Human Liver Mitochondrial Monoamine Oxidase. III. Kinetic Studies Concerning Time-Dependent Inhibitions*

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ABSTRACT: Kinetic data indicate that dissimilar mechanisms are responsible for *in vitro* inhibitions of human liver mitochondrial monoamine oxidase by agents representative of three major categories of inhibitors and that the apparent potency of a given inhibitor depends upon several conditions of the determinations.

Iproniazid and isopropylhydrazine inhibit the enzyme by a time-dependent mechanism. While substrates protect the enzyme from such time-dependent inhibitions, the kinetics of substrate oxidations by partially inactivated enzyme indicate noncompetitive inhibitions with respect to substrate. The time-dependent interactions of iproniazid or isopropylhydrazine with the enzymes are not simple second-order reactions, but appear to result from catalytic conversions of these hydrazines to noncompetitive inhibitors. Unlike these hydrazines, 2-phenylcyclopropylamines and 2-propynylamines are instantaneous, competitive inhibitors as well as time-dependent noncompetitive inhibitors. The time-dependent inhibitions are in keeping with simple second-order reactions. The effect of pH upon inhibitor constants of the instantaneous interactions and the second-order rate constants of the time-dependent reactions suggest that un-ionized species of these amine inhibitors interact with the enzyme. In respect to both types of inhibition, the *d* isomer of tranylcypromine may be considered to be primarily responsible for the inhibitory effects of the racemic mixture. *p*-Mercuribenzoate inhibits the enzyme activity by a time-dependent mechanism, consistent with a simple second-order reaction. This sulfhydryl reagent has no instantaneous effect upon the enzyme activity, however, and the kinetics of substrate oxidation by partially inactivated enzyme indicate the inhibition to be competitive with respect to substrate.

The preceding paper in this series (C. M. McEwen, Jr., G. Sasaki, and D. C. Jones, submitted for publication) reported that the un-ionized species of biogenic amines interact with the mitochondrial monoamine oxidase (EC 1.4.3.4) of human liver to form enzyme–substrate complexes. These kinetic data agreed with a previous report (McEwen *et al.*, 1968) concerning interactions of this partially purified enzyme with model substrates and substrate analogs, including ammonia and alcohols. The data of these studies were interpreted as evidence that the enzyme active center contains an electrophilic binding site and nonpolar areas for hydrophobic bonding. In this respect, instantaneous interactions even of complex substrates and competitive inhibitors with the enzyme may be described in relatively simple terms when the pK_a values of ionizable groups of the reactants are considered.

Reviews of the literature on time-dependent inhibitions of mitochondrial monoamine oxidase activities (Biel *et al.*, 1964; Zirkle and Kaiser, 1964: Burger and Nara, 1965) reveal conflicting reports concerning mechanisms and apparent potencies of identical mitochondrial monoamine oxidase inhibitors studied *in vitro*. Although the conflicting data probably result, at least in part, from different sources and purities of the mitochondrial monoamine oxidases studied (Hellerman and Erwin, 1968) they may also reflect diverse procedures that neglect variations in conditions (*e.g.*, time and pH values) of the studies. Despite the use of mitochondrial monoamine oxidase inhibitors in medicine, we are not aware of kinetic studies concerning time-dependent inhibitions of any human mitochondrial monoamine oxidase.

This report concerns the *in vitro* interactions of human liver mitochondrial monoamine oxidase with typical, time-dependent inactivators of its activities. The inactivators represent three major categories of mitochondrial monoamine oxidase inhibitors: (1) hydrazine derivatives, (2) derivatives of cyclopropylamine and propynylamine, and (3) reagents that react with protein sulfhydryl groups. Categories one and two include pharmacological agents used in clinical medicine, as well as simple analogs of these agents. Category three is considered in this study because mitochondrial monoamine oxidase activities from animal tissues are inhibited by sulfhydryl reagents (Blaschko, 1952; Nara *et al.*, 1966; Erwin and Hellerman, 1967). For these reasons, the present study provides additional data as evidence that the enzyme preparation has properties characteristic of usual mitochondrial monoamine oxidases.

Experimental Procedure

Materials. Partially purified mitochondrial monoamine oxidase (specific activity 44) was prepared from human liver by two Triton X-100 extractions of isolated mitochondria, ammonium sulfate fractionation of the second extract, extensive dialysis of the soluble activity, and a final centrifugation (27,000*g* for 2 hr) of the dialyzed preparation, as previously described (McEwen *et al.*, 1968).

Iproniazid (Marsilid phosphate) and isopropylhydrazine were gifts of Roche Laboratories, Nutley, N. J. Cyclopropylamines, including tranylcypromine (Parnate sulfate), were

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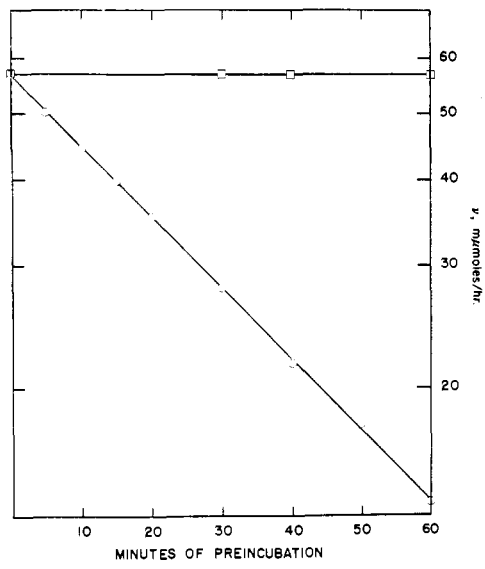

FIGURE 1: Time-dependent inhibition by iproniazid. Preincubation reaction mixtures containing 7 units of enzyme in 2.90 ml of 0.10 M sodium pyrophosphate buffer (pH 8.10) with (○) or without (□) 17.3 μM iproniazid were maintained at 25° for the indicated minutes. Following preincubation, 0.10 ml of 0.40 M veratrylamine in buffer was added to each mixture and initial rates of veratraldehyde production from 13.3 mM veratrylamine were measured at 25°.

