Synthesis, Transfer, and Specific Binding of Purified L-[^{35}S]Methionine-Labeled Rat Liver Mitochondrial Adenosine Triphosphatase and Its Subunits to Mitochondrial Inner Membrane†

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ABSTRACT: Rat liver mitochondrial ATPase, labeled with L-[^{35}S]methionine was isolated and purified. Time-dependent incorporation of L-[^{35}S]methionine was monitored with polyacrylamide electrophoresis. Cycloheximide inhibited methionine incorporation into ATPase 86%, which implies extramitochondrial ATPase synthesis on cytoribosomes of rat liver cells. [^{35}S]ATPase, dissociated by cold treatment into subunits, was transferred *in vitro* to the inner mitochondrial membrane to a greater extent than native [^{35}S]ATPase. Chloramphenicol did not affect the transfer of [^{35}S]microsomal proteins or native [^{35}S]ATPase to the inner mitochondrial membrane. Inner mitochondrial membrane specifically bound greater quantities of [^{35}S]ATPase or its subunits than did outer membrane.

The biogenesis of mitochondria is a challenging aspect of current work on cell organization, replication, and growth (Ashwell and Work, 1970; Haldar *et al.*, 1966; Freeman *et al.*, 1967; Kadenbach, 1966; Beattie *et al.*, 1966). It has been found that a very high percentage of mitochondrial proteins is synthesized on the cytoplasmic ribosomes with subsequent transfer to the mitochondria. This transfer process is enigmatic for it involves relatively large proteins penetrating a membrane that restricts transfer of small inorganic and organic molecules (Chappell and Haarhoff, 1966) and even protons (Mitchell and Moyle, 1969). Tzagoloff (1969) has shown that, in yeast, mitochondrial ATPase is synthesized by the cytoplasmic–ribosome protein synthesizing system and transferred to the mitochondria. Kadenbach (1966) reported a transfer of protein from microsomes to mitochondria *in vitro* but, as will be pointed out in this paper, there are reasons to question the interpretation of his data.

The development of methods for the isolation of highly purified rat liver mitochondrial ATPase (ATP phosphohydrolase, E.C. 3.6.1.3) (Lambeth and Lardy, 1971) and of methods reported in this paper to label this protein *in vivo* provided an opportunity to study the incorporation of this radioactive enzyme into isolated liver mitochondria. This enzyme is especially suitable for studying protein transfer into mitochondria, since it may be employed in its native state of molecular weight 360,000 (Lambeth and Lardy, 1971) or as subunits of maximum molecular weight 53,000 (Lambeth and Lardy, 1971; Senior and Brooks, 1971) derived by cold dissociation (Penefsky and Warner, 1965) of the parent enzyme.

Materials and Methods

Preparation of L-[^{35}S]*Methionine-Labeled Native and Cold-Dissociated ATPase.* ATPase was isolated by the procedure of Lambeth and Lardy (1971) from the livers of female rats, Badger Research Co. (hysterectomy-derived, Sprague–Dawley strain), 200–250 g, fasted for 24 hr before intraperitoneal injection of L-[^{35}S]methionine (specific radioactivity, either 0.51 or 20 Ci/mmole) (Amersham/Searle) and killed 2 hr later. Mitochondria for the ATPase isolation were separated at 15,000*g* for 5 min. Some microsomal contamination may be responsible for the relatively high radioactivity in the mitochondria shown in Table I.

Liver homogenates were prepared with 0.25 M sucrose (pH 7.4) at 1:6 (w/v). Microsomes, mitochondria, postmicrosomal supernatant fraction, mitochondrial inner and outer membranes, and soluble fraction were prepared according to Sottocasa *et al.* (1967).

Isolation, purification, and quantitative assay of labeled proteins were described previously (Hochberg *et al.*, 1972).

Identical quantities (300 μg) of isolated purified native mitochondrial ATPase, mitochondrial inner membrane, and postmicrosomal supernatant proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (7.5%, pH 7.1) (column size, 0.6 × 14 cm) as described by Schnaitman (1969). The protein bands were cut out, homogenized in 0.5 ml of 30% H_2O_2, and heated for 1–2 hr at 70°, placed quantitatively on circular Whatman No. 3MM paper, dried under heat, and counted as described previously (Hochberg *et al.*, 1972).

ATPase activity was determined as described by Lambeth and Lardy (1971).

The radioactive ATPase was stored at room temperature in a buffer (pH 7.3) containing 0.1 M KCl–20 mM potassium phosphate–2 mM ethylenediaminetetraacetate–4 mM ATP at 7.65 to 10.7 mg/ml. Enzyme specific activity was 100–110 (μmoles of P_i liberated times mg of protein^{-1} times min^{-1}). cd-ATPase[1] of the same original enzyme activity was prepared by storing ATPase in the absence of ATP for 3 hr at 0° immediately prior to use. Amounts of cd-ATPase bound to mitochondria were calculated assuming uniform labeling

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[1] Abbreviation used is: cd-ATPase, cold-dissociated ATPase or ATPase subunits.

TABLE I: Purification of ^{35}S-Labeled Rat Liver Mitochondrial ATPase.[a]

Purification Step	Total Protein (mg)	Radioactivity (cpm/mg)	ATPase Activity[b]
1. Mitochondria after freezing and thawing	1000	8100	4.0
2. Membrane-bound ATPase	400	7600	8.1
3. Solubilized ATPase	220	5100	11.4
4. Protamine sulfate fractionation	18.5	3300	81.0
5. DEAE–Sephadex chromatography	10.0	2000	104.0

[a] Rats were injected with 83.3 μCi (0.2 μmole) of L-[^{35}S]-methionine (specific radioactivity of 510 μCi/mole) for 2 hr. [b] μmoles of P_i liberated × mg of protein^{-1} × min^{-1}.

