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ANION INHIBITION OF MONOAMINE OXIDASE*

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Abstract—It was found that rat liver monoamine oxidase is inhibited by various anions and that the degree of inhibition depends on which of two substrates, serotonin or tyramine, is used to test the monoamine oxidase activity. In addition, the inhibition by anions is affected by pH and temperature in ways that differ with the two substrates: for example, with rising temperatures inhibition of tyramine deamination by salts increases while inhibition of serotonin deamination decreases. The differential inhibition of MAO by anions suggests the existence of more than one active site or more than one enzyme subunit in rat liver monoamine oxidase. The relative substrate specificity of the inhibitions by anions suggests that there might exist other substrate-specific inhibitors.

IT WAS reported earlier that the monoamine oxidase (MAO) located in the rat liver mitochondria[1] is inhibited by high concentrations of tyramine hydrochloride.[2] Another hydrochloride, namely that of tryptamine, has also induced a similar inhibition.[3] Therefore it seemed significant that neither the bases benzylamine and *m*-xylyenediamine, nor the creatinine sulfate complex of 5-hydroxytryptamine could be shown to inhibit this sytem. Still, presence versus absence of substrate-induced inhibition has been considered as reflecting the existence of at least two mitochondrial monoamine oxidases in rat liver,[3] although anions are necessarily present in all the buffers and in some substrates.

The present communication reports the inhibition of rat liver monoamine oxidase by some anions and differentiates this effect from inhibition by high concentrations of amines. The findings were evaluated as to their bearing on the issue of single versus multiple mitochondrial monoamine oxidases.

MATERIALS AND METHODS

Tissue. Liver was excised in the cold (4°) from freshly decapitated male Sprague-Dawley rats. It was forced through a tissue press, and the pulp was homogenized with a Teflon pestle routinely in triple-distilled water or occasionally in buffers to yield 10% (w/v) suspensions. In some instances the suspensions were dialyzed at 4° against at least 45 volumes of triple-distilled water for 18 hr to reduce both the anion concentration and the enzyme blank. When rat liver mitochondria were to be tested, the 10% rat liver homogenate was made up in 0·25 M sucrose, centrifuged for 10 min at 800 *g*, and the supernatant further centrifuged for 20 min at 5,000 *g*.[4]

Electrophoresis. The mitochondrial fraction was suspended in 0·005 M Tris maleate buffer, pH 7·4, containing 0·13% (v/v) of the detergent Igepal CO-630.[5] The mitochondria were solubilized by sonic oscillation in the Raytheon 10 KC oscillator at

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1 amp for 20 min. The ensuing solution was electrophoresed in a Beckman/Spinco model CP continuous flow paper electrophoresis apparatus with 0·005 M Tris maleate buffer, pH 7·4, containing 0·13% (v/v) of the detergent. The electrophoresis was run in a 4° cold room at 25 V/cm, with the sample feed rate set at 0·6 ml/hr. Thirty-two fractions were collected and tested for MAO; 0·02 M serotonin creatinine sulfate complex and 0·02 M tyramine HCl were used as substrates.

Reagents. The substrates tested were the following: (1) serotonin creatinine sulfate (Sigma Chemical Co., St. Louis, Mo.); (2) tyramine HCl (Nutritional Biochemicals Corp., Cleveland, Ohio); and (3) tyramine creatinine sulfate (prepared by the Regis Chemical Co., Chicago, Ill.). The latter had a melting point of 231°–233° and contained on analysis, C: 41·19%; H: 5·72%; N: 15·99%. Theoretical values calculated for $C_{12}H_{20}N_4O_6S$: C: 41·37; H: 5·79; N: 16·09. These substrates were dissolved in triple-distilled water, and 0·1 N NaOH was added until the desired pH was reached (Beckman glass electrode assembly). In some instances the substrates were dissolved in the buffers.

