AN ELECTRON-TRANSPORT SYSTEM ASSOCIATED WITH THE OUTER MEMBRANE OF LIVER MITOCHONDRIA

A Biochemical and Morphological Study

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ABSTRACT

Preparations of rat-liver mitochondria catalyze the oxidation of exogenous NADH by added cytochrome *c* or ferricyanide by a reaction that is insensitive to the respiratory chain inhibitors, antimycin A, amytal, and rotenone, and is not coupled to phosphorylation. Experiments with tritiated NADH are described which demonstrate that this "external" pathway of NADH oxidation resembles stereochemically the NADH–cytochrome *c* reductase system of liver microsomes, and differs from the respiratory chain-linked NADH dehydrogenase. Enzyme distributation data are presented which substantiate the conclusion that microsomal contamination cannot account for the rotenone-insensitive NADH–cytochrome *c* reductase activity observed with the mitochondria. A procedure is developed, based on swelling and shrinking of the mitochondria followed by sonication and density gradient centrifugation, which permits the separation of two particulate subfractions, one containing the bulk of the respiratory chain components, and the other the bulk of the rotenone-insensitive NADH–cytochrome *c* reductase system. Morphological evidence supports the conclusion that the former subfraction consists of mitochondria devoid of outer membrane, and that the latter represents derivatives of the outer membrane. The data indicate that the electron-transport system associated with the mitochondrial outer membrane involves catalytic components similar to, or identical with, the microsomal NADH–cytochrome b_5 reductase and cytochrome b_5.

INTRODUCTION

It is has been observed, first in 1951 by Lehninger (1–3) and by several investigators since (4–10), that preparations of rat-liver mitochondria catalyze a rapid oxidation of exogenous NADH in the presence of added cytochrome *c*. This "external" NADH–cytochrome *c* reductase reaction differs from the intramitochondrial, respiratory chain-linked, oxidation of NADH by cytochrome *c*, in that it is insensitive to the electron-transport inhibitors antimycin A (3–5), amytal (6–8), and rotenone (10), and is devoid of coupled phosphorylation (1–3, 7, 9, 10).

Liver microsomes are known to contain a highly active NADH–cytochrone *c* reductase

(11, 12), which also is insensitive to the above inhibitors, and is not coupled to phosphorylation. The question has, therefore, repeatedly been considered (3, 13–17) as to whether contaminating microsomes might be responsible for the "external" NADH–cytochrome *c* reductase activity found with preparations of rat-liver mitochondria. Early tissue fractionation studies by Hogeboom and Schneider (11, 12) indicated a dual distribution of NADH–cytochrome *c* reductase between mitochondria and microsomes, but the contribution of the respiratory chain to the mitochondrial enzyme activity measured was not clearly assessed. Later, however, de Duve et al. (5) found a similar distribution pattern in experiments in which the respiratory chain-linked oxidation of NADH was blocked by antimycin A.

Extensive investigations of the microsomal NADH–cytochrome *c* reductase system (see references 18, 19 for reviews) have revealed that this consists of two catalytic components: the flavoprotein NADH–cytochrome b_5 reductase, and cytochrome b_5. In the course of these investigations, Strittmatter and Ball (20, 21), and Chance and Williams (22) noted the occurrence of cytochrome b_5 in preparations of rat-liver mitochondria and attributed it to contaminating microsomes. In 1958–60, Raw and associates (23-26) reported similar observations with liver mitochondria from various species, but concluded, on the basis of the low ribonucleic acid content of their preparations, that the amount of the cytochrome found could not be accounted for by microsomal contamination. They also undertook a purification of this cytochrome, as well as of a flavoprotein catalyzing its reduction by NADH, from pig-liver mitochondria, and found that the two enzymes were similar to, but not identical with, the microsomal cytochrome b_5 and NADH–cytochrome b_5 reductase, respectively. However, the functional and cytochemical relationship of this electron-transport system to the mitochondrial respiratory chain remained unsettled.

This paper reports a study of the "external" NADH–cytochrome *c* reductase system of rat-liver mitochondria. Enzyme distribution data substantiating the conclusion that the system does not originate from microsomal contamination are presented. Experiments with tritiated NADH are described which demonstrate that the "external" NADH–cytochrome *c* reductase of mitochondria resembles stereochemically the microsomal system and differs from the respiratory chain-linked NADH dehydrogenase. A procedure is developed, based on swelling and shrinking of the mitochondria followed by sonic oscillation and density gradient centrifugation, which permits the separation of two particulate subfractions, one containing the respiratory chain, and the other the rotenone-insensitive NADH–cytochrome *c* reductase system. Morphological evidence supports the conclusion that the former subfraction consists of mitochondria devoid of outer membrane, and that the latter represents derivatives of the outer mitochondrial membrane. The data indicate that the electron-transport system associated with the mitochondrial outer membrane involves catalytic components similar to, or identical with, the microsomal NADH–cytochrome b_5 reductase and cytochrome b_5. Parts of this work have already been reported in a preliminary form (27–29).

EXPERIMENTAL

Preparation of Mitochondria and Microsomes

Albino rats weighing 150–200 g were used. The animals were starved overnight before sacrifice. Mitochondria were prepared by differential centrifugation from a 10% liver homogenate in 0.25 M sucrose. After sedimentation of the nuclear fraction at 600 *g* for 15 min, mitochondria were sedimented from the supernatant by centrifugation at 6,500 *g* for 20 min. The fluffy layer was carefully discarded, and the pellet was washed twice with ½ and ¼ the initial volume of 0.25 M sucrose. For separation of the microsomal fraction, the 6,500 *g* supernatant was centrifuged first at 15,000 *g* for 15 min. The sediment was discarded, and microsomes were sedimented from the supernatant at 105,000 *g* for 60 min. The surface of the pellet was rinsed with 0.25 M sucrose. Unless otherwise stated, mitochondria and microsomes were suspended in 0.25 M sucrose.

Continuous Density Gradient Centrifugation of Mitochondria and Microsomes

A linear density gradient was obtained in a 25-ml centrifuge tube (Spinco SW-25) by superimposing eleven layers of 2-ml of sucrose solutions with concentrations ranging between 1.18 and 2.28 M. After equilibration at 0°C for at least 12 hr, a continuous, linear gradient was formed. The linearity of the gradient was ascertained by adding 2,6-dichlorophenol-indophenol to the sucrose and reading the optical density at 610 mμ of 40 consecutive fractions separated from the tube after equilibration.

Suspensions of mitochondria (45 mg protein/2.5

ml) or microsomes (50 mg protein/2.5 ml) in 0.25 M sucrose were layered on top of the density gradient. When indicated, the suspensions were sonicated, in aliquots of 3.5 ml, for 15 sec at 3 amp with a Branson Sonifier at 0°C, prior to layering them on the gradient. Gradient centrifugation was carried out in a Spinco SW-25 rotor at 16,000 rpm for 12 hr. Sampling was performed by inserting a syringe needle into the bottom of the tube, and the volumes of the single fractions were calculated from the number of the drops.

Subfractionation of Mitochondria by Sonication Followed by Discontinuous Density Gradient Centrifugation

Mitochondria in 0.25 M sucrose (5–7 mg protein/ml) were subjected to sonic oscillation as described above. An amount of the sonicated suspension, corresponding to approximately 50 mg of protein (7–10 ml), was layered on top of a 1.18 M sucrose solution (15–18 ml; final volume, 25 ml), and centrifuged in a Spinco SW-25 rotor at 24,000 rpm for 3 hr. The procedure resulted in the separation of three subfractions: a pellet at the bottom of the tube ("heavy" subfraction); an interface band ("light" subfraction); and a supernatant in the 0.25 M sucrose portion of the gradient ("soluble" subfraction). The concentration of the lower sucrose layer was chosen so as to allow a tight packing of the pellet. Separation of the supernatant and of the interface band was done by means of a capillary with a U-shaped tip, in order to avoid turbulence. After removal of the interface band, the remaining lower sucrose layer (which was water-clear and free of protein) was decanted, the pellet was rinsed with 0.25 M sucrose, and suspended in the same solution.

The separated interface band contained, by necessity, a portion of the supernatant fraction. The volume of this portion was calculated by subtracting the volume of the separated supernatant from that of the original load (7–10 ml). In the experiments in which the discontinuous density gradient was used, the enzyme activities and the content of protein and cytochrome b_5 of the soluble and light subfractions were calculated, introducing a correction for the amount of supernatant present in the interface band.

Subfractionation of Mitochondria by Swelling and Shrinking Followed by Discontinuous Density Gradient Centrifugation

Mitochondria, prepared as described above, were suspended in 10 mM Tris-phosphate buffer, pH 7.5, by means of a teflon pestle fitted into the centrifuge tube. After 5 min at 0°C, the suspension was diluted with ⅓ volume of 1.8 M sucrose containing 2 mM ATP and 2 mM $MgSO_4$. Immediately, a visible increase in turbidity appeared in the suspension, due to shrinking of the mitochondria. After 5 min further at 0°C, 7–10 ml of the suspension (containing approximately 50 mg of protein) was layered on top of a solution of 1.18 M sucrose (15–18 ml; final volume 25 ml), and centrifuged in a Spinco SW-25 rotor at 24,000 rpm for 3 hr. Separation of the subfractions was done as described in the preceding section.

Subfractionation of Mitochondria by Combined Swelling-Shrinking and Sonication Followed by Discontinuous Density Gradient Centrifugation

Mitochondria were first swollen and shrunken as described in the preceding section. 5 min after the addition of the sucrose-ATP-$MgSO_4$ solution, the suspension was subjected to sonic oscillation and discontinuous density gradient centrifugation as indicated above. Subfractions were separated as already described.

Assays

NADH-, NADPH-, and succinate-cytochrome c reductase activities were measured spectrophotometrically at 30°C, by following the reduction of cytochrome *c* at 550 mμ. The assay mixture contained in 3 ml: 0.1 mM NADH or NADPH or 3 mM succinate, 0.1 mM cytochrome *c*, 0.3 mM KCN, 50 mM phosphate buffer, pH 7.5, and, when indicated, 1.5 μM rotenone. The reaction was started by the addition of the substrate.

NADH-ferricyanide reductase activity in subfractions from mitochondria and microsomes was measured spectrophotometrically at 30°C by following the reduction of ferricyanide at 420 mμ. The assay mixture contained in 3 ml: 0.5 mM NADH, 1 mM ferricyanide, 0.3 mM KCN, and 50 mM phosphate buffer, pH 7.5. The reaction was started by the addition of the enzyme.

Cytochrome c oxidase activity was measured polarographically at 30°C, using a Clark oxygen electrode. The reaction mixture contained in 3 ml: 0.1 mM cytochrome *c*, 16 mM ascorbate, and 75 mM phosphate buffer, pH 7.5. The reaction was initiated by the addition of ascorbate. In some experiments, the enzyme activity was measured spectrophotometrically by following the oxidation of reduced cytochrome *c* at 550 mμ. In this case, cytochrome *c* was reduced by the addition of crystals of sodium borohydride to a 3 mM cytochrome *c* solution. Upon neutralization with 100 mM of HCl, the excess reductant was eliminated. The final concentration of reduced cytochrome *c* in the cuvette was adjusted to 0.08 mM in 75 mM phosphate buffer, pH 7.5. A good agreement was

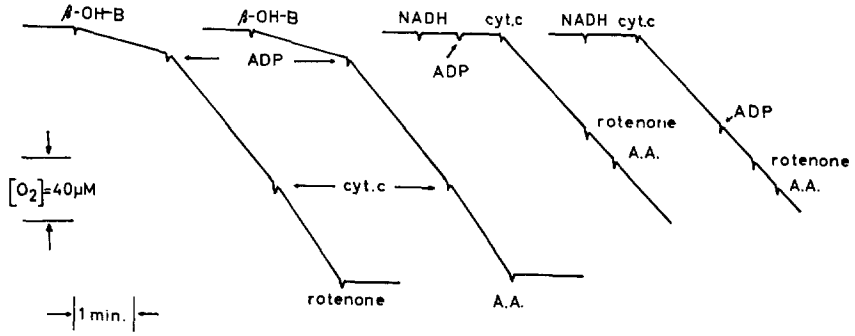


FIGURE 1 Aerobic oxidation of intra- and extramitochondrial NADH catalyzed by rat-liver mitochondria. Oxygen uptake was measured polarographically with a Clark oxygen electrode at 30°C. The assay mixture contained in 3 ml: 3 mg mitochondrial protein, either 3 mM β-hydroxybutyrate (β-OH-B) or 2 mM NADH, 50 mM Tris-HCl buffer, pH 7.5, 100 mM KCl, 8 mM $MgSO_4$, 25 mM phosphate buffer, pH 7.5; and, when indicated, 2 mM ADP, 0.05 mM cytochrome *c* (cyt. *c*), 1.5 μM rotenone, and 3 μM Antimycin A (A.A.) were added.

found between the initial rates measured with the two techniques.