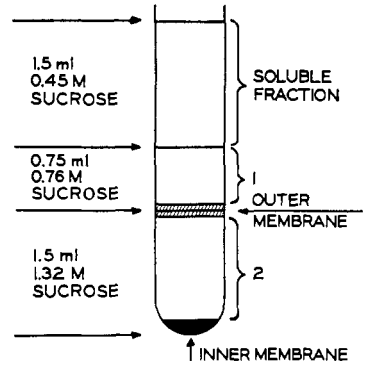

FIGURE 1: Discontinuous density gradient separation of mitochondrial membranes.

of all subunits or uptake of subunits in amounts proportional to their occurrence in the native enzyme.

Preparation of ^{35}S-Labeled Microsomes and Postmicrosomal Supernatant. Microsomes were pelleted (105,000*g* for 1 hr) from the supernatant fraction remaining after collecting labeled mitochondria. The labeled microsomes were suspended in a minimal volume of Kadenbach's medium B (1966) (0.15 M sucrose–70 mM KCl–9 mM $MgCl_2$–35 mM Tris-HCl, pH 7.6), and the labeled postmicrosomal supernatant was reserved for the "transfer reactions," (Table VI).

Nonradioactive Mitochondria, Microsomes, and Postmicrosomal Supernatant. Female rats, Badger Research Co. (hysterectomy-derived, Sprague–Dawley strain), 200–250 g, were either fasted or fed as noted in each experiment. Liver homogenates were prepared with 10 volumes of 0.25 M sucrose (pH 7.4)–20 mM Tris-HCl–1 mM EDTA for isolation of nonradioactive mitochondria–microsomes–postmicrosomal supernatant used in the "transfer reactions" (Table VI). Mitochondria were sedimented at 6500*g* for 5 min and washed three times with 0.25 M sucrose (pH 7.4)–20 mM Tris-HCl, and final sedimentation was at 15,000*g* for 10 min. Mitochondria were suspended in a minimal volume of washing medium. Microsomes, sedimented at 105,000*g* for 1 hr, were suspended in a minimal volume of medium B.

Microsomes for the "radioactive ATPase transfer reaction" (Table V) were prepared according to Kadenbach (1966) with some modifications. Liver was homogenized in 2.5 volumes of medium B. The homogenate was centrifuged at 15,000*g* for 10 min, and the supernatant fraction was centrifuged at 105,000*g* for 60 min. The sedimented microsomes were washed once and suspended in a minimal volume of medium B.

Preparation of Sonicated Mitochondria, Inner and Outer Mitochondrial Membranes, and Mitochondria Stripped of Their Outer Membrane. Mitochondria were subjected to sonic oscillation with a Branson oscillator at 3 A for 30 sec in 10 ml of 0.25 M sucrose (pH 7.4). Inner and outer mitochondrial membranes were prepared according to Sottocasa *et al.* (1967). Purity was monitored according to Hochberg *et al.* (1972). Mitochondria, stripped of their outer membrane, were prepared according to Parsons and Williams (1967).

All transfer reactions were performed at 37° for 1 hr in a Dubnoff shaker. Two-minute thermal equilibration was allowed before radioactive microsomes, postmicrosomal supernatant, ATPase, or cd-ATPase was added. The incubation system (Kadenbach, 1966) contained 115 mM sucrose–42 mM KCl–5.4 mM $MgCl_2$–30 mM Tris-HCl (pH 7.6)–2.5 mM phosphate. Cofactors (1 mM ATP, 0.2 mM GTP, and 10 mM α-ketoglutarate) were added when indicated. Intact and sonicated mitochondria, inner and outer mitochondrial membrane, and "stripped mitochondria" were incubated in 0.25 M sucrose (pH 7.4).

Sonication and Fractionation Procedures after Incubation. When appropriate, immediately after incubation, the reaction mixtures were centrifuged at 15,000*g* for 10 min; the mitochondria and "stripped mitochondria" were washed with 5 ml of 0.25 M sucrose (pH 7.4) and recentrifuged at 15,000*g* for 10 min. The 0.25 M sucrose wash of the "stripped mitochondria" contained no detectable radioactivity.

Mitochondria from each reaction mixture were mixed with 1.1 ml of "swelling medium," and, after 5 min, 0.4 ml of "shrinking medium" (Sottocasa *et al.*, 1967) was added. "Stripped mitochondria" were diluted with 1.5 ml of 0.25 M sucrose (pH 7.4). Mitochondria were sonicated at 10 A for 10 sec. Mitochondria and "stripped mitochondria" were layered on a gradient formed in a Spinco 305050 cellulose tube as shown in Figure 1. Gradients were centrifuged at 27,000 rpm for 180 min in a Spinco SW 39L rotor.

Incubated sonicated mitochondria were diluted to 1.5 ml with sucrose, pH 7.4, to produce a concentration of 0.45 M and layered directly on the gradient as shown in Figure 1.

Inner and outer membranes were diluted with 0.25 M sucrose (pH 7.4) to a total volume of 9 ml and centrifuged at 198,000*g* for 10 min in a Spinco 50 rotor.

Determination of Radioactivity. Sample aliquots were placed on paper as previously described (Hochberg *et al.*, 1972). Inner membranes were solubilized in a minimal volume of 1 N NaOH, an equal volume of Triton X-100 was added, and the total volume was counted.

Antibiotics and Hormones. Chloramphenicol (Sigma) was added to the "transfer reaction" in a concentration of 137 μg/ml and 374 μg/ml. Glucagon (Lilly) and thyroxine (Na salt) (Sigma) were dissolved in saline and injected intraperitoneally at 200 μg/100 g and 2 mg/100 g body weight, respectively.

All reagents and chemicals were of the highest purity available.

