group) appearing in CO_2, acetate, and formate. A methionine activating enzyme was shown to activate ethionine to form S-adenosyl-L-ethionine in yeast and rat liver mitochondria(9,10,11,12). Conceivably, a reaction of this type may occur prior to ethionine de-ethylation in resistant *E. coli*. Assuming an initial de-ethylation of ethionine, these labeled products may be explained on the basis of known pathways of 2-carbon metabolism.

Studies on the incorporation of C^{14}-ethionine by resistant cells indicate the methionine analogue is incorporated rapidly into protein (TCA precipitable) followed by a rapid decrease in radioactivity of this fraction and paper chromatographic analysis of the TCA insoluble fraction showed the presence of ethionine. These results indicate that ethionine is transferred to protein prior to its degradation. A de-alkylation of ethionine in peptide bond sequence followed by the formation of methionine, if true, would represent a unique process.

Summary. *E. coli* resistant to ethionine shows a 6 to 8 hour longer lag period than sensitive cells when grown on a glucose salts medium without ethionine. The lag period was reduced to that of sensitive cells when methionine, ethionine, cystathionine or homocysteine was added to the growth medium. When ethyl-1-C^{14}-ethionine was added to resistant cells there was a rapid increase of the tracer into both the amino acid pool and protein followed by a rapid decrease of label in both fractions. Formate, acetate, and CO_2 isolated from culture filtrates of resistant cells metabolizing ethyl-1-C^{14}-ethionine were labeled. No significant differences were noted between the incorporation of C^{14}-methionine by ethionine-resistant and sensitive cells. On the basis of these observations, it is concluded that ethionine resistance in *E. coli* is the result of the synthesis of enzymes capable of converting ethionine to methionine. The data indicate that ethionine is de-ethylated to the level of homocysteine followed by transmethylation to methionine.

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Comparative Studies in the Characterization of Monoamine Oxidases.* (28765)

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Since the early survey studies on amine oxidases(1,2) there have been diverse investigations on monoamine oxidases in various preparations from a number of tissues and species, but many aspects of monoamine oxi-

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dase characteristics and the quantitative significance of these enzyme activities remain uncertain. Monoamine oxidases are generally considered to be a closely related group of enzymes, insensitive to semicarbazide, which catalyze the oxidative deamination of amines that do not possess a methyl group on the α carbon(3). Intracellularly the major portion of monoamine oxidase activity is associated with the mitochondria(4-8), but appreciable activities have been reported in non-mitochondrial particulate fractions(5,6) or in the soluble state(9). Differences in substrate specificities or inhibitor sensitivities among preparations from different tissues or species (1,10,11) may reflect: (a) intrinsic differences in monoamine oxidases of diverse origin, (b) activity of other enzymes or (c) differences in accessibility of substrate or inhibitor to enzyme. Multiple substrate and inhibitor studies have variously suggested that a tissue may contain one(1,12) or more than one monoamine oxidase(13), and a changing pattern of substrate specificity during acetone fractionation of a tissue extract was also interpreted as evidence for 2 separate monoamine oxidases(14).

The present study was undertaken to provide further evidence on these points of uncertainty. Experiments with rat liver preparations were employed to define quantitatively some major monoamine oxidase activites, their intracellular localization and some characteristics of the reactions. Substrate specificity and multiple substrate experiments on particulate preparations from liver, kidney and heart of the rat and guinea pig were used to study the heterogeneity of monoamine oxidase within a given tissue and in different tissues.

