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The Labeling *in Vivo* of Monoamine Oxidase by ^{14}C-Pargyline: a Tool for Studying the Synthesis of the Enzyme

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SUMMARY

Pargyline (*N*-methyl-*N*-2-propynylbenzylamine), an irreversible inhibitor of monoamine oxidase *in vitro*, was found to bind irreversibly to the enzyme *in vivo*. The apparent covalent nature of the latter binding was established by exhaustive washing and treatment of the protein with trichloracetic acid. The inhibitor was selective for monoamine oxidase *in vivo* as well as *in vitro*, which permitted the determination of the turnover rates of monoamine oxidase in various subcellular fractions of rat liver. The half-life of mitochondrial monoamine oxidase was approximately 3.5 days, as determined by the rate of recovery of enzyme activity or by the decay of radioactivity after administration of sufficient 7-^{14}C-pargyline to inhibit the enzyme completely. Similarly, the half-life of microsomal monoamine oxidase was found to be approximately 1 day. Evidence is presented suggesting that radioactively labeled, inactive monoamine oxidase may be taken up by lysosomes. Cycloheximide completely prevented the recovery of monoamine oxidase activity in the microsomal fractions and markedly inhibited the rate of recovery of enzyme activity in the mitochondrial fractions.

INTRODUCTION

In recent years, considerable interest has developed in the submitochondrial localization of various membrane-bound enzymes and proteins, including monoamine oxidase (1), rotenone-insensitive NADH–cytochrome *c* reductase, and cytochrome b_5 (2). With the advent of techniques for the separation of outer and inner mitochondrial membranes as described by Schnaitman, Erwin, and Greenawalt (1), Sottocase *et al.* (2), and Parsons *et al.* (3), it has become possible to study protein turnover rates of these membranes as well as to investigate the mechanisms of catalysis and inhibition of monoamine oxidase (4, 5).

Until recently, determination of the turnover rates of mitochondrial membrane proteins has been dependent on the incorporation of radioactively labeled amino acids into these proteins and subsequently measuring the rates of disappearance of label. Utilizing this procedure, Beattie *et al.* (6) estimated the half-life of rat liver mitochondrial proteins to be approximately 8.5 days. After continuous administration of ^{14}C-carbonate, Swick *et al.* (7) estimated the half-life values for six rat liver mitochondrial proteins to be 4–6 days. In a study of turnover rates of heme components of outer and inner mitochondrial membrane proteins, Druyan *et al.* (8) observed that cytochrome b_5, associated with the outer membrane,

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possessed a half-life of 4.4 days, and obtained values of 4–6 days for hemes of the inner membrane proteins.

Another approach to the study of synthesis of proteins of the outer mitochondrial membrane involves the use of irreversible inhibitors of enzymes associated with this membrane. Barondes (9) determined the rate of regeneration of monoamine oxidase activity in rat brain mitochondria following the administration of 1-phenyl-2-hydrazinopropane (JB 516), an irreversible inhibitor of the enzyme *in vitro* (10). Since JB 516 and other hydrazines are not specific for monoamine oxidase (11), pargyline (*N*-methyl-*N*-2-propynylbenzylamine), a more specific, irreversible inhibitor of monoamine oxidase, was used in the present studies. Hellerman and Erwin (5) found that pargyline was specific for monoamine oxidase *in vitro* and that the active sites of the enzyme could be titrated with this compound. In addition, pargyline apparently becomes covalently bound to the enzyme. In preliminary studies, Erwin and Simon (12) observed that the rates of recovery of monoamine oxidase activity in rat liver mitochondria were similar following administration of sufficient iproniazid or pargyline for complete inhibition of the enzyme activity *in vivo*. The present studies were conducted in order to determine the nature and specificity of the binding of pargyline to monoamine oxidase *in vivo* and to utilize this inhibitor in studying the turnover rate of the enzyme in outer mitochondrial membranes and in the microsomal membranes.

