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.

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

A. A. Hochberg, R. N. Zahlten, F. W. Stratman, and H. A. Lardy*

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

† From the Department of Biochemistry and Institute for Enzyme Research, University of Wisconsin, Madison, Wisconsin 53706. *Received February 24, 1972.* This research was supported in part by grants from the National Institutes of Health and the National Science Foundation. F. W. S. held the Babcock Fellowship from the Department of Biochemistry, University of Wisconsin, during this research.

TABLE I: A Comparison of L-[*methyl*-^{14}C]Methionine and L-[1-*ethyl*-^{14}C]Ethionine Uptake into Rat Liver and Incorporation into Proteins of Different Cell Fractions.[a]

Fraction	% of Injected Radioactivity Found in Liver		Specific Radioactivity (cpm/mg of Protein)	
	[^{14}C]-Methionine	[^{14}C]-Ethionine	[^{14}C]-Methionine	[^{14}C]-Ethionine
Homogenate	10.1	3.0		
Supernatant 600*g*	8.0	2.1		
Supernatant 15,000*g*	5.5	1.7	1730	110
Mitochondria	0.9	0.3	1630	49

[a] Each of two rats were injected with the same molar and isotopic concentration of either [^{14}C]methionine or ethionine; 40 μCi (16 μmoles) of amino acid, specific activity of 2.5 Ci/mole, and sacrificed after 24 hr. See Materials and Methods for liver fractions and protein isolation.

all experiments. The rats were fasted for 24 hr before the injections at indicated times.

L-[1-*ethyl*-^{14}C]Ethionine was purchased from New England Nuclear and L-[^{35}S]ethionine was purchased from Amersham/Searle. All other chemicals were supplied from Mallinckrodt and Schwarz/Mann, in the highest available purity.

Fractionation Procedures. Liver homogenates were prepared as described previously (Hochberg *et al.*, 1972). Microsomes, postmicrosomal supernatant, mitochondria, and mitochondrial subfractions were prepared according to Sottocasa *et al.* (1967).

Purity of mitochondrial subfractions was monitored by assaying the marker enzyme, monoamine oxidase, according to Tabor *et al.* (1954) and Allman *et al.* (1968).

TABLE II: Distribution of Radioactivity Derived from L-[*methyl*-^{14}C]Methionine and L-[1-*ethyl*-^{14}C]Ethionine in Mitochondria and Postmitochondrial Supernatant.[a]

Extracts and Protein	% of Total Radioactivity	
	Mitochondria	15,000*g* Supernatant
5% Trichloroacetic acid soluble		
Methionine	5.6	23.9
Ethionine	82.5	83.1
10% Hot trichloroacetic acid soluble		
Methionine	36.8	24.0
Ethionine	7.6	5.9
Ethanol–ether		
Methionine	31.2	23.5
Ethionine	7.3	4.1
Soluble protein		
Methionine	26.3	28.5
Ethionine	2.5	6.8

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

TABLE III: Uptake and Incorporation of L-[^{35}S]Ethionine and of L-[1-*ethyl*-^{14}C]Ethionine into Rat Liver Fractions and Isolated Proteins.[a]

Cell Fraction[d]	% of Total Amount Injected Found in Fraction		
	1 Hr[b]	24 Hr[c]	
	[^{14}C]-Ethionine	[^{14}C]-Ethionine	[^{35}S]-Ethionine
Homogenate	5.6	6.5	3.7
600*g* Supernatant	4.9	4.2	2.6
6500*g* Supernatant	4.1	3.5	2.1
15,000*g* Supernatant	4.1	3.1	2.1
Microsomes	0.2 (63)	0.4 (210)	0.1 (2280)
Postmicrosomal supernatant	4.1 (28)	2.2 (110)	1.8 (1600)
Mitochondria	0.1 (18)	0.4 (115)	0.2 (930)
Soluble fraction	(8)	(67)	(1100)
Outer membrane	(10)	(88)	(1130)
Inner membrane	(18)	(99)	(910)