Glucose-6-phosphatase activity was measured at 37°C according to the technique described by Swanson (30). The inorganic phosphate set free during the incubation time was determined by the isobutanol-benzene extraction method as described by Lindberg and Ernster (31).

NADPH-linked lipid peroxidation was assayed essentially as described by Orrenius et al. (32). NADPH was generated from $NADP^+$ with isocitrate and isocitric dehydrogenase (Sigma Chemical Co., St. Louis). Malonaldehyde formation was measured by the thiobarbituric acid reaction according to Bernheim et al. (33). For the assay of this activity mitochondria and microsomes were suspended in 0.15 M KCl, to avoid the interference of sucrose with the malonaldehyde determination.

NADPH-linked oxidative demethylation was assayed with aminopyrine as substrate according to Orrenius (34).

Cytochrome b_5 was estimated from difference spectra between the oxidized and the NADH- or $Na_2S_2O_4$-reduced preparations. The experimental details are given in the legends of Figs. 4 and 5. An extinction coefficient of 160 mM^{-1} cm^{-1} for the difference in absorbancy at 424 and 405 mμ (cf. reference 35) was used to calculate the concentration of cytochrome b_5.

Cytochrome P_{450} was estimated from the difference spectra between the reduced ($Na_2S_2O_4$) and the reduced and carbon monoxide-treated preparations. An extinction coefficient of 91 mM^{-1} cm^{-1} for the difference in absorbancy at 450 and 490 mμ (cf reference 36) was used to calculate the concentration of cytochrome P_{450}.

Stereospecificity of NADH oxidation in mitochondria and microsomes, or subfractions thereof, was determined with tritiated NADH. The two stereo-isomers, NADH-4*A*-^{3}H and NADH-4*B*-^{3}H, were prepared from NAD^+-4-^{3}H by reduction with unlabeled UDPG + UDPG dehydrogenase (Sigma) and with unalbeled ethanol + alcohol dehydrogenase (Sigma), respectively, as previously described (37). NAD^+-4^{3}H (spec. activity 0.7 mc/mmole) was prepared by the method of Krakow et al. (38).[1] The detritiation of NADH was followed by measuring the amount of tritium in water isolated from the reaction mixture as described by Lee et al. (37). Further experimental details are indicated in the legends of Fig. 2 and Table IV.

Protein was determined either by the biuret method as described by Gornall et al. (39), or with the Folin reagent according to Lowry et al. (40). In both cases bovine serum albumin was used as a standard.

All chemicals were commercial products. Spectrophotometric determinations were carried out with a Beckman DK-2 recording spectrophotometer.

Electron Microscopy

Electron microscopic examination has been carried out on the "light" and "heavy" mitochondrial subfractions obtained by the combined swelling-shrinking and sonication procedure followed by discontinuous density gradient centrifugation.

The light subfraction was sedimented from the interface band by centrifugation at 105,000 *g* for 30 min. The supernatant was discarded and the pellet fixed in situ with 1% osmium tetroxide buffered at pH 7.2 with *s*-collidine (41). When the pellet was sufficiently hard, generally after 30 min, it was carefully loosened from the bottom of the tube, and

[1] We are greatly indebted to Professor H. D. Hoberman, New York, for a generous gift of NAD^+-4-^{3}H.

floated in the fixation fluid for another 2 hr. The pellet retained its plano-convex shape formed by the bottom of the tube which made the orientation easy. After fixation, the pellet was divided into small blocks. When the size of the pellet permitted, separate blocks were cut from the surface and the bottom, in both cases in the center of the pellet. The blocks were dehydrated in graded acetone solutions and embedded in Vestopal W. The pellet containing the heavy subfraction was treated in the same manner. Sections were cut with an LKB Ultrotome and stained with uranyl acetate and lead citrate.

Negative staining was performed with the method suggested by Parsons (42). A drop of 2% phosphotungstic acid was placed on a clean glass surface. A small amount of a solution of 1% serum albumin in water was added. A droplet of the heavy or light subfraction, suspended in 0.25 M sucrose, was collected with the tip of a fine glass needle and dipped into the phosphotungstic acid. The specimen rapidly spread in a very thin layer on the surface. After 2 min, copper grids covered by a thin film of Formvar, stabilized with a 40-A-thick coating of carbon, were floated on the surface and rapidly removed. The remaining fluid was sucked off with a filter paper and the grids were dried in air.

The specimens were examined with a Siemens Elmiskop I.

RESULTS

Oxidation of Intra- and Extramitochondrial NADH

The polarographic traces in Fig. 1 illustrate some characteristic features of the aerobic oxidation of intra- and extramitochondrial NADH as catalyzed by freshly made preparations of rat-liver mitochondria. The oxidation of intramitochondrial NADH, generated here by β-hydroxybutyrate as added substrate, was greatly stimulated by ADP, indicative of tightly coupled phosphorylation; it was unaffected by added cytochrome *c*; and it was completely inhibited by rotenone and antimycin A. The oxidation of externally added NADH, in contrast, showed no respiratory control by ADP; it was strongly enhanced by added cytochrome *c*; and it was insensitive to rotenone and antimycin A.

A difference with regard to the effect of inhibitors was also observed when ferricyanide, rather than oxygen, was used as the terminal oxidant for intra- and extramitochondrial NADH (Table I). The results with intramitochondrial NADH are analogous to those already reported by Pressman (43) and by Estabrook (44).

TABLE I

Effects of Rotenone and Antimycin A on the Oxidation of Endogenous and Exogenous NADH by Ferricyanide, Catalyzed by Rat-Liver Mitochondria

The reaction mixture was similar to that in Fig. 1, except that 1 mM KCN, 5 mM ferricyanide, 50 mM glucose, and 100 units crystalline yeast hexokinase (Sigma) were added. Final volume was 2 ml. The samples were incubated in test tubes in a shaking bath at 30°C for 20 min. The reaction was terminated by addition of 2 ml 10% perchloric acid, the samples were centrifuged, and the concentration of ferricyanide in the extracts was determined spectrophotometrically at 420 mμ.

Substrate	Additions	Ferricyanide reduced	Inhibition
		μmoles/min/mg protein	%
β-Hydroxybutyrate	—	0.149	
" "	Rotenone	0.001	99
" "	Antimycin A	0.008	95
NADH	—	4.44	0
"	Rotenone	4.47	0
"	Antimycin A	4.47	0

Stereospecificity of NADH Oxidation in Mitochondria and Microsomes

It has been shown in this laboratory (37, 45) that the respiratory chain–linked NADH dehydrogenase reaction specifically involves the 4*B* hydrogen atom of NADH, and, furthermore, that this enzyme catalyzes a rapid exchange of hydrogen atoms between NADH and water. Drysdale et al. (46) have demonstrated that NADH–cytochrome b_5-reductase, which is involved in the microsomal NADH–cytochrome *c* reductase reaction, is 4*A* specific with regard to NADH and does not catalyze an exchange of hydrogen atoms between the latter and water. In view of these facts, it was of interest to investigate the stereochemical properties of the oxidation of extramitochondrial NADH catalyzed by preparations of rat-liver mitochondria.

In the experiment recorded in Fig. 2 *a*, mitochondria were incubated in parallel runs in the presence of externally added 4*A*-^{3}H-NADH and 4*B*-^{3}H-NADH, respectively. The detritiation of the pyridine nucleotide was followed as a function

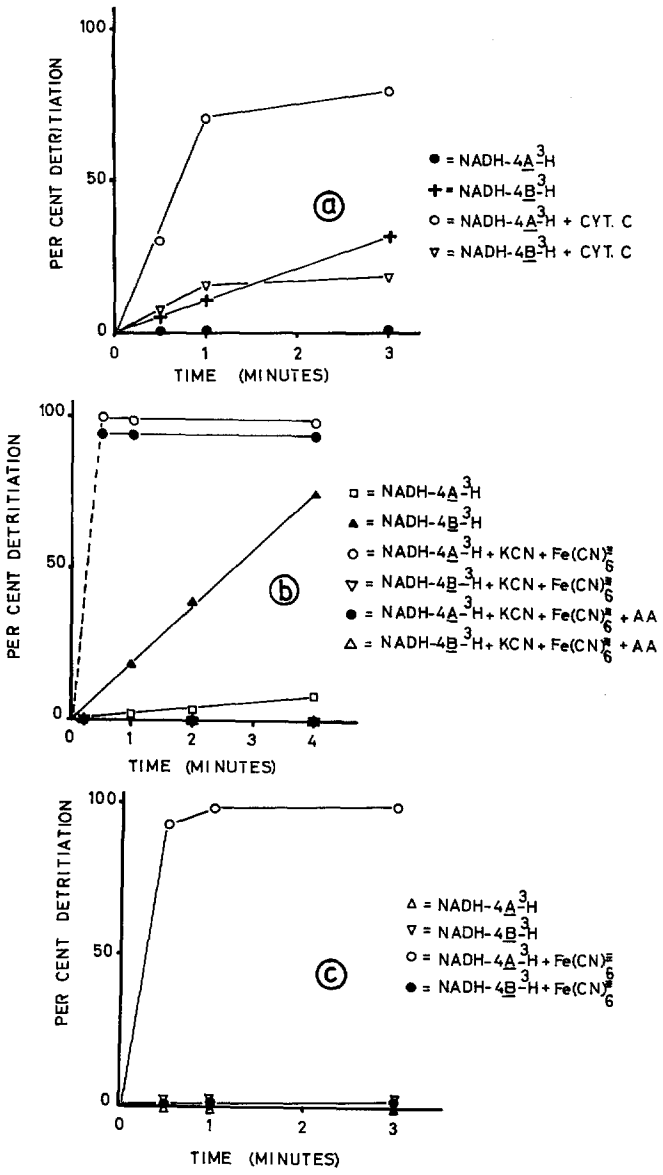


FIGURE 2 Stereospecificity of the oxidation of added NADH catalyzed by mitochondria and microsomes. *a*, Oxidation of NADH-4-^{3}H by intact rat-liver mitochondria under aerobic conditions; *b*, oxidation of NADH-4-^{3}H aerobically or by ferricyanide, catalyzed by intact rat-liver mitochondria; *c*, oxidation of NADH-4-^{3}H by ferricyanide, catalyzed by rat liver microsomes. Experimental conditions were as follows. The reaction mixture contained in 1.2 ml: 0.16 mM NADH-4*A*-^{3}H or 0.20 mM NADH-4*B*-^{3}H, prepared as described in the Experimental Section, 50 mM Tris-HCl buffer, pH 7.5, and 250 mM sucrose. In *a*, 2.1 mg mitochondrial protein, and, where indicated, 0.01 mM cytochrome *c* were added. In *b*, 5.8 mg mitochondrial protein, and where indicated, 0.9 mM ferricyanide, 0.2 mM KCN, and 3 μM antimycin A (A.A.) were added. In *c*, 2.0 mg microsomal protein, 0.9 mM ferricyanide, and 0.2 mM KCN were added. Reaction was carried out at 30°C and was started by the addition of the enzyme. At time intervals indicated, aliquots of 0.3 ml were removed, rapidly frozen, and handled for determination of ^{3}H in H_2O as described in ref. 37.

of time, in the absence and presence of added cytochrome *c*. In the absence of added cytochrome *c*, 4*A*-^{3}H-NADH was not detritiated at all, and 4*B*-^{3}H-NADH was detritiated at a slow and constant rate. When cytochrome *c* was added, there occurred a rapid detritiation of 4*A*-^{3}H-NADH, whereas the detritiation of 4*B*-^{3}H-NADH remained slow initially and ceased after 1 min of incubation. At this time, the sum of the percentages of tritium released from 4*A*-^{3}H- and 4*B*-^{3}H-NADH was close to 100. A similar picture was obtained when ferricyanide, in the presence of KCN, was used as the terminal electron acceptor (Fig. 2 *b*). As expected, the detritiation of 4*A*-^{3}H-NADH was not inhibited by antimycin A. In Fig. 2 *c*, an experiment similar to that shown in Fig. 2 *b* was carried out with liver microsomes. In accordance with the stereospecificity established by Drysdale et al. (46) for NADH–cytochrome-b_5 reductase, the microsomal oxidation of NADH by ferricyanide was accompanied by a release of tritium from 4*A*-^{3}H-NADH,

and the detritiation was dependent on the presence of ferricyanide.

