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Incorporation of L-[*methyl*-^{14}C]- and [^{35}S]Methionine into Mitochondrial Proteins†

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ABSTRACT: The time-dependent uptake and incorporation of L-[*methyl*-^{14}C]methionine into mitochondria and other liver cell fractions were studied in rats. The metabolic fate of this amino acid and its distribution in mitochondria and other rat liver cell fractions were investigated with both ^{14}C- and ^{35}S-labeled methionine. Methionine is demethylated *in vivo* and cysteine formed from its ^{35}S is incorporated into the cell proteins. The ratio of labeled cysteine:labeled methionine was the same in all proteins analyzed. The influence of cycloheximide and chloramphenicol on the distribution and incorporation of ^{14}C and ^{35}S derived from methionine is reported. A quantitative distribution of ^{14}C and ^{35}S, derived from methionine in different cell fractions, is proposed.

For studies of protein synthesis and metabolism, methionine possesses unique advantages over other amino acids. It is an essential amino acid that is involved in the initiation of polypeptide synthesis (Clark and Marker, 1966), it donates methyl groups for nucleic acids and lipids (Mudd and Cantoni, 1957), and there is an excellent structural analog available in ethionine. The half-life time of mitochondrial subfractions has been studied with [^{35}S]methionine (Fletcher and Sanadi, 1961; Bailey *et al.*, 1967; Brunner and Neupert, 1968). Protein synthesis in mitochondria has been examined by the time sequence of *in vivo* labeling (Fletcher and Sanadi, 1961; Beattie, 1969), by *in vitro* incorporation of radioactive amino acids (Roodyn, 1962; Kroon, 1965), by inhibition *in vivo* and *in vitro* through cycloheximide and chloramphenicol (Ashwell and Work, 1970), and also by labeling of specific proteins (Omura *et al.*, 1967; Gonzalez-Cadavid and Campbell, 1967).

Our studies have involved the incorporation of ^{14}C and ^{35}S from methionine into mitochondrial and other cell proteins for a number of objectives. We wished to obtain additional information about the mechanisms involved in the synthesis of mitochondrial proteins, to obtain specifically labeled, purified enzymes from these sources, to study their transport and incorporation into mitochondrial membranes, and finally to investigate the mechanism by which testosterone and related compounds exert their anabolic effects.

Materials and Methods

Female rats (200–250 g) from Badger Research Co. (hysterectomy-derived, Sprague–Dawley strain) were used for all experiments. The rats were fasted for 24 hr before injections at indicated times.

L-[*methyl*-^{14}C]Methionine was purchased from New England Nuclear, L-[^{35}S]methionine from Amersham/Searle. All other chemicals were supplied from Mallinckrodt and Schwarz/Mann in highest available purity.

Fractionation Procedure. Liver homogenates were prepared with 5 volumes of 0.25 M sucrose (pH 7.4). Microsomes, mitochondria, inner and outer mitochondrial membranes, and mitochondrial-soluble fractions were prepared according to Sottocasa *et al.* (1967) using their designated centrifical forces and times.

Purity of Mitochondrial Subfractions. Monoamine oxidase activity, a marker enzyme for outer membrane, was determined with the benzylaldehyde method as described by Tabor *et al.* (1954) and with the [^{14}C]tryptamine assay described by Allman *et al.* (1968). Native mitochondrial proteins were separated on polyacrylamide gel electrophoresis according to Schnaitman (1969).

The specific radioactivity of the ^{35}S-labeled total protein of mitochondria is appreciably higher than that of the proteins of the mitochondrial subfractions (Tables II and IV). This could arise because of the loss of some more highly labeled protein component during the mitochondrial fractionation.

Isolation and Purification of Labeled Proteins. Aliquots of each of the different cell fractions containing approximately 100 mg of protein were treated with equal volumes of 10% trichloroacetic acid (w/v) and sedimented at 1700*g* for 10

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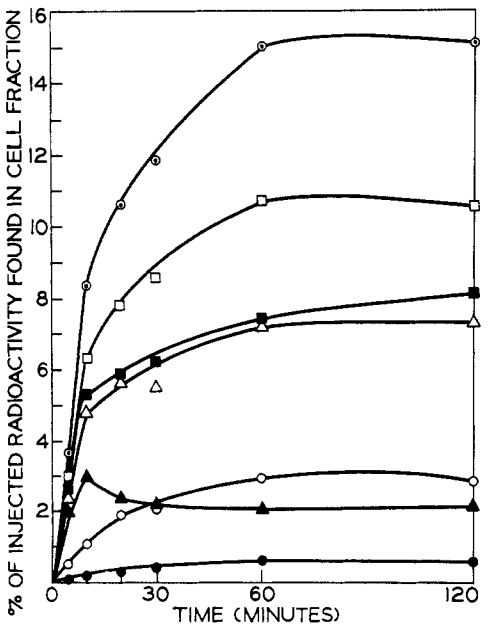

FIGURE 1: Uptake of radioactivity from L-[*methyl*-^{14}C]methionine into rat liver fractions. Each point represents one rat that was injected intraperitoneally with 83.3 μCi (8.96 μmoles) of L-[*methyl*-^{14}C]methionine, specific activity, 9.29 Ci/mole. Fractions were prepared as described in Materials and Methods. (⊙—⊙) whole homogenate; (□—□) 600*g*; (■—■) 6500*g*; (△—△) 15,000*g*; (▲—▲) 105,000*g*, supernatant fractions; (●—●) mitochondria; (○—○) microsomes. Livers were removed from rats at the indicated periods after injection of labeled methionine.

