6	99	0.29	
		3	16	4.6	27	225	6.7	86	0.26	
	0.25mM-sucrose/50mM-sodium Hepes	0	18	11	11	121	23	245	2.29	
		1	13	8.8	11	174	17	164	1.54	
		2	12	6.1	10	188	13	100	1.12	
		3	11	4.7	10	185	12	85	1.14	

Table 6. *Percoll fractionation of homogenate of rat liver*

The Percoll suspensions were prepared in either 0.25M-sucrose or 0.25M-sucrose/50mM-sodium Hepes, pH7.4. One half of each liver was homogenized in one of the above media and layered on top of the suspension in the same medium. For details of separation, see the Methods section. The density gradient of the Percoll suspension prepared in 0.25M-sucrose was determined by the use of coloured beads of known density (see the Methods section). Recoveries are expressed as percentages of the protein content and enzyme activities of the homogenate; the specific activities of glutamate dehydrogenase and glucose 6-phosphatase in the homogenate were set to 1.00. The results are the means from three rat livers.

Band	Distance from top* (cm)	Density*	Recovery†						Relative specific activity						Glucose 6-phosphatase glutamate dehydrogenase ratio
			Protein (%)		Glutamate dehydrogenase (%)		Glucose 6-phosphatase (%)		Glutamate dehydrogenase		Glucose 6-phosphatase		ratio		
			0	50	0	50	0	50	0	50	0	50			
	[Sodium Hepes] (mm)	...													
I	1.4	1.05	20	5	23	2	31	1.1	0.39	1.6	1.45	6.7			
II	2.5	1.063	2	13	4	20	3	1.8	1.40	1.1	0.65	0.75			
III	4.5	1.080	5	2	25	7	2	4.7	2.9	0.31	0.06	0.45			
IV	5.9	1.090	9	18	29	66	1	3.2	4.3	0.1	0.04	0.27			
V	7.4	1.11	4	0.5	12	0.5	0	2.8	—	—	—	—			
Pellet	8.3	—	2	2	3	2	0	0.6	—	—	—	—			
Total			42	40	96	97	37								

* The distance is from top to centre of band; density is at the centre of band for 0.25M-sucrose. The position of these bands in 0.25M-sucrose/50mM-sodium Hepes was slightly different.

† The contents of the top layer were not assayed.

and V appeared to consist solely of mitochondria, in part in the higher-salt medium as short chains. The distribution of protein (Table 6) closely paralleled the visual observation. In low-salt medium, glutamate dehydrogenase was nearly equally distributed between Bands I, III and IV. Band V was just on top of the erythrocyte pellet, and was sometimes difficult to separate from it. This band was more variable than the others. In the 50 mM-Hepes medium there was a substantial amount of glutamate dehydrogenase in Band II, but most of it (60–70%) was present in Band IV.

The distribution of glucose 6-phosphatase differed markedly in the two media. Without salt 28–34% of the activity was in Band I, and small amounts were in other fractions. With 50 mM-Hepes glucose 6-phosphatase was present roughly to an equal extent in Bands I, II and IV. The mitochondria in Bands I and II in low- and high-salt media, respectively, were associated with reticulum, whereas the major mitochondrial fraction in salt-free sucrose, Band IV, was low in glucose 6-phosphatase, with a relative specific-activity ratio of 0.04, as compared with a ratio of about 1.5 in Band I (Table 6).

Microscopic observations of quail liver fractions and the results of Table 2 indicated a more extensive aggregation and binding of reticulum to mitochondria in quail than in rat liver, and examination in Percoll gradients seemed desirable. The specific gravity of quail liver organelles was considerably less than those of rat, and the gradient was changed accordingly (see the Methods section). There was a brownish layer accounting for most of the soluble protein, four well-separated whitish bands and a small red pellet. A fifth band just on top of this pellet was very faint. Microscopic examination revealed mainly oil droplets in the top layer. Band I consisted of small unidentified organelles. The appearance of Bands II and III differed markedly in the two media. In the absence of salt or with 5 mM-sodium Hepes, Band II consisted of nuclei, mitochondria, and sheets of plasma membrane, but with 50 mM-sodium Hepes there were only a few nuclei and unidentified small organelles, and the nuclei were shifted into Band III, which also contained plasma membranes and mitochondria. In low-salt media mitochondria were present as single organelles, and some as short chains. With 50 mM-sodium Hepes, Band IV was narrow and rather dense and consisted of large mitochondrial chains and aggregates. The bottom pellet contained mainly erythrocytes.