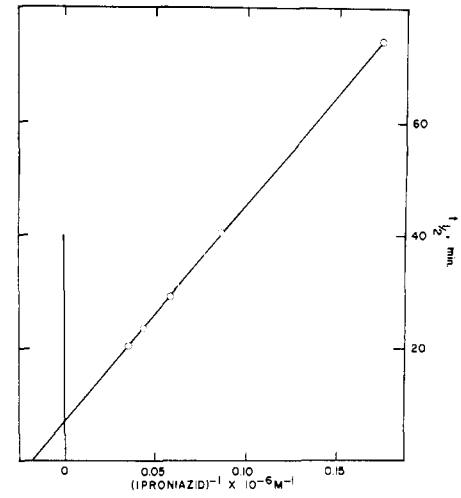

FIGURE 3: Inactivation half-times as a function of reciprocal concentrations of iproniazid. Reaction mixtures containing 7 units of enzyme and the indicated iproniazid concentrations in 2.90 ml of sodium pyrophosphate buffer (pH 8.10) were maintained at 25°. Following preincubation, 0.10 ml of 0.40 M veratrylamine in buffer was added to each mixture and initial rates of veratraldehyde production were measured at 25°. Pseudo-first-order plots (*e.g.*, Figure 1) were used to determine minutes of preincubation that produced 50% inhibition of veratraldehyde production.

kindly supplied by Dr. Charles L. Zirkle of Smith Kline and French Laboratories, Philadelphia. Pargyline hydrochloride was generously provided as Eutonyl by Abbott Laboratories, North Chicago. Propargylamine hydrochloride (Aldrich Chemical Co., Milwaukee) was recrystallized twice from absolute ethanol before use. *p*-Mercuribenzoate (Sigma Chemical Co., St. Louis) was used without purification. Benzylamines were purified before use as previously described (McEwen *et al.*, 1968). All inorganic compounds were analytical reagents. Double-distilled water, containing less than 20 ppb of copper, was used throughout.

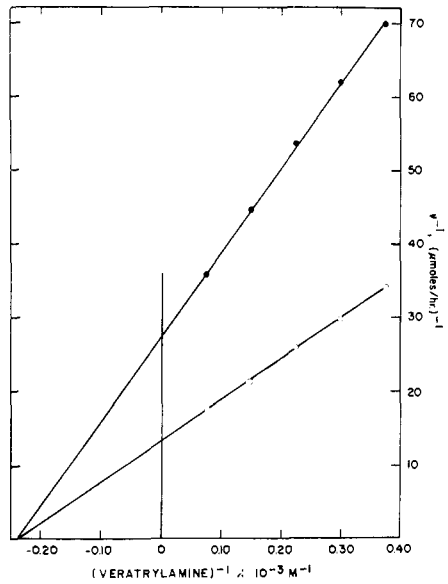

FIGURE 2: Lineweaver–Burk plot of the time-dependent, iproniazid inhibition. Reaction mixtures containing 7 units of enzyme with (●) or without (○) 17.3 μM iproniazid in 2.90 ml of 0.10 M sodium pyrophosphate buffer (pH 8.10) were maintained at 25° for 30 min. Following preincubation, 0.10 ml of buffer containing veratrylamine was added to each mixture and initial rates of veratraldehyde production from indicated veratrylamine concentrations were measured at 25°.

Methods. Initial rates of oxidations of benzylamines were determined spectrophotometrically with the use of a Cary Model 15 recording spectrophotometer, fitted with a thermostatically controlled cuvet chamber, which maintained reaction mixtures at 25.0 ± 0.2°, and cuvettes with 1.0-cm light paths. The production of aromatic aldehydes during the enzymic oxidations was measured directly as previously described (McEwen *et al.*, 1968). Unpublished data kindly provided by Dr. Charles L. Zirkle of Smith Kline and French Laboratories and by Dr. H. G. Schoepke of Abbott Laboratories indicate the pK_a values, at 25° in aqueous media, for 2-phenylcyclopropylamine, 2-(*n*-pentyl)cyclopropylamine, and pargyline to be 8.20, 8.15, and 6.60, respectively. Other dissociation constants, at 25° in aqueous media, were taken from publications cited by Perrin (1965). The Model G Beckman pH meter was used to determine pH values of reaction mixtures. We assume the variation of these measurements to be ±0.02 pH unit. In this respect, we were unable to detect any pH changes during the enzyme reactions studied in the buffered mixtures described in this report.

Results

Time-Dependent Inhibitions by Iproniazid and Isopropylhydrazine. Preincubation of the enzyme with low concentrations (5.8–28.8 μM) of iproniazid, *N*-isopropyl-*N*-isonicotinylhydrazine (Zeller *et al.*, 1952), in the absence of substrate, at pH 8.10 and 25°, significantly decreased initial rates of veratrylamine oxidation. On the other hand, these low concentra-

TABLE I: Inhibitor Constants for Instantaneous Competitive Inhibitions by Cyclopropylamines.[a]

Cyclopropylamine Inhibitor	Inhibitor Concn (μM)	pH Value	Substrate	Inhibitor Constants	
				K_i[b] (μM)	$\tilde{K}_i$[c] (μM)
Tranylcypromine (*dl-trans*-2-Phenylcyclopropylamine)	4.0	6.70	*N*-Methylbenzylamine	4.5	0.15
	2.0	7.12	*N*-Methylbenzylamine	1.8	0.14
	0.20	8.18	*N*-Methylbenzylamine	0.27	0.13
	0.13	9.02	*N*-Methylbenzylamine	0.15	0.13
	0.10	8.72	Benzylamine	0.17	0.13
	0.67	7.51	Veratrylamine	0.83	0.14
	0.50	7.58	Veratrylamine	0.67	0.13
	0.20	8.11	Veratrylamine	0.30	0.13
	0.040	8.72	Veratrylamine	0.17	0.13
d-trans-2-Phenylcyclopropylamine	0.20	8.15	*N*-Methylbenzylamine	0.14	0.067
	0.067	8.11	Benzylamine	0.16	0.070
	0.13	8.11	Veratrylamine	0.16	0.069
l-trans-2-Phenylcyclopropylamine	6.7	8.11	Veratrylamine	8.2	3.6
dl-cis-2-Phenylcyclopropylamine	0.20	8.15	*N*-Methylbenzylamine	0.17	0.080
	0.27	8.11	Benzylamine	0.18	0.081
	0.10	8.11	Veratrylamine	0.18	0.079
	0.067	8.72	Veratrylamine	0.11	0.082
2-(*n*-Pentyl)cyclopropylamine	200	8.15	Veratrylamine	230	120

[a] Initial rates of benzaldehyde or veratraldehyde production at 25° and the indicated pH values were measured with 3.0-ml reaction mixtures containing 0.10 M sodium pyrophosphate buffer, 7 units of monoamine oxidase, and substrate concentrations above and below the relevant, apparent K_m value (McEwen *et al.*, 1968) with and without the given concentrations of inhibitors. [b] Derived from Lineweaver–Burk plots which revealed the inhibitions to be strictly competitive. [c] Derived from the apparent K_i values with the use of eq 1.