Mitochondrial swelling was studied with 4 mg of mitochondrial protein in a final volume of 1.3 ml of medium adjusted

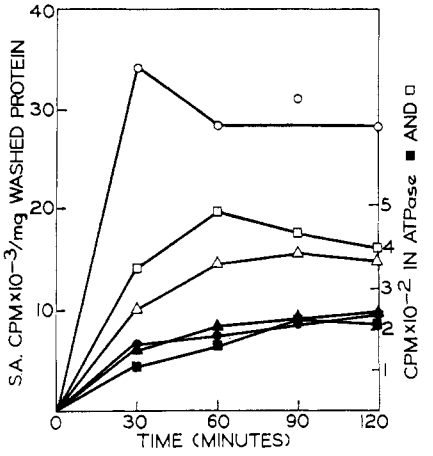

FIGURE 2: Time-dependent incorporation of L-[^{35}S]methionine into proteins of different cell fractions and rat liver ATPase. ●—●, mitochondria; ○—○, microsomes; △—△, postmicrosomal supernatant fraction; ▲—▲, inner membrane; ■—■, mitochondrial ATPase subunits (bands 10 and 11 from inner membrane, Figure 3); □—□, postmicrosomal supernatant-ATPase subunits (bands 10 and 11, Figure 3). Rats were injected with L-[^{35}S]methionine (153 μCi, 7.5 μmoles, specific radioactivity 20 Ci/mmole). Labeled mitochondrial, microsomal, and postmicrosomal supernatant proteins were isolated as described in Materials and Methods. Mitochondrial inner membrane and postmicrosomal supernatant proteins were separated by sodium dodecyl sulfate-gel electrophoresis. The protein band which had the same mobility as the major subunits of isolated purified mitochondrial ATPase was counted as described.

to different osmolarity and containing cofactors as noted in the legend to Figure 4. Light absorption changes at 565 nm were followed in 1-cm cells with a Gilford spectrophotometer Model 2000, thermostated at 37°.

Result

L-[^{35}S]Methione-Labeled Native ATPase. Total protein, radioactivity, and ATPase activity for each purification step of ^{35}S-labeled native mitochondrial ATPase are shown in Table I. Specific radioactivity of the protein fractions diminished and the ATPase activity increased 26-fold during purification. These mitochondria were collected at 15,000*g* for 10 min; the retained microsomes probably contributed to the high initial apparent specific activity of the mitochondria and may be responsible for the diminishing specific activity during purification.

The incorporation of L-[^{35}S]methionine into mitochondria, mitochondrial subfractions, microsomes, postmicrosomal supernatant, and isolated native mitochondrial ATPase is compared in Table II. The specific radioactivity of the major cell components was highest in the microsomes, lower in the postmicrosomal supernatant and lowest in the mitochondria (6500*g* × 10 min). The inner membrane had the highest specific radioactivity of the three mitochondrial subfractions; however, the differences were nonsignificant since standard deviations overlapped. Isolated native ATPase specific radioactivity was similar to that of the three mitochondrial subfractions; however, it was lower than that of the whole mitochondria. This results from a loss of more highly radioactive soluble proteins in fraction 1 of the gradient (Figure 1).

Table III shows the distribution of radioactivity derived from L-[^{35}S]methionine in the labeled proteins from different cell components. The ratio of cysteine to methionine appears to be lower in the inner membrane fraction and is distinctly lower in the ATPase as compared with other liver cell fractions.

Microsomes incorporated L-[^{35}S]methionine at a faster rate and to a greater extent than any other cell component or fraction (Figure 2). Postmicrosomal supernatant-ATPase subunits and the postmicrosomal supernatant total proteins were similar in rates of incorporation and total activity. Mitochondria, isolated mitochondrial ATPase subunits, and inner membranes were similar and the lowest in their rate of incorporation and total activity.

Cycloheximide inhibited by 85–96% the incorporation of L-[^{35}S]methionine into all cell components and isolated native ATPase (Table IV). There was no significant difference in the total amount of injected radioactivity (free and bound) found in the liver homogenate of the cycloheximide-treated rats as compared to the controls (not shown).

TABLE II: Incorporation of L-[^{35}S]Methionine into Mitochondria, Microsomes, Postmicrosomal Supernatant, and Isolated Mitochondrial ATPase.[a]

Component	Specific Radioactivity (cpm/mg of protein) Mean	S[c]
Mitochondria	3860	±47
Microsomes	9700	±490
Postmicrosomal supernatant	6100	±30
Mitochondrial		
Soluble fraction	2500	±340
Outer membrane	2300	±380
Inner membrane	2900	±220
ATPase[b]	2500	±0

[a] For the incorporation studies, three individual rats were used. Rats were injected with 83.3 μCi (0.2 μmole) of L-[^{35}S]methionine (specific radioactivity of 510 μCi/μmole) for 2 hr. [b] Mitochondrial ATPase was prepared from nine pooled livers. One determination. [c] S = standard deviation.

TABLE III: Distribution of Radioactivity Derived from L-[^{35}S]Methionine in the Labeled Proteins.[a]

Component	% of Total Radioactivity in Protein Hydrolysates: Cysteic acid	Methionine Sulfone
Mitochondria	15.1	84.9
Microsomes	17.3	82.7
Postmicrosomal supernatant	16.5	83.4
Mitochondrial:		
Soluble fraction	17.1	82.8
Outer membrane	16.5	83.4
Inner membrane	13.2	86.2
ATPase	6.3	93.2

[a] Rats were injected as described in Table II.

TABLE IV: Effect of Cycloheximide on the Incorporation of L-[^{35}S]Methionine into Mitochondria, Microsomes, Postmicrosomal Supernatant, Inner Mitochondrial Membrane, and Mitochondrial and Postmicrosomal Supernatant ATPase.[a]

Components	Specific Radioactivity (cpm/mg of protein) Control	Cyclo-heximide	% Inhibition
Mitochondria	9,500	670	93.0
Microsomes	28,200	1580	94.4
Postmicrosomal supernatant	14,800	500	96.6
Inner membrane	9,500	1120	88.2
Mitochondrial ATPase	190	27	85.9
Postmicrosomal supernatant ATPase[b]	410	36	91.1

[a] Rats were injected with radioactivity for 2 hr as described in Figure 2. Cycloheximide (10 mg/rat) was injected together with the L-[^{35}S]methionine. Labeled cell fraction proteins were isolated as described. Mitochondrial and postmicrosomal supernatant-ATPase were separated as described. [b] ATPase is given as cpm in the separated protein bands 10 and 11.