Methods. Enzyme preparations. Freshly excised tissues from adult male Wistar rats or from male Coulson guinea pigs were homogenized in ice-cold 0.25 M sucrose — 0.001 M EDTA†, pH 7.0 ("sucrose-EDTA") with a Potter-Elvehjem homogenizer. Employing the method of deDuve *et al*(15) with slight modifications, portions of 20% homogenates were fractionated by differential centrifugation to yield nuclei, mitochondria, lysosome, microsome and supernatant fractions. The supernatant fraction was then diluted with an equal volume of water and taken to 65% saturation with $(NH_4)_2SO_4$ at pH 7; the resulting precipitate was collected by centrifugation and labeled "supernatant $(NH_4)_2SO_4$ precipitate." The "total particulate preparation" was obtained by centrifuging a 20% homogenate at 100,000 $\times$ g for one hour and then washing the sediment once by resuspension in the original volume of "sucrose-EDTA" and recentrifugation. All preparations were resuspended in "sucrose-EDTA" and stored at —10°C until assay; monoamine oxidase activities were stable for several weeks in the frozen state.

Assays. Monoamine oxidase activities were determined by manometric assay of oxygen consumption at 37°. The standard reaction mixture contained 0.067 M phosphate buffer, pH 7.0, 0.05 M semicarbazide, suitably diluted enzyme preparation, and 0.02 M neutralized substrate, which was tipped in from a sidearm after equilibration; KOH was added to the center well. A linear period of oxygen uptake, normally 10-40 minutes after substrate addition, was employed to calculate activities. Activities reported in this paper have been corrected for rates of oxygen uptake obtained in controls without added substrate; these endogenous rates were on the order of 10 μl O_2/hr/100 mg protein in the total particulate preparation, about 20 for liver homogenates, and less than 5 for homogenate fractions. Control experiments with added catalase established that the level of catalase present in any preparation with significant monoamine oxidase activity was sufficient to insure degradation of any hydrogen peroxide formed.

Succinic oxidase activities were assayed manometrically with added cytochrome c(16) and protein content by the spectrophotometric assay of Lowry *et al*(17).

Results. Intracellular distribution of monoamine oxidase. Table I shows the results of a typical fractionation of rat liver homogenate. Both monoamine oxidase activity, with tyramine as substrate, and succinic oxidase activity were largely recovered in the mito-

† EDTA, ethylenediamine tetraacetic acid.

TABLE I. Intracellular Distribution of Monoamine Oxidase and Succinic Oxidase in Rat Liver.

Tissue fraction	Protein, mg	Monoamine oxidase		Succinic oxidase	
		$\mu l\ O_2$	%	$\mu l\ O_2$	%
Homogenate	286.5	532	100.0	17,670	100.0
Nuclei	25.0	37	6.9	860	4.9
Mitochondria	97.5	408	76.7	14,510	82.2
Lysosomes	20.3	16	3.0	620	3.5
Microsomes	50.1	67	12.6	800	4.5
Supernatant $(NH_4)_2SO_4$ ppt	86.3	8	1.5	620	3.5
Recovery	279.2	—	100.7	—	98.6

The homogenate and fractions were prepared and assayed as described under *Methods*. Monoamine oxidase assays were carried out with 0.02 M tyramine as substrate. Protein content is expressed as mg protein/fraction derived from 1 g wet wt of liver, and oxidative activities as $\mu l\ O_2$/hr/fraction derived from 1 g wet wt of liver.

chondrial fraction. The minor monoamine oxidase activities found in the nuclear, lysosomal, or "supernatant $(NH_4)_2SO_4$ precipitate" fractions appeared to represent contamination by mitochondria or mitochondrial fragments, as these fractions contained similarly low activities for succinic oxidase, which is known to be localized in the mitochondria (16,18). However, the microsome fraction also contained significant monoamine oxidase activity, which was in excess of the degree of mitochondrial contamination indicated by the level of succinic oxidase activity. Monoamine oxidase activity with serotonin as substrate showed a similar pattern of recovery in the mitochondrial and microsomal fractions (data not tabulated). Other experiments showed that the solution remaining after the "supernatant $(NH_4)_2SO_4$ precipitation" contained less than 3% of the homogenate protein and no significant monoamine oxidase or succinic oxidase activity.