tions of iproniazid had no detectable, instantaneous effect upon initial rates of veratrylamine oxidation, and inclusion of substrate in the preincubation mixtures prevented the inhibition. At fixed iproniazid concentrations, time-dependent inhibitions always appeared to follow pseudo-first-order kinetics (*e.g.*, Figure 1) similar to those originally observed with the monoamine oxidase of intact rat liver mitochondria (Davison, 1957). After preincubation of the partially purified human enzyme for 30 min at 25° in the presence of 17.3 μM iproniazid (Figure 2), a Lineweaver–Burk plot (Lineweaver and Burk, 1934) indicated that the inhibition was noncompetitive with respect to substrate. A Dixon plot (Dixon and Webb, 1958) relating inverse initial velocities of 3.3 and 13.3 mM veratrylamine oxidation to variable (0–28.8 μM) iproniazid concentrations in otherwise identical preincubation mixtures and under the same conditions confirmed the inhibition to be strictly noncompetitive. Apparent, time-dependent inhibitor constants (K_i values) derived from both plots were identical (16 μM) and agreed well with the iproniazid concentration (14 μM) that produced a 50% inhibition of tyramine oxidation by the monoamine oxidase of intact rat liver mitochondria after preincubation of the inhibitor with mitochondria for 30 min in the absence of substrate (Davison, 1957).

This time-dependent, noncompetitive inhibition nevertheless appeared to be reversible. For example, preincubation of seven units of enzyme with 17.3 μM iproniazid in 2.90 ml of 0.10 M sodium pyrophosphate buffer (pH 8.10) for 30 min at 25° caused a 52% inhibition of the oxidation of 13.3 mM veratrylamine. On the other hand, preincubation of seven units of enzyme with 17.3 μM iproniazid in 0.30 ml of the same buffer for 30 min at 25° and subsequent dilution of this preincubation mixture in order to measure the oxidation of 13.3 mM veratrylamine in a 3.0-ml reaction mixture at 25° (*i.e.*, the same assay conditions) revealed only a 13% inhibition.

Inactivation half-times ($t_{1/2}$ values) of the enzyme (Webb, 1963) at 25° in sodium pyrophosphate buffer (pH 8.10) were studied with respect to iproniazid concentrations in the preincubation mixtures. Unexpectedly, pseudo-first-order rate constants or the equivalent *inverse* inactivation half-times were not proportional to iproniazid concentrations. On the other hand, inactivation half-times were related to inverse iproniazid concentrations (Figure 3).

It has been suggested that the iproniazid inhibition of mitochondrial monoamine oxidases may be a consequence of the conversion of iproniazid into free isopropylhydrazine and that the active inhibitor is indeed isopropylhydrazine (Schwartz, 1962; Seiden and Westley, 1963; Smith *et al.*, 1963; Biel *et al.*, 1964). This hydrazine in 1.0–4.0 μM concentrations had no instantaneous effect upon the oxidation of 13.3 mM veratrylamine at pH 8.10 and 25°, but decreased the enzyme

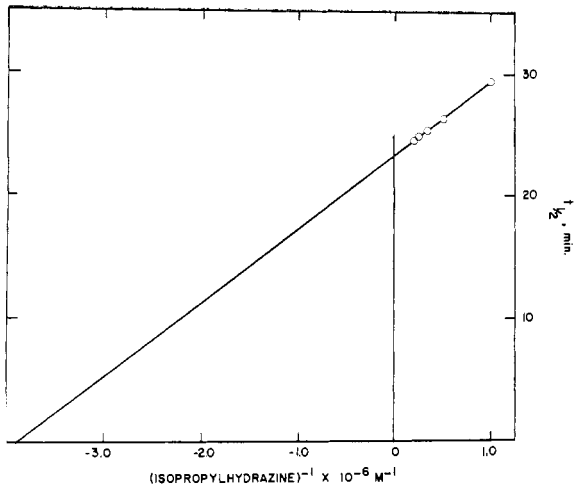

FIGURE 4: Inactivation half-times as a function of reciprocal concentrations of isopropylhydrazine. Reaction mixtures containing 7 units of enzyme and the indicated isopropylhydrazine concentrations in 2.90 ml of 0.10 M sodium pyrophosphate buffer (pH 8.10) were maintained at 25° for 5–30 min. Following preincubation, 0.10 ml of 0.40 M veratrylamine in buffer was added to each mixture and initial rates of veratraldehyde production were measured at 25°.

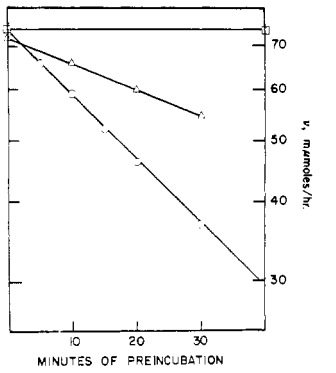

FIGURE 5: Time-dependent inhibitions by *d-trans*-2-phenylcyclopropylamine. Preincubation reaction mixtures containing 7 units of enzyme in 2.0 ml of 0.10 M sodium pyrophosphate buffer (pH 8.11) plus no further additions (□), or 0.10 μM *d-trans*-2-phenylcyclopropylamine with (△) and without (○) 0.47 mM benzyl alcohol, were maintained at 25° for the indicated minutes. Following preincubation, 1.0 ml of 30 mM *N*-methylbenzylamine in buffer was added to each mixture and initial rates of benzaldehyde production from 10 mM *N*-methylbenzylamine concentrations were measured at 25°.

activity by pseudo-first-order reactions like those of iproniazid after periods of preincubation at 25° and pH 8.10 in the absence of substrate. Inactivation half-times were again found to be reciprocal functions of hydrazine concentrations (Figure 4). In other words, the isopropylhydrazine inhibition appeared to depend upon a system that can be saturated. In keeping with less detailed reports (Davison, 1957; Biel *et al.*, 1964) concerning other mitochondrial monoamine oxidases, isopropylhydrazine may appear to be a more potent inhibitor than iproniazid. For example, a comparison of the data of Figures 3 and 4 reveals that 9.0 μM iproniazid and 3.6 μM isopropylhydrazine produced identical inactivation half-times (25 min) at pH 8.10. Such comparisons however, do not consider complex factors (see Discussion) which determine the relative potencies of these time-dependent Inhibitors.