Additional reagents used were: (1) the inorganic salts added to the reaction mixture as indicated (Baker Chemical analyzed reagents); (2) the buffers 0·1 M sodium phosphate, 0·04 M sodium citrate, or 0·05 M Tris maleate; (3) detergent: Igepal CO-630 (polyethyleneoxy derivative obtained from General Aniline & Film Corp., New York, N.Y.); (4) melanin, prepared from tyrosine (L. Light & Co., Ltd., Colabrook, England) and (5) for microdiffusion: saturated potassium carbonate, boric acid indicator solution,[6] and 0·0200 N HCl made up from constant-boiling hydrochloric acid.[7]

Enzymatic reaction. The reaction mixture consisted of 1 ml of homogenate and 1 ml of substrate. The inorganic salts were incorporated directly into the tissue suspension, whereas the buffers, when present, were added with either the suspension or the substrate. The reaction mixture was incubated in a 25-ml stoppered Erlenmeyer flask placed in a constant-temperature water bath shaking at 82 osc/min. The gaseous phase was air. Since the substrate-induced inhibition was readily demonstrable at temperatures higher than 20°,[2] the present reactions were run at 37° for 1 hr. Thereafter, 1 ml of the mixture was transferred into the outer ring of a Conway unit[8] containing 1 ml of the boric acid indicator solution in the central well. The enzymatic reaction was terminated, and the diffusion of ammonia was initiated by introduction into the outer ring of 1 ml of the potassium carbonate solution. The unit was sealed, the contents of the outer ring were mixed, and microdiffusion was continued without shaking for 3 hr at 23°. All determinations were made in duplicate, and the average values are reported after correction for tissue and reagent blanks. At the completion of the diffusion period the contents of the central well were titrated with the 0·0200 N HCl delivered from a Thomas-Rehberg microburette. The enzyme units were computed according to an earlier convention.[2] The reaction velocity was linear with regard to time and enzyme concentration. Recoveries of 96·2% to 98·2% of N H_4Cl standards were obtained both in the presence and absence of the salts tested in this work.

In a replication experiment six samples from the same 10% rat liver homogenate were incubated with serotonin creatinine sulfate at pH 6·5 at 37°. The mean value and standard deviation were 34·67 $\pm$ 0·33 μmoles NH_3/hr/g liver, which was very similar to the experiment with tyramine published earlier.[2] Nitrogen determinations were

performed on all tissue suspensions with the method of Hiller *et al.*[9] Excess of the detergent interfered with the analysis for nitrogen, but dialysis against distilled water removed this excess.

RESULTS

Effect of salts on the activity quantified with tyramine. Figure 1 shows the effect of substrate concentration on monoamine oxidase activity from the same rat liver homogenate in the presence and absence of various buffers at pH 7·2. For contrast with this substrate, some results with serotonin are also shown in Fig. 1. As the tyramine concentration was increased, a maximal enzyme activity was obtained between 5 and 15 mM, whereas with higher concentrations there occurred a proportionately more pronounced diminution of the activity. The diminution of the average reaction velocity by these higher concentrations of substrate will be referred to hereafter as "substrate-inhibition."

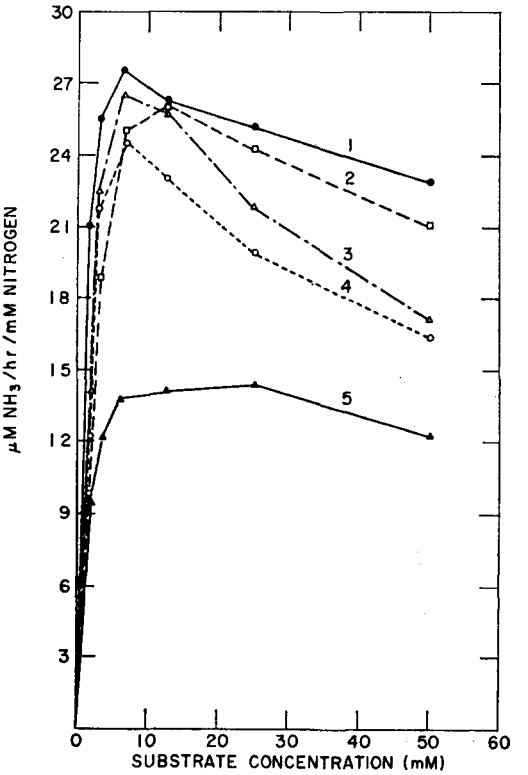

FIG. 1. Effect of buffers on monoamine oxidase activity at pH 7·2. Aliquots of a dialyzed 10% rat liver homogenate in distilled water were reacted with varying concentrations of two substrates, tyramine and serotonin, in the presence of the following buffers: curve 1*, tyramine creatinine sulfate, no buffer; curve 2†, tyramine creatinine sulfate, Tris maleate buffer, 0·025 M; curve 3†, tyramine HCl, Tris maleate buffer, 0·025 M; curve 4†, tyramine creatinine sulfate, phosphate buffer, 0·025 M; curve 5*, serotonin creatinine sulfate, Tris maleate buffer, 0·025 M.