METHODS AND MATERIALS

Rat livers were removed immediately after decapitation and placed in sufficient 0.25 M sucrose to give a 20% suspension. Homogenization was performed by 10 passes of a tightly fitting Teflon pestle in a glass homogenizer. After dilution with an equal volume of 0.25 M sucrose, the suspension was centrifuged at 600 × *g* for 10 min, and the resulting supernatant fluid (hereafter termed "homogenate") was decanted. Mitochondria were isolated essentially as described by Schnaitman, Erwin, and Greenawalt (1), except that in some cases the pink, fluffy layer obtained with the mitochondrial fraction, after the initial sedimentation and two washes, was not decanted. Outer and inner mitochondrial membranes from mitochondrial preparations not containing the pink, fluffy layer were prepared by the procedure of Schnaitman *et al.* (1), utilizing digitonin. Microsomal membranes were isolated from the supernatant fluid, after the initial mitochondrial sedimentation, by centrifugation at 160,000 × *g* for 1 hr.

For the preparation of lysosomes, a modification of the method of Ragab *et al.* (13) was employed. An aliquot of the mitochondrial fraction (approximately 50 mg of protein per 0.5 ml) containing the pink, fluffy layer was layered over 5 ml of 0.6 M sucrose in a 10-ml tube and centrifuged at 6000 × *g* for 10 min. The nonsedimented lysosomes were decanted and sedimented by centrifugation at 20,000 × *g* for 10 min.

For estimation of the degree of cross-contamination of the mitochondria by microsomal and lysosomal fractions, the following enzyme activities were determined on the separated fractions: glucose 6-phosphatase at pH 6.5, by the method of Swanson (14); β-glucuronidase, as described by Gianetto and DeDuve (15); and succinate dehydrogenase, according to the method of Arrigoni and Singer (16). Cross-contamination of the inner and outer mitochondrial membranes was determined from the activities of monoamine oxidase [according to Deitrich and Erwin (17)], kynurenine hydroxylase (18), and succinate dehydrogenase. Contamination of the inner and outer mitochondrial membranes with microsomes or lysosomes was estimated as described above. All enzyme assays were performed with a Gilford recording spectrophotometer as described in the figures and tables. Protein determinations were conducted according to the biuret procedure, using three times crystallized bovine serum albumin as a standard.

In studies with 7-^{14}C-pargyline hydrochloride, aliquots of each fraction were placed in liquid scintillation vials containing 10 ml of scintillation fluid [consisting of 7 g of 2,5-diphenyloxazole and 0.42 g of *p*-bis[2-(5-phenyloxazolyl)]benzene per liter

of toluene] and 2 ml of Beckman Biosolve BBS III. Protein determinations and enzyme assays were performed on each fraction. Radioactivity was measured in a Beckman liquid scintillation spectrometer and was corrected for quenching by the use of internal standards.

Sprague-Dawley rats of either sex, weighing 225–250 g, were employed in all experiments. Each rat was treated by injection with 20 mg of pargyline per kilogram of body weight or with 7-^{14}C-pargyline (specific activity, 1 μCi/mg). The animals were killed at various times after administration as indicated in the tables and figures. The 7-^{14}C-pargyline was kindly supplied by Abbott Laboratories. All other reagents were of the highest purity available.

In studies utilizing inhibitors of protein synthesis, male rats were treated with cycloheximide, 1 mg/kg intraperitoneally twice daily for two doses, and then with 0.5 mg/kg twice daily. The last dose of inhibitor was given 90 min before death.

RESULTS

Nature and specificity of pargyline binding in vivo. Inasmuch as Hellerman and Erwin (5) had observed that pargyline became covalently bound to the highly purified beef kidney monoamine oxidase *in vitro*, it was of interest to determine the nature of pargyline binding *in vivo*. As shown in Table 1, the specific activity (counts per minute per milligram of protein) of various rat liver fractions obtained at 12, 120, and 168 hr following the administration of 7-^{14}C-pargyline was not decreased when these fractions were washed three times with isotonic sucrose solutions containing 0.01 M unlabeled pargyline. Also, the specific activities were not decreased when the fractions were washed three times with 10% trichloracetic acid. These findings indicate that pargyline becomes essentially covalently bound to these fractions *in vivo*.