Instantaneous and Time-Dependent Inhibitions by 2-Substituted Cyclopropylamines. The oxidations of *N*-methylbenzylamine, benzylamine, and veratrylamine (3,4-dimethoxybenzylamine) were found to be instantaneously inhibited in a competitive fashion by 2-substituted cyclopropylamines at all pH values tested (Table I). Apparent inhibitor constants (K_i values) derived for a particular inhibitor from Lineweaver–Burk plots were found to vary with the pH values of the determinations. The variations in K_i values for a particular inhibitor were typical of the simple case of the nonprotonated species of the cyclopropylamines interacting with the enzyme to form enzyme–inhibitor complexes (Dixon and Webb, 1958; Webb, 1963). This simple interaction may be described (McEwen *et al.*, 1968) by the following modification of the Henderson–Hasselbalch equation

$$\tilde{K}_i = \frac{K_i}{1 + \text{antilog}\,(pK_a - pH)} \qquad (1)$$

where K_i is the apparent inhibitor constant at a given pH value; pK_a is the ionization constant of the inhibitor; and $\tilde{K}_i$ is a constant value for a particular inhibitory amine, which is independent of pH. On the other hand, $\tilde{K}_i$ values derived for dissimilar 2-substituted cyclopropylamines were found to be different. The *d*-(+) isomer of *trans*-2-phenylcyclopropylamine was found to have a $\tilde{K}_i$ value equal to one-half the $\tilde{K}_i$ value determined for the racemic *trans* mixture, tranylcypromine. The $\tilde{K}_i$ value determined for the *l*-(−) isomer, moreover, was 50 times greater than that of the *d*-(+) isomer of *trans*-2-phenylcyclopropylamine. These data indicate that the *d*-(+) isomer of tranylcypromine is primarily responsible for the instantaneous inhibition by the racemic mixture. The $\tilde{K}_i$ value determined for racemic *cis*-2-phenylcyclopropylamine is also less than that determined for the racemic *trans* mixture, tranylcypromine. Although 2-(*n*-pentyl)cyclopropylamine and the analogous tranylcypromine have similar pK_a values and hydrophobic properties, the remarkably different affinities of these inhibitors for the enzyme (Table I) was not unexpected. In comparison with tranylcypromine, 2-(*n*-pentyl)cyclopropylamine is reported to be a poor inhibitor of animal mitochondrial monoamine oxidases (Zirkle and Kaiser, 1964).

Tranylcypromine, its isomers, and racemic *cis*-2-phenylcyclopropylamine were also found to cause time-dependent inactivations of the enzyme at 25° in the absence of substrate. These time-dependent inhibitions were found to follow pseudo-first-order kinetics (*e.g.*, Figure 5) and were prevented by the inclusion of saturating benzylamine substrates in the preincubation mixtures. They were also retarded if less saturating concentrations of benzyl alcohol, an electronic analog of un-ionized benzylamine, were included in preincubation mixtures (Figure 5). After preincubation of the enzyme, for 30 min at pH 8.11 and 25°, in the presence of 2-phenylcyclopropylamines and in the absence of substrate, Lineweaver–Burk plots concerning the oxidation of veratrylamine (Figure 6) indicated mixed types of inhibition. These mixed inhibitions appeared to result from simple combinations of instantaneous, competitive inhibitions with time-dependent, noncompetitive inhibitions. Because the time-dependent inhibitions were found to follow pseudo-first order kinetics, the concentrations, [I],

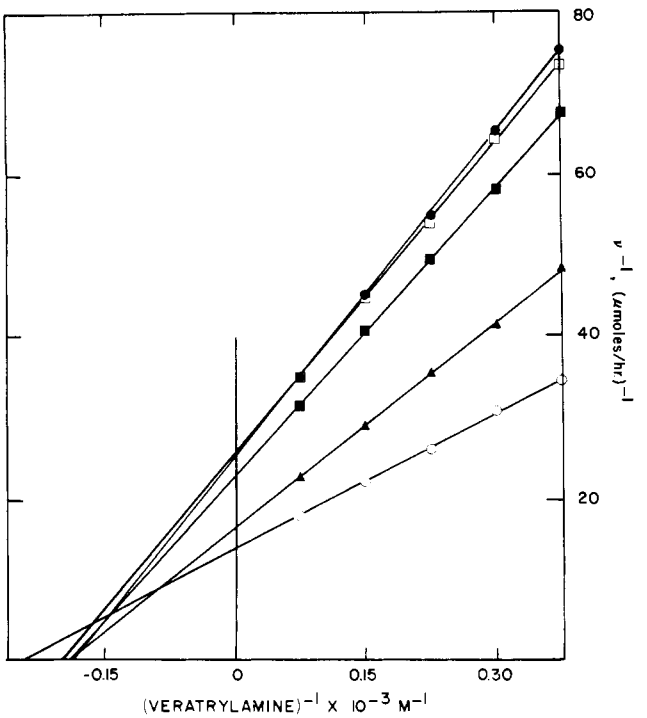

FIGURE 6: Lineweaver–Burk plots of time-dependent inhibitions by 2-phenylcyclopropylamines. Preincubation reaction mixtures containing 7.0 units of enzyme, 0.080 μM *d-trans*-2-phenylcyclopropylamine (●), 0.20 μM tranylcypromine (□), 4.0 μM *l-trans*-2-phenylcyclopropylamine (■), 0.080 μM *dl-cis*-2-phenylcyclopropylamine (▲), or no inhibitor (○) in 2.0 ml of 0.10 M sodium pyrophosphate buffer (pH 8.11) were maintained at 25° for 30 min. Following preincubation, 1.0 ml of buffer containing veratrylamine was added to each mixture and initial rates of veratraldehyde production from final, indicated veratrylamine concentrations were measured at 25°.

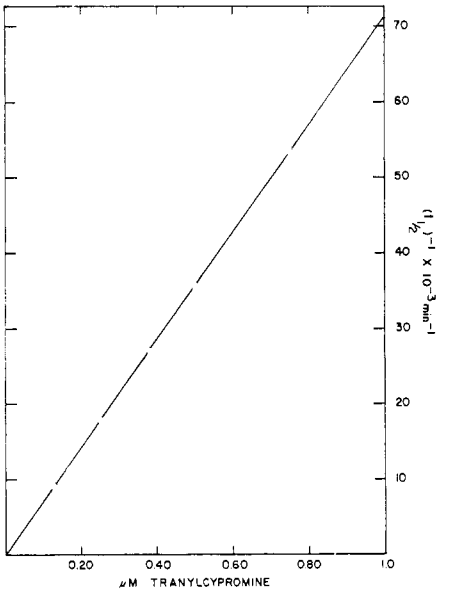

FIGURE 7: Relation of inverse half-times of enzyme inactivation to tranylcypromine concentrations. Preincubation mixtures containing 7.0 units of enzyme and the indicated tranylcypromine concentrations in 2.0 ml of 0.10 M sodium pyrophosphate buffer (pH 7.58) were maintained at 25° for 5–40 min. Following preincubation, 1.0 ml of 30 mM benzylamine in buffer was added to each preincubation mixture and initial rates of benzaldehyde production were measured at 25°. Inactivation half-times were derived from plots like those included in Figure 5.

of a given 2-phenylcyclopropylamine may be assumed to remain constant during preincubation and apparent K_i values for instantaneous inhibitions may be derived (Dixon and Webb, 1958; Webb, 1963) from the data of Figure 6 with the use of the following equation

$$K_i = \frac{[I]}{\frac{-1/K_m}{-1/K_p} - 1} \qquad (2)$$

where $-1/K_m$ equals the intercept on the abscissa in the absence of inhibitor and $-1/K_p$ equals the intercept on the abscissa in the presence of a given 2-phenylcyclopropylamine. These derivations provided apparent K_i values (*i.e.*, 0.30 μM for tranylcypromine, 0.16 μM for *d-trans*-2-phenylcyclopropylamine, 8.1 μM for *l-trans*-2-phenylcyclopropylamine, and 0.18 μM for racemic *cis*-2-phenylcyclopropylamine) that did not differ from apparent K_i values determined from instantaneous inhibitions at pH 8.11 (Table I).