* Result observed in two experiments.

† Result observed in three experiments.

The highest activity at optimal concentrations and the least inhibition by high concentrations were found when tyramine creatinine sulfate was the substrate and no buffer was present. The pH remained 7·2 during the course of these tests. In this laboratory this substrate has always yielded higher overall enzyme activity than other tyramine salts and was therefore routinely used. (See Fig. 1 for comparison with tyramine HCl.)

The lowest MAO activity with all concentrations of tyramine tested occurred in the presence of the phosphate buffer. Tris maleate yielded the highest activity among the buffers tested and was therefore adopted for all subsequent experiments. With 50 mM tyramine HCl, the enzymatic activity was less by about 38% than the maximal activity level; this compared to 19% inhibition with 50 mM tyramine creatinine sulfate. Several attempts were made to eliminate the "substrate inhibition" by varying the salts of the tyramine base (phosphate, sulfate, acetate) with the temperature fixed at 37° and the pH at 7·.2 The substrate inhibition was greatest when tyramine phosphate was

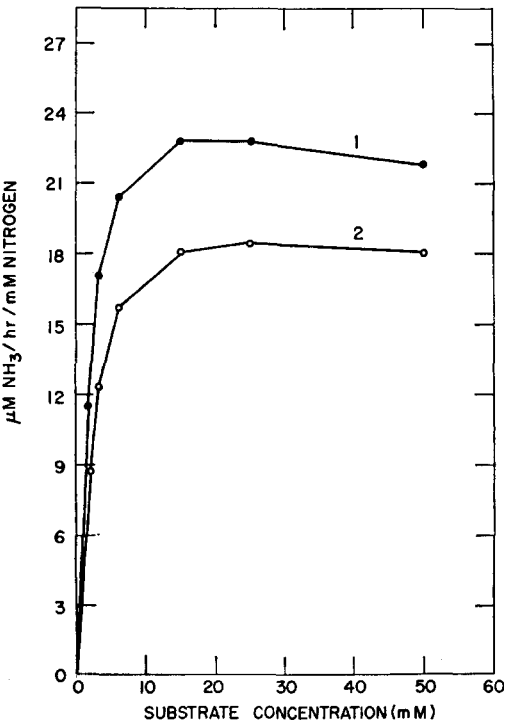

FIG. 2. Effect of tyramine creatinine sulfate concentration on monoamine oxidase activity at pH 6·5 in the presence and absence of 0·20 M NaCl. Incubation was carried out with a 10% rat liver homogenate in Tris maleate buffer with different concentrations of the substrate tyramine creatinine sulfate in the absence (1) and presence (2) of 0·20 M NaCl.

used; when the pH was lowered to 6·5 (Tris maleate buffer) the inhibition by tyramine creatinine sulfate was reduced to about 4% (Fig. 2). These tests showed that the inhibition by substrate concentrations higher than 12 mM was pH-dependent, buffer-dependent, and temperature-dependent.

Since tyramine HCl yielded lower reaction velocities than did tyramine creatinine sulfate (Fig. 1), an inhibitory effect of the chloride ion was sought by adding the anion initially in the form of NaCl. In the presence of 0·20 M NaCl, lower activities were encountered at all concentrations of tyramine creatinine sulfate tested (Fig. 2).

Table 1 shows the effects of different salts on the rate of the MAO reaction. The chlorides of sodium and potassium exerted virtually identical degrees of inhibition. The same was true when sodium phosphate was compared to potassium phosphate

TABLE 1. INHIBITION OF TYRAMINE MONOAMINE OXIDASE BY DIFFERENT SALTS*

Salt added	Concn. of salt (moles)	% of control
	pH 7·2	
KCl	0·20	82
NaCl	0·05	94
NaCl	0·20	85
K phosphate buffer	0·20	75
Na phosphate buffer	0·20	74
Na phosphate buffer	0·10	86
Na phosphate buffer	0·05	98
Na phosphate buffer	0·025	98
KNO_3	0·20	80
$NaNO_3$	0·20	80
KNO_3	0·50	60
Tris maleate buffer	0·20	98
Creatinine sulfate	0·05	96
	pH 6·5	
NaCl	0·04	95
NaCl	0·20	80
KCl	0·08	89