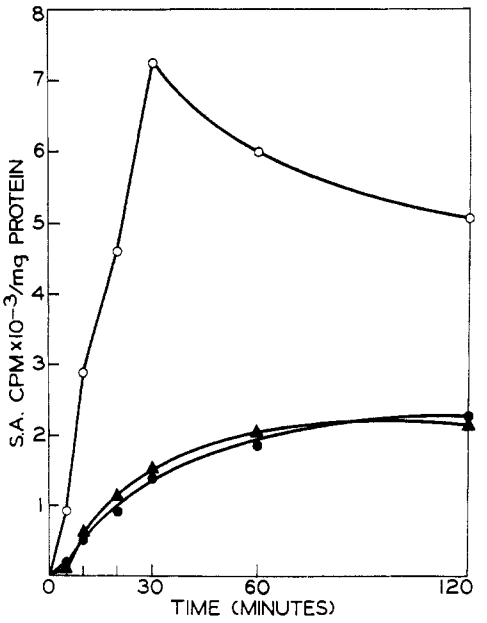

FIGURE 2: Time course of L-[*methyl*-^{14}C]methionine incorporation into mitochondrial, microsomal, and postmicrosomal supernatant proteins. Rats were injected as in Figure 1. (●—●) mitochondria; (○—○) microsomes; (▲—▲) postmicrosomal supernatant.

min; the supernatant fraction (5% trichloroacetic acid soluble) was saved, and the sediment was washed three times with 5% trichloroacetic acid, followed by suspension in 5 ml of 10% trichloroacetic acid, and heating for 30 min at 90° in a water bath. The suspension was centrifuged at 1700*g* for 10 min, the supernatant fraction (hot 10% trichloroacetic acid soluble) collected, and the sediment washed three times with 5% trichloroacetic acid. The precipitate was treated with ethanol–ether, 1:1 (v/v), heated in a water bath for 15 min at 40°, and centrifuged. The supernatant fraction (ethanol–ether soluble) was removed, and the protein precipitate was washed with hot ethanol–ether and with ether and air-dried.

The washed protein was dissolved first in 1 N NaOH followed by Triton X-100 (1:1, v/v), and reprecipitated with 10% trichloroacetic acid. The remaining protein was dissolved in 1 N NaOH, and the specific radioactivity determined. The supernatant solution of the last washing at every fractionation stage contained no measurable radioactivity. The first and second washings were also discarded because of their relatively low content of radioactivity.

Counting of Labeled Material. Sample aliquots were placed on circular Whatman No. 1 filter paper disks, dried under heat, and counted in 10 ml of toluene containing 0.1 g of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene and 4 g of 2,5-diphenyloxazole per liter with a Packard scintillation spectrometer, Model 3310; counting efficiency was 60%.

Protein Analysis. Proteins were determined with the biuret method (Layne, 1957). Radioactivity in N- and C-terminal amino acids of radioactive proteins was determined according to Suttie (1962). Performic acid oxidation of washed radioactive proteins was done according to Moore (1963). The performic acid was removed over P_2O_5 *in vacuo*. The dried, oxidized protein was hydrolyzed with 6 N HCl in a sealed ampoule and heated at 110° for 18 hr. The hydrolysate was dried *in vacuo* over NaOH.

Chromatography. Descending paper chromatography of the radioactive protein hydrolysate (*ca.* 10 mg of protein hydrolyzed) was performed on Whatman No. 3MM chromatography paper with butanol–acetic acid–water (60:15:25, volume basis). Figures on the abscissa designate the strip number. Paper strips (1 cm) were cut, and radioactivity was measured as described above.

Column chromatography was used to characterize methionine sulfone. Protein (performate oxidized) hydrolysate (40 mg) was separated at 50° on a 3.8 × 50 cm column, packed with sulfonated polystyrene resin (Bio-Rad), and eluted with 0.2 N citrate buffer (pH 3.3). The methionine sulfone peak was concentrated to minimal volume and counted.