In all cases of time-dependent 2-phenylcyclopropylamine inhibitions pseudo-first-order rate constants or the equivalent, *inverse* inactivation half-times were proportional to inhibitor concentrations, [I], in the preincubation mixtures. An example of these simple second-order kinetics is presented in Figure 7. Apparent second-order rate constants (k values) were derived (Webb, 1966) from such plots with the use of the equation

$$k = \frac{\ln 2}{t_{1/2}[I]} = 0.693\,\frac{(t_{1/2})^{-1}}{[I]} \qquad (3)$$

where $(t_{1/2})^{-1}/[I]$ equals the linear slope of each plot. In the case of tranylcypromine, k values were found to differ with respect to the pH values of the preincubation mixtures (Table II). Second-order rate constants computed with respect to the nonprotonated species of tranylcypromine ($\bar{k}$ values) were derived from apparent k values with the use of the appropriate relationship

$$\bar{k} = k[1 + \text{antilog}\,(pK_a - pH)] \qquad (4)$$

where pK_a refers to the dissociation constant of 2-phenylcyclopropylamines (8.20). In the case of tranylcypromine, the $\bar{k}$ values were found to be independent of the pH values of preincubation mixtures (Table II). For this reason it was assumed that nonprotonated species of other 2-phenylcyclopropylamines tested were responsible for the time-dependent enzyme inactivations (*e.g.*, the noncompetitive components of the time-dependent inhibitions). In keeping with the data concerning instantaneous inhibitions (Table I), the inhibitory effect of *d-trans*-2-phenylcyclopropylamine, estimated from both k and $\bar{k}$ values (Table II), was found to be twice that of the racemic tranylcypromine and more than fifty times that of the *l* isomer. We interpret these data as evidence that the *d* isomer of tranylcypromine is primarily responsible for the time-dependent inhibition by the racemic mixture. On the other hand,

TABLE II: Second-Order Rate Constants for Time-Dependent, 2-Phenylcyclopropylamine Inhibitions.[a]

Inhibitor	pH	Assay[b] Substrate	Substrate Concn (mM)	Second-Order Rate Constants k[c] (M^{-1} min^{-1})	$\tilde{k}$[d] (M^{-1} min^{-1})
dl-trans-2-Phenylcyclopropylamine	7.58	Benzylamine	10.0	0.50×10^5	2.6×10^5
dl-trans-2-Phenylcyclopropylamine	8.11	Benzylamine	1.0	1.1×10^5	2.4×10^5
dl-trans-2-Phenylcyclopropylamine	8.72	Benzylamine	1.0	1.8×10^5	2.4×10^5
dl-trans-2-Phenylcyclopropylamine	8.11	*N*-Methylbenzylamine	1.0	1.1×10^5	2.5×10^5
dl-trans-2-Phenylcyclopropylamine	8.11	Veratrylamine	13.3	1.2×10^5	2.6×10^5
d-trans-2-Phenylcyclopropylamine	8.11	Benzylamine	1.0	2.4×10^5	5.4×10^5
d-trans-2-Phenylcyclopropylamine	8.11	*N*-Methylbenzylamine	10.0	2.3×10^5	5.2×10^5
d-trans-2-Phenylcyclopropylamine	8.11	Veratrylamine	13.3	2.4×10^5	5.4×10^5
l-trans-2-Phenylcyclopropylamine	8.11	Veratrylamine	13.3	0.04×10^5	0.09×10^5
dl-cis-2-Phenylcyclopropylamine	8.11	Benzylamine	1.0	0.68×10^5	1.5×10^5
dl-cis-2-Phenylcyclopropylamine	8.11	Veratrylamine	13.3	0.70×10^5	1.6×10^5

[a] Preincubation reaction mixtures containing 7.0 units of enzyme and variable concentrations of the indicated 2-phenylcyclopropylamine inhibitor, but no substrate, in 2.0 ml of 0.10 M sodium pyrophosphate buffer were maintained at 25° and given pH values for 5–40 min (*e.g.*, Figure 7). [b] Following preincubation periods substrate in 1.0 ml of the same buffer was added to each mixture and initial rates of benzaldehyde or veratraldehyde production at 25° were measured with indicated final substrate concentrations, and at the pH values of preincubation mixtures. [c] Derived with the use of eq 3 from linear plots of $(t_{1/2})^{-1}$ *vs.* [I]. [d] Derived from k values with the use of eq 4.

racemic *cis*-2-phenylcyclopropylamine appeared to be a less effective, time-dependent inhibitor than tranylcypromine.

Instantaneous and Time-Dependent Inhibitions by 2-Propynylamines. Pargyline (*N*-benzyl-*N*-methyl-2-propynylamine) and its parent compound, propargylamine (2-propynylamine), were found to be instantaneous, competitive inhibitors of the enzyme (Table III). Apparent inhibitor constants (K_i values) derived for pargyline and propargylamine were found to depend upon the pH values of the determinations in the same manner as that described for the instantaneous inhibitions by 2-substituted cyclopropylamines. In other words, $\tilde{K}_i$ values derived for these inhibitors using eq 1 suggested that the competitive inhibitions were due to an interaction of the nonprotonated species of the 2-propynylamines with the free enzyme. A Dixon plot concerning the instantaneous effects of variable (0–0.40 μM) concentrations of pargyline upon veratrylamine oxidation in 0.10 M sodium pyrophosphate buffer (pH 7.51) was found to be typical of a simple competitive inhibition and provided a K_i value (0.12 μM) that was identical to the apparent inhibitor constant for pargyline at pH 7.51 derived from a Lineweaver–Burk plot (Table III). Pargyline has a much more marked affinity for the enzyme than its parent compound, propargylamine. Its $\tilde{K}_i$ value is also less than that of *N*-methylbenzylamine (4.1 μM) (McEwen *et al.*, 1968), another parent compound. Although its propargyl residue may contribute to the affinity of pargyline for the enzyme, the $\tilde{K}_i$ value of propargylamine is distinctly greater than that (21 μM) of its saturated analog, *n*-propylamine (McEwen *et al.*, 1968).