It is evident from the above results that the oxidation of external NADH by either cytochrome *c* or ferricyanide, catalyzed by preparations of rat-liver mitochondria, involves specifically the 4*A*-hydrogen atom of NADH. Furthermore, since no detritiation of 4*A*-^{3}H-NADH was observed in the absence of an added electron acceptor, it appears that the NADH dehydrogenase responsible for this reaction does not catalyze an appreciable exchange of hydrogen atoms between the reduced pyridine nucleotide and water. These stereochemical properties are identical with those found for microsomal NADH oxidation. The relatively slow detritiation of 4*B*-^{3}H-NADH observed with mitochondria under aerobic conditions (cf. Fig. 2 *a*) may reflect a limited access of the added NADH to the intramitochondrial, respiratory chain-linked NADH dehydrogenase, which has been shown to be 4*B*-specific (37, 45).

Distribution of Enzymes in Mitochondria and Microsomes

The finding that the oxidation of external NADH catalyzed by our mitochondrial preparations displayed stereochemical properties identical with those of microsomal NADH oxidation, made it important to evaluate whether, and to what extent, it might originate from contaminating microsomes. To this end, the mitochondrial and microsomal fractions, isolated from the same liver homogenate, were compared with regard to a number of enzymic activities that are well established to be exclusively microsomal. These included the glucose-6-phosphatase (47), and the NADPH-linked drug-hydroxylation (48) and lipid-peroxidation (49–51) reactions. As illustrated by data in Table II, all of these activities were 20–25 times higher (on the protein basis) in the microsomal than in the mitochondrial fraction, indicating that the extent of microsomal contamination in the latter was in the range of 4–5% of the total protein. In contrast, the rotenone-insensitive NADH cytochrome *c* reductase activity was as an average only 2.7 times higher in the microsomal than in the mitochondrial fraction, and would thus require a microsomal contamination amounting to as much as 37% of the total protein if the enzyme were entirely of microsomal origin. In separate experiments, it was ascertained that addition of mitochondria to microsomes did not inhibit the various microsomal enzyme activities here investigated, and that the rotenone-insensitive NADH cytochrome *c* reductase activities of the combined mitochondrial and microsomal fractions were additive. Hence, the enzyme activities recorded in Table II for the mitochondrial fraction are unlikely to result from a selective activation of microsomal NADH–cytochrome *c* reductase, or from a selective inactivation of the other microsomal enzymes here investigated, by mitochondria.

Isopycnic Centrifugation of Mitochondria and Microsomes

In a further attempt to localize cytochemically the mitochondrial rotenone-insensitive NADH–

TABLE II

Comparison of the Mitochondrial and Microsomal Fractions of Rat-Liver Homogenate with Respect to Variou Enzymic Activities

Mean values ± SEM. Number of experiments in parentheses.

Fraction	NADH-cyt. *c* reductase (rotenone-insensitive)	Glucose-6-phosphatase	NADPH-linked lipid peroxidation	NADPH-linked oxid. demethylation
	μmoles cyt. c/min/mg protein	*μmoles P_i/20 min/mg protein*	*μmoles malonaldehyde/min/mg protein*	*μmoles formaldehyde/20 min/mg protein*
Microsomes	0.62 ± 0.043 (7)	5.54 ± 0.31 (6)	0.25 ± 0.016 (5)	0.393 ± 0.015 (3)
Mitochondria	0.23 ± 0.007 (7)	0.23 ± 0.029 (6)	0.01 ± 0.006 (5)	0.019 ± 0.001 (3)
Microsomes / Mitochondria	2.7	23.5	25.0	20.7

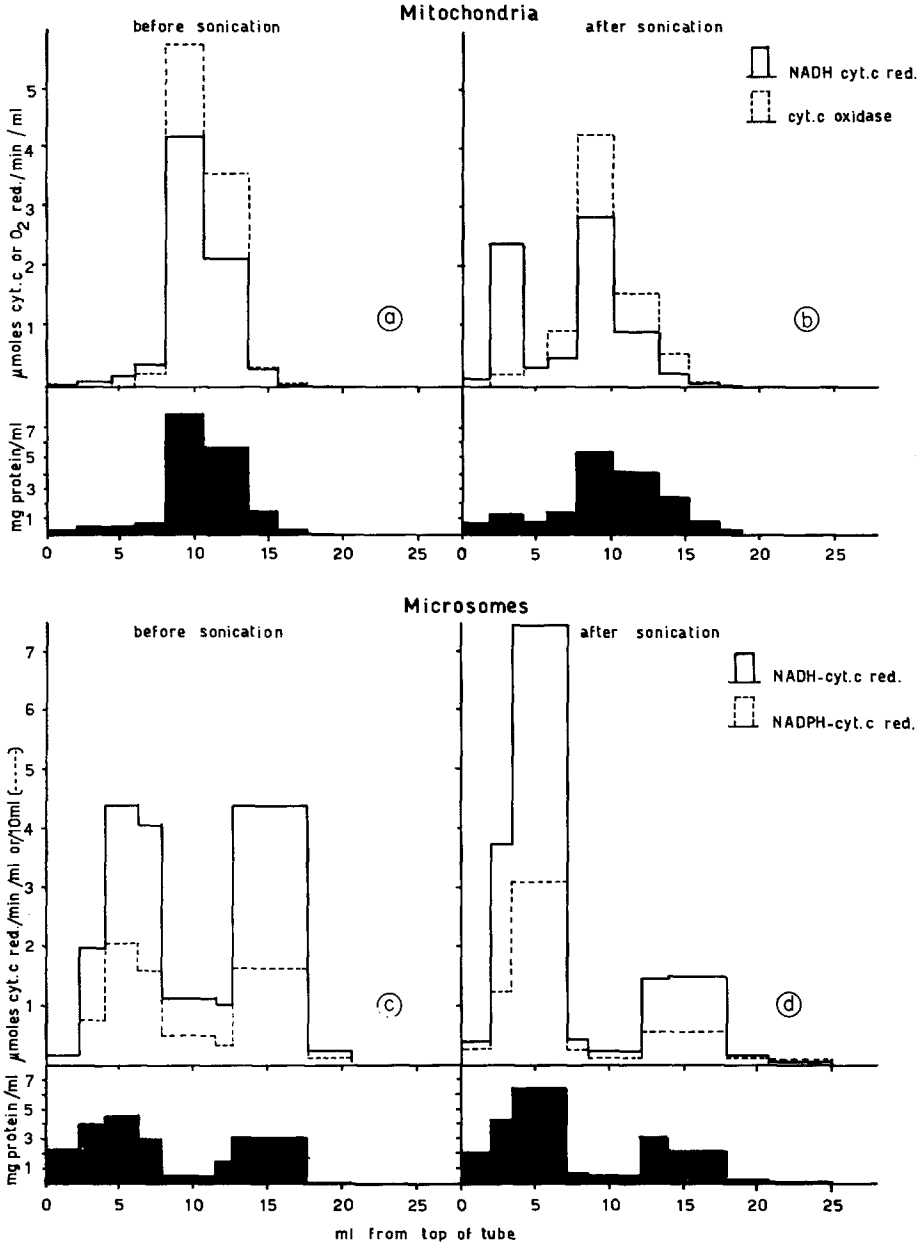


FIGURE 3 Distribution of total protein and of rotenone-insensitive cytochrome *c* reductase, cytochrome *c* oxidase, and NADPH–cytochrome *c* reductase activities upon continuous density gradient centrifugation of mitochondria and microsomes before and after sonication.

cytochrome *c* reductase, the mitochondrial and microsomal fractions were subjected to isopycnic centrifugation on a linear sucrose gradient ranging between 1.18 and 2.30 M sucrose (Fig. 3). Such experiments were performed both with the native preparations, and with preparations that had been exposed to a brief ultrasonic treatment. The cytochrome *c* oxidase activity was used as a marker for mitochondria, and the NADPH–cytochrome *c* reductase activity as a marker for

TABLE III

Comparison between the Light Subfractions from Sonicated Mitochondria and Microsomes with Regard to Various Enzymes

The light mitochondrial subfraction corresponds to the volume between 1.84 and 4.19 ml, and the light microsomal subfraction to the volume between 3.40 and 7.15 ml, of the gradients from the top of the tube as indicated in Fig. 3.

Light subfraction from	Rotenone-insensitive NADH-cyt. *c* reductase	NADH-$K_3Fe(CN)_6$ reductase	Cyt.b_5	NADPH-cyt.*c* reductase	Cytochrome P_{450}	Glucose-6-phosphatase
	μmoles cyt. c/min/mg protein	*μmoles $K_3Fe(CN)_6$/min/mg protein*	mμmoles/mg protein	*μmoles cyt.c red./min/mg protein*	*mμmoles/mg protein*	*μmoles P_i/20 min/mg protein*
Mitochondria	1.34	6.58	0.31	0.001	0.00	0.00
Microsomes	1.05	2.50	0.51	0.044	0.33	3.44

microsomes.[2] The untreated mitochondrial fraction (Fig. 3 *a*) was recovered as a single band in the gradient. The cytochrome *c* oxidase and rotenone-insensitive NADH–cytochrome *c* reductase activites closely paralleled the distribution of the total protein. Upon sonication (Fig. 3 *b*), there appeared a second, light, band in the gradient, which amounted to ca. 10% of the total protein. It was practically devoid of cytochrome *c* oxidase activity, but contained some 30% of the total rotenone-insensitive NADH–cytochrome *c* reductase activity; the latter was about 5 times higher, on the protein basis, than that found in the heavy subfraction. The light subfraction was likewise devoid of succinate–cytochrome *c* reductase and rotenone-sensitive NADH–cytochrome *c* reductase activities (cf. Table V). The untreated microsomal fraction (Fig. 3 *c*) appeared as two bands in the gradient (presumably corresponding to the "smooth" and "rough" microsomal vesicles (58)), one heavier and one ligher than the mitochondria, both of which exhibited NADH– and NADPH–cytochrome *c* reductase activities. Upon sonication (Fig. 3 *d*), the light microsomal subfraction increased at the expense of the heavy one (probably because of the detachment of ribosomes from the rough vesicles), and there was a corresponding shift in the content of the cytochrome *c* reductases, with unchanged ratios of NADH– and NADPH–cytochrome *c* reductase activities. It may be noticed that the light microsomal subfraction was slightly heavier than the light mitochondrial subfraction obtained upon sonication.