In order to determine the specificity of pargyline binding *in vivo*, the percentage recoveries of monoamine oxidase activity in various subcellular fractions, isolated from livers of untreated rats, were compared with the distribution of covalently bound ^{14}C-pargyline in similar fractions isolated from livers of animals which had received the monoamine oxidase inhibitor. As shown in Table 2, the percentage recoveries of monoamine oxidase activity in the mitochondrial and microsomal fractions were virtually the same as the recoveries of ^{14}C in these fractions. Also, the enzyme activities and radioactivity found in the outer and inner mitochondrial membrane fractions

TABLE 1

Apparently covalent nature of ^{14}C-pargyline binding to mitochondrial and microsomal monoamine oxidase in vivo

Rats were given ^{14}C-pargyline (specific activity, 0.2 μCi/μmole), 20 mg/kg of body weight intraperitoneally, and their livers were removed at the times indicated after injection. Fractions were isolated and counted as described in the text. Also, each fraction was washed three times by resuspension in approximately 30 volumes of 0.25 M sucrose containing 0.01 M pargyline hydrochloride. In separate experiments, the 12-hr samples of each fraction were precipitated with 10% trichloracetic acid and washed three times by centrifugation and resuspension in 10% trichloracetic acid. After the final centrifugation, fractions were resuspended and counted. Protein determinations were performed before and after washings. Values represent the averages of three separate experiments. In all studies, sufficient protein was used to give at least 300 cpm total with the microsomal fractions and at least 1000 cpm total with other fractions.

Fraction	Time after ^{14}C-pargyline	Specific radioactivity		
		Before exchange wash	After exchange wash	After trichloracetic acid wash
	hr	*cmp/mg protein*		
Mitochondria	12	87	85	82
Microsomes	12	15	13	16
Outer mitochondrial membranes	12	112	134	126
Mitochondria	120	35	33	
Outer mitochondrial membranes	120	53	70	
Mitochondria	168	30	29	
Outer mitochondrial membranes	168	40	49	

TABLE 2

Distribution of monoamine oxidase activity and ^{14}C-pargyline labeled in vivo in various fractions of rat liver

Monoamine oxidase activity in various fractions from the livers of untreated rats was determined as described in the text with *p*-dimethylaminobenzylamine as substrate. Counts per minute were determined on various fractions from livers of animals 12 hr following the administration of ^{14}C-pargyline (specific activity, 0.2 μCi/μmole), 20 mg/kg intraperitoneally. The extent of cross-contamination of the various fractions was determined as described in the text. Mitochondrial fractions contained less than 10% of the total glucose 6-phosphatase activity and approximately 20% of the total β-glucuronidase activity. The microsomal fractions contained no detectable succinate dehydrogenase, and the lysosomal fractions possessed less than 5% of the total succinate dehydrogenase activity. The outer mitochondrial membrane preparations contained less than 5% of the total succinate dehydrogenase activity, whereas, as shown below, the inner mitochondrial membranes possessed approximately 18% of the monoamine oxidase activity. Corrections were made for contamination of mitochondria with lysosomes and microsomes; however, no other corrections were necessary.

Fraction	Monoamine oxidase recovery	Monoamine oxidase activity[a]	Radioactivity[a]	Recovery of radioactivity	Protein recovery
	%			%	%
Homogenate	100	0.532 (0.003)	7112 (35)	100	100
Mitochondria	53.9	0.287 (0.010)	3406 (90)	51	21.7
Microsomes	9.0	0.048 (0.002)	529 (17)	8	17.4
Outer mitochondrial membranes	37.7	0.201 (0.016)	2346 (130)	33	7.8
Inner mitochondrial membranes	18.0	0.096 (0.004)	1180 (38)	16	17.7
Lysosomes	0.5	0.0027 (0.0003)	715 (92)	10	4.7
144,000 × *g* supernatant	0		71	1	41.0
Total recovery	63.4			70	84.8

[a] Monoamine oxidase activity and radioactivity are expressed as change in absorbance at 255 mμ per minute and counts per minute per gram of liver, wet weight, respectively. Values in parentheses represent the specific enzyme activity and radioactivities per milligram of protein and are averages of four separate determinations.

paralleled one another. The yields of protein obtained in various fractions were not similar to the recoveries of enzyme activity or radioactivity. It should be noted that the percentage recovery of radioactivity (^{14}C-pargyline) in the lysosomal fraction was considerably greater than that of monoamine oxidase in this fraction.

As shown in Table 2, the total recoveries of monoamine oxidase activity, radioactivity, and protein were 63.4%, 70%, and 84.8%, respectively. The difference in recoveries of monoamine oxidase activity and ^{14}C is due primarily to the presence of ^{14}C in the lysosomal fraction, which contains little monoamine oxidase activity. Since most of the protein loss occurs in the washing of the mitochondrial fractions and inasmuch as monoamine oxidase activity and ^{14}C are localized primarily in these fractions, less monoamine oxidase activity or radioactivity than protein would be recovered. As noted in Table 2, changes in the specific activities, as well as recoveries, of the different fractions are similar for monoamine oxidase and radioactivity. These results indicate that pargyline is selective for monoamine oxidase in binding to these subcellular fractions of rat liver *in vivo*.