* The experiments were performed in the presence of the substrate tyramine creatinine sulfate, 0·02 M; Tris maleate buffer 0·025 M; dialyzed 10% rat liver homogenate (w/v); and the salts as indicated in the table. The total volume was 2 ml. The pH of the substrate was adjusted with 0·1 N NaOH. The incubation of the reaction was at 37° in a shaking water bath. Wherever inhibition was observed the experiment was repeated at least once.

or sodium nitrate to potassium nitrate. This suggested that the inhibition was a function of the respective anions rather than the cations. As can also be seen in Table 1, sodium and potassium phosphate buffer, 0·20 M, which has an ionic strength of 0·49, resulted in about 25% inhibition. However, potassium nitrate, 0·50 M, with almost identical ionic strength (0·50), caused a 40% inhibition. Therefore the inhibition was independent of the ionic strength of the added salts.

In order to determine whether osmotic pressure changes affected the enzymatic activity there were added to the tyramine–MAO reaction mixture, at pH 7·4, eight different concentrations of sucrose up to 0·9 M. Sucrose did not inhibit the enzyme activity.

Effects of salts on activity as tested with serotonin creatinine sulfate. The effect of different salts on the reaction rate of MAO was studied also with serotonin creatinine sulfate because this substrate induced less inhibition at superoptimal concentrations than did tyramine (Fig. 1). As is shown in Table 2, among the salts tested the chlorides

TABLE 2. INHIBITION OF SEROTONIN MONOAMINE OXIDASE BY DIFFERENT SALTS*

Salt added	Conc. of salt (moles)	% of control			
		pH 5·8	pH 6·5	pH 7·2	pH 8·3
KCl	0·01	94		99	
	0·02	86		98	
	0·04	72		96	
	0·08	61	66	90	99
	0·20		42	79	88
NaCl	0·01	91			
	0·02	86			
	0·04	72			
	0·08	60	66	89	100
	0·20		40	70	84
K phosphate buffer	0·08	92		100	
	0·20			82	
KNO_3	0·08		80	88	
	0·20		46	78	
$NaNO_3$	0·20		43		
K_2SO_4	0·08	93		100	
	0·20			90	
K citrate buffer	0·08	95		98	
	0·20			83	
Na acetate buffer	0·08	93	96	98	
	0·20		93	86	

* The experiments were performed in the presence of the substrate serotonin creatinine sulfate 0·019 M; Tris maleate buffer, 0·025 M; dialyzed 10% rat liver homogenate w/v; and the salts as indicated in the table. The total volume was 2 ml. The pH of the substrate was adjusted with 0·1 N NaOH. The incubation of the reaction was at 37° in a shaking water bath. Whenever inhibition was observed, the experiment was repeated at least once.

and nitrates yielded the greatest degrees of inhibition. Greater inhibition was seen with lower pH, whereas there was probably less inhibition at pH 8·3. Table 2 demonstrates also that the inhibition by chloride ion was again proportional to the concentration of the anion: KCl and NaCl gave identical degrees of inhibition. Potassium nitrate (0·20 M) and sodium nitrate (0·20 M) also yielded about the same percentage inhibition, whereas sodium acetate at the same molarity and ionic strength produced much less inhibition. KCl (0·20 M) with an ionic strength of 0·2 resulted in 21% inhibition, whereas there was no inhibition by K_2SO_4 (0·08 M) with an ionic strength of 0·24. Therefore, as was the case with tyramine, it seemed that the inhibition of the MAO reaction was caused by the anions. Furthermore, it was independent of the ionic strength of the reaction mixture.

Incubation of the sodium chloride with the enzyme for 60 min prior to testing induced only 8% more inhibition than had resulted from the addition of the salt at the initiation of the enzyme reaction.

In order to diminish binding of anions by proteins other than the enzyme, mitochondrial fractions were also tested for chloride inhibition. These were found to be

inhibited to the same degree as the homogenate. None of the salts tested accelerated the reactions.