Total particulate preparation. Because of the particulate distribution of monoamine oxidase activity, "total particulate preparations" described under *Methods* were used for studies on monoamine oxidase characteristics. With all tissues examined, including liver, kidney and heart of rat and guinea pig, essentially all of the homogenate monoamine oxidase activity with tyramine was recovered in the "total particulate preparation"; less than 5% was found in the residual

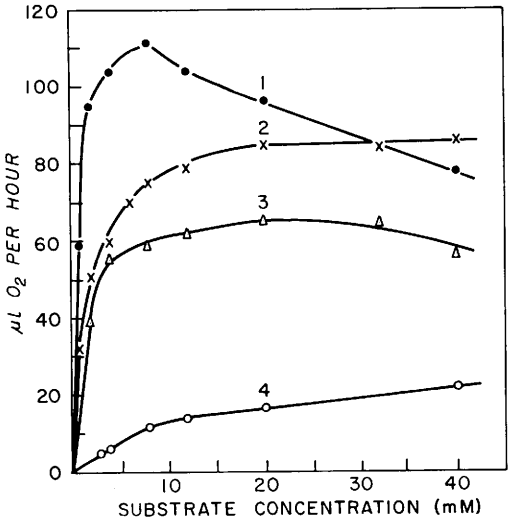

FIG. 1. Effect of substrate concentration on monoamine oxidase activity. Manometric assays were carried out on aliquots of rat liver total particulate preparation as described under *Methods*, with varying concentrations of the following substrates: Curve 1, tyramine; curve 2, serotonin; curve 3, benzylamine; curve 4, m-xylylenediamine.

soluble fraction. Preliminary studies with rat liver total particulate preparations in the standard monoamine oxidase assay showed that tyramine, serotonin, benzylamine and m-xylylenediamine were attacked rapidly, while cadaverine was not attacked at a rate sufficient for quantitative manometric studies. In experiments in which the reaction was permitted to go to completion with tyramine, serotonin or benzylamine as substrate, oxygen consumption was 0.85-0.95 of the theoretical 1.0 atom of oxygen consumed per molecule amine in the oxidative deamination reaction. If semicarbazide was omitted from the reaction mixture, further oxidation of the substrates occurred.

The effect of varying substrate concentrations on monoamine oxidase activity is shown in Fig. 1. The curves indicate that the 0.02 M substrate concentrations routinely employed in this study gave high but, with some substrates, not maximal activity levels.

Comparative studies on substrate specificity. As is shown in Table II, the total particulate preparations from liver, kidney and heart of rat and guinea pig showed both species and tissue differences in the rates at which various amines are attacked. The gen-

TABLE II. Monoamine Oxidase Activities of "Total Particulate Preparations" from Rat and Guinea Pig Tissues.

Tissue	Oxygen uptake with substrate indicated: Tyramine	Serotonin	Benzylamine	m-Xylylenediamine
Rat liver	274 ± 22 (4)	257 ± 10 (4)	176 ± 3 (4)	45 ± 9 (4)
" kidney	48 ± 4 (5)	107 ± 12 (4)	24 ± 7 (4)	19 ± 8 (6)
" heart	129 ± 13 (3)	311 ± 3 (3)	4 ± 2 (2)	0 ± 5 (2)
Guinea pig liver	489 ± 17 (6)	472 ± 35 (5)	43 ± 7 (5)	27 ± 7 (6)
" " kidney	330 ± 30 (4)	427 ± 15 (4)	15 ± 3 (2)	16 ± 3 (2)
" " heart	33 ± 10 (3)	95 ± 2 (3)	7 ± 3 (2)	11 ± 3 (2)

Total particulate preparations were prepared and assayed as described under *Methods*. Substrates were added in 0.02 M concentration. Monoamine oxidase activities are expressed as μl O_2/hr/100 mg protein in the total particulate fraction. Each value tabulated is the mean and standard deviation of data obtained in the number of experiments indicated in parentheses.

eral levels of monoamine oxidase activity varied among the tissues and, more significantly, the relative rates at which various substrates were attacked in a given tissue were markedly different for the various tissues.