Results

Distribution of Radioactivity from L-[*methyl*-^{14}C]*Methionine in Rat Liver Fractions.* The rates of uptake of radioactivity from intraperitoneally injected L-[*methyl*-^{14}C]methionine into rat liver fractions are shown in Figure 1. Extremely rapid labeling of the homogenate and the four supernatant fractions probably reflects the ingress of free methionine. Uptake of radioactivity into microsomes involves a smaller fraction of the total uptake and that into mitochondria is the least. Maximum uptake into all fractions is reached at 60 min, maintained until 120 min (Figure 1), and decreases slowly from 2 to 24 hr (not shown).

When the time-dependent incorporation of L-[*methyl*-^{14}C]-methionine into mitochondrial proteins is compared with the incorporation into microsomal proteins (Figure 2), striking differences appear, *i.e.*, the specific radioactivities of the microsomal proteins reach their highest peak by 30 min, and slowly decline thereafter. Mitochondrial and postmicrosomal supernatant proteins increase in radioactivity in a parallel manner over 120 min.

The labeling of the mitochondrial outer membrane pro-

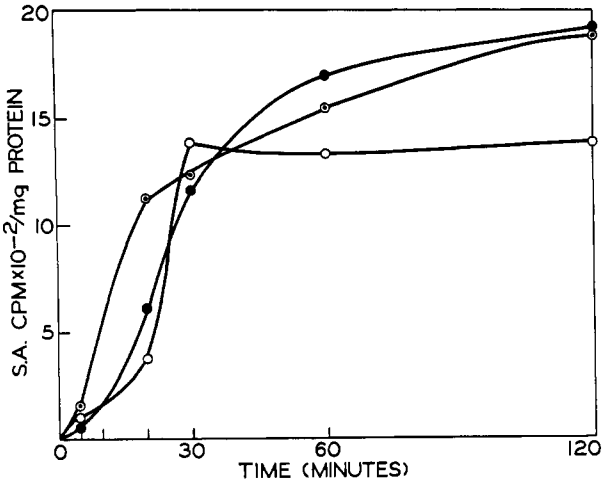

FIGURE 3: Time course of L-[*methyl*-^{14}C]methionine incorporation into proteins of the mitochondrial-soluble fraction and outer and inner membranes. Rats were injected as in Figure 1. Mitochondrial subfractions were prepared as described in Materials and Methods. (●—●) soluble fraction; (○—○) outer membrane; (⊙—⊙) inner membrane.

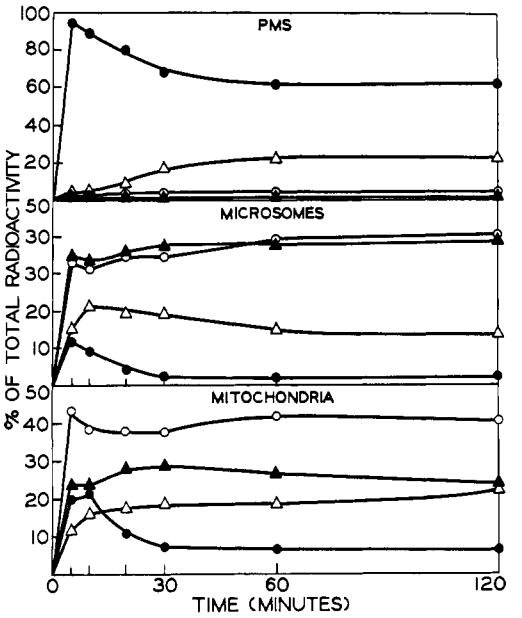

FIGURE 4: Distribution of radioactivity derived from L-[*methyl*-^{14}C]-methionine into different cell fractions. Rats were injected as in Figure 1. (●—●) 5% trichloroacetic acid soluble; (○—○) 10% hot trichloroacetic acid soluble; (▲—▲) ethanol–ether-soluble fractions; (△—△) protein.

teins reaches its maximum after 30 min and stays constant over the following 90 min (Figure 3), which is similar to that found in microsomes. The inner membrane proteins were labeled somewhat more rapidly than either the outer membrane or soluble mitochondrial proteins. After the initial 20 min of rapid incorporation, the inner membrane and soluble proteins slowly gained radioactivity throughout the remaining 2-hr period of observation.

The distribution of radioactivity in the various extracts of the postmicrosomal supernatant, microsomes, and mitochondria for the 120-min time study are presented in Figure 4. The 5% trichloroacetic acid supernatant fraction contains acid-soluble compounds including free radioactive amino acids. The 10% hot trichloroacetic acid fraction represents mainly radioactivity incorporated into nucleic acids. The ethanol–ether fraction contains all the soluble lipids and possibly lipoproteins.

In the postmicrosomal supernatant, maximum free acid soluble radioactivity is reached after 5 min, followed by a slight decrease to a constant plateau. Almost no radioactivity appeared in the hot 10% trichloroacetic acid or ethanol–ether extracts of the postmicrosomal supernatant.

The most rapid labeling of the microsomal fraction occurred in the 10% hot trichloroacetic acid and ethanol–ether fractions. Their rapid initial labeling was followed by a long, constant plateau. Protein labeling is slower and less; 5% trichloroacetic acid soluble radioactivity is very low.