Further observations indicating that pargyline selectively binds to monoamine oxidase are presented in Table 3. In these studies *in vitro* it was observed that the specific activities of the ^{14}C-pargyline bound to the mitochondrial, microsomal, outer mitochondrial membrane, and inner mitochondrial membrane fractions were remarkably similar to the values obtained in the

TABLE 3

Distribution of covalently bound ^{14}C-pargyline in various fractions of rat liver in vitro

The various subcellular fractions were isolated as described in the text. ^{14}C-Pargyline (specific activity, 0.2 μCi/μmole) was added to various subcellular fractions or homogenates to give a final concentration of 10^{-5} M pargyline. The final volume of each subcellular fraction was 1 ml, and the final volume of the 20% homogenate was 10 ml. After incubation of the subcellular fractions with ^{14}C-pargyline for 30 min, approximately 10 volumes of isotonic sucrose solution containing 0.01 M unlabeled pargyline were added to each fraction, and, after mixing, the fractions were centrifuged as described in the text. This washing procedure was repeated four times, and aliquots of the resulting resuspended fractions were taken for determination of radioactivity. The liver homogenate was incubated for 30 min with ^{14}C-pargyline, and the fractions then were separated and counted.

Fraction	Radioactivity	
	In liver tissue fractions[a]	After incubation of homogenate with ^{14}C-pargyline[b]
	cpm/g tissue	
Mitochondria	3650	3042
Microsomes	548	876
Outer mitochondrial membrane	2190	2640
Inner mitochondrial membrane	1145	1320
Lysosomes	240	324

[a] Represents counts per minute contained in various fractions obtained from 1 gram of liver tissue, wet weight.

[b] Represents the counts per minute in various fractions obtained from 1 gram of liver tissue when the homogenate was incubated with ^{14}C-pargyline, as above, and the fractions then were separated and counted.

studies *in vivo* (Table 2). The total activity (counts per minute per gram of tissue, wet weight) in the lysosomal fraction, labeled *in vitro*, was considerably lower than the values observed when this fraction was isolated from the livers of rats which had received ^{14}C-pargyline, possibly indicating that the lysosomes rapidly accumulated

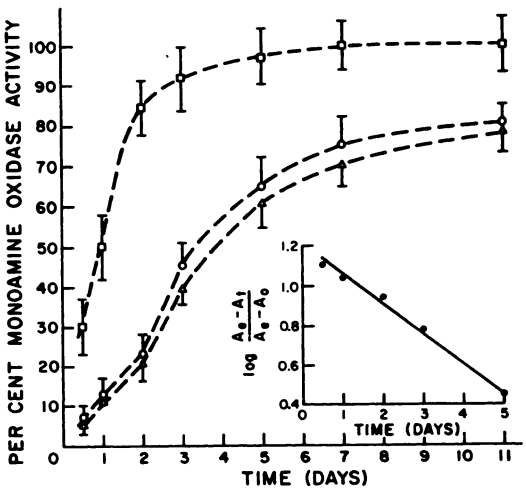

FIG. 1. *Rate of recovery of monoamine oxidase activity in preparations from rat liver following administration of pargyline in vivo*

Mitochondrial, outer mitochondrial membrane, and microsomal membrane fractions were prepared as described in the text from animals receiving 20 mg of pargyline per kilogram of body weight. Monoamine oxidase activity and protein were determined as described in the text. Values are the means ± standard errors of at least six experiments. Results obtained with microsomes (□- - -□), mitochondria (△- - -△), and outer mitochondrial membranes (○- - -○) are as illustrated. In the inset, a plot of log $[(A_\infty - A_t)/(A_\infty - A_0)]$ with respect to time, according to the method of Swick *et al.* (7), is shown. A_∞, A_t, and A_0 refer to mitochondrial monoamine oxidase activity after 80% recovery from inhibition (i.e., at 11 days), monoamine oxidase activity at any given time, and initial monoamine oxidase activity (i.e., at 12 hr), respectively. The slope (k-value) equals 0.37 day^{-1}, representing a half-life of 1.8 days.

radioactively labeled monoamine oxidase *in vivo* (Table 2).