Gel electrophoretic separation of radioactive inner membrane, postmicrosomal supernatant, and isolated native ATPase in the presence of sodium dodecyl sulfate are illustrated in Figure 3. Radioactivity was determined in all three major protein bands of purified ATPase. Three times as much radioactivity was found in bands 10 and 11 of isolated native ATPase as in all other portions of the gel. Radioactivity was found in protein bands 10 and 11 of both inner membrane and postmicrosomal supernatant which corresponds to the major subunits of isolated native radioactive ATPase.

Transfer and Binding of ^{35}S-Labeled ATPase. Radioactive ATPase protein was transferred to mitochondria in all reactions. In contrast to the suggestion of Kadenbach (1966), microsomes did not increase the transfer of ^{35}S-labeled native ATPase from the reaction medium to the inner mitochondrial membrane in the presence of all other components and cofactors (reaction A *vs.* B, Table V). Chloramphenicol did not significantly inhibit the transfer of labeled native ATPase from the reaction medium to the inner mitochondrial membrane (reaction D *vs.* A, Table V). Maximum transfer of ^{35}S-labeled native ATPase occurred when cofactors were absent (reaction C, Table V) which may be related to the fact that mitochondrial swelling was greatest in the absence of the cofactors (Figure 4).

Thirty per cent of the radioactivity of ^{35}S-labeled proteins of the microsomes were transferred to the mitochondria in the absence of postmicrosomal supernatant and cofactors (reaction a, Table VI). The addition of postmicrosomal supernatant (reaction b) or cofactors (reaction c) or both (reaction d) reduced the total radioactivity transferred from microsomes to mitochondria. Chloramphenicol (reaction e) failed to inhibit this transfer of microsomal proteins. Labeled postmicrosomal supernatant proteins (reaction f) were transferred to the mitochondria when all components and cofactors were present but only to a small extent (1.2%). Maximum transfer occurred in the absence of cofactors which is again ascribed to the swelling of mitochondria (Figure 4) occurring under these conditions.

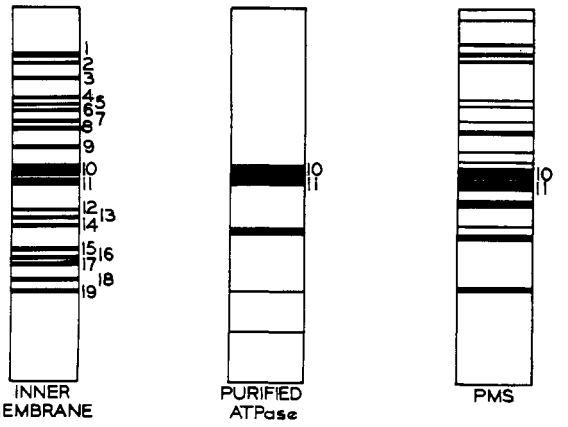

FIGURE 3: Polyacrylamide gel electrophoresis of inner mitochondrial membrane, mitochondrial ATPase, and postmicrosomal supernatant fraction (7.5% acrylamide, 0.1% sodium dodecyl sulfate, pH 7.1; 300 μg of protein/gel; column size, 0.6 × 14 cm).

The transfer of cd-ATPase from the reaction medium to the inner mitochondrial membrane was greatly increased in comparisons with the transfer of the native enzyme (Table VII). The addition of microsomes, postmicrosomal supernatant, and cofactors inhibited the transfer of both native ATPase and cd-ATPase to the inner mitochondrial membrane. The cofactors greatly diminish mitochondrial swelling (Figure 4).

TABLE V: Requirements for the Transfer of ^{35}S-Labeled Native Purified ATPase from the Reaction Medium to the Inner Membrane of Isolated Intact Mitochondria.[a]

Reaction	Microsomes	Cofactors	Labeled ATPase in Total Mitochondrial Fraction (μg) Soluble Mean	Soluble S[b]	Inner Membrane Mean	Inner Membrane S
A	+	+	1	±1.2	6.3	±4.9
B	−	+	5	±6.3	4.3	±2.5
C	+	−	2	±2.5	16.0	±1.0
D	+	+	0	0	3.3	±0.6

[a] Triplicate incubations contained 2 ml of the standard reaction mixture, mitochondria (8.7 mg of protein), postmicrosomal supernatant (10.1 mg of protein) and the native purified ^{35}S-labeled ATPase, 1.1 mg of protein (specific radioactivity 3000 cpm/mg of protein, enzyme specific activity 100–110 μmoles of $P_i \times min^{-1} \times mg^{-1}$), and when indicated: microsomes, 1.68 mg of protein, and cofactors. When microsomes and cofactors were omitted, their volume was replaced by medium B. Reaction D included chloramphenicol (137 μg/ml). Total volume in the reaction mixture was 2.4 ml. Mitochondria were reisolated, sonicated, and subfractionated on sucrose gradient (Figure 1). No radioactivity was detected in the outer membrane fraction. Fractions 1 and 2 were not assayed for radioactivity. Cell components were derived from rats fasted 24 hr. These are mean values with indicated standard deviations. Reaction mixtures contained 1240 (±120) and supernatant (unbound) 1200 (±74) μg of labeled ATPase. [b] S, standard deviation.

TABLE VI: Transfer of ^{35}S-Labeled Proteins from Microsomes or Postmicrosomal Supernatant to Isolated Intact Mitochondria.[a]

Reaction	Post-microsomal Super-natant	Cofactors	Radioactivity in Total Fraction: Supernatant (cpm) (unbound) Mean	Range[b]	Mitochondrial Fractions (cpm): Soluble Mean	Soluble Range	Outer Mean	Outer Range	Inner Mean	Inner Range	% of Total Radioactivity Transferred to Mitochondria
a	−	−	15,800	1,800	2570	800	2350	170	8990	3970	30.9
b	+	−	16,100	120	1590	360	1180	600	7300	520	24.6
c	−	+	23,000	7,200	2230	480	2610	870	4840	1080	19.1
d	+	+	22,800	2,400	1260	550	1160	160	5400	810	17.4
e	+	+	21,500	1,900	1670	440	1380	290	4220	800	16.8
f	+	+	423,800	25,000	2120	310	630	320	2520	1500	1.2