The most apid and highest labeling of mitochondria with methyl-^{14}C takes place in the hot 10% trichloroacetic acid soluble fraction, whereas the ethanol–ether soluble fraction and the proteins are similar but lower. The free acid soluble radioactivity reaches a peak at 10 min and returns to a low but constant value.

The specific radioactivities of the proteins of mitochondria, 15,000*g* supernatant fraction, and postmicrosomal supernatant are relatively constant from 2 to 8 hr and decrease slowly during the next 16 hr (Figure 5); however, the microsomal proteins decrease somewhat more extensively. The percentage of radioactivity in the different extractions (5% trichloroacetic acid, 10% trichloroacetic acid, ethanol–ether) of the mitochondria, microsomes, and postmicrosomal supernatant remained constant over the 24-hr period (not shown).

There was no change in specific radioactivities of the proteins or the percentage of radioactivity of the different extractions (5% trichloroacetic acid, 10% trichloroacetic acid, ethanol–ether) of the inner and outer membrane and soluble fraction of the mitochondria (not shown).

It was shown by paper chromatography of hydrolysates that in all protein fractions methionine is the only amino acid which contains ^{14}C in significant amounts (Figure 6). Additional chromatography on a Beckman Amino Acid Analyzer gave essentially the same resolution. Approximately 3 and

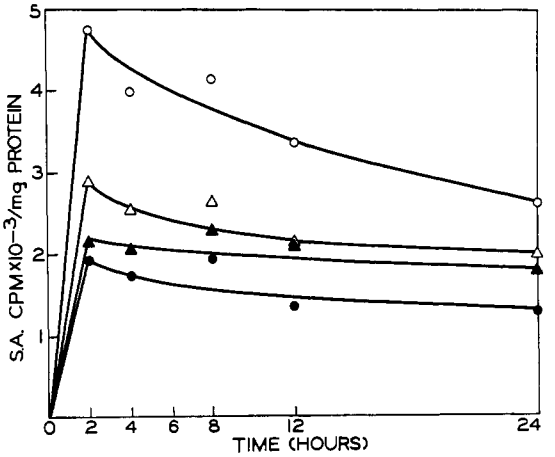

FIGURE 5: Time-dependent incorporation of L-[*methyl*-^{14}C]methionine into proteins of mitochondria, microsomes, postmicrosomal supernatant, and 15,000*g* supernatant. Livers of two rats were pooled for each indicated time after injection of 100 μCi (10.8 μmoles) of L-[*methyl*-^{14}C]methionine, specific activity, 9.29 Ci/mole. (●—●) mitochondria; (○—○) microsomes; (▲—▲) postmicrosomal supernatant; (△—△) 15,000*g* supernatant.

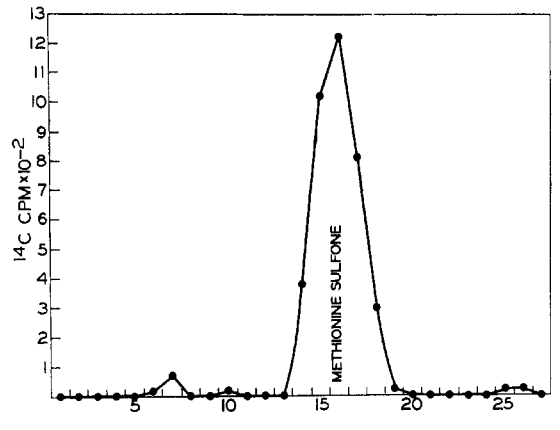

FIGURE 6: Paper chromatographic analysis of the hydrolysate of rat liver mitochondrial radioactive protein isolated after injecting L-[*methyl*-^{14}C]methionine for 24 hr. Rats were injected as stated in Figure 5. For detail, see Materials and Methods. Figures on abscissa designate the strip number (1 cm/strip) from the origin.

1.5% of the radioactivity in mitochondrial protein were found in the N- and C-terminal position, respectively.

Distribution of Radioactivity from L-[^{35}S]*Methionine in Rat Liver Fractions.* Microsomal proteins have the highest specific radioactivity from L-[^{35}S]methionine after 24 hr, when compared with all the other protein fractions (Table I).

Fractionation and extraction procedures applied to mitochondria, microsomes, and postmicrosomal supernatant (Table II) show approximately the same distribution of radioactivity in the hot 10% trichloroacetic acid and ethanol–ether soluble proteins of microsomes and mitochondria.

Paper chromatographic analysis (Table III) of the hydrolysates of performic acid oxidized protein showed two major radioactive peaks that were identified as cysteic acid and methionine sulfone. The ratio of the radioactivities of these two amino acids was about 1:3 in all purified proteins.

The distribution of the radioactivity from the protein hydrolysate of ^{35}S-labeled mitochondrial proteins is shown in Figure 7; it resembles the chromatographic resolution obtained from all other protein fractions.