As shown in Table III, the light mitochondrial subfraction strikingly differed from the light microsomal subfraction in that it exhibited no appreciable glucose-6-phosphatase and NADPH–cytochrome *c* reductase activities, and contained no cytochrome P_{450}. Both the NADH–cytochrome *c* reductase and NADH-ferricyanide reductase activities were higher (on the protein basis) in the light mitochondrial than in the light microsomal subfraction. In addition, the light mitochondrial subfraction contained an appreciable amount of cytochrome b_5, as shown by the difference spectra in Fig. 4. Reduction with NADH and with sodium dithionite resulted in virtually identical difference spectra with absorption maxima at 556, 526, and 434 mμ which are typical of cytochrome b_5 (cf. reference 18). These data indicated that the light mitochondrial subfraction contained both an enzymically reducible cytochrome b_5 and an NADH–cytochrome b_5 reductase. The latter conclusion is further supported by the data in Table IV which show that the NADH-ferricyanide reaction catalyzed by the light mitochondrial fraction was 4*A* specific with respect to NADH and that there occurred no detritiation of 4*A*-^{3}H-NADH when the fraction was incubated in the presence of NADH alone (i.e., there occurred no exchange of hydrogen atoms between NADH and water). These properties are identical with

[2] Early enzyme distribution studies in several laboratories (5, 52–55) indicated the occurrence of NADPH–cytochrome *c* reductase in both mitochondria and microsomes. Later work (56), however, has revealed that the enzyme is localized exclusively in the microsomes. The apparent occurrence of NADPH-cytochrome *c* reductase in mitochondria may be explained by the presence of pyridine nucleotide transhydrogenase (57), which, in conjunction with the mitochondrial NADH–cytochrome *c* reductase and NAD^+, would catalyze the oxidation of NADPH by cytochrome *c*.

those of NADH–cytochrome b_5 reductase (46). The small extent of detritiation of 4*B*-^{3}H NADH, both in the absence and presence of ferricyanide, shows that only little respiratory chain-linked NADH dehydrogenase was present in the light mitochondrial subfraction.

Subfractionation of Mitochondria by Sonication and Discontinuous Density Gradient Centrifugation

Results similar to those described above were obtained when the separation was performed by discontinuous, rather than continuous, density gradient centrifugation (0.45 and 1.18 M sucrose). By this method, three subfractions could be distinguished: a brown, tightly packed pellet at the bottom of the tube (heavy subfraction); a pinkish-yellow band at the interface between the two sucrose layers (light subfraction); and a slightly yellow supernatant in the 0.45 M sucrose layer (soluble subfraction). Various biochemical parameters of the three subfractions are presented in Table V. The heavy subfraction contained about 84% of the total protein, and the light and soluble subfractions about 8% each. Cytochrome *c* oxidase, succinate–cytochrome *c* reductase, and rotenone-sensitive NADH–cytochrome *c* reductase activities were found almost exclusively in the heavy subfraction (93–99% of total activities recovered). In contrast, 55% of the rotenone-insensitive NADH–cytochrome *c* reductase activity was found in the light subfraction, with a specific activity exceeding that of heavy subfraction by 13-fold. The soluble subfraction exhibited a marginal rotenone-insensitive NADH–cytochrome *c* reductase activity, and was completely devoid of respiratory chain-linked enzyme activities. Cytochrome b_5 was found in both the light and the soluble subfractions, with a 2.7-fold concentration (on the protein basis) in the former. The amount of cytochrome b_5 found in the two subfractions was 64 mμmoles/g of total mitochondrial protein.

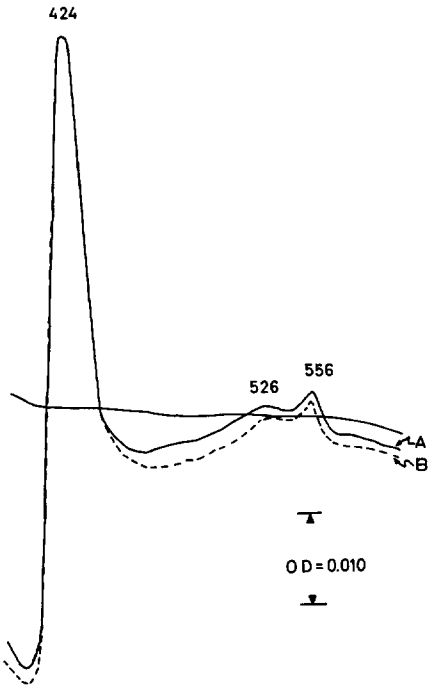


FIGURE 4 Difference spectrum of the light mitochondrial subfraction obtained by sonication and continuous density gradient centrifugation. Both cuvettes contained in 3 ml: 3.6 mg protein, 150 mM phosphate buffer pH 7.5, and 1.5 μM rotenone. Reduction was obtained by adding to one of the cuvettes, either 50 μM NADH (trace *A*), or a few grains of $Na_2S_2O_4$ (trace *B*). The data are from the same experiment as those in Table IV.

TABLE IV

Stereospecificity of NADH Oxidation by Ferricyanide, Catalyzed by the Light Mitochondrial Subfraction

The reaction mixture contained in 2.5 ml: light mitochondrial subfraction 0.22 mg protein, 0.15 mM NADH-4*A*-^{3}H or 0.2 mM NADH-4B-^{3}H, 50 mM Tris-HCl buffer pH 7.5, 250 mM sucrose, 0.2 mM KCN, and, when indicated, 0.9 mM ferricyanide. After complete oxidation of NADH (as monitored spectrophotometrically at 340 mμ) in the two ferricyanide-containing samples, all four samples were rapidly frozen, and tritium in water isolated from the reaction mixtures was determined as described in reference 37.

Tritiated NADH added	% ^{3}H in H_2O	
	− Ferricyanide	+ Ferricyanide
NADH-4*A*-^{3}H	0.9	92.6
NADH-4*B*-^{3}H	1.7	4.2

Subfractionation of Mitochondria after Swelling and Shrinking

It was concluded from the foregoing results that liver mitochondria contain an NADH–cyto-

TABLE V

Protein Content and Some Enzymic Parameters of Mitochondrial Subfractions Obtained by Sonication and Discontinuous Density Gradient Centrifugation

Subfraction	Total protein	Cytochrome *c* oxidase /mg Protein	Cytochrome *c* oxidase Total	Succ.-cyt.*c* reductase /mg Protein	Succ.-cyt.*c* reductase Total	Rotenone-sens. NADH-cyt.*c* red. /mg Protein	Rotenone-sens. NADH-cyt.*c* red. Total	Rotenone-insens. NADH-cyt. *c* red. /mg Protein	Rotenone-insens. NADH-cyt. *c* red. Total	Cytochrome b_5 /mg Protein	Cytochrome b_5 Total
	mg	mμmoles cytochrome *c* oxidized or reduced/min								mμmoles	
Heavy	35.2	0.960	33.8	0.246	8.7	0.287	10.1	0.30	10.7		
Light	3.5	0.343	1.2	0.029	0.1	0.200	0.7	3.95	13.8	0.572	2.00
Soluble	3.3	0	0	0	0	0	0	0.22	0.7	0.212	0.70

TABLE VI

Protein Content, Rotenone-Insensitive NADH-Cytochrome c Reductase Activity, and Concentration of Cytochrome b_5 in Mitochondrial Subfraction Obtained by Swelling and Shrinking Followed by Differential or Discontinuous Density Gradient Centrifugation

Exp. No.	Fraction	Total protein	Rotenone-insensitive NADH-cyt.*c* red. /mg Protein	Rotenone-insensitive NADH-cyt.*c* red. Total	Cytochrome b_5 /mg Protein	Cytochrome b_5 Total
		mg	μmoles cyt.*c*/min		mμmoles	
1	Mitochondria after swelling-shrinking					
	Before centrifugation	53.3	0.542	28.8		
	After centrifugation at 10,000 *g* for 10 min:					
	1st pellet (heavy subfract.)	46.6	0.642	29.8		
	1st supernatant	6.5	0.350	2.3	0.838	4.57
	After centrifugation of 1st supernatant at 105,000 *g* for 60 min:					
	2nd pellet (light subfract.)	1.6	1.500	2.4		
	2nd supernatant (soluble subfract.)	4.8	0.014	0.1		
	Sum (heavy + light + soluble)	53.0		32.3		
2	Mitochondria after swelling-shrinking and discontinuous density gradient centrifugation (0.45 and 1.17 M sucrose) at 90,000 *g* for 3 hr:					
	Heavy subfract.	40.0	0.410	16.5		
	Light subfract.	1.3	1.308	1.7	0.716	0.93
	Soluble subfract.	4.7	0	0	0.850	3.99

chrome *c* reductase system, consisting of NADH–cytochrome b_5 reductase and cytochrome b_5, which is associated with a particulate mitochondrial subfraction that is devoid of the enzymes of the respiratory chain. Since it is generally accepted that the respiratory chain is located in the inner mitochondrial membrane (see reference 59 for review), the outer mitochondrial membrane appeared to be a logical candidate as the site of the mitochondrial NADH–cytochrome b_5 reductase-cytochrome b_5 system.

Parsons (59) has reported that exposure of liver mitochondria to hypotonic phosphate buffer causes a swelling of both mitochondrial mem-

branes, and subsequent treatment with ATP and Mg^{++} results in a selective shrinkage of the inner membrane. In the experiment shown in Table VI, the mitochondria were exposed to swelling and shrinking as described in the Experimental section. The resulting heavy and light subfractions were separated either by differential centrifugation (Exp. 1), or by discontinuous density gradient centrifugation (Exp. 2). In both cases, a heavy subfraction, a light subfraction, and a soluble subfraction were obtained, containing about 87, 3, and 10% of the total protein, respectively. The light and soluble subfractions were completely devoid of respiratory chain components (including cytochrome oxidase, succinate–cytochrome *c* reductase, and rotenone-sensitive NADH–cytochrome *c* reductase). The light subfraction contained about 10% of the total rotenone-insensitive NADH–cytochrome *c* reductase, with a specific activity of two and one-half to three times that of the heavy subfraction. The soluble subfraction exhibited practically no rotenone-insensitive NADH–cytochrome *c* reductase activity. Cytochrome b_5 was found in both the light and the soluble subfractions, in approximately equal concentrations. The amount of cytochrome b_5 in the two subfractions corresponded approximately to 100 mμmoles cytochrome b_5/g of total mitochondrial protein.

Subfractionation of Mitochondria by Combined Swelling-Shrinking and Sonication, Followed by Discontinuous Density Gradient Centrifugation

Since both the sonication and the swelling-shrinkage method resulted only in a partial separation of the rotenone-insensitive NADH–cytochrome *c* reductase from the respiratory chain–containing, heavy mitochondrial subfraction, it was decided to combine the two procedures. Mitochondria were first exposed to swelling and shrinking and then subjected to sonication (cf. Experimental section). Subsequent centrifugation on a discontinuous density gradient (0.45 and 1.18 M sucrose) at 90,000 *g* for 3 hr again resulted in three subfractions: a tightly packed, brown pellet at the bottom of the tube (heavy subfraction); a pinkish-yellow interface layer (appearing occasionally as a double layer) (light subfraction); and a clear, yellow supernatant occupying the 0.45 M sucrose fraction of the gradient (soluble subfraction).

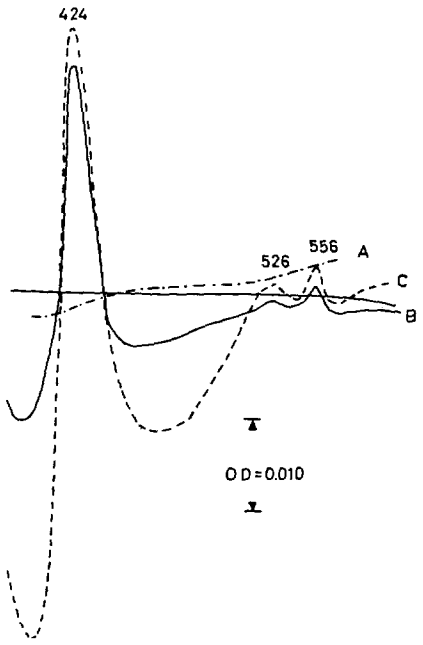


FIGURE 5 Difference spectra of the heavy, light, and soluble subfractions obtained by the combined swelling-shrinking and sonication procedure followed by discontinuous density gradient centrifugation. Each cuvette contained in 2.5 ml: 200 mM sucrose, 200 mM Tris-HCl buffer pH 7.5, and, in the case of the heavy subfraction, 0.05% sodium deoxycholate. In the case of the heavy subfraction (7.50 mg protein/cuvette; trace *A*) and the light subfraction (4.22 mg protein/cuvette; trace *B*), 3 mM succinate and 1.5 μM rotenone were added to both cuvettes, followed by the addition of 50 μM NADH to one of the cuvettes. In the case of the soluble subfraction (3.40 mg protein/cuvette; trace *C*), no succinate or rotenone was added, and reduction was carried out by adding a few grains of $Na_2S_2O_4$ to one of the cuvettes.