[a] Duplicate incubations contained 2 ml of the standard reaction mixture; mitochondria (8 mg of protein) and microsomes, 1.6 mg of protein, containing, in experiments a–e inclusive, 45,000 cpm of ^{35}S. In experiment f, nonlabeled microsomes were used. Other additions include: postmicrosomal supernatant, 10 mg of protein in experiment f this fraction was labeled with 434,000 cpm of ^{35}S; cofactors. Chloramphenicol, 274 μg/ml, was present in experiment e. Sucrose, 0.25 M (pH 7.4), replaced postmicrosomal supernatant or cofactors and medium B replaced microsomes when these three fractions were omitted. Total volume in the reaction mixture was 2.7 ml. Labeled microsomes and postmicrosomal supernatant were prepared from rats fasted for 24 hr prior to intraperitoneal injection of L-[^{35}S]methionine (244 μCi/0.83 μmole was given in 1 ml of saline to each rat). Unlabeled mitochondria, microsomes, and postmicrosomal supernatant were prepared from rats fasted for 24 hr. Reaction mixtures contained 45,000 (±4800) and 434,000 (±27,500) cpm of ^{35}S for microsomes and postmicrosomal supernatant, respectively. Mitochondria were reisolated, sonicated, and subfractionated on sucrose gradient (Figure 1). [b] Range, the difference between the highest and lowest values.

Exposure of the inner mitochondrial membrane by sonication prior to incubation increases the specific association of ^{35}S-labeled native ATPase and cd-ATPase with the inner mitochondrial membrane when compared to intact mitochondria (Table VIII). cd-ATPase associated with the inner mitochondrial membrane of sonicated mitochondria to a greater extent than does native ATPase.

Purified inner mitochondrial membrane associated with four times the amount of ^{35}S-labeled native ATPase and cd-ATPase as did the purified outer mitochondrial membrane (Table IX). However, there was no difference between native ATPase and cd-ATPase binding to either the purified inner or the outer mitochondrial membrane which differs from the results obtained with sonicated mitochondria or "stripped mitochondria." Differences in binding in Tables VIII, IX, and X may be due to differences in exposure of the inner side of the inner membrane produced by various treatments.

Purified isolated mitochondria, stripped of their outer membrane and washed prior to gradient separation, associated with twice as much ^{35}S-labeled cd-ATPase as native ATPase (Table X).

Transfer of native as well as of cd-ATPase to the inner membrane of whole mitochondria was not facilitated (Table XI) by pretreatment of rats with glucagon or thyroxin.

The influence of osmolarity, cell proteins, cofactors, and hormones on mitochondrial swelling is shown in Figure 4. Identical reaction mixtures as used in the presence of ^{35}S-labeled native ATPase and cd-ATPase were used to study mitochondria over a 60-min period. Changes in osmolarity in the presence of cofactors did not affect mitochondrial swelling. Mitochondria alone in sucrose or with postmicrosomal supernatant and microsomes reached maximum swelling by 15 and 30 min, respectively. However, the addition of cofactors inhibited mitochondrial swelling in the presence of postmicrosomal supernatant and microsomes. Glucagon treatment appeared to reduce mitochondrial swelling to a greater extent than thyroxin treatment.

Discussion

Synthesis of Labeled Native ATPase. One of the aims of research in mitochondrial protein synthesis is to trace the incorporation of a radioactive amino acid into a defined protein rather than into total mitochondrial proteins (Roodyn *et al.*, 1961, 1962; Freeman *et al.*, 1967; Kadenbach, 1966). Antibiotics have served as useful tools in the elucidation of the mechanism of mitochondrial protein synthesis (Kroon, 1963; Ashwell and Work, 1968; Loeb and Hubby, 1968). Despite all efforts, it has not yet been precisely determined which and how many of the mitochondrial membrane and enzyme proteins are built in the mitochondria or are made by the cytoribosomes and transferred to the mitochondria.

We investigated the metabolism of L-[^{14}C]methionine and L-[^{35}S]methionine in rat liver (Hochberg *et al.*, 1972) and established that maximum incorporation into mitochondria and mitochondrial subfractions occurs *in vivo* after 2 hr. We have confirmed the incorporation of methionine into a defined protein by the isolation of native ATPase with labeled ^{35}S from methionine and by observing time-dependent incorporation by the use of gel electrophoresis. Isolated labeled native ATPase had similar specific radioactivity (Table II) and shows a similar time dependent incorporation of ^{35}S from methionine (Figure 2) as that of the inner membrane. These facts indicate that the mitochondrial proteins and ATPase labeled with ^{35}S from methionine have similar origins. Further substantiation of the hypothesis of Tzagoloff (1969), which indicates that synthesis of ATPase in yeast is of cytoribosomal origin, was shown in rat liver by the almost complete inhibition of

TABLE VII: Requirements for the Transfer of ^{35}S-Labeled Native Purified ATPase and Cold-Dissociated ATPase from the Reaction Medium to the Inner Membrane of Isolated Intact Mitochondria.[a]

Enzyme	Microsomes	Postmicrosomal Supernatant	Cofactors	Enzyme Bound by Inner Membrane (μg) Mean	Range
ATPase	−	−	−	3.8	0.8
ATPase	−	−	+	2.5	0.1
ATPase	+	+	+	1.6	0.6
cd-ATPase	−	−	−	34.6	2.4
cd-ATPase	−	−	+	12.4	1.1
cd-ATPase	+	+	+	8.6	6.8

[a] Duplicate incubations contained 2 ml of the standard reaction mixture; mitochondria (8.2 mg of protein); cold-dissociated or native purified ^{35}S-labeled ATPase, 0.4 mg of protein (specific radioactivity 5300 cpm/mg, specific activity 100–110 μmoles of $P_i \times min^{-1} \times mg^{-1}$); and when indicated: microsomes, 1.75 mg of protein; postmicrosomal supernatant, 9.8 mg of protein; and cofactors. When omitted, equal volumes of sucrose 0.25 M (pH 7.4), and medium B replaced postmicrosomal supernatant and microsomes, respectively. Total volume in the reaction mixture was 2.66 ml. Mitochondria, microsomes, and postmicrosomal supernatant were prepared from rats fasted for 12 hr. No radioactivity was detected in the soluble and outer membrane fractions of the mitochondria after separation on the gradient as in Figure 1. Fractions 1 and 2 were not assayed for radioactivity. Reaction mixtures contained 380 (±58) and supernatant (unbound) 320 (±57) μg of labeled ATPase or cd-ATPase.