Inhibition of L-[*methyl*-^{14}C]*Methionine and* L-[^{35}S]*Methionine Incorporation into Protein with Cycloheximide and Chloramphenicol.* Protein synthesis inhibition studies were performed with cycloheximide and chloroamphenicol *in vivo* and, when combined with complete cell fractionation and isolation of mitochondrial membranes, were expected to give some indication of the localization of the synthesis of methionine-containing mitochondrial proteins in the cell compartment. The uptake and distribution of radioactivity in cell fractions of all six treatments (Table IV) were in the same range as shown previously in Figure 1.

Total inhibition of L-[*methyl*-^{14}C]methionine incorporation into all cell proteins was observed when cycloheximide was injected 30 min before and nearly complete inhibition when injected together with the labeled methionine (Table IV). Partial inhibition resulted when cycloheximide was injected 30 min after methionine. Chloramphenicol gave also, to a lesser degree, *in vivo* inhibition of L-[*methyl*-^{14}C]methionine into mitochondrial-soluble fraction and inner membrane proteins.

The incorporation of L-[^{35}S]methionine was not inhibited completely with cycloheximide (Table IV), however similar extents of inhibitions occurred within the same time periods as with L-[^{14}C]methionine. The inhibition by chloramphenicol of the incorporation of L-[^{35}S]methionine into mitochondria

TABLE II: Distribution of Radioactivity in Extracts and Proteins of Different Cell Fractions after L-[^{35}S]Methionine Injection.[a]

Extracts and Protein	% Total Radioactivity in Cell Fraction: Mitochondria	Microsomes	Postmicrosomal Supernatant
5% Trichloroacetic acid soluble	10.0	3.7	52.8
10% Trichloroacetic acid soluble	2.5	1.7	1.2
Ethanol–ether soluble	5.4	5.8	1.6
Protein	81.4	88.6	44.2

[a] Rats were injected as stated in Table I.

TABLE I: Incorporation of ^{35}S from L-[^{35}S]Methionine into Proteins of Different Cell Fractions.[a]

Cell Fraction	Specific Radioactivity (cpm/mg of Protein)
Mitochondria	4300
Microsomes	5600
Postmicrosomal supernatant	3800
Mitochondrial	
Soluble fraction	3600
Outer membrane	3400
Inner membrane	3600

[a] Each of four rats was injected with 133 μCi (62.5 μmoles) of L-[^{35}S]methionine, specific activity 2.13 Ci/mole, for 24 hr.

TABLE III: Distribution of ^{35}S in Rat Liver Mitochondrial Proteins after Injection of L-[^{35}S]Methionine.[a]

Cell Fraction	Cysteic Acid (%)	Methionine Sulfone (%)
Mitochondria	28	72
Microsomes	29	71
Postmicrosomal supernatant	26	74
Mitochondrial		
Soluble fraction	19	81
Outer membrane	21	79
Inner membrane	19	81

[a] Rats were injected as stated in Table I. The isolated proteins were oxidized, hydrolyzed, and chromatographed as described in Materials and Methods.

TABLE IV: Effect of Cycloheximide and Chloramphenicol on the Incorporation of L-[*methyl*-^{14}C]Methionine or L-[^{35}S]Methionine into Cell Proteins.[a]

Treatment	% Inhibition											
	Mitochondrial											
	Total		Soluble Fraction		Outer Membrane		Inner Membrane		Microsomes		Postmicrosomal Supernatant	
	^{14}C	^{35}S	^{14}C	^{35}S	^{14}C	^{35}S	^{14}C	^{35}S	^{14}C	^{35}S	^{14}C	^{35}S
1. Control	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0
2. Cycloheximide,[b] 30 min before isotope	>99	95.2	>99	94.2	>99	88.5	>99	94.2	>99	93.4	>99	97.7
3. Cycloheximide, together with isotope	96.8	94.2	94.9	91.4	81.0	85.6	82.9	90.7	95.9	91.4	>99	96.3
4. Cycloheximide, 30 min after isotope	56.3	46.5	42.1	17.8	44.9	28.6	52.6	36.9	42.7	29.5	17.0	37.2
5. Chloramphenicol,[c] 30 min before isotope	48.0	21.3	34.9	<1.0	12.9	18.8	45.9	10.0	13.0	<1.0	8.0	19.3
6. Chloramphenicol, together with isotope	49.0	26.0	54.8	<1.0	18.6	13.0	48.2	9.0	18.0	<1.0	9.0	12.6

[a] Rats were injected with 83.3 μCi (37 μmoles) of L-[*methyl*-^{14}C]methionine, specific radioactivity 2.25 Ci/mole, or with 83.3 μCi (0.6 μmole) of L-[^{35}S]methionine, specific activity 531 Ci/mole, for 60 min. Two rats were used for each treatment and their livers were pooled before homogenization. [b] Cycloheximide, 10 mg/rat, injected intraperitoneally. [c] Chloramphenicol, 260 mg/rat, injected intraperitoneally.

and the outer and inner membrane components was similar, whereas inhibition of L-[^{14}C]methionine incorporation in the outer membrane was one-half that of the mitochondria and inner membrane (Table IV).