After preincubation with the enzyme for 30 min at pH 8.11 and in the absence of substrate (*e.g.*, conditions of Figure 6), 0.10 μM pargyline and 0.10 mM propargylamine were found to cause mixed types of inhibitions similar to those observed with 2-phenylcyclopropylamines. If the 2-propynylamine concentrations are assumed not to change during the preincubations, the inhibitor concentrations present in the assays of residual enzyme activity were found to account for the competitive components of the time-dependent inhibitions. For example, apparent K_i values at pH 8.11 derived for propargylamine (0.23 mM) and pargyline (0.12 μM) from Lineweaver–Burk plots with the use of eq 2 agreed well with the apparent K_i values obtained for the instantaneous inhibitions by these agents at the same pH value (Table III). Again, like 2-phenylcyclopropylamines, 2-propy-

TABLE III: Inhibitor Constants for Instantaneous Competitive Inhibitions by 2-Propynylamines.[a]

2-Propynylamine Inhibitor	Inhibitor Concn (μM)	pH Value	Substrate	Inhibitor Constants K_i (μM)	$\tilde{K}_i$ (μM)
Propargylamine	400	7.51	Veratrylamine	640	120
(2-Propynylamine)	200	8.11	Veratrylamine	230	110
	100	8.72	Veratrylamine	110	94
	200	8.15	*N*-Methylbenzylamine	230	120
Pargyline	0.16	7.48	Veratrylamine	0.13	0.11
(*N*-Benzyl-*N*-methyl-	0.32	7.51	Veratrylamine	0.12	0.11
2-propynylamine)	0.080	8.72	Veratrylamine	0.11	0.11
	0.080	8.72	Veratrylamine	0.11	0.11
	0.013	8.61	*N*-Methylbenzylamine	0.12	0.12

[a] Derived from Lineweaver–Burk plots under conditions given in footnotes of Table I.

nylamines caused time-dependent inhibitions which follow pseudo-first-order kinetics when the enzyme was preincubated with fixed inhibitor concentrations at 25° and simple second-order kinetics when the enzyme was preincubated with variable inhibitor concentrations at 25° (*e.g.*, Figure 8). As expected, saturating concentrations of benzylamine, *N*-methylbenzylamine, and veratrylamine included in the preincubation mixtures prevented the time-dependent inhibitions, and second-order rate constants derived for the time-dependent inhibitions did not depend upon the substrate used to assay residual enzyme activity (Table IV). The data of Table IV also suggest that the nonprotonated species of pargyline is responsible for its time-dependent inhibitions and we assume that eq 4 also obtains with respect to the time-dependent inhibitions caused by propargylamine. Pargyline is a far more effective, time-dependent inhibitor of the enzyme than its parent compound, propargylamine.

Time-Dependent Inhibition by p-Mercuribenzoate. Initial rates of oxidation of 0.02–1.0 mM concentrations of benzylamine at pH 8.11 are not altered by 2.0–20 μM concentrations of *p*-mercuribenzoate instanteously. The same mercurial concentrations, however, do cause time-dependent inhibitions of benzylamine oxidation if the enzyme and the mercurial are preincubated at pH 8.11 and 25° in the absence of benzylamine. These inhibitions follow pseudo-first-order kinetics for at least 40 min. As indicated by Figure 9, enzyme inactivation half-times ($t_{1/2}$ values), derived from such linear, pseudo-first-order plots, are inversely proportional to *p*-mercuribenzoate concentrations included in preincubation mixtures. From eq 3 and the data of Figure 9, an apparent second-order rate constant of 1.7×10^3 M^{-1} min^{-1} is obtained for the time-dependent inhibition of benzylamine oxidation at pH 8.11. As indicated by the Lineweaver–Burk plot of Figure 10, the time-dependent inhibition of benzylamine oxidation by 2.0 μM *p*-mercuribenzoate at pH 8.11 is competitive with respect to substrate included in the assay for residual enzyme activity. That is, the time-dependent inhibition appears to be due to a decreased affinity of the enzyme for benzylamine. The combined data are consistent with the hypothesis that maximal inactivation in the absence of substrate is associated with the production of an enzyme with an infinite K_m value for benzylamine, but that benzylamine included in the preincubation mixtures prevents this loss of enzyme affinity for substrate.

Discussion

In agreement with the original kinetic studies of Davison (1957) concerning the mitochondrial monoamine oxidase of rat liver, we have found that the human liver mitochondrial monoamine oxidase is inhibited by iproniazid in a time-dependent manner indicative of a pseudo-first-order reaction and that the inhibition is reduced when preincubation mixtures are diluted before measurement of residual enzyme activity. In keeping with reports concerning rat liver (Gorkin *et al.*, 1962)

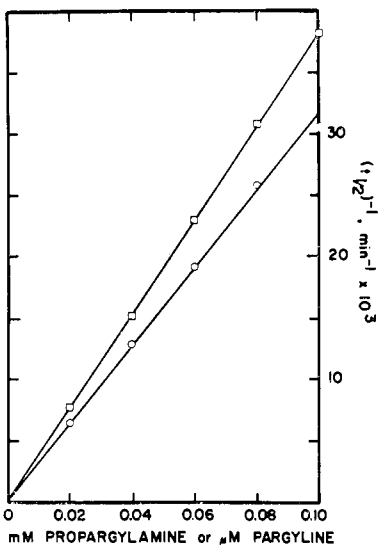

FIGURE 8: Relation of inverse half-times of enzyme inactivation to concentrations of 2-propynylamines. Preincubation mixtures containing 7.0 units of enzyme and the indicated concentrations of propargylamine (□) or pargyline (○) in 2.0 ml of 0.10 M sodium pyrophosphate buffer (pH 8.11) were maintained at 25° for 5–40 min. Following preincubation, 1.0 ml of 3.0 mM benzylamine in buffer was added to each mixture and initial rates of benzaldehyde production were measured at 25°.