[a] Values in parentheses are specific radioactivities (cpm/mg of protein) of the isolated proteins. [b] Each of three rats was injected with 100 μCi (39 μmoles) of L-[1-*ethyl*-^{14}C]ethionine (specific activity 2.59 Ci/mole) and sacrificed after 1 hr. [c] Each of two rats was injected with 30 μCi (12.5 μmoles) of L-[1-*ethyl*-^{14}C]ethionine (specific activity 2.4 Ci/mole) and each of three rats with 250 μCi (81 μmoles) of L-[^{35}S]ethionine (specific activity 3.0 Ci/mole) and sacrificed after 24 hr. [d] Fractions were isolated as described in Materials and Methods.

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

Counting of Labeled Material. Sample aliquots were placed on circular Whatman No. 1 filter paper disks, dried, and counted as described previously (Hochberg *et al.*, 1972).

Protein Analysis. Proteins were determined with the biuret method (Layne, 1957). Performic acid oxidation, hydrolysis of oxidized protein, and end-group amino acid analysis were performed as described elsewhere (Hochberg *et al.*, 1972).

Chromatography. The descending paper chromatographic (*ca.* 10 mg of protein hydrolyzed) procedure has been described (Hochberg *et al.*, 1972). Column chromatography was used to characterize ethionine sulfone. Forty mg of trichloroacetic acid and ethanol–ether washed protein (performate oxidized) hydrolysate was separated at 30° on a 3.8 × 50 cm column, packed with sulfonated polystyrene resin (Bio-Rad), and eluted with 0.2 N Na-citrate buffer (pH 3.1). The ethionine sulfone peak was concentrated to minimal volume and counted.

Results

The amount of L-[1-*ethyl*-^{14}C]ethionine taken up (Table I) was always 30% of the L-[*methyl*-^{14}C]methionine under comparable conditions. However, the distribution of ^{14}C derived from ethionine in the different cell compartments was

TABLE IV: Distribution of Radioactivity Derived from L-[^{35}S]Ethionine and from L-[1-*ethyl*-^{14}C]Ethionine in Different Cell Fractions.[a]

Extracts and Protein	Time (hr)	Isotope	% Total Radioactivity Measured in Cell Fractions		
			Mitochondria	Microsomes	Postmicrosomal Supernatant
	24	ES[b]	82.0	63.3	90.7
5% Trichloroacetic acid	24	EC[c]	82.5	42.7	95.6
	1	EC[c]	98.9	96.3	99.7
	24	ES	2.0	2.5	0.6
10% Hot trichloroacetic acid	24	EC	7.6	21.8	0.9
	1	EC	0.0	1.8	0.0
	24	ES	0.8	1.8	0.2
Ether–ethanol	24	EC	6.0	21.6	0.8
	1	EC	0.0	0.0	0.0
	24	ES	15.0	32.0	8.4
Protein	24	EC	4.0	13.7	2.6
	1	EC	1.0	1.7	0.3

[a] Rats were injected as indicated in Table III. [b] ES = L-[^{35}S]ethionine. [c] EC = L-[1-*ethyl*-^{14}C]ethionine.

similar to that of [^{14}C]methionine. The specific radioactivity of the proteins after ethionine injection was *ca.* 3% of that following administration of labeled methionine in the case of mitochondria and *ca.* 6% in the 15,000*g* supernatant fraction proteins. The ratios of the specific radioactivity of mitochondrial and supernatant fraction (15,000*g*) proteins were quite different in a comparison of ethionine (1:0.5) with methionine (1:1).

Over 80% of the radioactivity derived from [^{14}C]ethionine is found in the 5% trichloroacetic acid soluble fraction of mitochondria and supernatant, where only 6% (mitochondrial) and 24% (supernatant) are detected from methionine (Table II). The percentage of ^{14}C derived from ethionine in the proteins is positively correlated with the specific radioactivity shown in Table I, where a ratio of 2:1 exists between 15,000*g* supernatant fraction and the mitochondria.