The particulate fractions accounted for about half of the protein content of the homogenate and two-thirds of the glucose 6-phosphatase, but the recovery of mitochondria, as assayed by glutamate dehydrogenase, was nearly quantitative (Table 7). In salt-free medium most mitochondria were recovered

in Band III, but those in Band IV had the highest specific activity and the lowest content of glucose 6-phosphatase (Table 7). With 50 mM-sodium Hepes, the yield of glutamate dehydrogenase in Bands III and IV was roughly the same, but the specific activity was greater in Band IV. Band III contained one-third of the glucose 6-phosphatase of the liver homogenate and also the great bulk of nuclei (see above), thus probably accounting, at least in part, for the lower specific activity of the glutamate dehydrogenase. The association of glucose 6-phosphatase with mitochondria as measured by relative enzyme ratio in the last column of Table 7 was much greater than in rat liver.

To examine what the removal of endoplasmic reticulum will do to the distribution of mitochondria in the density gradient, a homogenate from quail liver was used. Nuclei were removed as described in the Methods section, and a fraction sedimenting between 300g and 10000g was collected and washed three times with salt-free sucrose. About 85% of the glutamate dehydrogenase was recovered in the washed pellet, and the relative ratio of glucose 6-phosphatase to glutamate dehydrogenase was decreased to 0.34. In this experiment (Table 8) the Percoll gradient was prepared in 0.25 M-sucrose/0.06 M-mannitol (as used by Reinhart *et al.*, 1982) and about 15 mM in K⁺ (see Table 8). With the homogenate 64% of glutamate dehydrogenase and 36% of the glucose 6-phosphatase were recovered in Band IV, and approx. 15% of each enzyme in Bands II and III. With washed mitochondria 90% of the recovered glutamate dehydrogenase was in Band IV, but there was less glucose 6-phosphatase, with the ratio declining to 0.28, indicating a stronger binding of reticulum to mitochondria in quail than in rat liver. With both rat (see above) and quail liver, removal of reticulum decreases markedly the heterogeneity with respect to rate of sedimentation or density of the mitochondrial fractions.

Phase-contrast microscopy

Most of the mass in the 300g pellet obtained by layering over 0.35 M-sucrose consisted of nuclei (Fig. 2). There were a substantial number of mitochondria when the medium contained 50 mM-sodium Hepes. When livers were pre-perfused and homogenized with 0.25 M-sucrose (without buffer), the fraction appeared to be a rather pure preparation of nuclei. The low- and high-speed pellets appeared to be composed of mitochondria, with very few other particles. There was a marked difference in the mitochondrial fractions prepared with 5 mM- or 50 mM-sodium Hepes. With the former the mitochondria in all fractions were virtually single organelles (Fig. 3a). In the presence of 50 mM-sodium Hepes or 20 mM-NaCl or -KCl, the mitochondria formed linear chains of from three to ten

Table 7. Percoll fractionation of homogenate of quail liver

Percoll suspensions were made in either 0.25M-sucrose or 0.25M-sucrose/50mM-sodium Hepes, pH7.4. One half of each liver was homogenized in 10 vol. of either of these media, and 2ml of each homogenate was layered on top of the Percoll suspension made in the same medium. For details of the Percoll separation, see the Methods section. Densities were determined as described in Table 6 and the Methods section. The recoveries of the enzymes, the relative specific activity and the specific-activity ratio are expressed as in Table 6. Results are the means for two bird livers.

Band	Distance from top* (cm)	[Sodium Hepes] (mm)	Density*	Recovery						Relative specific activity						Glucose 6-phosphatase glutamate dehydrogenase ratio
				Protein (%)		Glutamate dehydrogenase (%)		Glucose 6-phosphatase (%)	Glutamate dehydrogenase		Glucose 6-phosphatase		glutamate dehydrogenase			
				0	50	0	50		0	50	0	50	0	50		
I	1.5	1.035	10	3	1	0	19	8	—	2.5	3.6	—	—	—		
II	2.5	1.048	7	3	12	3	14	7	1.2	1.0	2.4	1.40	2.57	—		
III	4.3	1.065	23	25	63	40	25	33	3.4	1.9	1.4	0.40	0.63	—		
IV	6.0	1.076	3	14	17	53	3	21	6.3	4.2	0.9	0.15	0.39	—		
V and pellet			3	2	3	2	4	3	—	—	—	—	—	—		
Total			46	47	96	98	65	72								

* In 0.25M-sucrose; approximate distance and density at centre of band.