Rate of return of monoamine oxidase activity in vivo following administration of pargyline. As shown in Fig. 1, following complete inhibition of monoamine oxidase activity *in vivo*, the activity in the mitochondrial and outer mitochondrial membrane fractions returned to 50% of the normal level in approximately 3.5 days. The rate of return of enzyme activity in the microsomal fraction was considerably more rapid than in the mitochondrial fractions, with 50% of the normal activity being

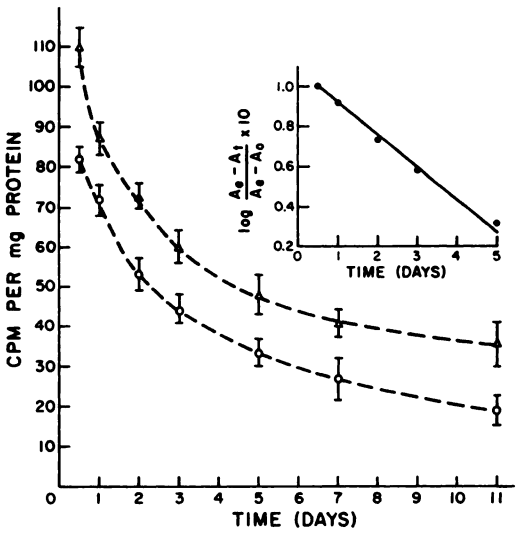

FIG. 2. *Rate of disappearance of radioactivity from mitochondria and outer mitochondrial membranes following administration of ^{14}C-pargyline in vivo*

The fractions were prepared as described in the text from the livers of rats receiving 20 mg of ^{14}C-pargyline (0.2 μCi/μmole) per kilogram of body weight. Radioactivity was determined as described in the text. Each point represents the mean ± standard error for six degrees of freedom. The results obtained with mitochondria (O- - -O) and outer mitochondrial membranes (△- - -△) are as illustrated. Corrections for cross-contamination were made as described in Table 2. In the inset, a plot of log $[(A_e - A_t)/(A_e - A_0)]$ with respect to time, according to the method of Swick *et al.* (7), is shown for the mitochondrial fractions (Fig. 1). The slope (k-value) equals 0.38 day^{-1}, representing a half-life of 1.8 days.

attained approximately 1 day after the administration of pargyline. When the data were plotted according to the method of Swick *et al.* (7) (Fig. 1), a slope (k-value) of 0.37 day^{-1} was obtained. This value represents a half-life of 1.8 days for the mitochondrial monoamine oxidase. Since it was obtained at 80% recovery from inhibition (Fig. 1), rather than at equilibrium, this value may be somewhat lower than the actual half-life.

The rate of disappearance of radioactivity from the mitochondrial fraction and the outer mitochondrial membrane fraction isolated from the livers of animals receiving ^{14}C-pargyline is shown in Fig. 2. It was observed that approximately 3.5 days were required for the specific radioactivity (counts per minute per milligram of protein) to decrease by 50% of the 12-hr value. Again, however, if the data were plotted according to the method of Swick *et al.* (7) (Fig. 2), a rate constant of 0.38 day^{-1} was obtained, representing a half-life of 1.8 days for the radioactive pargyline associated with monoamine oxidase (Tables 1, 2, and 3). These results are in excellent agreement with those obtained in Fig. 1 for the rate of return of monoamine oxidase activity. As shown in Fig. 3, radioactivity associated with the microsomal membranes decreased to 50% of the 12-hr value in approximately 1 day. These results also are in excellent agreement with those obtained in Fig. 1.

Effect of inhibitors of protein synthesis. Figure 4 shows that cycloheximide effectively decreased the rate of return of monoamine oxidase activity in the mitochondrial fraction. However, the recovery of activity was not completely blocked. The enzyme activity returned to approximately 20% of the control value 4 days after pargyline administration, compared to 55% in the absence of