The data in Tables VII and VIII and Fig. 5 illustrate some biochemical properties of the three subfractions. From the data in Tables VII and VIII, respectively, the distribution of the total protein was 57.2 and 59.5% in the heavy subfraction, 9.0 and 9.0% in the light subfraction, and 33.8 and 31.5% in the soluble subfraction. As shown in Table VII, the recovery of the various enzyme activities was close to 100%, except for the succinate–cytochrome *c* reductase activity, which was only about 70%. Over 90% of the total respiratory chain activities recovered, in-

TABLE VII

Protein Content and Some Enzymic Parameters of Mitochondrial Subfractions Obtained by the Combined Swelling-Shrinking and Sonication Procedure Followed by Discontinuous Density Gradient Centrifugation

Fraction	Total protein	Cytochrome *c* oxidase		Succ.-cyt.*c* reductase		Rotenone-sens. NADH-cyt.*c* red.		Rotenone-insens. NADH-cyt.*c* red.	
		μmoles Cytochrome *c* oxidized or reduced/min							
		/mg Protein	Total	/mg Protein	Total	/mg Protein	Total	/mg Protein	Total
	mg								
Mitochondria after swelling-shrinking and sonication *Before* centrifugation	145.6	0.742	108.0	0.351	51.1	0.151	22.0	0.390	56.8
After centrifugation:									
Heavy subfract.	81.0	1.310	106.0	0.421	34.1	0.257	20.8	0.097	7.9
Light subfract.	12.8	0.719	9.2	0.109	1.4	0.140	1.8	3.040	38.9
Soluble subfract.	47.9	0.058	2.8	0	0	0	0	0.109	5.2
Sum (heavy + light + soluble)	141.7		118.0		35.5		22.6		52.0
% Recovery	97.1		109.2		69.5		102.7		91.5

TABLE VIII

Protein and Cytochrome b_5 Content of Mitochondrial Subfractions Obtained by the Combined Swelling-Shrinking and Sonication Procedure Followed by Discontinuous Density Gradient Centrifugation

Subfraction	Total protein	Cytochrome b_5 mμmoles	
		/mg Protein	Total
	mg		
Heavy	65.7	0	0
Light	9.9	0.152	1.50
Soluble	34.7	0.478	16.56
Sum (heavy + light + soluble)	110.3	0.163	18.06

cluding cytochrome *c* oxidase, succinate–cytochrome *c* reductase, and rotenone-sensitive NADH–cytochrome *c* reductase, were found in the heavy subfraction. The remainder of these activities was associated with the light subfraction, practically none being found in the soluble subfraction. On the protein basis, the three activities were two to four times higher in the heavy than in the light subfraction. Conversely, about 75% of the rotenone-insensitive NADH–cytochrome *c* reductase activity was recovered in the light subfraction, with a specific activity exceeding that of the heavy subfraction ca. 30-fold. The soluble subfraction again exhibited only marginal rotenone-insensitive NADH–cytochrome *c* reductase activity.

The cytochrome b_5 content of the three subfractions was estimated from difference spectra of the type shown in Fig. 5. In the case of the heavy and light subfractions, which contained respiratory chain components, succinate and rotenone were added to both cuvettes, followed by the addition of NADH to one of them. When the samples had became anaerobic, the difference spectrum was recorded. The heavy subfraction revealed no appreciable deviation from the base line. The light subfraction exhibited a difference spectrum typical of cytochrome b_5, with absorption maxima at 556, 526, and 424 mμ. Further addition of sodium dithionite to either subfraction did not reveal the presence of any enzymically nonreducible cytochrome b_5. In the case of the soluble fraction, which was devoid of both respiratory chain enzymes and NADH–cytochrome b_5 reductase, no succinate and rotenone were added, and reduction was performed by adding sodium dithionite to one of the cuvettes. The difference spectrum showed the α-, β-, and γ-bands of reduced cytochrome b_5. Minima in the 450–500 mμ region, which are seen in the differ-

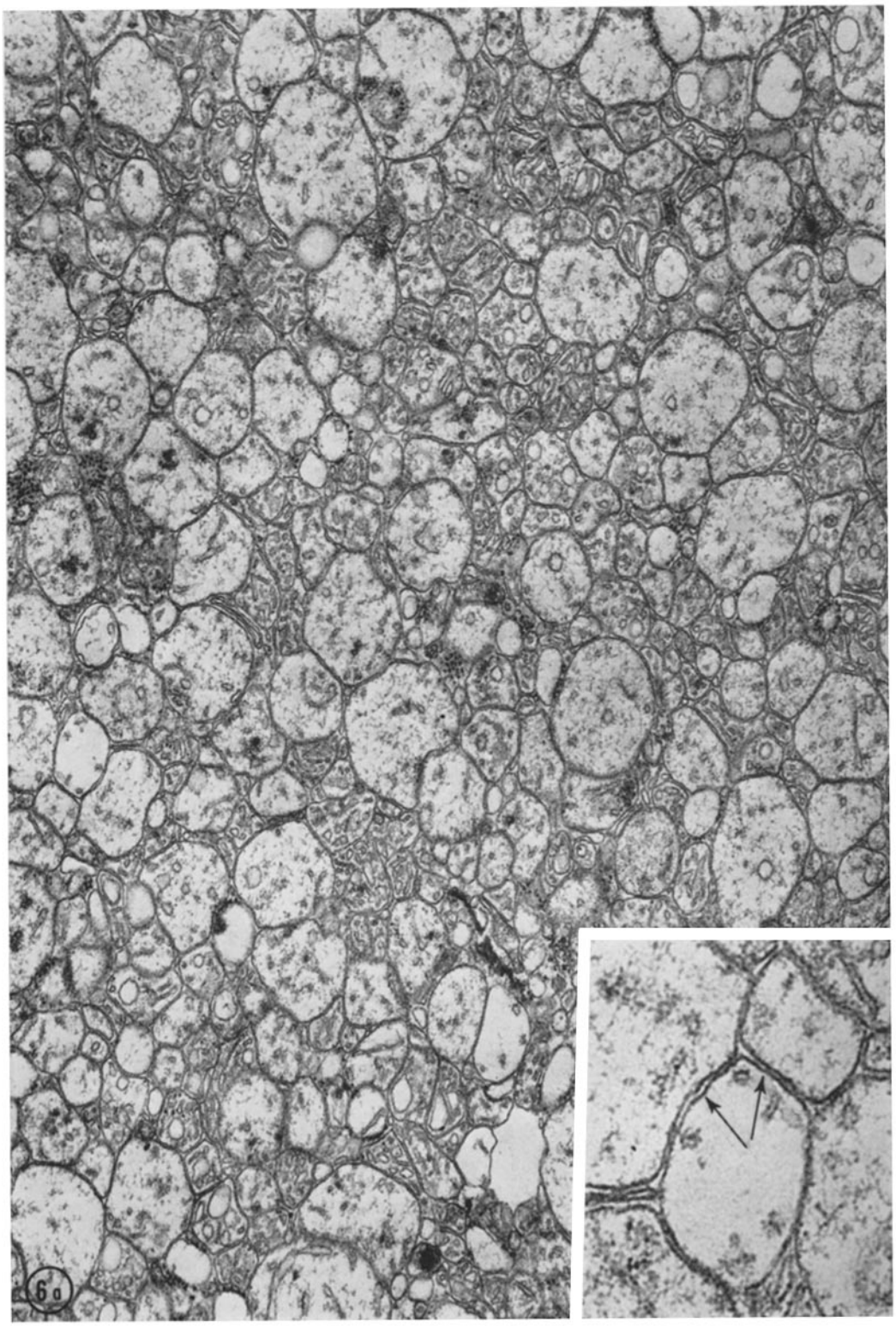
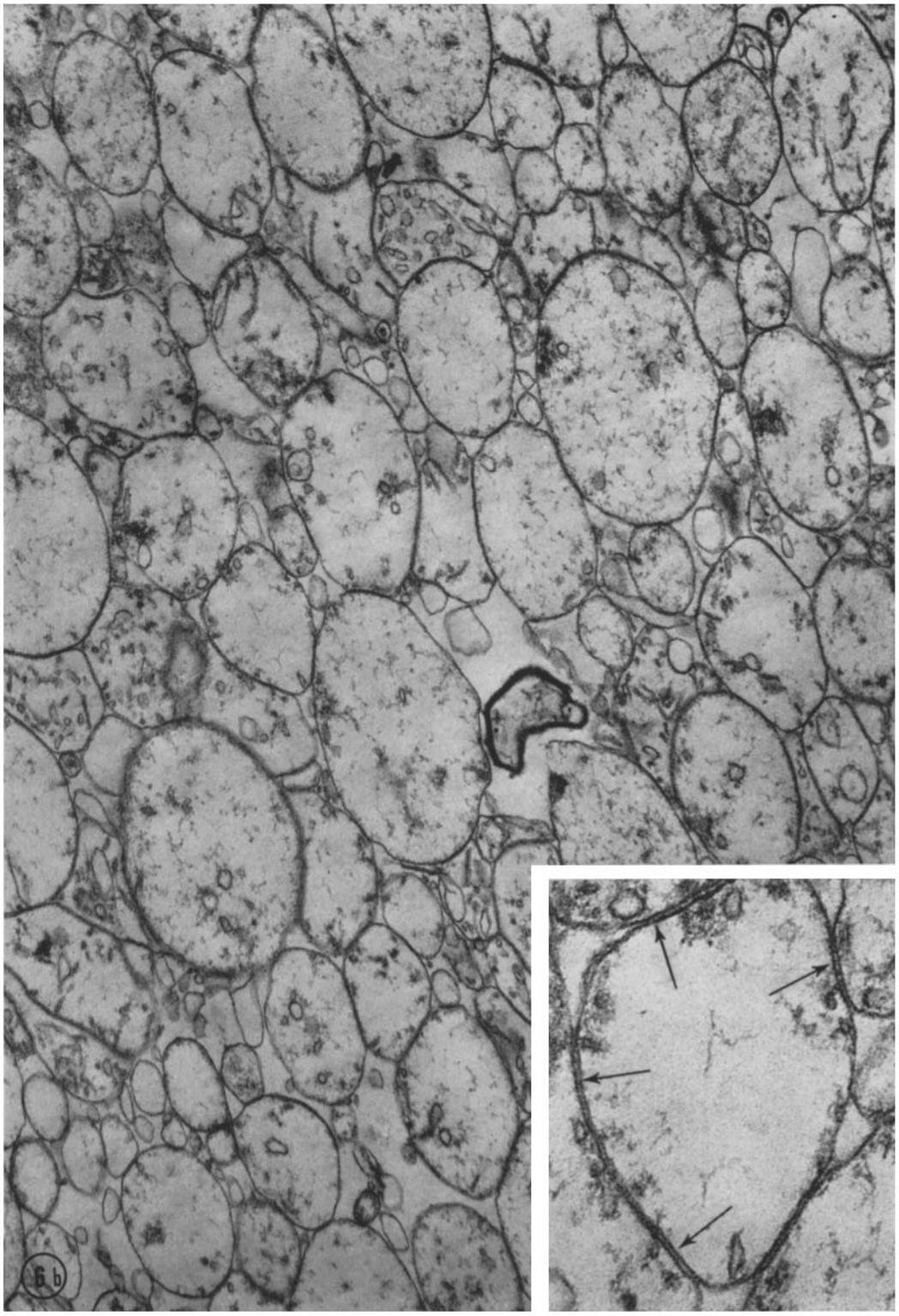


FIGURE 6 Electron micrographs of section of the heavy mitochondrial subfraction obtained by the combined swelling-shrinking and sonication procedure followed by discontinuous density gradient centrifugation. Fig. 6 *a*, Lower part of pellet. Fig. 6 *b*, Upper part of pellet. × 30,000.

Insets: Higher magnifications of same, showing that apparent "double" membranes (arrows) are derived from different adjacent vesicles which are bordered by single membranes. × 80,000.