Comparison of inhibition by chloride to that by substrate. To determine whether inhibition by chloride was reversible, 0·08 M NaCl was incubated for 20 min at 37° with a 10% rat liver homogenate in Tris maleate buffer, pH 6·5, followed by dialysis at 4° for 18 hr. The dialysis bath consisted of either Tris maleate buffer, pH 6·5, or this buffer plus 0·08 M NaCl. The inhibition produced by the NaCl proved completely reversible when tested with both serotonin creatinine sulfate and tyramine HCl. Similar experiments were undertaken also with regard to the inhibition by the substrate (Fig. 3). After incubation of three separate aliquots of the same 15% rat liver

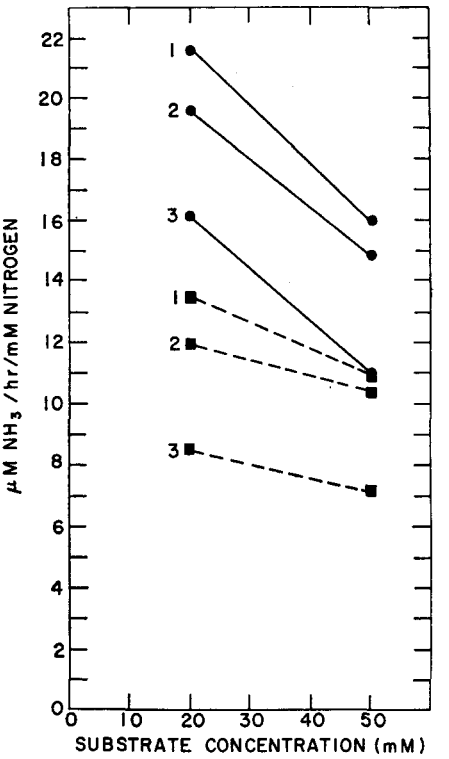

FIG. 3. Effect of dialysis on the substrate (or product) inhibition of monoamine oxidase. Three separate aliquots of a 15% rat liver homogenate in Tris maleate buffer, 0·025 M, pH 7·4 were incubated for 30 min at 37° with: distilled water (1); tyramine HCl 0·075 M (2); and serotonin creatinine sulfate, 0·075 M (3). After dialysis against distilled water for 18 h the enzyme activity was tested with the substrates tyramine HCl (——) and serotonin creatinine sulfate (– –) at two concentrations, 20 mM and 50 mM.

homogenate with (1) distilled water, (2) tyramine HCl (0·075 M), or (3) serotonin creatinine sulfate (0·075 M), all three aliquots were dialyzed separately against distilled water for 18 hr. The dialyzed homogenates were then tested for MAO activity with two concentrations each of tyramine HCl and serotonin creatinine SO_4. Preincubation with serotonin creatinine SO_4 had irreversibly inhibited the enzyme to a larger extent than had tyramine HCl. This decrease in enzyme activity following incubation

with the substrates persisted regardless of whether tyramine or serotonin was used to test the enzyme activity. Furthermore, the inhibition caused by the substrate (or product) persisted after dialysis, as tested against two concentrations of substrate (20 mM and 50 mM). It remained of the same degree as encountered previously (Fig. 1). Thus the anion inhibition was completely reversible, whereas the inhibition caused by the substrate or its products was irreversible.

As described by several investigators,[10, 11] a brown melanin-like pigment is formed during the MAO reaction. The intensity and shade of the pigment depend on the substrate. The homogenates which were incubated with serotonin and tyramine prior to dialysis developed a dark khaki color which did not disappear after dialysis, since it adhered firmly to the particles of the suspension. The homogenates incubated with serotonin creatinine sulfate were even darker brown than the ones incubated with tyramine HCl. The possibility arose that these pigments might cause the irreversible "substrate" inhibition. To investigate this possibility, melanins were prepared by autoxidation of the respective substrate solutions in air at pH 0·6 for 1 week: The insoluble brown precipitates formed were dialyzed against distilled water. These pigments were added to rat liver mitochondria in concentrations up to 0·35% (w/v), and the suspension was submitted to sonication in a Raytheon 10 KC oscillator at 1 amp for 20 min. Neither the serotonin melanin nor the tyramine melanin inhibited the MAO reaction, as tested by both serotonin and tyramine as substrates; but they did not seem to adhere to the particles of the tissue suspension. Identical results became evident when commercially obtained melanin, prepared from tyrosine, was tested.