There was a greater amount of 5% trichloroacetic acid soluble radioactivity and an inhibition of incorporation into protein when cycloheximide was injected before or with L-[^{14}C]methionine (Table V). One can conclude that cycloheximide inhibits protein synthesis which results in accumulation of free nonutilized L-[^{14}C]methionine but does not inhibit the transfer of methyl groups from methionine to nucleic acids (10% hot trichloroacetic acid soluble) and lipids (ethanol–ether soluble). Administration of chloramphenicol had no appreciable effect on the amount of ^{14}C derived from methionine found in the extracts or proteins. The per cent of total radioactivities in ethanol–ether extracts was not significantly altered by any of the treatments.

Radioactivity from L-[^{35}S]methionine was greater in the 5% trichloroacetic acid soluble fraction and less in the proteins, when cycloheximide was given before or at the same time (Table VI). The mitochondrial and the microsomal ethanol–ether and 10% hot trichloroacetic acid soluble fractions contained twice the radioactivity when cycloheximide was given with or before the L-[^{35}S]methionine. However, chloramphenicol had no measurable effect on ^{35}S radioactivity in the various extracts.

Discussion

The uptake of radioactivity from methionine into fractions of the 24-hr fasted (*i.e.*, methionine deficient) (Allison *et al.*, 1947) rat liver is very rapid (Figure 1) until 60 min. Then follows a period of apparent equilibrium between the radioactivity of the proteins and the amino acids with retention of a constant specific activity (Figure 2) of the proteins. Between 8 and 24 hr, the specific radioactivity in the protein declines slowly. This reflects the methionine deficient status of the 24-hr fasted rat. The microsomal fraction incorporates L-[^{14}C]-methionine (Figure 2) earlier and the time curve is quite different from that of mitochondria or postmicrosomal super-

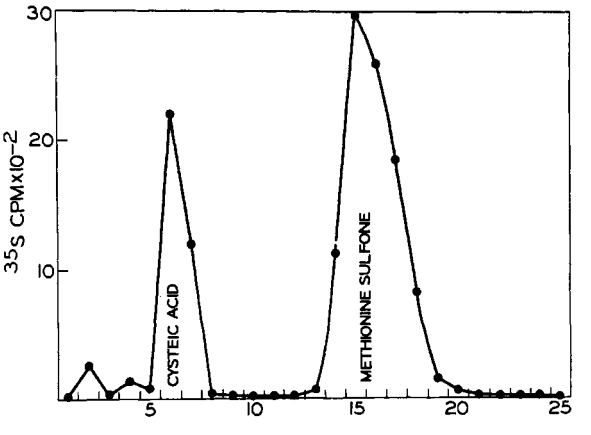

FIGURE 7: Paper chromatographic analysis of the hydrolysate of rat liver mitochondrial radioactive protein isolated after injection of L-[^{35}S]methionine for 24 hr. Rats were injected as stated in Table I. For detail, see Materials and Methods. Figures on abscissa designate the strip number (1 cm/strip) from the origin.

TABLE V: Effect of Cycloheximide and Chloramphenicol on the Distribution of Radioactivity Derived from L-[*methyl*-^{14}C]Methionine in Different Cell Fractions.[a]

	% Total Radioactivity					
	Treatment					
Extracts and Protein	1	2	3	4	5	6
5% Trichloroacetic acid soluble						
Mitochondria	7.3	16.7	16.1	12.8	10.5	9.5
Microsomes	5.4	16.1	8.6	6.5	4.9	3.4
Postmicrosomal supernatant	61.5	>99.0	98.9	71.2	62.0	54.2
10% Hot trichloroacetic acid soluble						
Mitochondria	38.8	55.0	48.4	43.5	40.4	39.8
Microsomes	41.6	51.6	45.8	43.3	44.8	40.9
Postmicrosomal supernatant	0.5	1.0	0.6	1.6	3.4	1.6
Protein						
Mitochondria	23.7	<1.0	10.9	18.6	19.0	25.8
Microsomes	21.4	<1.0	7.0	19.2	25.2	22.0
Postmicrosomal supernatant	37.1	<1.0	0.5	27.1	33.7	41.4

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

TABLE VI: Effect of Cycloheximide and Chloramphenicol on the Distribution of Radioactivity Derived from L-[^{35}S]-Methionine in Different Cell Fractions.[a]