6 b

ence spectra of both the light and the soluble subfractions, presumably originate from reduced flavoproteins. In complementary experiments with the combined light and soluble subfractions, it was ascertained that the cytochrome b_5 found in the soluble subfraction was quantitatively reducible by NADH. Treatment of the soluble subfraction with pyridine yielded a pyridine hemochrome spectrally identical with that reported by Raw et al. (23) for mitochondrial cytochrome b_5.

Quantitative data concerning the distribution of cytochrome b_5 among the three subfractions, based on the spectra recorded in Fig. 5, are summarized in Table VIII. The heavy subfraction was virtually devoid of cytochrome b_5. Of the total cytochrome b_5, only about 8% was recovered in the light subfraction, the remainder being in the soluble subfraction. On the protein basis, the concentration of cytochrome b_5 was 3 times higher in the latter than in the former. The total amount of cytochrome b_5 was 163 mμmoles/g mitochondrial protein.

Electron Microscopy

Electron microscopic examination of the heavy and light subfractions obtained by the combined swelling-shrinking and sonication procedure revealed the following (Figs. 6–10):

The osmium tetroxide–fixed material from the heavy mitochondrial subfraction (fig. 6) consisted of large vesicles, with a diameter ranging between 1500 and 15,000 A. Each vesicle was bordered by a single membrane. In many instances, the vesicles contained smaller, round, or elongated profiles bordered by a single membrane of the same thickness as that of the surrounding vesicle, and probably representing sections of cristae. Smaller vesicles with abundant inner structure were concentrated in the lower part (Fig. 6 *a*), and larger vesicles with little inner structure in the upper part (Fig. 6 *b*) of the pellets. Negatively stained specimens of the same subfraction showed mitochondrial images in a stage of bursting, with protrusions of unfolding cristae (Fig. 8). At higher magnifications (Fig. 10 *a*), a coating of mushroom-like repeating units, similar to those first described by Fernández-Morán (60), could be discerned on the surface of the cristal membranes.

The osmium tetroxide–fixed light subfraction (Fig. 7) consisted of relatively small vesicles, with a diameter ranging between 600 and 4000 A. The vesicles were bordered by a single membrane. Inner structures were only rarely seen. Larger vesicles were concentrated in the lower part (Fig. 7 *a*), and smaller vesicles in the upper part (Fig. 7 *b*) of the pellets. The light subfraction as observed with the negative-staining technique consisted of more or less flattened vesicles (Fig. 9). The outer surface was slightly irregular, but higher magnification (Fig. 10 *b*) revealed no mushroom-like repeating units.

DISCUSSION

The data reported in this paper seem to leave little doubt that the so called "external" NADH–cytochrome *c* reductase system found in preparations of liver mitochondria is a true mitochondrial constituent and cannot be explained on the basis of microsomal contamination. The mitochondrial preparations exhibited only marginal activities of glucose-6-phosphatase and of other exclusively microsomal enzymes, and their rotenone-insensitive NADH–cytochrome *c* reductase activity was almost 10 times higher, in relation to these activities, than the corresponding ratios found with liver microsomes (cf. Table II). The rotenone-insensitive mitochondrial NADH–cytochrome *c* reductase activity was 2.7 times lower than the microsomal one, which is in good agreement with earlier data of Hogeboom and Schneider (11, 12) and de Duve et al. (5). The cytochrome b_5 content of the mitochondria was 163 mμmoles/g protein (cf. Table VIII), which is 3.7 times lower than the value of 600 mμmoles/g protein reported by Strittmatter (18) for rat-liver microsomes.[3] If the extent of microsomal contamination of the mitochondrial preparations were as high as indicated by the rotenone-insensitive NADH–cytochrome *c* reductase activity or the cytochromal b_5 content—37 or 27% of the total protein—it should be possible to separate the two elements by continuous density gradient centrifugation, in view of the

[3] A cytochrome b_5 content of 1100 mμmoles/g microsomal protein has been reported by Klingenberg (35), but that value relates to KCl-washed microsomes, which probably are depleted of their soluble protein content. The value of 600 mμmoles/g protein, quoted above, is in good agreement with those which can be calculated from more recent data of Dallner (750 mμmoles/g protein; reference 58), and from data obtained here (cf. Table III) for the light microsomal subfraction (500 mμmoles/g protein).

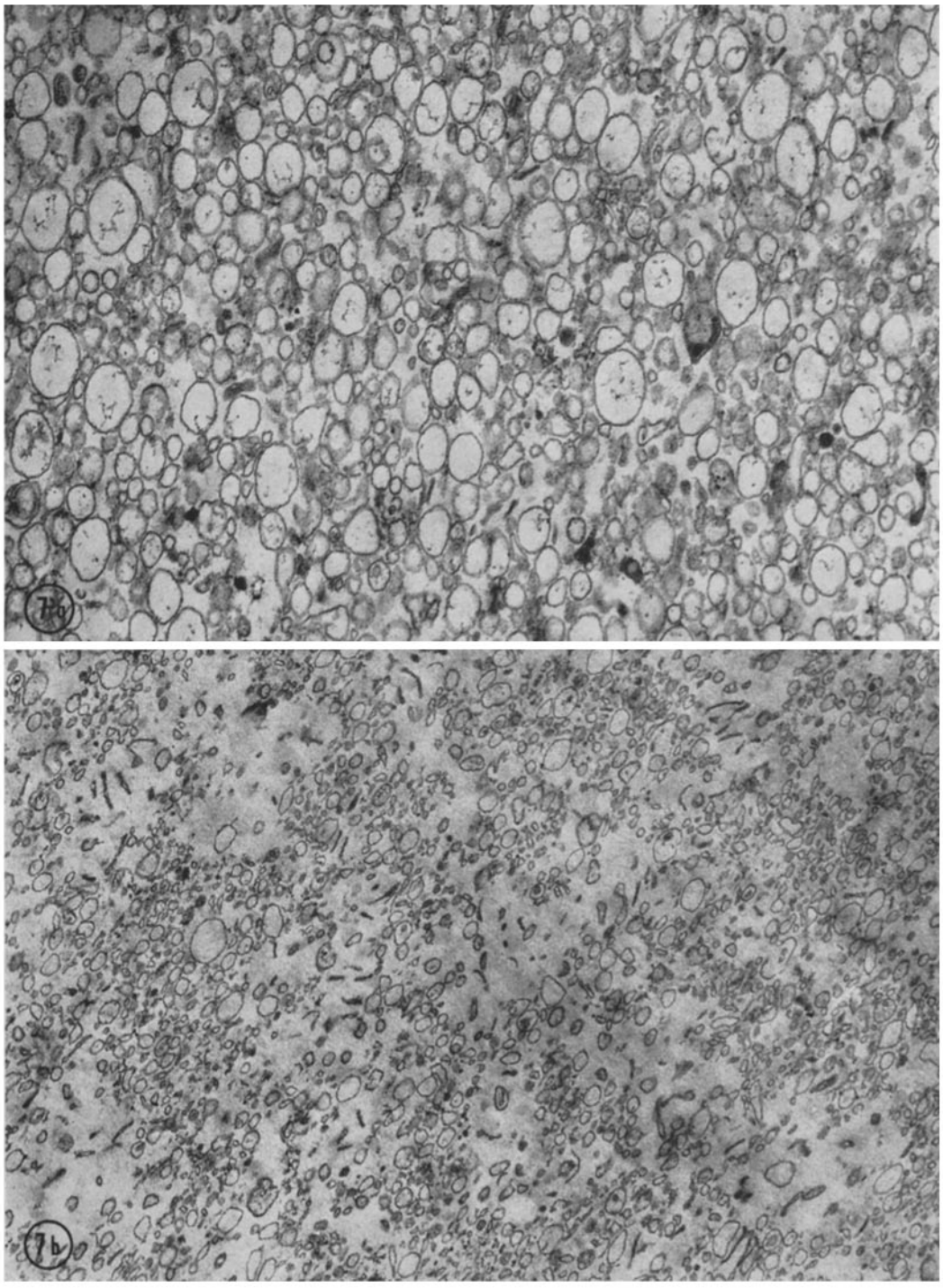


FIGURE 7 Electron micrographs of sections of the light mitochondrial subfraction obtained by the combined swelling-shrinking and sonication procedure followed by discontinuous density gradient centrifugation. × 30,000. Fig. 7 *a*, Lower part of pellet. Fig. 7 *b*, Upper part of pellet.

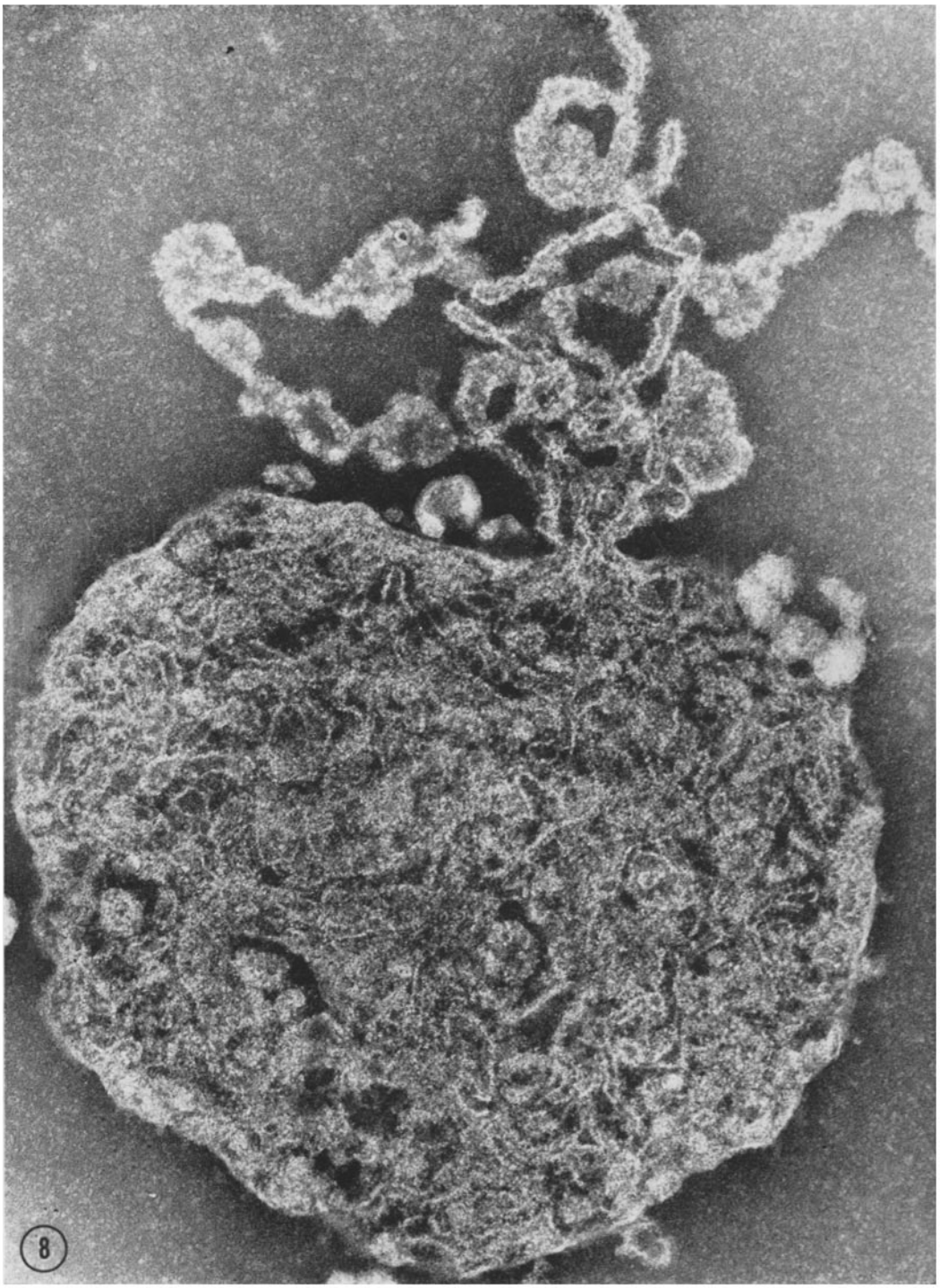


FIGURE 8 Negatively stained specimen of heavy mitochondrial subfraction obtained by the combined swelling-shrinking procedure followed by discontinuous density gradient centrifugation. × 86,000.