TABLE IV: Second-Order Rate Constants for Time-Dependent, 2-Propynylamine Inhibitions.[a]

Inhibitor	pH	Assay: Substrate	Assay: Substrate Concn (mM)	Second-Order Rate Constants: k (M^{-1} min^{-1})	Second-Order Rate Constants: $\tilde{k}$ (M^{-1} min^{-1})
Propargylamine	8.11	Benzylamine	1.0	2.6×10^2	5.2×10^2
Propargylamine	8.11	Veratrylamine	6.7	2.6×10^2	5.2×10^2
Propargylamine	8.11	Veratrylamine	13.3	2.6×10^2	5.2×10^2
Pargyline	7.58	*N*-Methylbenzylamine	3.3	2.0×10^5	2.3×10^5
Pargyline	8.11	Benzylamine	1.0	2.2×10^5	2.3×10^5
Pargyline	8.11	Veratrylamine	13.3	2.2×10^5	2.3×10^5
Pargyline	8.61	*N*-Methylbenzylamine	0.33	2.2×10^5	2.3×10^5

[a] Conditions and derivations described in footnotes of Table II.

and rat brain (Seiden and Westley, 1963) mitochondrial monoamine oxidases, the time-dependent effect of iproniazid upon the human liver enzyme indicates a strictly noncompetitive type of inhibition. In keeping with Webb's interpretation (Webb, 1963) of Davison's data, the iproniazid inhibition of the human enzyme does not follow simple second-order kinetics. Unexpectedly, however, inactivation half-times could be inversely related to iproniazid concentrations in a manner analogous to Michaelis–Menten kinetics. The data as a whole are consistent with the hypothesis that the mitochondrial preparation catalyzes the conversion of iproniazid into a product that is a noncompetitive inhibitor of the mitochondrial monoamine oxidase of human liver. It has been suggested (Zeller *et al.*, 1955; Schwartz, 1962; Smith *et al.*, 1963; Seiden and Westley, 1963; Biel *et al.*, 1964) that isopropylhydrazine may be the inhibitory product and that mitochondrial monoamine oxidase may be responsible for its production from iproniazid. Certain observations of this investigation do not support these hypotheses: the relationship of isopropylhydrazine concentrations to enzyme inactivation half-times indicates that isopropylhydrazine inhibits the enzyme by a mechanism similar to that of iproniazid and that this mechanism involves the conversion of isopropylhydrazine to a noncompetitive inhibitor of the enzyme.

We are aware of opinions (Zeller and Sarkar, 1962; Bloom, 1963) that time-dependent inhibitions of animal mitochondrial monoamine oxidases by hydrazine derivatives arise from chemical reactions between enzyme and inhibitor analogous to the transformations of normal substrates by the enzyme and

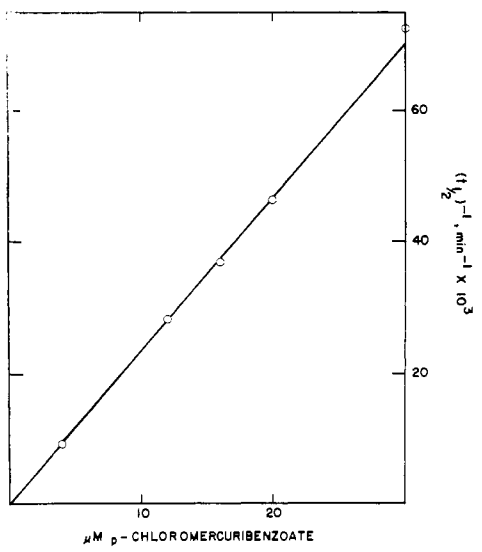

FIGURE 9: Relation of inverse half-times of enzyme inactivation to *p*-mercuribenzoate concentrations. Preincubation mixtures containing 7.0 units of enzyme and the indicated *p*-mercuribenzoate concentrations in 2.0 ml of 0.10 M sodium pyrophosphate buffer (pH 8.11) were maintained at 25° for 5–40 min. Following preincubation, 1.0 ml of 3.0 mM benzylamine in buffer was added to each mixture and initial rates of benzaladehyde production were measured at 25°.

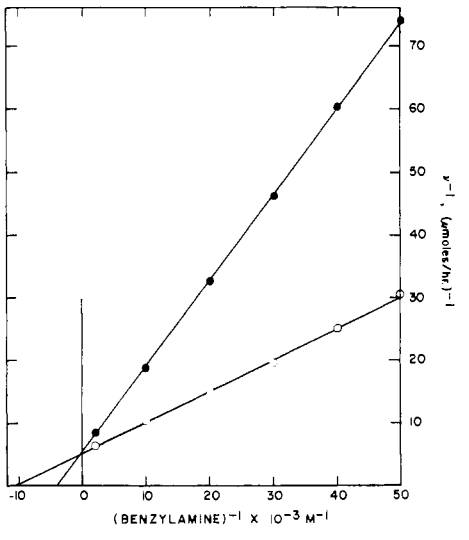

FIGURE 10: Lineweaver–Burk plot of the time-dependent *p*-mercuribenzoate inhibition. Reaction mixtures containing 7.0 units of enzyme with (●) or without (○) 2.0 μM *p*-mercuribenzoate in 2.0 ml of 0.10 M sodium pyrophosphate buffer (pH 8.11) were maintained at 25° for 20 min. Following preincubation, 1.0 ml of buffer containing sufficient benzylamine to provide the indicated, final substrate concentrations was added to each mixture and initial rates of benzaldehyde production were measured at 25°.

that, in effect, hydrazine inhibitors are pseudo substrates of the enzyme. If hydrazine derivatives are pseudo substrates, they should also be instantaneous, competitive inhibitors of the enzyme. To our knowledge, only Davison (1957) has reported hydrazine derivatives (*i.e.*, iproniazid and isopropylhydrazine) to interact with a mitochondrial monoamine oxidase in this manner. In keeping with a larger body of evidence, we did not detect instantaneous, competitive inhibitions of the monoamine oxidase of human liver mitochondria by concentrations of iproniazid or isopropylhydrazine that produced time-dependent inhibitions. The kinetic data of Figures 3 and 4 indicate that, if iproniazid and isopropylhydrazine do form reversible enzyme–inhibitor complexes, the corresponding apparent K_i values at pH 8.10 should amount to 58 and 0.26 μM, respectively. Concentrations of these inhibitors used in this study should have allowed us to detect instantaneous, competitive inhibitions if the time-dependent inhibitions stem from hydrazine interactions analogous to substrate transformations. Certainly, the finding that iproniazid and isopropylhydrazine inhibitions are prevented by substrate should not be interpreted as evidence that these hydrazines are pseudo substrates because it is quite possible that substrate prevents the interactions of inhibitory intermediates with the enzyme. Nevertheless, because mitochondrial monoamine oxidases may be copper–proteins (Nara *et al.*, 1966), because cupric ions oxidize hydrazine derivatives in a manner that has been presented (Green, 1964) as a model of the mechanism by which these compounds inhibit mitochondrial monoamine oxidases, and finally because there is no evidence that protein-bound copper is involved in the binding of substrates to any mitochondrial monoamine oxidase, it is still possible that time-dependent inhibitions by hydrazine derivatives are caused by other enzyme interactions which are not analogous to the formation of enzyme–substrate complexes.