* In 0.25M-sucrose; approximate distance and density at centre of band.

Table 8. Percoll fractionation of washed quail mitochondria

The Percoll gradient was that in Table 7, as described in the Methods section, except that the suspensions were made in 0.21M-mannitol/0.06M-sucrose/0.01M-KCl/0.01M-potassium Hepes (pH7.4)/1mM-dithioerythritol (mannitol medium). One half of each liver was homogenized in the mannitol medium and one half in the 0.25M-sucrose/5mM-sodium Hepes (pH7.4) medium ('sucrose medium') to obtain washed mitochondria. The nuclear pellet was removed from the homogenate in the sucrose medium as described in the Methods section, and the supernatant centrifuged at 10000g_{max} for 10min. The mitochondrial pellet was washed in 3 x 20 vol. of the sucrose medium by centrifugation at the original speed, and the washed pellet taken up in the mannitol medium. The homogenate and the washed mitochondrial suspension were layered on top of the Percoll gradient and centrifuged as described in the Methods section. The units are expressed as in Table 7.

	Homogenate						Washed mitochondria					
	Distribution			Relative specific activity			Distribution			Relative specific activity		
	Protein (%)	Glutamate dehydrogenase (%)	Glucose 6-phosphatase (%)	Glutamate dehydrogenase	Glucose 6-phosphatase	Glucose 6-phosphatase	Protein (%)	Glutamate dehydrogenase (%)	Glucose 6-phosphatase (%)	Glutamate dehydrogenase	Glucose 6-phosphatase	Glucose 6-phosphatase
Top	55	1	25	—	—	—	2	0	0	—	—	—
Band I	2	0.5	4	—	—	—	0	0	0	—	—	—
Band II	12	15	17	1.03	1.38	—	2	1	3	—	—	—
Band III	9	17	15	1.85	1.72	—	2	7	3	—	—	—
Band IV	21	64	36	2.86	1.70	—	24	73	22	3.83	1.07	—
Pellet	2	0.5	1	—	—	—	1	1	1	—	—	—
Recovery	101	98	98	—	—	—	31	82	29	—	—	—

Fig. 3. *Phase-contrast microphotographs of the mitochondrial 1000 g pellet from rat liver*
For details see the Methods section. (a) and (b) show preparations from one liver, homogenized in either 0.25 M-sucrose/5 mM-sodium Hepes (a) or 0.25 M-sucrose/50 mM-sodium Hepes (b); (c) shows a preparation from another liver homogenized in 0.25 M-sucrose/50 mM-KCl. Magnification $\times 400$. Note absence of nuclei, erythrocytes and 'debris' in the 1000 g pellets. Also note that in (b) and (c) there are gaps between mitochondria in the chains and the larger aggregates, so that the organelles do not touch each other.

Fig. 4. *Phase-contrast microscopy of mitochondria from quail liver prepared in 0.25 M-sucrose/5 mM-sodium Hepes*
Magnification $\times 400$.

organelles (Fig. 3b). There was no direct contact, but a small gap, between the organelles. The chain-like nature became apparent by concerted Brownian

movement of the organelles, which, however, made photographic representation difficult, owing to blurring, especially at higher magnification. At higher salt concentrations (30–50 mM-NaCl or -KCl), the chains were larger and often twisted, as a ball of string (Fig. 3c). With quail hepatocytes, the extent of aggregation in 5 mM-sodium Hepes buffer was more pronounced than in those from rat, as shown in Fig. 4, and in 50 mM buffer the aggregates were quite large.

The aggregation of mitochondria is reversible. When salt solutions were added to mitochondrial preparations made in sucrose, aggregation occurred. With 10 mM-NaCl or -KCl, short linear aggregates were visible after 2–3 h in the cold. The speed and extent of aggregation increased with the salt concentration, and at 30 mM-NaCl or -KCl extensive aggregation was apparent within 1 h. There was no marked visual difference whether the univalent cations were Na^+ , K^+ , Tris or triethanolamine. The addition of 2.5 mM- MgCl_2 or - CaCl_2 caused immediate extensive aggregation (results not shown). When a large volume of 0.25 M-sucrose was added to mitochondria prepared with medium containing 50 mM-NaCl or -KCl, there was rapid dispersal of the aggregates, and after a few minutes most of the mitochondria appeared as free organelles.