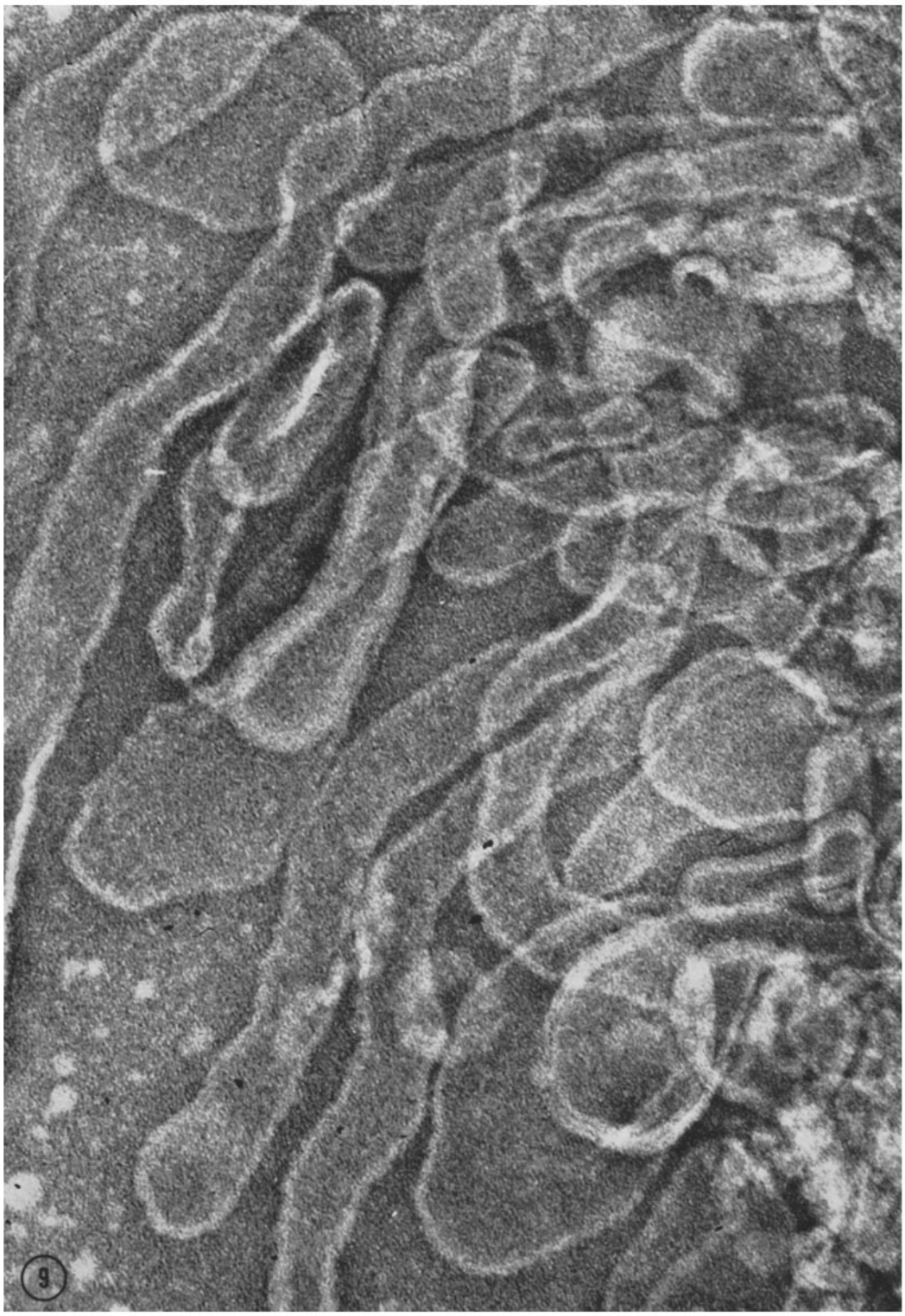


FIGURE 9 Negatively stained specimen of light mitochondrial subfraction obtained by the combined swelling-shrinking procedure followed by discontinuous density gradient centrifugation. × 165,000.

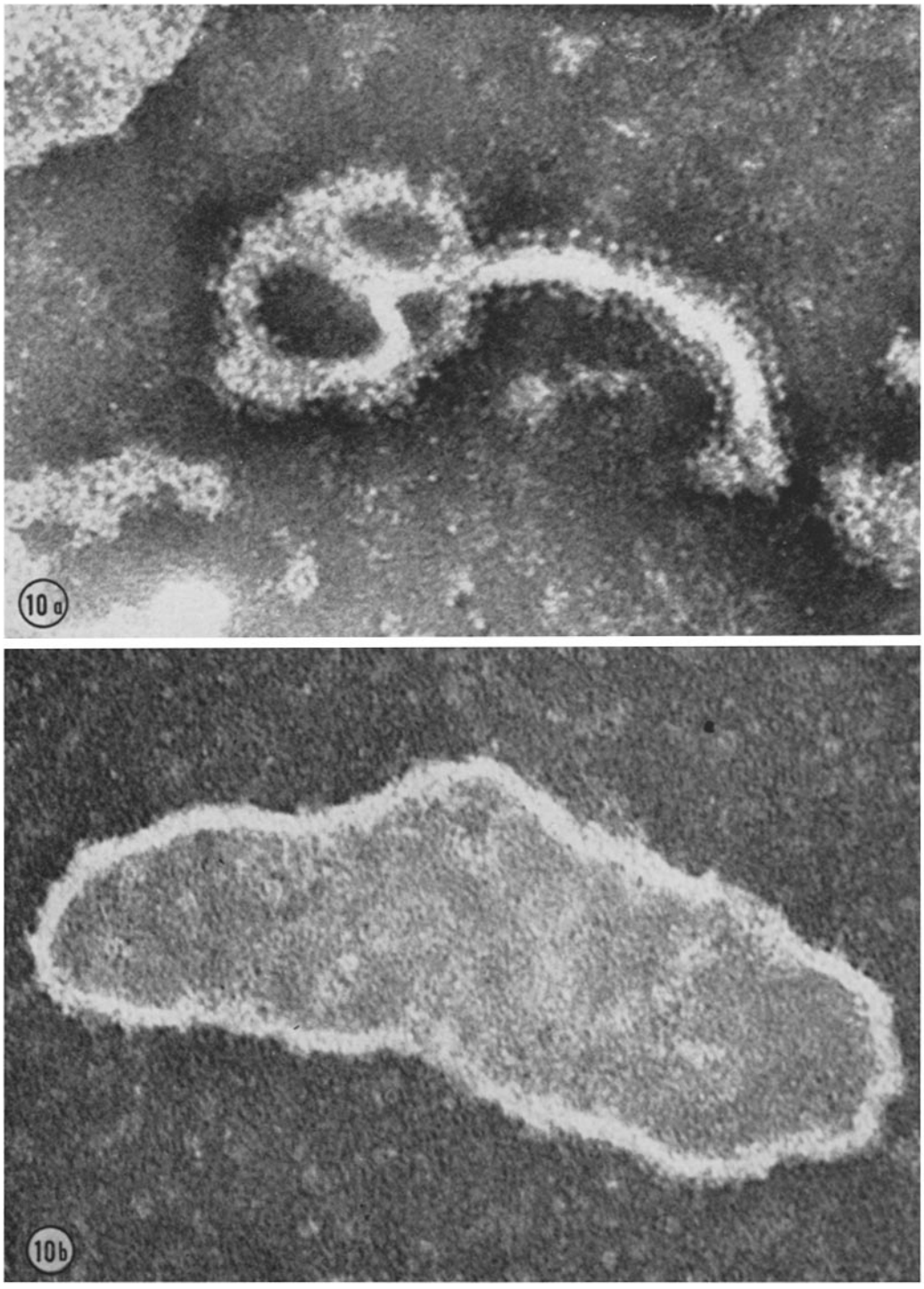


FIGURE 10 Negatively stained specimens of heavy and light mitochondrial subfractions obtained by the combined swelling-shrinking procedure followed by discontinuous density gradient centrifugation. Fig. 10 *a*, Heavy subfraction. × 240,000. Fig. 10 *b*, Light subfraction. × 278,000.

difference in density between mitochondria and microsomal vesicles. Such a separation did not occur (cf. Fig. 3 *a*). Brief sonication followed by continuous density gradient centrifugation did result in a particulate subfraction with a high concentration of rotenone-insensitive of NADH–cytochrome *c* reductase. However, this subfraction differed from microsomes both in density and in being devoid of other microsomal enzymes. When microsomes were exposed to the same treatment, the resulting subfractions retained their original ratios of various enzymic activities. Although in electron micrographs of liver cells, endoplasmic membranes are frequently seen closely adjacent to mitochondria, there is no indication that these segments of the endoplasmic reticulum would differ in enzymic composition from the rest of the system. In fact, glucose-6-phosphatase has been demonstrated histochemically to be abundantly present also in those endoplasmic membranes surrounging mitochondria (61).

Our results strongly suggest that the rotenone-insensitive NADH–cytochrome *c* reductase system present in liver mitochondria is associated with the outer mitochondrial membrane. The principle arguement in favor of this conclusion is the finding that the system can be concentrated in a particulate mitochondrial subfraction which is devoid of respiratory chain components. Separation of such a subfraction from the bulk of the mitochondrial structure has been achieved either by sonication of the mitochondria under suitable conditions, or by exposing them to swelling and shrinkage, a treatment which, according to morphological observations of Parsons (59), would be expected to lead to a disruption and detachment of the outer mitochondrial membrane. By combining the two procedures, we have been able to obtain two particulate subfractions: a heavy subfraction, containing the bulk of the respiratory chain components and only little rotenone insensitive NADH–cytochrome *c* reductase; and a light subfraction, poor in respiratory chain components and containing the majority of the rotenone-insensitive NADH–cytochrome *c* reductase. Electron microscopic examination revealed a very distinct difference between the heavy and light subfractions, with little intermixing between the two. The heavy subfraction consisted of relatively large vesicles bounded by a single membrane and containing tubular profiles coated by mushroom-like repeating units on their surface. This picture is consistent with the interpretation that the heavy subfraction consists of mitochondria devoid of outer membrane. The light subfraction consisted of relatively small, mostly flattened, empty vesicles, without any mushroom-like repeating units on their surface. These features are consistent with those described by Parsons (59) for the outer mitochondrial membrane in situ, if one assumes that this membrane, once fragmented and detached from the mitochondria, rearranges into vesicles. The localization of the rotenone-insensitive NADH–cytochrome *c* reductase system in the outer mitochondrial membrane accounts logically for the phenomenon that, in the intact mitochondrion, this system is readily accessible to exogenous NADH and inaccessible to endogenous NADH. From our stereochemical data, we can also conclude that this system indeed is entirely responsible for the oxidation of "external" NADH by cytochrome *c* catalyzed by isolated rat-liver mitochondria, as first suggested by Raw et al. (26). Preliminary data obtained with tritiated NADH indicate that a similar enzyme system is also present in mitochondria from rat skeletal muscle, Ehrlich ascites tumor cells,[4] and yeast (62).