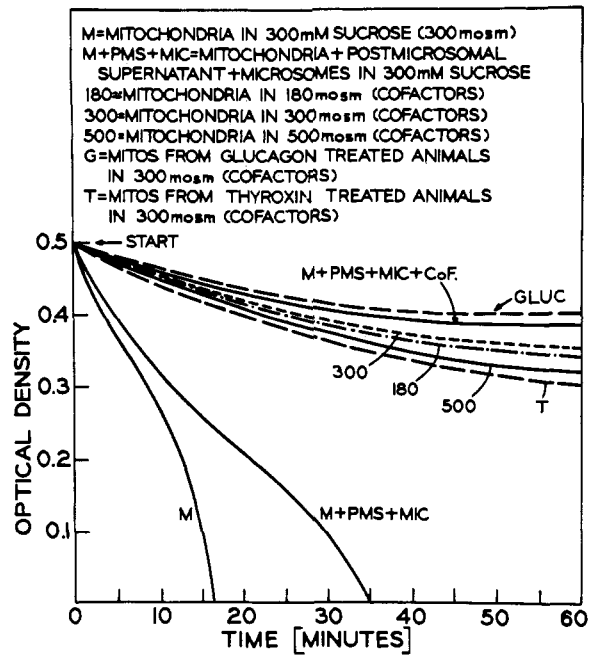

FIGURE 4: Mitochondrial swelling monitored through light absorption changes at 565 nm.

ATPase synthesis by cycloheximide in our experiment (Table IV). The same results are evident for postmicrosomal supernatant, since incorporation of ^{35}S into protein bands corresponding to the major components of isolated labeled native ATPase (Figure 3) was strongly inhibited by cycloheximide (Table IV).

The high ratio of cysteine to methionine in the inner membrane in comparison to that of the isolated native ATPase (Table III) reflects the low content of cysteine in rat liver ATPase (D. O. Lambeth and H. A. Lardy, unpublished data; Catterall and Pederson, 1971).

The rapid and high incorporation of ^{35}S from methionine into the microsomal fraction, the time-dependent change in ^{35}S radioactivity from methionine among cell components, and the inhibition of incorporation of ^{35}S from methionine by cycloheximide indicates that ATPase is synthesized in a manner similar to that reported for yeast, *i.e.*, on the cytoribosomes of the rat liver and transferred to the mitochondrial inner membrane.

Transfer and Binding of Labeled Native ATPase and Its Subunits. The transfer of ^{35}S-labeled proteins from the microsomes to the mitochondria *in vitro* is dependent on the integrity of the mitochondrial membranes (Figure 4). When cofactors are absent, this integrity is destroyed, thereby allowing labeled proteins to bind to the mitochondrial components. The addition of postmicrosomal supernatant did not stabilize the membranes; however, it seems that some labeled microsomal proteins combined in an unknown fashion with postmicrosomal supernatant proteins. Therefore, Kadenbach's (1966) assumption that detachment of labeled proteins from microsomes is greater when mitochondria replace the cell sap and the ATP-generating system is not valid since the incorporation of radioactivity under this condition may have been due to the binding of labeled proteins, which were still attached to microsomes, to the inner membrane from swollen and ruptured mitochondria.

Postmicrosomal supernatant proteins, labeled with ^{35}S from methionine were transferred to mitochondrial components in only minute amounts (1.2%), substantiating Kadenbach's data (1966), but not necessarily his conclusion that microsomes are essential. One may question whether the radioactivity transferred from the postmicrosomal supernatant to mitochondria was in part ATPase of molecular weight 380,000 (Lambeth and Lardy, 1971; Catterall and Pederson, 1971) or subunits of ATPase. Since there was no indication of the presence of the 380,000 molecular weight ATPase in rat liver postmicrosomal supernatant as determined in our laboratory by the use of aurovertin (Lardy *et al.*, 1964), one must assume that only subunits are present. Furthermore, only very small quantities of ^{35}S-labeled native purified ATPase or labeled microsomal proteins are transferred and bound to the mitochondrial inner membrane unless the mitochondrial inner membrane integrity is destroyed through swelling.

Chloramphenicol (Kroon, 1963; Ashwell and Work, 1968) had no effect on the transfer of ^{35}S-labeled proteins from microsomes to mitochondrial inner membranes or the transfer of ^{35}S-labeled native purified ATPase to the inner membrane. These results are at variance with the inhibition shown in yeast cells during derepression (Tzagoloff, 1969). However, ATPase activity accumulates in the postmicrosomal supernatant in yeast under chloramphenicol inhibition. In our laboratory, polyacrylamide gel separation of rat liver postmicrosomal supernatant proteins indicated that ATPase was present, but it may be only an inactive form since no ATPase

TABLE VIII: Transfer of ^{35}S-Labeled Native Purified ATPase and Cold-Dissociated ATPase from Sucrose Medium (0.25 M, pH 7.4) to Inner Membranes of Intact and Sonicated Mitochondria.[a]

		Labeled ATPase or cd-ATPase in Total Fraction (μg)									
		Separation of Mitochondrial Membranes									
		Soluble		Fraction 1		Outer		Fraction 2		Inner	
Mitochondria	Enzyme	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range
Intact	ATPase	0	0			0	0			2.2	0.6
Intact	cd-ATPase	0	0			0	0			8.1	2.0
Sonicated	ATPase	180	17	84	2	3.9	5.8	0	0	14.6	4.0
Sonicated	cd-ATPase	120	29	56	3	14.0	6.0	0	0	34.1	8.0