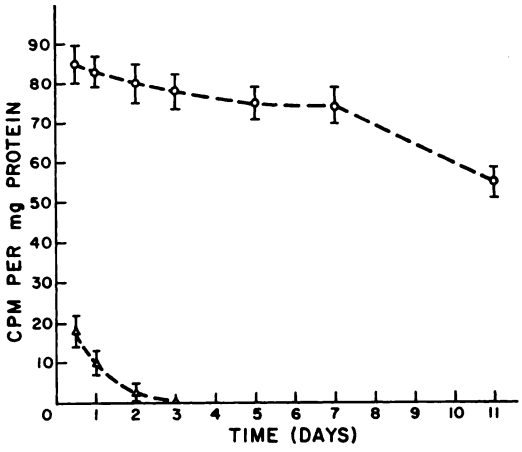

FIG. 3. *Rate of disappearance of radioactivity from microsomal and lysosomal fractions in vivo following administration of ^{14}C-pargyline*

Fractions were prepared as described in the text from animals treated as described in Fig. 2. The results with microsomes (△- - -△) and lysosomes (O- - -O) are as illustrated.

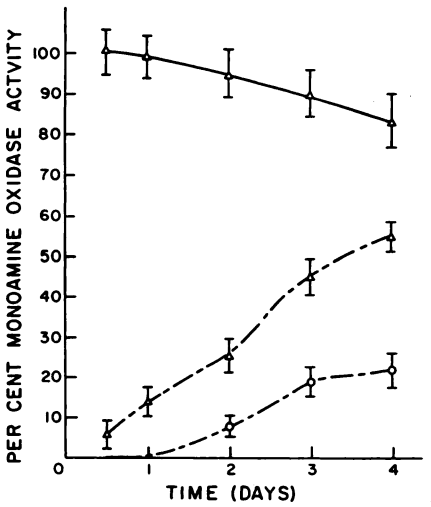

FIG. 4. *Effect of cycloheximide on recovery of monoamine oxidase activity in rat liver mitochondria after pargyline administration*

Cycloheximide was administered as described in the text and in Table 4. Pargyline (20 μCi/μmole) was administered at zero time, and at the times indicated animals were killed and liver mitochondria were isolated as described in the text. Control animals received only cycloheximide. The results with cycloheximide alone (△——△), cycloheximide plus pargyline (○- - -○), or pargyline alone (△- - -△) are as illustrated. Values represent the means ± standard errors and represent percentages of the total monoamine oxidase activity per gram of liver (wet weight).

cycloheximide. As shown in Fig. 5, cycloheximide completely blocked the return of monoamine oxidase activity in the microsomal membrane fractions. Although the regeneration of monoamine oxidase activity in microsomes and mitochondria was inhibited by cycloheximide, this compound had relatively little effect on endogenous monoamine oxidase levels (Figs. 4 and 5). As a result of these observations, experiments were conducted to determine whether cycloheximide would alter protein synthesis in the rat liver. The data presented in Table 4 show that treatment with cycloheximide decreased the incorporation of ^{14}C-leucine into liver protein by 40%. These results, obtained 90 min after administration of cycloheximide, are in agreement with those reported by Yeh and Shils (19). These authors found that 7 hr after the administration of 0.5 mg of cycloheximide per kilogram of body weight to female rats, protein synthesis was inhibited approximately 60%, and 12 hr after administration of the inhibitor, amino acid incorporation returned to normal. Since, in the present studies, cycloheximide was administered every 12 hr, it is probable that there existed periods when protein synthesis was inhibited followed by periods when protein synthesis was relatively intact. Such results might explain the relatively minor decreases in endogenous monoamine oxidase activity observed when cycloheximide was administered.

Since it appeared that mitochondrial and microsomal monoamine oxidase may not be similar enzymes, the kinetic properties of the enzymes in these fractions were investigated (Table 5). The K_m values for benzylamine, *p*-dimethylaminobenzylamine, and kynuramine were obtained from observed reaction rates. K_i values for serotonin, norepinephrine, and tyramine were obtained using *p*-dimethylaminobenzylamine as substrate with both the mitochondrial and microsomal enzymes. As shown in Table 5, little difference was observed in the values

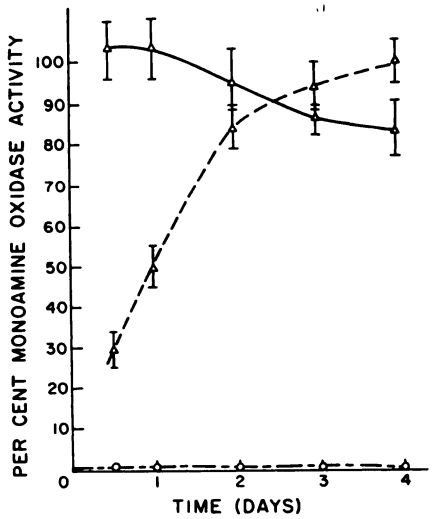

FIG. 5. *Effect of cycloheximide on recovery of monoamine oxidase activity in rat liver microsomes after pargyline administration*

Experiments were performed as described in Fig. 4. Results with cycloheximide alone (△——△), cycloheximide plus pargyline (○- - -○) and pargyline alone (△- - -△) are as illustrated.