There was a similar distribution after either 1 or 24 hr of ^{14}C from ethionine in all cell fractions except mitochondria and microsomes which contained greater amounts of radioactivity after 24 hr (Table III). This indicates that uptake of ethionine into the cell compartment is similar regardless of the time exposed but incorporation into protein is time dependent.

The incorporation of L-[1-^{14}C]ethionine into the mitochondrial, microsomal, and postmicrosomal supernatant proteins (Table III) is extremely low after 1 hr compared with methionine (Hochberg *et al.*, 1972). After 24 hr of L-[1-*ethyl*-^{14}C]ethionine exposure (Table III), the ^{14}C radioactivity found in all the protein fractions is higher than that found after 1 hr. A comparison between L-[^{35}S]ethionine and L-[1-^{14}C]ethionine is not valid since they were injected in different concentrations and specific radioactivities.

The distribution of ^{35}S and ^{14}C from ethionine in various extracts of cell fractions (Table IV) indicates that in the microsomes and mitochondria, a smaller percentage of the ^{14}C and of ^{35}S was incorporated into the protein and relatively more ^{14}C than of ^{35}S was found in the 10% hot trichloroacetic acid soluble and ethanol–ether fractions. Microsomes had a smaller percentage of ^{14}C than ^{35}S radioactivity in the 5% trichloroacetic acid soluble fractions whereas in mitochondria the relative distribution did not differ.

The chromatographic separation (Figure 1) of the hydrolysates of oxidized mitochondrial protein gave three distinct peaks of ^{35}S-labeled amino acids, which were identified as cysteic acid, methionine sulfone, and ethionine sulfone. The relative radioactivity measured in the peaks 24 hr after ethionine injection shows that the ^{35}S from ethionine is predominately distributed between cysteic acid and methionine sulfone. A relatively small amount of ^{35}S is detectable as ethionine incorporated into proteins. The relative distribution of radioactivity into all the other cell protein hydrolysates is shown in Table V.

The chromatographic separation (Figure 2) of L-[1-^{14}C]-ethionine-derived radioactive amino acids in mitochondrial proteins indicates one major peak of [^{14}C]ethionine sulfone and a smaller peak of an unidentified metabolite (X_1). The

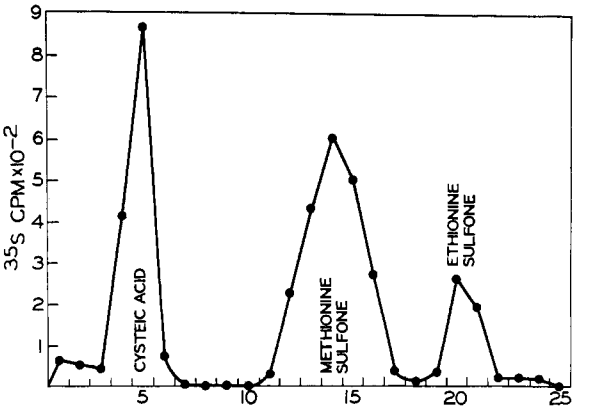

FIGURE 1: Paper chromatographic analysis of the hydrolysate of rat liver mitochondrial protein isolated after injection of L-[^{35}S]-ethionine. Rats were injected as described in footnote *a* of Table III. Procedures are described in Materials and Methods. Figures on abscissa designate the strip number (1 cm/strip) from the origin.

TABLE V: Per Cent of Total Radioactivity in the Chromatographed, Hydrolyzed Rat Liver Cell Proteins, after Injection of L-[^{35}S]Ethionine or L-[1-*ethyl*-^{14}C]Ethionine.[a]

Protein	Isotope	Cysteic Acid	Methionine Sulfone	Ethionine Sulfone	Unidentified Compounds
Mitochondrial	^{35}S	36	51	13	
Mitochondrial	^{14}C			64	36
Microsomes	^{35}S	39	50	11	
Microsomes	^{14}C			46	53[b]
Postmicrosomal supernatant	^{35}S	40	49	10	
Postmicrosomal supernatant	^{14}C			44	55[b]
Mitochondrial soluble fraction	^{35}S	37	53	10	
Mitochondrial soluble fraction	^{14}C			49	51
Outer membrane[c]	^{35}S	35	52	12	
Inner membrane	^{35}S	32	51	16	
Inner membrane	^{14}C			64	35