Type of inhibition by anions. When the data from Fig. 2 were plotted according to the method of Lineweaver-Burk,[12] the sodium chloride inhibition of the tyramine creatinine sulfate reaction at pH 6·5 was found to be noncompetitive. From the plot in the absence of the inhibitor, the Michaelis-Menten constant (K_m) was found to be $2{\cdot}4 \times 10^{-3}$ M, which gives a value of K_i for sodium chloride of $5{\cdot}5 \times 10^{-1}$ M. At pH 6·5 in the presence and absence of 0·20 M KCl, the inhibition of monoamine oxidase was also noncompetitive when serotonin creatinine sulfate was the substrate. The K_i in this case was $2{\cdot}6 \times 10^{-1}$ M.

Modifications of the anion inhibition. Since it has been established that the substrate inhibition by tyramine HCl is temperature-dependent,[2] the effect of temperature on the anion inhibition was also investigated. Figure 4 shows the logarithm of the percentage inhibition by anions plotted against the reciprocal of the absolute temperature. However, the inhibition of tyramine MAO followed an endothermic pattern while serotonin MAO inhibition by KCl followed an exothermic one. It is significant that with tyramine both the substrate inhibition[2] and the anion inhibition increased with rising temperatures.

It was of interest to determine whether the inhibition of the MAO reaction by anions was dependent upon the integrity of the subcellular organelles which contain the enzyme. The detergent Igepal was added to the 10% rat liver homogenate in Tris maleate buffer, 0·025 M, pH 6·5, to make a 2% (v/v) solution. The inhibition of the tyramine creatinine sulfate (0·02 M)–monoamine oxidase reaction with 0·08 M KCl was the same regardless of whether the enzyme was solubilized or not. On the other hand the inhibition of the serotonin creatinine sulfate (0·02 M)–monoamine oxidase reaction with 0·08 M KCl was 28% with the original homogneate and 44% when the homogenate was solubilized by the detergent. In addition, the detergent itself had

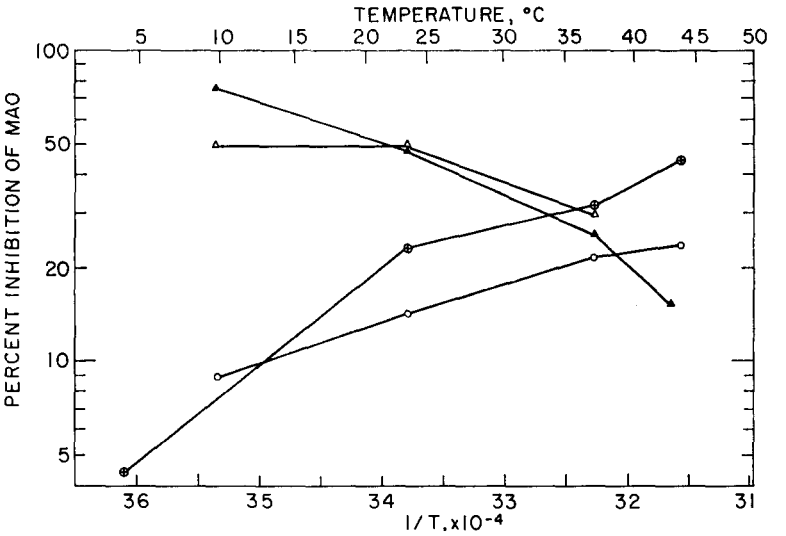

FIG. 4. Percentage inhibition of monoamine oxidase by anions as a function of temperature.
⊕— Tyramine HCl, 0·02 M; phosphate buffer, 0·20 M, pH 7·4.
○— Tyramine HCl, 0·02 M; KCl 0·02 M, pH 6·5.
△▲ Serotonin creatinine sulfate, 0·02 M; KCl 0·08 M, pH 6·5.

Note that the ordinate scale is logarithmic, and the abscissa scale is proportional to the reciprocal of the absolute temperature.

caused some slight diminution of the MAO activity with both substrates. Identical results were obtained when the concentration of Igepal was reduced to 0·5%.