2-Cyclopropylamines and 2-propynylamines, studied in this report, were found to inhibit the mitochondrial monoamine oxidase activity of human liver in similar fashions. Unlike iproniazid and isopropylhydrazine, these amines cause instantaneous, competitive, as well as time-dependent inhibitions. In certain respects, determinants of the instantaneous and the time-dependent inhibitions are similar. For example, the un-ionized species of the *d* isomer of tranylcypromine accounts for both instantaneous and time-dependent inhibitions caused by the racemic mixture. A more careful examination of the data of Tables I–IV, however, reveals that the potency of a given compound as an instantaneous inhibitor may have no relation to its potency as a time-dependent inhibitor. For example, *dl-cis*-2-phenylcyclopropylamine is a more effective, instantaneous inhibitor than tranylcypromine, but a less effective, time-dependent inhibitor than the racemic *trans* mixture. Furthermore, the simple second-order reactions of 2-cyclopropylamines and 2-propynylamines with the enzyme do not suggest that they are mediated by the formation of reversible enzyme–inhibitor complexes responsible for the instantaneous, competitive inhibitions by these compounds. While these amine inhibitors appear to inactivate the enzyme activity by a mechanism that is different from that of the hydrazine derivatives studied, the kinetic data of this report do not dispute evidence that hydrazines, 2-cyclopropylamines, and 2-propynylamines may inactivate mitochondrial monoamine oxidases, by presumably time-dependent interactions, at the same enzyme site (Hellerman and Erwin, 1968).

As pointed out previously (Zirkle and Kaiser, 1964; Burger and Nara, 1968), available data concerning 2-cyclopropylamine and 2-propynylamine inhibitions of the mitochondrial monoamine oxidases of animal tissues *in vitro* are ambiguous, in that different reports indicate that both types of agents may lead to inhibitions that are either competitive or noncompetitive with respect to substrate. To our knowledge, the data of the present report indicate, for the first time, that 2-cyclopropylamines and 2-propynylamines may cause both competitive and noncompetitive inhibitions of the same enzyme activity. The type of inhibition depends upon the time the enzyme is exposed to the inhibitor in the absence of substrate. This condition may well explain previous conflicting reports concerning the kinetics of these inhibitors as well as the apparent *in vitro* potencies of individual 2-cyclopropylamines and 2-propynylamines, particularly when these apparent potencies are expressed as I_{50} values.

p-Mercuribenzoate has been repeatedly reported to be an inhibitor of mitochondrial monoamine oxidase activities of animal tissues (Blaschko, 1952; Gorkin, 1966). Recent data (Erwin and Hellerman, 1967; Hellerman and Erwin, 1968) concerning the mitochondrial monoamine oxidase of bovine kidney clearly indicate that the inhibition of this highly purified enzyme by *p*-mercuribenzoate is associated with its interaction with enzyme sulfhydryl groups. Nevertheless, to our knowledge, no study has described in detail the kinetics of inhibition of mitochondrial monoamine oxidases by the sulfhydryl reagent. The data of the present study indicate that *p*-mercuribenzoate inhibits the mitochondrial monoamine oxidase of human liver by a time-dependent, second-order reaction and that this inhibition is characterized by simple competitive kinetics. In keeping with these findings, time-dependent interactions of *p*-mercuribenzoate with protein sulfhydryl groups are reported to be not unusual, and competitive inhibitions by mercurials have been observed with a surprisingly large number of enzymes (Webb, 1966). If animal mitochondrial monoamine oxidases are inhibited in the same manner, it is not surprising that *p*-mercuribenzoate concentrations reported to inhibit these enzymes may vary by a factor of 1000 under different assay conditions. It has been proposed (Erwin and Hellerman, 1967) that the inhibition of benzylamine oxidation by *p*-mercuribenzoate may be due to changes in conformation of the mitochondrial monoamine oxidase of bovine kidney, that multiple sulfhydryl groups of this enzyme react with this inhibitor, but that the rate-limiting step of these reactions is an initial interaction of the inhibitor with a single sulfhydryl group. The kinetic data of the present report concerning the *p*-mercuribenzoate inhibition of the human liver mitochondrial monoamine oxidase agree with these proposals.

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The Heterogeneity of Histones. I. A Quantitative Analysis of Calf Histones in Very Long Polyacrylamide Gels*

Sakol Panyim and Roger Chalkley

ABSTRACT: The heterogeneity of calf histones is limited. The histones are divided into five major electrophoretic groups, several of which are further subdivided to make a total of twelve species of histone molecule. The heterogeneity described is probably not due to impurity (though this is hard to assess), to failure of extraction, to degradation during extraction, or to multiple polymerization through disulfide bonds.

The relative amounts of each of the twelve components is reported. No significant differences in relative quantitation are observed for bovines of varying age and sex. A tissue-specific lysine-rich histone is reported; other major groups of histone show tissue specificity only in terms of variation in the amount of each histone fraction present.

Calf thymus histones are thought to be a mixture of relatively few homogeneous proteins. Johns has described five major components separable by chemical means (Johns, 1964; Phillips and Johns, 1965). Kinkade and Cole (1966) have shown that one of these groups (the lysine-rich histones) is further divided into three or four components.

Histones, it has been argued, may play a role in the regulation of genetic activity of higher organisms. As a method of studying this matter several workers analyzed the similarity or otherwise of histones from different organs and various species (Bustin and Cole, 1968; Fambrough *et al.*, 1968; Hnilica *et al.*, 1966; McGillivray, 1968), and from active and inactive chromatin (Littau *et al.*, 1964). There were, however, a number of severe problems encountered in this work: nucleoprotein preparations are often contaminated by highly active proteolytic enzymes (Reid and Cole, 1964; Furlan and Jericijo, 1967; Panyim *et al.*, 1968); there was a lack of resolution in the various techniques for separating histones (Shepherd and Gurley, 1966; Rasmussen *et al.*, 1962; Cruft, 1961) and there was a need for a more precise quantitation of histone fractions which were only insufficiently resolved. Cole and his coworkers have largely solved these problems for the lysine-rich (F1) histone fractions; however, their procedure is lengthy, and precise quantitation difficult because of overlap of column effluent fractions and moreover at this time is restricted to only one of the five groups of histones described by Johns.

We wanted to determine whether any other of the five major components of calf thymus histones showed further subdivision. Also, we wanted to quantitate all the various subfractions of an unseparated histone preparation in a single experimental system to facilitate comparison studies between the entire histone complement of different organs of a given creature and between histones of different species.

We have used a gel electrophoretic technique (Panyim and Chalkley, 1969) which, coupled with microdensitometric scanning and electronic curve analysis, permits us to reliably quantitate histone fractions which differ in electrophoretic

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