[a] Rats were injected as described in Table IV. For details, see Materials and Methods. [b] Microsomal and postmicrosomal supernatant proteins contained two unknown compounds, X_1 and X_2, in ratios of 1:3.8 and 1:3.6, respectively, which are reported as one value. [c] Not enough protein for analysis of ^{14}C.

chromatograms gave the same picture essentially for all subfractions, except microsomes and postmicrosomal supernatant which contained two unidentified metabolites (X_1 and X_2) (Table V).

Approximately 5.4 and 3% of the radioactivity in mitochondrial protein were found in the N- and C-terminal positions, respectively.

Discussion

It is well known from previous publications that ethionine is incorporated into liver proteins (Fowden *et al.*, 1967). In this study, we tried to establish the extent of the ethionine incorporation into subcellular fractions as well as to trace the metabolic fate of this molecule in the rat liver cell. This information is important for understanding the known effects of ethionine on mitochondrial metabolism (Vogt and Farber, 1970) and protein synthesis (Farber *et al.*, 1964).

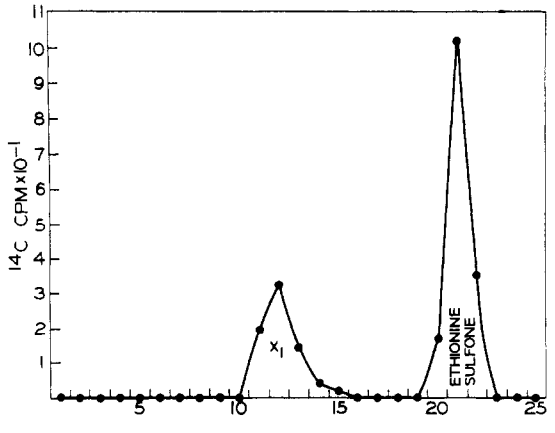

FIGURE 2: Paper chromatographic analysis of the hydrolysate of rat liver mitochondrial protein isolated after injection of L-[1-*ethyl*-^{14}C]ethionine. Rats were injected as described in footnote *a* of Table III. Procedures are described in Materials and Methods. Figures on abscissa designate strip number (1 cm/strip) from the origin.

It has been shown that [^{14}C]- or [^{35}S]ethionine is incorporated as ethionine into different cell proteins (Figures 1 and 2). The incorporation into the mitochondrial inner membrane (Table V) is of special interest, because replacement of methionine with ethionine in the proteins could change extensively the properties of the membrane and could explain partially the effect of ethionine on mitochondria metabolism.

The comparison between methionine and ethionine, when injected at the same molar and isotopic concentration (Table I), indicates that the normal amino acid (methionine) penetrates more rapidly and is incorporated to a greater extent into the proteins. However, there is a considerable pool of methionine, together with a large C-1 pool that will dilute the methyl label from methionine whereas no such pool exists for ethionine. The very low affinity of the methionine-activating enzymes (Schlenk and Tillotson, 1954; Mudd and Mann, 1963) for ethionine can explain the finding that 80% of the radioactivity derived from ethionine is not incorporated but is found in the 5% trichloroacetic acid soluble fraction, and that ethionine is incorporated to a greater extent after 24 hr than at 1 hr after injection (Table II, III).