An important, but still unresolved problem concerns the relationship of the "external" NADH–cytochrome *c* reductase of mitochondria and the NADH cytochrome b_5 reductase–cytochrome b_5 system of microsomes. As first shown by Raw and associates (23–26), liver mitochondria do contain a hemoprotein with the spectral characteristcs of cytochrome b_5 and an NADH dehydrogenase capable of reducing this hemoprotein. Our data confirm these results and show, in addition, that the mitochondrial system exhibits the same stereochemical properties with respect to NADH as does the corresponding microsomal enzyme. Whether the mitochondrial hemoprotein and its reductase indeed are identical with the microsomal cytochrome b_5 and NADH–cytochrome b_5 reductase, cannot be decided with certainty at this time. Concerning the reductase, Raw and Mahler (25) have found that the mitochondrial enzyme is more sensitive to dicoumarol than the micorsomal one. We have confirmed this finding. A differential sensitivity to -SH reagents has likewise been reported by Avi-Dor et al. (17). Regarding the mitochondrial hemoprotein, Raw et al. (23) have reported that this is

[4] E. E. Gordon and G. L. Sottocasa. Unpublished results.

not reducible by cysteine, in contrast to microsomal cytochrome b_5. This observation also has been confirmed in the course of the present work. Raw et al. (23) have further found that treatment of mitochondria with 10% ethanol releases the cytochrome b_5–like hemoprotein, whereas the same treatment of microsomes does not release any cytochrome b_5. In line with this finding is our observation that hypotonic treatment solubilizes a large part of the cytochrome b_5–like hemoprotein of mitochondria, but not cytochrome b_5 from microsomes. Apparently, this hemoprotein is less firmly bound to mitochondria than cytochrome b_5 is bound to microsomes. Finally, when this work was completed, Parsons *et al.* (63) reported that an isolated outer membrane fraction from liver mitochondria exhibited a low-temperature difference spectrum similar to that of microsomal cytochrome b_5, but differing from the latter in the location of the α_1 and α_2 peaks. It will require further work to decide whether these different features reflect real differences in the native enzyme molecules, or whether they can be explained on the basis of a different composition of, or binding to, the mitochondrial and microsomal membranes, as well as the possible modifications of the enzyme molecules that may accompany their release from the two types of membrane. At any event, the occurrence of a similar or identical electron-transport system in the outer mitochondrial and the endoplasmic membranes seems to open up interesting prospectives regarding both the interrelationship of these two cytoplasmic elements and the metabolic function of the enzyme system itself.

The procedures described in this paper for the separation of the inner and outer mitochondrial membranes may be useful in the future for studies of the chemical and enzymic compositions of different mitochondrial compartments. With the combined swelling-shrinking and sonication procedure, which resulted in the quantitatively best separation of the inner and outer membranes, about 58% of the total protein was recovered in the heavy subfraction, 9% in the light subfraction, and 33% in the soluble subfraction. Morphologically, the heavy subfraction consisted of mitochondria, with a more or less well preserved inner structure, but without an outer membrane. This subfraction apparently represents the inner membrane system and presumably part of the matrix. The finding that this subfraction contained the bulk of the respiratory chain enzymes is consistent with the generally accepted concept that the inner mitochondrial membrane is the site of the respiratory chain. The light subfraction, as already discussed, most probably represents vesiculated derivatives of the outer mitochondrial membrane, and any enzyme concentrated in this fraction may, therefore, be concluded to be a constituent of this membrane. The rotenone-insensitive NADH–cytochrome *c* reductase may serve in the future as a suitable marker of the liver-mitochondrial outer membrane. The soluble subfraction probably includes primarily any material present between the two mitochondrial membranes, as well as part of the matrix. In addition, it may contain material released from both the inner and outer membranes. Further information regarding the enzymic composition of the three subfractions has been reported briefly elsewhere (64, 65), and will be the subject of a forthcoming paper.

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REFERENCES

1. LEHNINGER, A. L. 1951. *J. Biol. Chem.* **190**:345.
2. LEHNINGER, A. L. 1951. *Phosphorus Metabol.* **1**:344.
3. LEHNINGER, A. L. 1955. *Harvey Lectures.* **49**:176.
4. PRESSMAN, B. C., and C. DE DUVE. 1954. *Arch. Intern. Physiol.* **62**:306.
5. DE DUVE, C., B. C. PRESSMAN, R. GIANETTO, R. WATTIAUX, and F. APPELMANS. 1955. *Biochem. J.* **60**:604.
6. ERNSTER, L., H. LÖW, and O. LINDBERG. 1955. *Acta Chem. Scand.* **9**:200.
7. ERNSTER, L., O. JALLING, H. LÖW, and O. LINDBERG. 1955. *Exptl. Cell Res. Suppl.* **3**:124.
8. ERNSTER, L. 1956. *Exptl. Cell Res.* **10**:721.
9. MALEY, G. F. 1957. *J. Biol. Chem.* **224**:1029.
10. ERNSTER, L., G. DALLNER, and G. F. AZZONE. 1963. *J. Biol. Chem.* **238**:1124.

11. HOGEBOOM, G. H. 1949. *J. Biol. Chem.* **177**:847.
12. HOGEBOOM, G. H., and W. C. SCHNEIDER. 1950. *J. Nat. Cancer Inst.* **10**:983.
13. SCHNEIDER, W. C., and G. H. HODGEBOOM. 1956. *Ann. Rev. Biochem.* **25**:201.
14. SLATER, E. C. 1958. *Advan. Enzymol.* **20**:147.
15. ERNSTER, L., and O. LINDBERG. 1958. *Ann. Rev. Physiol.* **20**:13.
16. ERNSTER, L. 1959. *Biochem. Soc. Symp.* **16**:54.
17. AVI-DOR, Y., A. TRAUB, and J. MAGER. 1958. *Biochim. Biophys. Acta.* **30**:164.
18. STRITTMATTER, C. F. 1961. *In* Hematin Enzymes. J. E. Falk, R. Lemberg, and R. K. Morton, editors. Pergamon Press, Oxford. 461.
19. STRITTMATTER, P. 1963. *In* The Enzymes. P. D. Boyer, H. A. Lardy, and K. Myrbäck, editors. Academic Press, Inc., N. Y. **8**:113.
20. STRITTMATTER, C. F., and E. G. BALL. 1952. *Proc. Nat. Acad. Sci.* **38**:19.
21. STRITTMATTER, C. F., and E. G. BALL. 1954. *J. Cellular Comp. Physiol.* **43**:57.
22. CHANCE, B., and G. R. WILLIAMS. 1955. *J. Biol. Chem.* **217**:395.
23. RAW, I., R. MOLINARI, D. FERREIRA DO AMARAL, and H. R. MAHLER. 1958. *J. Biol. Chem.* **233**: 225.
24. MAHLER, H. R., I. RAW, R. MOLINARI, and D. FERREIRA DO AMARAL. 1958. *J. Biol. Chem.* **233**:230.
25. RAW, I., and H. R. MAHLER. 1959. *J. Biol. Chem.* **234**:1867.
26. RAW, I., N. PETRAGNANI, and O. CAMARGO-NOGUEIRA. 1960. *J. Biol. Chem.* **235**:1517.
27. SOTTOCASA, G. L., and L. ERNSTER. 1965. 2nd Meeting Federation of European Biochemical Societies, Vienna. Academic Press Inc., London. 112. (Abstr.)
28. SOTTOCASA, G. L., and B. KUYLENSTIERNA. 1966. 3rd Meeting Federation of European Biochemical Societies, Warsaw. Academic Press, London, and Publ. Polish Acad. Sci., Warsaw. 118. (Abstr.)
29. SOTTOCASA, G. L. *In* Round-Table Discussion on Mitochondrial Structure and Compartmentation, Bari. 1966. E. Quagliariello, E. C. Slater, S. Papa, and J. M. Tager, editors. Adriatica Editrice, Bari. In press.
30. SWANSON, M. A. 1950. *J. Biol. Chem.* **184**:647.
31. LINDBERG, O., and L. ERNSTER. 1955. *Methods Biochem. Anal.* **3**:1.
32. ORRENIUS, S., G. DALLNER, and L. ERNSTER. 1964. *Biochem. Biophys. Res. Commun.* **14**:329.
33. BERNHEIM, F., M. L. BERNHEIM, and K. M. WILBUR. 1948. *J. Biol. Chem.* **174**:257.
34. ORRENIUS, S. 1965. *J. Cell Biol.* **26**:713.
35. KLINGENBERG, M. 1958. *Arch. Biochem. Biophys.* **75**:376.
36. OMURA, T., and R. SATO. 1964. *J. Biol. Chem.* **239**:2370.
37. LEE, C. P., N. SIMARD-DUQUESNE, L. ERNSTER, and H. D. HOBERMAN. 1965. *Biochim. Biophys. Acta.* **105**:397.
38. KRAKOW, G., J. LUDOWIEG, J. H. MATHER, W. M. NORMORE, L. TOSI, S. UDAKA, and B. VENNESLAND. 1963. *Biochemistry.* **2**:1009.
39. GORNALL, A. G., C. J. BARDAWILL, and M. M. DAVID. 1949. *J. Biol. Chem.* **177**:751.
40. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL. 1951. *J. Biol. Chem.* **193**: 265.
41. BENNETT, H. S., and J. M. LUFT. 1959. *J. Biophys. Biochem. Cytol.* **6**:113.
42. PARSONS, D. F. 1963. *J. Cell Biol.* **16**:260.
43. PRESSMAN, B. C. 1955. *Biochim. Biophys. Acta.* **17**: 274.
44. ESTABROOK, R. W. 1961. *J. Biol. Chem.* **236**:3051.
45. ERNSTER, L., H. D. HOBERMAN, R. L. HOWARD, T. E. KING, C. P. LEE, B. MACKLER, and G. L. SOTTOCASA. 1965. *Nature.* **207**:940.
46. DRYSDALE, G. R., M. J. SPIEGEL, and P. STRITTMATTER. 1961. *J. Biol. Chem.* **236**:2323.
47. HERS, H. G., and C. DE DUVE. 1950. *Bull. Soc. Chim. Biol.* **32**:20.
48. BRODIE, B. B., J. AXELROD, J. R. COOPER, L. GAUDETTE, B. N. LADU, C. MITOMA, and S. UDENFRIEND. 1955. *Science.* **121**:603.
49. HOCHSTEIN, P., and L. ERNSTER. 1963. *Biochim. Biophys. Res. Commun.* **12**:388.
50. HOCHSTEIN, P., and L. ERNSTER. 1964. *In* Ciba Foundation Symposium on Cellular Injury, London. 1963. A. V. S. de Reuck and J. Knight, editors. Churchill, London. 123.
51. HOCHSTEIN, P., K. NORDENBRAND, and L. ERNSTER. 1964. *Biochem. Biophys. Res. Commun.* **14**:323.
52. HOGEBOOM, G. H., and W. C. SCHNEIDER. 1950. *J. Biol. Chem.* **186**:417.
53. REYNAFARJE, B., and V. R. POTTER. 1957. *Cancer Res.* 17:1112.
54. VIGNAIS, P. V., and P. M. VIGNAIS. 1957. *J. Biol. Chem.* **229**:265.
55. ERNSTER, L. 1958. *Acta Chem. Scand.* **12**:600.
56. PHILLIPS, A. H., and R. G. LANGDON. 1962. *J. Biol. Chem.* **237**:2652.
57. KAPLAN, N. O., S. P. COLOWICK, and E. F. NEUFELD. 1953. *J. Biol. Chem.* **205**:1.
58. DALLNER, G. 1963. *Acta Pathol. Microbiol. Scand. Suppl.* **166**:
59. PARSONS, D. F. 1965. *Intern. Rev. Exp. Pathol.* **4**:1.
60. FERNÁNDEZ-MORÁN, H. 1962. *Circulation.* **26**: 1039.
61. ORRENIUS, S., and J. L. E. ERICSSON. 1966. *J. Cell Biol.* **31**:243.

62. OHNISHI, T., G. L. SOTTOCASA, and L. ERNSTER. *Bull. Soc. Chim. Biol.* In press.
63. PARSONS, D. F., G. R. WILLIAMS, W. THOMPSON, D. F. WILSON, and B. CHANCE. *In* Round Table Discussion on Mitochondrial Structure and Compartmentation, Bari. 1966. E. Quagliariello, E. C. Slater, S. Papa, and J. M. Tager, editors. Adriatica Editrice, Bari. In press.
64. ERNSTER. L. *In* Round Table Discussion on Mitochondrial Structure and Compartmentation, Bari. 1966. E. Quagliariello, E. C. Slater, S. Papa, and J. M. Tager, editors. Adriatica Editrice, Bari. In press.
65. SOTTOCASA, G. L., B. KUYLENSTIERNA, L. ERNSTER, and A. BERGSTRAND. 1967. *Meth. Enzymol.* **10.** In press.