A 10% rat liver homogenate in Tris maleate buffer, 0·025 M, pH 6·5, was sonicated by the Raytheon 10 KC oscillator at 1 amp for 20 min in order to fragment the mitochondria, which contain almost all of the monoamine oxidase.[1] This manipulation had no effect on the inhibition of monoamine oxidase by 0·08 M KCl.

Electrophoresis. The inhibition of MAO by anions depended on the type of substrate. Furthermore, the detergent affected primarily the deamination of serotonin. Therefore electrophoresis of mitochondrial solutions was used in the search for two separate enzymes. When electrophoresed in 0·005 M Tris maleate buffer at pH 7·4, the MAO activity moved toward the anode. Almost all the MAO activity was found in 3 of the 32 collecting test tubes, but there was no significant separation of the tyramine reaction from the serotonin reaction in any of the tubes. The two activities also remained inseparable by electrophoresis when 0·23 M barbital buffer at pH 8·6, and 0·005 M Tris maleate buffer at pH 5·9 were used. The MAO was concentrated only eight times over the original 10% homogenate.

DISCUSSION

Among the enzymes which become inhibited by superoptimal concentrations of their substrates, some have been investigated also for their sensitivity to various anions. The list of enzymes which are inhibited by both anions and high concentrations of their substrates includes urease,[13, 14] acetylcholinesterase,[15, 16] carboxypeptidase,[17, 18] and fumarase,[19, 20] In the case of the monoamine oxidase studied here, these two

inhibiting factors are linked to each other by virtue of the fact that the amines used as substrates often contain anions. This source introduces anions as a variable, even when other sources are controlled rigorously. Therefore, when the inhibition of monoamine oxidase by anions was first observed, it was considered worthy of further definition.

The present data indicate that the anions rather than the cations of the inorganic salts tested had induced the inhibition under discussion; inhibition of identical magnitude at the same ionic strength was induced by the chlorides and by the nitrates of two alkali metals. On the other hand, phosphate and nitrate induced different inhibition when tested at similar ionic strengths. The use of phosphate buffer recommended earlier[1, 2] was now shown to result in lower activities than Tris maleate buffer. Furthermore, superoptimal concentrations of the tyramine hydrochloride (the substrate used earlier) yielded much higher inhibitions than did tyramine creatinine sulfate. The ranking of the degree of inhibition induced by each of the anions tested did not duplicate either the Hofmeister or the Bucher series.[21] However, none of the present monoamine oxidase preparations was purified enough to minimize the concentration of inactive macromolecules known to bind the substrates[22] and anions[23] investigated here.

Several findings differentiated the inhibition induced by the anions from the inhibition due to superoptimal concentrations of substrate. The former could be induced in both the tyramine and the serotonin systems, whereas the latter was present chiefly with tyramine. The substrate inhibition was evident in the absence of inhibitory anions, but it disappeared when either the pH or the temperature was reduced. By contrast, the anion inhibition increased when the pH was reduced, but this happened only when serotonin was the substrate. Furthermore, the substrate (or product) inhibition could not be reversed by dialysis, whereas the anion-induced inhibition was readily reversible. Hence it became evident that these two phenomena were produced by different mechanisms. These mechanisms could be defined only to the extent of showing that the anion-induced inhibition was a noncompetitive one.

The discussion thus far has also pointed out what may be the major conclusion of this work: there were considerable differences between the tyramine reaction and the serotonin reaction in almost all experiments presented here. The dissimilarity between these two reactions became strikingly evident when the change in the anion inhibition with changing temperature was studied in both systems. Indeed, the effect of temperature was in opposite directions: an exothermic pattern was followed by the serotonin and an endothermic pattern by the tyramine system.

Striking differences among MAO systems deaminating different substrates have been observed by others.[3,24] An important difference is the greater heat stability of the oxidation of serotonin by comparison to that of tyramine in rat liver mitochondria.[3] These differences might bear on the thesis that mitochondrial MAO consists of several enzymes.[24, 25] Our inability to separate two MAO activities by electrophoresis contrasts with the successful chromatographic separation of two other mitochondrial MAO systems by Gorkin.[25] Still, the monoamine oxidase inhibitors studied to date have been nonspecific in that they indiscriminately reduced the deamination of all the substrates. On the other hand, the anion inhibition discussed here is unique in that it has shown a considerable degree of substrate specificity under certain conditions. Hence, it is possible that there might be developed similar but more potent substrate-specific inhibitors for monoamine oxidase.

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