The differences in the relative incorporation of methionine and ethionine into mitochondria in comparison with postmitochondrial supernatant proteins could be explained on the basis that some ethionine-labeled proteins are not transferred to the mitochondria (Kadenbach, 1966). Chromatographic separation of proteins labeled with [^{14}C]ethionine shows that ethionine is the major radioactive component of the protein; however, the ethyl group or part of it was transferred to two unidentified metabolites (X_1 and X_2) in the microsomes and postmicrosomal supernatant. Unidentified metabolite (X_1) corresponds closely to the chromatographic mobility of arginine (Orenstein and Marsh, 1968; Friedman *et al.*, 1969). A small amount of ethionine is directly incorporated into protein but much more of it is deethylated to homocysteine which is converted to cysteine (Stekol *et al.*, 1950) (Table V and Figure 1) or methylated to methionine. This deethylation is confirmed by the radioactivity (originating from the ethyl group) found in the hot 10% trichloroacetic acid and the ethanol–ether soluble fractions.

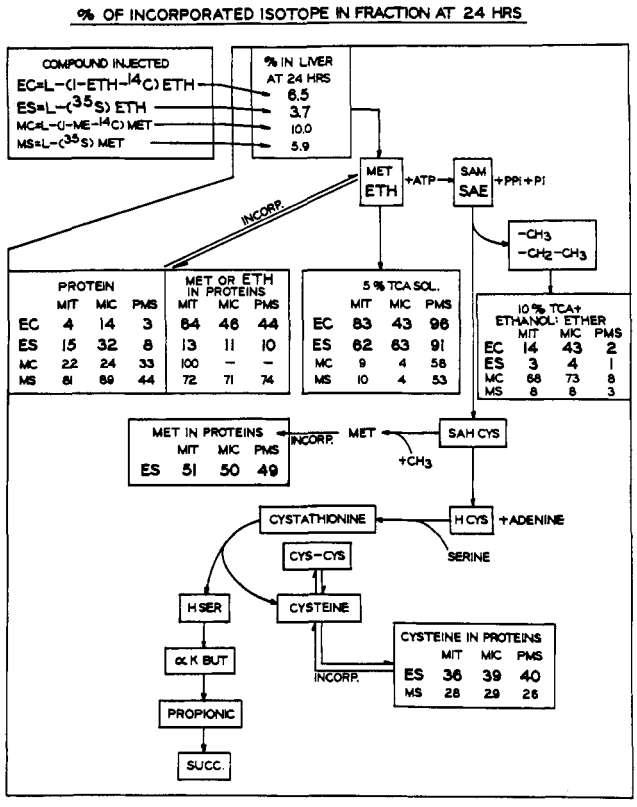

FIGURE 3: Quantitative distribution of [^{14}C]- and [^{35}S]methionine and -ethionine in rat liver cell fractions and components. For example, 3.7% of the radioactivity from intraperitoneally injected [^{35}S]ethionine (ES) is found in the liver after 24 hr. Of the total radioactivity in the mitochondria, 82% is soluble in 5% trichloroacetic acid, 3% is soluble in hot 10% trichloroacetic acid or ethanol–ether, and 15% is in the washed proteins, for a total of 100%. Of the total radioactivity in the protein, 13% is ethionine, 51% is methionine, and 36% is cysteine, for a total of 100%. Data represent the mean value from three or more rats for each compound. EC, 30 μCi, 12.5 μmoles in each of two rats. ES, 250 μCi, 81 μmoles in each of three rats. MC, 100 μCi, 10.8 μmoles in each of three rats. MS, 133 μCi, 62.5 μmoles in each of four rats. SAM, *S*-adenosylmethionine; SAE, *S*-adenosylethionine; SAH CYS, *S*-adenosylhomocysteine; HCYS, homocysteine; HSER, homoserine; αKBUT, α-ketobutyrate; SUCC, succinate; CYS-CYS, oxidized cysteine; INCORP, incorporation.

The low amount of ethionine incorporated into the protein is a direct result of the small percentage of the ethionine taken up by the cell and the deethylation pathway.

The ratio of cysteine:methionine:ethionine derived from [^{35}S]ethionine is constant in the different cell fraction proteins; therefore, these radioactive proteins might have the same origin (Table V).

Figure 3 summarizes the metabolic pathways in the rat liver cell for methionine from the preceding paper (Hochberg *et al.*, 1972) and for ethionine from this paper.

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