Michaelis constants. Total particulate preparations were assayed with various concentrations of substrates over the range 0.002-0.40 M, and Michaelis constants (K_m) were determined from plots of v/[S] against [S](19). For the rat liver monoamine oxidase, values for K_m were 8.5×10^{-4} M with tyramine, 1.4×10^{-3} M with serotonin, 1.8×10^{-3} M with benzylamine, and 1.5×10^{-2} M with m-xylylenediamine; for the guinea pig liver enzyme, 1.6×10^{-3}M with tyramine, and 2.7×10^{-3} M with serotonin; for the guinea pig kidney enzyme, 3.2×10^{-4} M with tyramine and 1.9×10^{-3} M with serotonin.

Lysis of particles. Subjection of total particulate preparations from rat liver, guinea pig liver, or guinea pig kidney to either hypotonic electrolyte media or repeated freezing and thawing to lyse particulate structures did not result in increased monoamine oxidase activity; the activity of the original preparations therefore apparently was not limited by accessibility of substrate to the particulate site of the enzyme. After lytic treatment of preparations from rat liver and guinea pig kidney, all of the monoamine oxidase activity was still sedimented by centrifugation at 100,000 $\times$ *g* for one hour; with the guinea pig liver preparation, about 10% of the activity was no longer sedimented by such centrifugation after the lytic treatment.

Multiple substrate studies. As is shown in Table III, Part A, for total particulate preparations from 3 tissues, the monoamine oxidase activity in the presence of 2 substrates was not additive. In most instances, the activity with a combination of substrates lay between the levels observed with the substrates singly, and the activity level for a given combination reflected the relative K_m values of the 2 substrates. For the tyramine-serotonin combinations, the activity was below that of the less active substrate; these results may have reflected the inhibition observed with high concentrations of tyramine (Fig. 1). This suggestion is supported by the data of Table III, Part B, where use of lower substrate concentrations for the tyramine-serotonin experiments resulted in activities for the combination which were intermediate between the levels for the substrates separately.

Heat inactivation. The data of Table IV indicate that pre-treatment of the enzyme preparation at elevated temperature can cause markedly different degrees of inactivation of monoamine oxidase activity toward different substrates.

Discussion. The present results on intracellular distribution concur with previous findings that monoamine oxidase of liver is localized largely in the mitochondria. However, the microsome fraction also consistently contained monoamine oxidase activity in excess of that predicted by mitochondrial contamination, as measured by succinic oxidase activity in the microsomes. To the degree

TABLE III. Effects of Substrate Combinations on Monoamine Oxidase Activity.

Substrates	Activity relative to tyramine oxidation in particulate preparations from: Rat liver	Guinea pig liver	Guinea pig kidney
A			
.02 M tyramine	(100)	(100)	(100)
.02 M serotonin	91 ± 5	97 ± 7	127 ± 4
.02 M benzylamine	64 ± 1	9 ± 1	3 ± 1
.02 M m-xylylenediamine	17 ± 3	6 ± 1	3 ± 3
.02 M tyramine + .02 M serotonin	70 ± 7	80 ± 6	78 ±13
.02 M tyramine + .02 M benzylamine	64 ± 4	41 ± 3	40 ± 3
.02 M tyramine + .02 M m-xylylenediamine	92 ± 2	87 ± 4	109 ± 5
.02 M serotonin + .02 M benzylamine	83 ± 9	51 ± 9	67 ±11
B			
.01 M tyramine	110 ± 2	103 ± 2	102 ± 2
.01 M serotonin	69 ± 2	86 ± 1	100 ± 1
.01 M tyramine + .01 M serotonin	103 ± 2	98 ± 2	101 ± 4