To examine the nature of the mitochondrial-reticular structure in the intact cell, isolated hepatocytes from female Japanese quail were treated with a Polytron homogenizer. In the sucrose/50 mM-Hepes medium, the cells largely broke up after 10 s at three-quarters of maximal speed; at that time the

Fig. 5. Phase-contrast microscopy of homogenates of hepatocytes from Japanese quail

Cells were treated in a Polytron homogenizer in 0.25 M-sucrose/5 mM-sodium Hepes, pH 7.4, and examined by phase contrast. (a) Treated for 10s at three-quarters speed at 50 mM-sodium Hepes. Note large aggregates of mitochondria and a few chains. (b) Treated 20s at half speed in 5 mM-sodium Hepes. Only free mitochondria are present. (c) After a few minutes after addition of 50 mM-NaCl to the preparation in (b). Note large aggregates similar to those in (a). Magnification $\times 400$.

extruded cell contents appeared as a large mitochondrial network. There were some mitochondrial chains, but few if any free mitochondria (Fig. 5a). Disintegration for about 60s produced chains of from 10 to over 30 mitochondria. The yield of free mitochondria was small even after 2–3 min of homogenization, a treatment leading to substantial damage as measured by leakage of glutamate dehydrogenase. On the other hand, in the salt-free sucrose, treatment at lower speeds for 10–20s yielded a preparation of free mitochondria (Fig. 5b). On addition of 50 mM-NaCl these aggregated to large structures (Fig. 5c). These findings indicate that the mitochondrial aggregates are not artifacts caused by secondary 'clumping' by salt after the preparation, but that they are present in the intact cell.

Electron microscopy

Negative or positive stain does not reveal internal detail, but serves to show overall structures and cell aggregates. There is no fixing, dehydration, embedding and sectioning, and preparations can be examined and photographed within 1 h of the application to the grids. Unfixed preparations stained with uranyl acetate or sodium phosphotungstate revealed the existence of structures ranging from a few mitochondria to long multi-branched chains (Fig. 6a). The mitochondrial preparations were applied on the grid in 0.25 M-sucrose/5 mM-sodium Hepes, but in drying on the

grid the salt concentration increased to a variable extent, probably accounting for the occurrence of structures with various degrees of aggregation. Mitochondria were rarely in direct contact. At higher magnification (Figs. 6b and 6c) the connections between mitochondria resembled short necks, thin tubes, or thin strands and sheets. Membranous structures, probably reticulum vesicles detached from mitochondria, are shown in Fig. 6(d). It appears that the intermitochondrial connecting membranes are plastic in that they can be deformed. The micrographs strongly support the conclusions derived from visual phase-contrast microscope observations. Mitochondria and reticulum appear to constitute a large branched network, probably multidimensional, in the cell.

Discussion

Association of mitochondria and reticulum

Several workers (Lewis & Tata, 1973; Shore & Tata, 1977a,b; Pickett *et al.*, 1981; Meier *et al.*, 1981; Cascarano *et al.*, 1982) found that on sedimentation of liver homogenates a fraction of the reticulum remained bound to mitochondria. This could only be completely removed by drastic means, such as by mechanical shearing (Lewis & Tata, 1973) or digitonin (Pickett *et al.*, 1981; Cascarano *et al.*, 1982), procedures which strip off the mitochondrial outer membrane. Several ultrastructural studies by electron microscopy have shown a

Fig. 6. *Electron micrographs of rat liver mitochondria stained with uranyl acetate*

For details see the text. Magnifications: (a), $\times 2600$; (b), $\times 6200$; (c), $\times 33\,000$; (d), $\times 24\,000$. Note chains and branched structures in (a) and (b); note connection by thick 'necks', by thin tubules and by hyaline membranes in (c) and (d). In (d), note reticulum breaking away from mitochondria and detached fragments of reticular membrane.

continuity between reticulum and mitochondria. Tubular connections between mitochondria were noted in nerve axons and acinar pancreatic cells of mice (Robertson, 1960). Franke & Kartenbeck (1971) described tubular connections of endoplasmic reticulum between mitochondria in *Tetrahymena* and liver cells, and similar observations were reported by Morr *et al.* (1971) in liver and onion root cells, and by Brucker & Grove (1971) in several groups of fungi. More recently, Montisano *et*

al. (1982) examined transmission electron micrographs of serial sections of liver mitochondria *in situ*. They found that at least 81% of the mitochondria showed at least one point of contact and probably of fusion between reticulum and the mitochondrial outer membrane. A continuum between reticulum and mitochondrial outer membrane was suggested by Franke & Kartenbeck (1971), and more recently by Meier *et al.* (1981) and Montisano *et al.* (1982). Cascarano *et al.* (1982) suggested a model for

mitochondrial-reticulum attachment. It appears that most investigators dismissed these observations as artifacts, and the evidence for a mitochondrial-reticular structure has generally been discounted by biochemists and cell biologists. We found no mention of the above studies in any of the reviews or monographs on cell organelles, including the most recent ones.