TABLE 4

Inhibition of protein synthesis in rat liver by cycloheximide

Male rats were treated with cycloheximide as described in the text. The experiment was terminated 64 hr after the first dose of drug. The last dose was given 90 min before ^{14}C-leucine (2.5 μCi/100 g) was given intraperitoneally. The animals were killed 30 min later, and their livers were removed. A 10% homogenate in 0.25 M sucrose was prepared and centrifuged at 600 × *g*. One milliliter of the supernatant fluid was added to 1 ml of 0.6 N perchloric acid. The precipitated protein was washed three times with 0.33 N perchloric acid by centrifugation, and the precipitate was dissolved in 1 ml of "NCS" solubilizer[a] and counted by scintillation. Both internal and external standards were used to correct for quenching, which was found to be uniform in all samples. Values are calculated from the disintegrations per minute incorporated into protein per gram of liver, wet weight.

Treatment	Radioactivity			Incorporation
	Total	Acid-precipitated	Acid-soluble[b]	
		dpm/g liver		*% control*
Control	133,011	99,032	33,979	
Cycloheximide	122,344	59,711	62,633	60

[a] Amersham/Searle, Chicago.

[b] Acid-soluble values represent the difference between total and acid-precipitated disintegrations per minute. Values represent those obtained from a typical experiment.

TABLE 5

Kinetic properties of mitochondrial and microsomal monoamine oxidase

The mitochondrial and microsomal fractions were isolated as described in the text. The K_m and K_i values were obtained by plotting the data obtained by a Lineweaver-Burk plot. With benzylamine, *p*-dimethylaminobenzylamine, or kynuramine, the activity of monoamine oxidase in the fractions was determined by the methods of Tabor *et al.* (20), Deitrich and Erwin (17), and Weissbach *et al.* (21), respectively. K_i values for tyramine, serotonin, and epinephrine were determined by adding these substrates in a final concentration of 1 mM and assaying the enzyme activity with *p*-dimethylaminobenzylamine as substrate.

Substrate	Mitochondrial monoamine oxidase		Microsomal monoamine oxidase	
	$K_m \times 10^4$	$K_i \times 10^4$	$K_m \times 10^4$	$K_i \times 10^4$
Benzylamine	1.6		0[a]	
p-Dimethylaminobenzylamine	2.2		3.0	
Kynuramine	0.12		0.15	
Tyramine		2.8		1.1
Serotonin		7.6		9.0
Epinephrine		18.0		15.0

[a] Monoamine oxidase activity, as measured by the increase in absorbance at 250 mμ due to benzaldehyde formation, could not be observed in the microsomal fractions.

when either mitochondrial or microsomal monoamine oxidase was utilized. The major difference was that benzaldehyde formation by microsomal preparations could not be observed with benzylamine as substrate.

DISCUSSION

As noted in Figs. 1 and 2, the rates of return of monoamine oxidase activity after pargyline administration and the rates of disappearance of radioactivity were markedly different in the mitochondrial and microsomal fractions. These results indicate that monoamine oxidases in these fractions possess distinctly different turnover rates. There are at least two possible explanations for these observations: either (a) the microsomal enzyme is a precursor for the mitochondrial enzyme, or (b) the enzymes are distinctly different, possessing different rates of synthesis and degradation. Data presented in Figs. 4 and 5 show that although cycloheximide completely prevents the recovery of enzyme activity in the microsomal fraction, the activity in the mitochondrial fraction was approximately 20% of the normal value after 5 days. If a similar proportion of enzyme activity had returned in the microsomal fraction, the assay employed would have detected it. These results support the conclusion that monoamine oxidase ac-