	% Total Radioactivity					
	Treatment					
Extracts and Proteins	1	2	3	4	5	6
5% Trichloroacetic acid soluble						
Mitochondria	27.5	79.5	77.5	34.4	32.4	26.7
Microsomes	5.5	62.8	51.9	12.4	8.1	5.5
Postmicrosomal supernatant	41.2	95.3	91.2	64.8	58.4	47.4
10% Hot trichloroacetic acid soluble						
Mitochondria	1.7	2.7	2.6	1.6	2.7	2.5
Microsomes	2.5	2.1	4.3	3.9	3.1	2.8
Postmicrosomal supernatant	2.5	0.7	1.0	2.1	2.0	3.0
Ethanol–ether soluble						
Mitochondria	1.9	4.6	4.7	2.3	1.7	1.8
Microsomes	2.5	7.9	6.5	2.3	2.0	1.4
Postmicrosomal supernatant	1.3	1.2	0.7	1.1	1.1	1.2
Protein						
Mitochondria	68.6	13.1	15.1	61.5	63.1	68.8
Microsomes	89.4	27.1	37.1	81.2	86.6	90.0
Postmicrosomal supernatant	54.9	3.0	7.0	31.9	38.4	48.2

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

natant. Similar results were obtained when the *in vivo* metabolism of cytochrome *c* was studied (Gonzales-Cadavid and Campbell, 1967). When L-[^{35}S]methionine was used, the same rapid incorporation into the microsomal fraction occurred in relation to postmicrosomal supernatant or mitochondrial fractions as was obtained with L-[^{14}C]methionine (not shown). The very low turnover of the mitochondrial proteins (Brunner and Neupert, 1968) could explain the plateau which is reached in the incorporation of L-[^{14}C]methionine (Figures 2 and 5) into mitochondrial proteins. The incorporation of ^{14}C or ^{35}S into mitochondrial subfractions (Figure 3) shows that the components of the mitochondria (inner and outer membranes and soluble fractions) have similar specific radioactivities. The time curve is in accordance with the finding of Beattie (1969).

When L-[^{14}C]methionine is used (Figure 4) the hot 10% trichloroacetic acid and the ethanol–ether extractions are radioactive and contain at least 70% of the total radioactivity found in the mitochondria or in the microsomal fraction, compared to the 7% found in these fractions in the case of L-[^{35}S]methionine (Table I). The mitochondria and the microsomes are higher in L-[^{14}C]methionine radioactivity than the postmicrosomal supernatant which probably reflects the difference in amount of nucleic acids and lipids in those fractions (Figure 4).

It is of interest to note, that the pattern of distribution of the radioactivity in the extractions of the mitochondria is the same as that of the microsomes, but different from that of the postmicrosomal supernatant. The methylations of the microsomal and mitochondrial fractions had similar time curves. The distribution of radioactivity (not shown) in the different extracts and the specific radioactivity (Figure 5) in the purified proteins from 2 to 24 hr are not changed and can be explained on the basis of the low turnover of the components. The per cent of the total radioactivity found in the proteins in the presence of L-[^{35}S]methionine (Table I) is higher than that obtained after L-[^{14}C]methionine injection (not shown), because the ^{35}S-labeled proteins (Figure 7) contain also [^{35}S]cysteine, which implies that demethylation probably occurred *via* the *S*-adenosylmethionine pathway (Mudd and Cantoni, 1957).

The effect of cycloheximide on protein synthesis is well known (Sisler and Siegel, 1967) and both cycloheximide and chloramphenicol were used as tools in mitochondrial protein synthesis studies *in vivo* and *in vitro* (Kroon, 1963; Ashwell and Work, 1968; Gonzales-Cadavid *et al.*, 1970; Firkin and Linnane, 1969). The fact that cycloheximide inhibits nearly completely methionine incorporation into mitochondria, mitochondrial subfractions, microsomal, and postmicrosomal supernatant proteins (Table III and IV) and increases the amount of the radioactivity isolated in the 5% trichloroacetic acid soluble fraction (Table IV and V) supports those reports that cycloheximide affects cytoribosomal protein synthesis (Ashwell and Work, 1970). Thus, it would appear that at least 90% of the methionine-containing proteins are synthesized on the cytoribosomes (Table III and IV) and are then transferred to the mitochondria (Kadenbach, 1966). Supporting evidence for the "transfer" are the results of Tzagoloff (1969) on the synthesis of yeast mitochondrial ATPase and Stratman *et al.* (1972) with radioactive rat liver ATPase. Chloramphenicol, when injected into rats, does not influence the per cent of radioactivity in the 5% trichloroacetic acid soluble or the protein fractions (Tables IV and V).

The use of chloramphenicol *in vivo* as an inhibitor of mitochondrial protein synthesis does not reveal any correlation to *in vitro* experiments as has been shown for cycloheximide in cytoribosomal protein synthesis. The inhibitory effect of chloramphenicol *in vivo* is much less and shows discrimination against incorporation of ^{14}C from the methyl group of methionine in comparison to ^{35}S of methionine. A possible explanation is that chloramphenicol *in vivo* is a more potent inhibitor of methylation of mitochondrial proteins, but it does not effectively inhibit incorporation of cysteine derived from methionine in mitochondrial proteins.