Our evidence for an attachment of reticulum to mitochondria in mammalian and avian livers is based on the enzyme content of fractions separated by rate sedimentation and density-gradient centrifugation in media of various ionic strengths, and on observations by phase and electron microscopy. Our results show that from one-third to one-half of the endoplasmic reticulum as assayed with glucose 6-phosphatase is not recovered as conventional microsomal fractions, but remains attached to mitochondria. The fraction of bound reticulum is even greater in quail liver. The extent of binding is not uniform; mitochondrial subfractions separated by sedimentation or isopycally differ markedly in their content of reticulum. The attachment depends greatly on ionic strength, and it increases with cation concentration.

Since association of the organelles is found after differential and isopycnic centrifugation, it is most unlikely that the association is fortuitous or an 'artifact'. The former procedure discriminates by sedimentation rate, which is a function mainly of particle size and shape; with the second procedure density alone determines the pattern. Free microsomal fractions sediment at a much lower rate than mitochondria and their density is less, appearing in Percoll at the very top of the gradient. The higher the reticular content, the less the specific gravity of the mitochondrial fractions; conversely, when most of the reticulum is stripped off, most of the mitochondria are recovered as a single band of high density (Table 8). The effect of reticular content on the sedimentation rate is more complex. At high ionic strength, when there is aggregation of mitochondria into chains, the sedimentation rate is increased and significant amounts of mitochondria come down even in the nuclear pellet (Tables 1 and 2). However, the reticular content of the high-speed pellet is much higher than that of the low-speed pellet. This may be due to viscous drag of organelles with appendages of membrane material. Free liver mitochondria also differ in size and shape. The combination of factors, such as size, shape, density and viscous drag, make interpretation of sedimentation rates difficult. When mitochondria are stripped of most of the reticulum, they sediment at quite uniform rates, and are recovered at relatively low speed (see the Results section).

Heterogeneity of mitochondria and reticulum

It has long been known that liver mitochondria

vary in size (de Duve, 1967) and that they sediment at gravitational force ranging from several hundred to 10000g. Reinhart *et al.* (1982) and we obtain several bands of mitochondria in Percoll media. Our results suggest that, at least in part, this difference in density may be due to the extent of attached endoplasmic reticulum.

It is unclear at present whether there is functional heterogeneity among the mitochondrial fractions. The distinction between 'low-speed' and 'high-speed' pellets is of course arbitrary; different yields may be obtained by choosing other gravitational forces or times of centrifugation. However, our results (Tables 1-3) show a large difference in the specific activity of matrix enzymes and in oxygen uptake, which appears to be too extensive to be accounted for by the dilution of mitochondrial protein with reticulum or by the presence of other organelles or proteins. Also, we observed that the ratios of the activities of two enzymes of urea synthesis, ornithine carbamoyltransferase and carbamoyl phosphate synthase, were 1.5-2 times higher in the high-speed than in the low-speed pellet (results not shown). As 20% of liver mass consists of non-parenchymal cells, definitive studies require mitochondrial preparations from isolated hepatocytes.

We have shown that the recovery of glucose 6-phosphatase in the postmitochondrial supernatant and the distribution of reticulum between the microsomal fraction and that sedimenting with mitochondria depend on ionic strength. Similar observations were reported by Meier *et al.* (1981). Numerous ultrastructural studies by transmission electron microscopy show two types of endoplasmic reticulum in liver cells. In areas devoid of mitochondria the reticulum consists of regular parallel plates of lamellae. In areas dense with mitochondria the reticulum is irregular, with single lamellae in close contact and intertwined with mitochondria. We suggest the possibility that the microsomal vesicular fraction recovered conventionally at 100000g is derived largely from the reticulum of the parallel stacks. The reticulum associated with mitochondria appears quite stable in media of high ionic strength, but is labile and breaks up in low-salt media, and can be largely extracted with salt-free sucrose.

We have compared the enzyme profile of the reticulum in the post-mitochondrial supernatant recovered in a 100000g pellet with that eluted from mitochondria by salt-free sucrose. Glucose 6-phosphatase, NADPH-cytochrome *c* reductase, rotenone-insensitive NADH-cytochrome *c* reductase, cytochrome *P*-450 and cytochrome *b*₅, and UDP-glucuronyltransferase were present in both fractions, although the distribution patterns were different (results not shown). It is, however, well known that the distribution of enzymes of the reticulum is not topographically uniform (Dallner

et al., 1968); it remains to be seen whether there are functional differences between the two types of reticulum.