tivity observed in the microsomal fraction is not due to contamination by the outer mitochondrial membrane. It is also possible to conclude from these data that the microsomal and mitochondrial enzymes are distinctly different proteins and that the microsomal enzyme may not be a precursor for the mitochondrial enzyme. However, another possibility is that cycloheximide may prevent the binding of newly synthesized monoamine oxidase to the microsomal fraction, allowing the enzyme to be transferred directly to the mitochondrial fraction. The evidence available does not permit a distinction between these two possibilities or, for that matter, other possibilities. Recently, Youdim and Sandler (22) reported that monoamine oxidases may exist in mitochondria as isozymes, and evidence that mitochondrial monoamine oxidase may be different from the microsomal enzyme has been reported (23). As shown in Table 5, the kinetic constants for various substrates for the microsomal monoamine oxidase and the mitochondrial enzyme were quite similar; the only significant difference was the inability to detect benzaldehyde formation by the microsomal fraction with benzylamine as substrate.

The data presented in Fig. 3 show that the specific radioactivity in the liver lysosomal fractions was maximal 12 hr following the administration of ^{14}C-pargyline. Although after 12 hr the specific radioactivity in the microsomal and mitochondrial fractions decreased rapidly, the radioactivity associated with the lysosomal fraction did not decrease appreciably until after 7–11 days. Since evidence presented in Table 3 indicates that the lysosomes do not bind pargyline *in vitro* sufficiently to account for the amount of label in this fraction *in vivo*, it is possible that the high specific radioactivity in the lysosomal fractions obtained after the administration of labeled pargyline was due to the ingestion of radioactively labeled microsomal and mitochondrial monoamine oxidase by the lysosomes *in vivo*. The long lag period in the decrease of radioactivity in the lysosomal fraction could be accounted for by the inability of the lysosome to degrade completely the monoamine oxidase with its covalently bound pargyline.

In studies with a highly purified bovine kidney monoamine oxidase preparation, Hellerman and Erwin (5) observed that pargyline became covalently bound to the active site of the enzyme *in vitro*. These investigators also demonstrated that, with bovine kidney preparations of various specific activities, pargyline was selective for monoamine oxidase. In addition, it was shown that pargyline was initially a substrate for the enzyme and, in the process of being oxidized by the enzyme, became covalently bound. The present studies have extended to systems *in vivo* the covalent nature of the binding of pargyline to monoamine oxidase. Since this compound becomes covalently bound to monoamine oxidase *in vivo*, apparently because of the active site-directed nature of the inhibitor, it provides a useful tool for studying the rate of synthesis of monoamine oxidase in various subcellular fractions, as well as providing some evidence as to the means of degradation and disposition of this enzyme. Another advantage of the use of pargyline in studying the turnover rates of monoamine oxidase protein is that the nature of the binding renders recycling of the inhibitor improbable.

The half-life of approximately 1 day for the monoamine oxidase associated with the microsomal membrane is somewhat shorter than the value of 2.3–2.5 days for the half-life of microsomal cyctochrome b_5 as reported by Druyan *et al.* (8). However, the findings presented above are in agreement with other reports that the microsomal membrane proteins turn over more rapidly than proteins of the outer mitochondrial membrane (8, 24).

Inasmuch as monoamine oxidase has been demonstrated to be localized on the outer mitochondrial membrane (1), the use of pargyline as an irreversible inhibitor of the enzyme has enabled us to determine the turnover rate of a specific protein associated with the outer mitochondrial membrane. The results indicate that the half-life of monoamine oxidase in the outer mitochondrial membrane may be as small as 1.8 days and no longer than 3.5 days. These half-lives are shorter than those reported for some mitochondrial proteins, particularly those associated with the inner mitochondrial mem-

branes (7, 8). Also, Druyan *et al.* (8) observed that the half-life of cytochrome b_5 associated with the outer mitochondrial membrane was 4.4 days. The somewhat shorter half-life values obtained for microsomal and mitochondrial monoamine oxidase in the present studies may be due to a more rapid turnover of the irreversibly inhibited enzyme than of the normal endogenous protein. Such results would be expected if the rates of synthesis of the enzyme were regulated by a substrate or product feedback mechanism. Nevertheless, the half-life values for monoamine oxidase obtained in the present study are sufficiently similar to those obtained by other investigators (7, 8) to indicate that the outer mitochondrial membrane proteins may turn over as a unit. However, the data are also consistent with the possibility of a "point-for-point" replacement of monoamine oxidase in the outer mitochondrial membrane.

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