Total particulate preparations were prepared and assayed for monoamine oxidase as described under *Methods*. Assays were carried out with substrates added in the final concentrations indicated above. For each individual preparation, activities were calculated as percentage of the activity obtained with that preparation when 0.02 M tyramine was used as substrate. Each value tabulated represents the mean and standard deviation of percentages obtained from 2-6 experiments.

that this measure of mitochondrial contamination is quantitatively valid, monoamine oxidase appears to have a dual localization, as suggested by deDuve *et al*(5), with a low but significant percentage localized in cellular particulates that sediment with the microsome fraction. The level of any soluble monoamine oxidase present in the tissues studied was very minor compared to the level of particulate enzyme and was not unambiguously demonstrable by the manometric procedures employed. The present finding that an appreciable portion of particulate monoamine oxidase of guinea pig liver, but not of rat liver or guinea pig kidney, was solubilized by lytic treatment of the total particulate preparations may explain a previous finding (9) that guinea pig liver is a particularly rich source of soluble monoamine oxidase; in that previous study the tissues were homogenized in water, under conditions which would result in lysis of mitochondria.

Since the various tissues showed markedly different relative rates of activity with different substrates, the monoamine oxidase complement of various tissues appears to be qualitatively different; the tissue differences in K_m values similarly suggest qualitatively different enzymes. These differences might indicate that various tissues (a) contain a different single monoamine oxidase, (b) contain a different group of monoamine oxidases, or (c) contain different proportions of several monoamine oxidases common to several tissues. Failure of lytic treatment to alter levels of activity in total particulate preparations from the 3 tissues examined suggests that differential accessibility of substrate to the intracellular site of the enzymes was not a major factor in the tissue differences observed.

The apparent competition for enzyme in the multiple substrate experiments suggests that each tissue contains a single enzyme active on all 4 substrates examined. The differences in relative substrate affinities of monoamine oxidase preparations from differ-

TABLE IV. Differential Heat Inactivation of Monoamine Oxidase Activities with Various Substrates.

Substrate	Heat treatment at 55°C	60°C
Tyramine	63.3	38.0
Serotonin	95.4	76.4
Benzylamine	40.5	.8
m-Xylylenediamine	39.6	—

A rat liver total particulate preparation was incubated for 10 min at temperatures indicated, then quickly chilled and assayed for monoamine oxidase activity with various substrates as described under *Methods*. Results are given as percentage of the original activity with that substrate which remained after heat treatment.

ent tissues may reflect minor differences in the chemical groupings or conformation about the active centers of the enzymes; alternately, the properties of essentially identical enzymes may be differently influenced in different cell types by components of the particulate mitochondrial matrix with which the monoamine oxidases are associated. The differential destruction by heat of activity toward various substrates may appear to suggest the presence of separate enzymes for the different substrates, but the results could, alternately, mean that conformational or gross structural changes induced by heat denaturation have quantitatively different effects upon either combination or reactivity of the enzyme with different substrates. The present conclusions do not extend to and are not altered by the possible presence in the tissues of other enzymes with quantitatively minor amine oxidase-like activities.

Summary. Fractionation of rat liver homogenates suggests a dual intracellular localization of monoamine oxidase, with the major portion in the mitochondria and a smaller but significant portion sedimenting with the microsomal fraction. Comparative studies on substrate specificities and Michaelis constants of monoamine oxidase preparations from liver, kidney and heart of rat and guinea pig indicate marked tissue differences in monoamine oxidases. Multiple substrate studies and other data support the view that each tissue contains a single, distinctive major monoamine oxidase.

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Genetic Control of Albumin Phenotypes in Horses.* (28766)

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Evidence that a minimum of 6 codominant autosomal alleles control transferrin phenotypes in horses has recently been reported from this laboratory(1). During further studies, using a starch-gel procedure recommended by Kristjansson(2), we have observed individual variation in the patterns of proteins which migrate in the albumin and post-albumin regions of the gels. Three

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