References

Allison, J. B., Anderson, J. A., and Seeley, R. D. (1947), *J. Nutr. 33*, 361.

Allman, D. W., Bachmann, E., Orme-Johnson, N., Tan, W. C., and Green, D. E. (1968), *Arch. Biochem. Biophys. 125*, 981.

Ashwell, M. A., and Work, T. S. (1968), *Biochem. Biophys. Res. Commun. 32*, 1006.

Ashwell, M. A., and Work, T. S. (1970), *Annu. Rev. Biochem. 39*, 251.

Bailey, E., Taylor, C. B., and Bartley, W. (1967), *Biochem. J. 104*, 1026.

Beattie, D. S. (1969), *Biochem. Biophys. Res. Commun. 35*, 67.

Brunner, G., and Neupert, W. (1968), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 1*, 153.

Clark, B. F. C., and Marker, K. A. (1966), *J. Mol. Biol. 17*, 394.

Firkin, F. C., and Linnane, A. W. (1969), *Exp. Cell. Res. 55*, 68.

Fletcher, M. J., and Sanadi, D. R. (1961), *Biochim. Biophys. Acta 51*, 356.

Gonzales-Cadavid, N. F., Bello, E. M. A., and Ramirez, J. L. (1970), *Biochem. J., 118*, 577.

Gonzalez-Cadavid, N. F., and Campbell, P. N. (1967), *Biochem. J. 105*, 443.

Kadenbach, B. (1966), *Biochim. Biophys. Acta 134*, 430.

Kroon, A. M. (1963), *Biochim. Biophys. Acta 72*, 391.

Kroon, A. M. (1965), *Biochim. Biophys. Acta 108*, 275.

Layne, E. (1957), *Methods Enzymol. 3*, 450.

Moore, S. (1963), *J. Biol. Chem. 238*, 235.

Mudd, S. H., and Cantoni, G. L. (1957), *J. Biol. Chem. 231*, 481.

Omura, T., Siekevitz, P., and Palade, G. E. (1967), *J. Biol. Chem. 242*, 2389.

Roodyn, D. B. (1962), *Biochem. J. 85*, 177.

Schnaitman, C. A. (1969), *Proc. Nat. Acad. Sci. U. S. 63*, 412.

Sisler, H. D., and Siegel, M. R. (1967), *in* Antibiotics: Mechanism of Action I, Gottlieb, D., and Shaw, P. D., Ed., Berlin, Springer/Verlag, p 283.

Sottocasa, G. L., Kuylenstierna, B., Ernster, L., and Bergstrand, A. (1967), *Methods Enzymol. 10*, 448.

Stratman, F. W., Hochberg, A. A., Zahlten, R. N., and Lardy, H. A. (1972), *Biochemistry 11*, 3154.

Suttie, J. W. (1962), *Biochem. J. 84*, 382.

Tabor, C. W., Tabor, H., and Rosenthal, S. M. (1954), *J. Biol. Chem. 208*, 645.

Tzagoloff, A. (1969), *J. Biol. Chem. 244*, 5027.

Incorporation of L-[1-*ethyl*-^{14}C]- and L-[^{35}S]Ethionine into Mitochondrial Proteins†

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ABSTRACT: The uptake and the incorporation of L-[1-*ethyl*-^{14}C]ethionine, and L-[*methyl*-^{14}C]methionine into different rat liver organelles was compared by injection of the same molar and isotopic concentration. Ethionine is incorporated as such into mitochondria and mitochondrial subfractions *in vivo*. The incorporation into various cell proteins was determined. Ethionine is in part deethylated *in vivo* and the homocysteine formed is methylated to methionine. The ratio among methionine:cysteine:ethionine derived from [^{35}S]ethionine was similar in proteins from different cell fractions. Quantitative relations for the distribution of the ethyl group and the sulfur atom from ethionine are proposed.

Ethionine, an antimetabolite of methionine, synthesized by Dyer (1938), replaces methionine in proteins and ethylates nucleic acids and lipids (Stekol and Weiss, 1950; Levine and Traver, 1951; Farber *et al.*, 1964). The ability of the cell to incorporate ethionine into proteins is well documented (Fowden *et al.*, 1967). Farber *et al.* (1964) have discussed the various means by which ethionine also inhibits protein synthesis *in vivo*. *In vitro* studies with mitochondria from ethionine-treated female rats by Vogt and Farber (1970) suggest that the change observed in mitochondrial metabolism is correlated to the decreased ATP level in the liver. The purpose of our study was to trace the *in vivo* incorporation of ethionine into rat liver cell organelles and to determine the metabolic fate of the ethyl and sulfur moieties of the ethionine molecule in various cell organelles.

Materials and Methods

Female rats (200–250 g) from Badger Research Co. (hysterectomy-derived, Sprague–Dawley strain) were used for

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