[a] Duplicate incubations contained 1 ml of total volume of 0.25 M sucrose (pH 7.4), and when indicated: mitochondria (8 mg of protein) or sonicated mitochondria (8 mg of protein) and cd-ATPase or native purified ^{35}S-labeled ATPase, 0.4 mg of protein of the same radioactive and enzyme activity as shown in Table VII. Mitochondria were prepared from rats fasted 24 hr. Reaction mixtures and supernatant (unbound) contained 310 (±21) and 300 (±39) μg of labeled ATPase or cd-ATPase, respectively.

activity was detected. Studies on transfer of ATPase in intact liver cells with chloramphenicol have not been reported. However, we (Hochberg *et al.*, 1972) have shown that chloramphenicol does preferentially inhibit the *in vivo* incorporation of ^{14}C from methionine into rat liver mitochondrial inner membrane (46–48%), whereas only slight inhibition (9–10%) occurs when the methionine is labeled with ^{35}S. Similar, but minor, inhibition for both isotopes occurred in postmicrosomal supernatant (10–16%) and microsomes (0–15%). On the other hand, cycloheximide inhibits completely the *in vivo* incorporation of both ^{14}C and ^{35}S from methionine. If one accepts the existence of mitoribosomes, despite the fact that purity of these preparations must be questioned (Malkin, 1971a,b), then our data suggest that chloramphenicol specifically inhibits some reaction of the metabolism of the methyl group of the methionine on the mitoribosomal level. The synthesis of protein from [^{14}C]-methionine which was inhibited by chloramphenicol may be related to the subunit proteins postulated to be the membrane factor synthesized by mitoribosomes in yeast (Tzagoloff, 1971). The fact that ATPase is not synthesized in cycloheximide-treated animals could also reflect mitochondrial synthesis under cytoplasmic control. Significantly greater quantities of ^{35}S-labeled cd-ATPase than native ATPase were transferred from the reaction mixture and bound to the mitochondrial inner membrane of intact or sonicated mitochondria under all conditions. The cold treatment (0° for 3 hr) of native purified ATPase is known to cause dissociation into subunits (Penefsky and Warner, 1965). The increased transfer of ATPase subunits is most likely a reflection of their smaller molecular weight (Lambeth and Lardy, 1971; Catterall and Pedersen, 1971), which allowed them to permeate the outer

TABLE IX: Transfer of ^{35}S-Labeled Native Purified ATPase and Cold-Dissociated ATPase from Sucrose Medium (0.25 M, pH 7.4) to Isolated Outer or Inner Mitochondrial Membranes.[a]

		Enzyme Bound by 6 mg of Membrane Protein	
Mitochondrial Membrane	Enzyme	Mean (μg)	Range (μg)
Outer	ATPase	32	2
	cd-ATPase	38	1
Inner	ATPase	162	4
	cd-ATPase	158	34

[a] Duplicate incubations contained 1 ml of total volume of 0.25 M sucrose (pH 7.4), and when indicated: outer membrane, 6 mg of protein; or inner membrane, 6 mg of protein. Cold-dissociated and native purified ^{35}S-labeled ATPase, 0.4 mg of protein, of the same radioactive and enzyme activity as shown in Table VII. Mitochondrial membranes were prepared from rats fasted 24 hr. Reaction mixtures contained 310 (±30) μg of labeled ATPase or cd-ATPase.

TABLE X: Transfer of ^{35}S-Labeled Native Purified ATPase and Cold-Dissociated ATPase from Sucrose Medium (0.25 M, pH 7.4) to Isolated Mitochondria Stripped of Their Outer Membrane.[a]

	Enzyme Bound by Isolated Mitochondria Stripped of Their Outer Membrane	
Enzyme	Mean (μg)	Range (μg)
ATPase	3.3	0.6
cd-ATPase	7.4	4.7

[a] Duplicate incubations contained a total volume of 1 ml of sucrose, 0.25 M (pH 7.4), and isolated mitochondria stripped of their outer membrane, 7 mg of protein. Cold-dissociated and native purified ^{35}S-labeled ATPase, 0.4 mg of protein, of the same radioactive and enzyme activity as shown in Table VII, was added. "Stripped mitochondria" were isolated from rats fasted for 24 hr. After incubation, mitochondria were centrifuged through the gradient shown in Figure 1. No radioactivity was detected in the area of the gradient where soluble or outer membrane would have been isolated. Reaction mixtures contained 300 (±28) and supernatant (unbound) 300 (±37) μg of labeled ATPase or cd-ATPase.

and inner mitochondrial membranes. Increased exposure or permeability to subunits of ATPase was also observed in the absence of cofactors when mitochondria swell and rupture.

Disruption of the mitochondria by sonication prior to incubation increased the binding of ATPase subunits to inner mitochondrial membrane when compared to native ATPase. The inner mitochondrial membrane of disrupted mitochondria also bound 3 to 4 times as much ATPase or ATPase subunits as that of intact mitochondria.

The difference in degree of [^{35}S]ATPase binding to outer membrane and soluble fractions of mitochondria, which were sonicated prior to incubation, as compared to membranes of incubated intact mitochondria could be due to reaggregation of membrane particles during the time of incubation, thereby preventing complete separation *via* the sucrose gradient.

The binding of native ATPase or ATPase subunits to the inner membrane was specific when compared to the outer membrane. A higher binding of ATPase subunits to inner membrane is obtained as compared to the native ATPase under all conditions except in the case of isolated outer and inner membranes. Exposure of the inner side of the inner membrane removes the restriction for permeation, allowing equal quantities of ATPase or subunits to bind.

The greater binding of ATPase subunits to the mitochondria from which the outer membrane had been stripped suggests that the subunits can permeate to the inner side of the inner membrane whereas native ATPase cannot. The increase in binding to isolated inner membranes also suggests that the inner side of the inner membrane is the main binding site for ATPase. The similar results from intact mitochondria incubated in sucrose or in reaction medium with cofactors also suggests that the inner membrane is the barrier to the permeation of native ATPase.

Hormones are known to alter mitochondrial configuration (Tapley, 1956) and possibly membrane function (Tapley and Cooper, 1956; Rosa, 1971), but treating rats with glucagon or thyroxine failed to alter the transfer and binding of either ATPase subunits or native ATPase to the inner mitochondrial membrane when compared to untreated controls.