Cell fractionation

Our findings are relevant for designing methods for the isolation of mitochondria and reticulum. To obtain mitochondria by differential centrifugation with a minimum content of reticulum, the use of salt-free minimally buffered media is essential; 5 mM-sodium or -potassium Hepes introduces only about 2 mM of cations and provides adequate buffering capacity. In virtually all conventional procedures, a so-called 'nuclear' pellet, sedimenting at 600–1000 *g*, is discarded and the mitochondria are harvested by centrifuging the supernatant at 6000–10000 *g*. We have found that even at 600 *g* (results not shown), depending on the buffer and salt content of the sucrose, some 30–50% of the mitochondrial-matrix enzymes are discarded. Moreover, the specific activity of the discarded mitochondria and their capacity for oxygen uptake is much higher than those sedimenting at lower rates (Tables 1–3). We show here that by applying the method of Shore & Tata (1977a) most of the erythrocytes, nuclei, plasma membrane and any 'debris' can be readily and rapidly removed with a small loss of mitochondria, and centrifuging the supernatant at 8000–10000 *g* yields a pellet containing at least 90% of the mitochondrial enzymes. This fraction can be freed of most of the reticulum by washing two or three times with salt-free sucrose, with retention of some 80% of the total liver glutamate dehydrogenase.

Also, it has been generally assumed that the conventional microsomal preparation (centrifugation of postmitochondrial supernatant at 100000 *g*) is representative of the total endoplasmic reticulum. We show here that from one-third to one-half (depending on ionic strength) of the endoplasmic reticulum remains in the mitochondrial fraction.

Mitochondrial-reticular cytostructure

The micrographs showing continuity of endoplasmic reticulum with the mitochondrial outer membrane (Franke & Kartenbeck, 1971; Morré *et al.*, 1971), the more recent studies by Meier *et al.* (1978, 1981) and Cascarano and co-workers (Pickett *et al.*, 1981; Montisano *et al.*, 1982; Cascarano *et al.*, 1982), and our own studies, support a concept of a cell structure of liver in which mitochondria are linked together by a reticular network. The most direct visual evidence of a mitochondrial-reticular cytostructure was provided by transmission and scanning electron micrographs of hepatocytes from which the plasma membrane was stripped off with digitonin (Fiskum *et al.*, 1980; Cook *et al.*, 1983). The soluble proteins are lost, but,

rather than disintegrating, the cell shape remains largely intact. As shown clearly in the scanning electron micrographs (Cook *et al.*, 1983), the roughly spherical cell remnant was composed of nucleus and mitochondria, which were enveloped and held together by a heavy continuous network of membranes. The membranes appeared to be in intimate contact with the mitochondrial and nuclear outer membranes. Fiskum *et al.* (1980) suggested that the mitochondria may be held together by a network of actin fibres. This is unlikely, since actin constitutes 1–2% of liver protein (Fisher & Phillips, 1979), and is associated mainly with the plasma membrane, and Fiskum *et al.* (1980) found that it is nearly all extracted by the digitonin treatment. The scanning electron micrographs show the membrane network to be far too massive to consist of minor cell constituents such as actin fibres or microfilaments, and it appears to consist of endoplasmic reticulum. It appears thus that, in the cell, mitochondria and nucleus constitute a structure connected by a membranous network composed of the endoplasmic reticulum. A continuity of endoplasmic reticulum with the nuclear outer envelope was previously proposed by Morré *et al.* (1974). Our observations that in high-salt media the nuclear fraction contains a substantial amount of mitochondria and reticulum (Tables 1 and 2), and that when the concentration of sodium Hepes in Percoll was increased to 50 mM there was a shift of the nuclear fraction from Band I to Band II of a higher density and higher mitochondrial content (Table 6), is suggestive of attachment, but further studies are required.

The studies mentioned above and our own results call for a revision of the accepted model of the structure of the liver cell. There is strong evidence of a cytostructure linking together as a continuum a part of the endoplasmic reticulum, mitochondria, and probably the nucleus. Free mitochondria do not occur in the ionic environment of the cytosol, and the simple organelles are an artifact of preparation. The physiological significance of such a structure remains to be established. A function in the synthesis of proteins and phospholipids and their transport into mitochondria is an attractive speculation.

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