Integrity of the mitochondrial membrane system is of utmost importance in evaluating transfer or exchange reactions (Tapley, 1956). Our studies indicate that conclusions cannot be drawn unless swelling studies of mitochondria are conducted with all reaction mixtures. This is clearly evident where native ATPase was not significantly bound to the inner membrane unless the integrity of the mitochondrial membrane system was affected. Further studies were carried out to evaluate swelling of mitochondria under conditions used by Swanson (1971) for transfer of polynucleotides from the reaction mixture into the mitochondria. Mitochondria ruptured within 5 min under Swanson's conditions, suggesting that the incorporation of polynucleotides may have depended on specific association with mitochondrial binding sites that were exposed after rupture occurred. In fact, the reported time-dependent and linear uptake of polynucleotides corresponds closely to the time-dependent swelling of mitochondria observed.

The transfer of ATPase subunits and specific binding to inner membranes indicates that molecular weight is important. Our isolated ATPase may not be the true or biologically effective ATPase nor are the ATPase protein species produced by cold treatment necessarily those that are biologically transferred from microsomes to mitochondria. It is impossible to create *in vitro* conditions which simulate the active mobility of cell organelles *in vivo*, thus our transfer reactions may not reflect biological transfer within the cell. In eukaryotics, at least, there is a real question as to whether mitochondria have been purified to a high enough degree to prove that mitochondria really synthesize protein *in vitro* (Malkin, 1971-a,b). Data from our laboratory indicate that the current methodology used in evaluating *in vitro* mitochondrial protein synthesis is not accurate or meaningful, since denatured mitochondria incorporate some amino acids to the same or to a greater extent than intact mitochondria (unpublished data). Thus, the biological significance of mitochondrial protein synthesis and the mechanism of transfer of proteins through mitochondrial membranes is still not clearly understood.

TABLE XI: Transfer of ^{35}S-Labeled Native Purified ATPase and Cold-Dissociated ATPase from the Reaction Medium to the Inner Membrane of Intact Mitochondria from Non-fasted Hormone-Treated Rats.[a]

Treatment	Enzyme	Enzyme Bound by Inner Membrane	
		Mean (μg)	Range (μg)
None	ATPase	1.7	0.8
	cd-ATPase	13.4	0.0
Glucagon	ATPase	0.8	0.3
	cd-ATPase	10.8	0.3
Thyroxine	ATPase	2.6	2.6
	cd-ATPase	13.2	1.4

[a] Duplicate incubations contained 2 ml of the standard reaction mixture plus cofactors; microsomes, 1.6 mg of protein; mitochondria, 8 mg of protein; and postmicrosomal supernatant, 10 mg of protein. Total volume in the reaction mixture was 2.53 ml. Cell organelles were prepared from rats that had been injected intraperitoneally with glucagon (200 μg/100 g body weight) or thyroxine, Na salt, (2 mg/100 g of body weight), one hour before sacrifice and from normal saline injected controls. Cold-dissociated and native purified ^{35}S-labeled ATPase, 0.4 mg of protein, of the same radioactive and enzyme activity as shown in Table VII, was added. No radioactivity was detected in the soluble or outer membrane fraction from the gradient. Reaction mixtures contained 240 (±52) and supernatant (unbound) 240 (±48) μg of labeled ATPase or cd-ATPase.

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Poly(5-chlorocytidylic acid)†

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ABSTRACT: 5-Chlorocytidine diphosphate has been obtained in high yield by the chlorination of cytidine diphosphate using tetrabutylammonium iodotetrachloride. 5-Chlorocytidine diphosphate has been polymerized with polynucleotide phosphorylase and the resultant poly(5-chlorocytidylic acid) has been characterized. Poly(5-chlorocytidylic acid), which is more stable toward hydrolysis by pancreatic ribonuclease than the unchlorinated polynucleotide, possesses considerable secondary structure in acid solution and forms a 1:1 hybrid with poly(I). The thermal stability of the hybrid is considerably higher than that of poly(I)·poly(C).

The discovery that double-stranded polyribonucleotides *e.g.*, poly(I)·poly(C) can induce the formation of interferon (Field *et al.*, 1967; Colby, 1971) has encouraged the synthesis of polyribonucleotides which have been modified in the base (Michelson and Monny, 1967), the sugar (Hobbs *et al.*, 1971), and the phosphate backbone (Eckstein, 1970). Little systematic work has, however, been carried out on the relationship between structure and biological activity in these compounds.

Chemical modification of the pyrimidine bases in the polyribonucleotides can be readily accomplished as electrophilic attack occurs at the 5 position in pyrimidines and, for example, halogenation of pyrimidine nucleotides gives the 5-halogenonucleotides. Conversion of the latter to the pyrophosphates followed by polymerization with polynucleotide phosphorylase leads to 5-halogenopyrimidine polyribonucleotides (Michelson and Monny, 1967). The direct bromination of poly(C) to poly(5BrC) has been reported (Means and Fraenkel-Conrat, 1971) and the latter has also been prepared by the polymerization of 5BrCDP (Howard *et al.*, 1969). Introduction of a halogen atom in the 5 position of a pyrimidine has a pronounced effect on the physical properties of the polynucleotide and, for example, poly(5BrC) and poly(5IodoC) possess considerable secondary structure in acid solution (Michelson and Monny, 1967). 5-Substituted cytidine polyribonucleotides form stable hybrids with poly(I) (Ross *et al.*, 1971) and these are inducers of interferon (De Clercq *et al.*, 1970; Colby and Chamberlin, 1969). In the present investigation the hitherto unknown poly(5ClC) has been prepared and the physical properties of the polynucleotide and its complex with poly(I) have been investigated. Biological studies on the poly(5ClC)·poly(I) complex are in progress.

Materials and Methods

Materials. CDP was synthesized from the phosphoromorpholidate (Moffatt and Khorana, 1961) and was converted by ion-exchange chromatography into the trisodium salt before use. UDP, pancreatic ribonuclease type IA (EC 2.7.7.-16), and *Crotalus adamanteus* venom were purchased from Sigma Chemical Corp. Polynucleotide phosphorylase (poly-

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