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Inhibition of Aldehyde Oxidation by Adrenergic and Adrenolytic Substances.* (20285)

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The metabolic disposal of epinephrine and related amines is usually attributed, in part at least, to the monoamine oxidase system. This system oxidizes amines to the corresponding aldehydes which then will be converted to the acid. The studies reported in this paper show that the enzyme present in guinea pig liver which converts aromatic aldehydes into the corresponding acids is inhibited reversibly by adrenergic amines and irreversibly by adrenergic blocking agents.

Methods. Enzyme activity was measured by means of an ultraviolet spectrophotometric method. Fig. 1 shows that salicylaldehyde, salicylic acid and saligenin (salicylalcohol) have distinct absorption peaks in the ultraviolet. The molar extinction coefficient of salicylaldehyde is 2300 at 325 mμ (the peak at 260 mμ was not used because of its proximity to the absorption zone of trichloracetic acid), that of salicylic acid 4450 at 303 mμ. These values are valid only for the present experimental conditions since the spectra are very sensitive to pH changes(1). The substrate used in all experiments was salicylaldehyde (Eastman-Kodak). The enzyme was prepared from guinea pig liver homogenized in 0.1 M phosphate buffer at pH 7.4. The

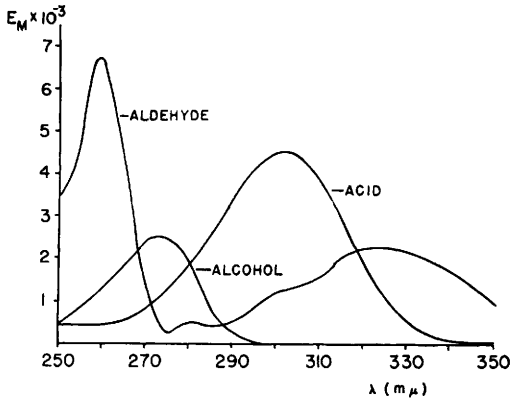

FIG. 1. Ultraviolet absorption spectrum of salicylaldehyde, salicylic acid and saligenin, in the presence of trichloracetic acid in proportions specified in the text. λ = wavelength in mμ; E_M = molar extinction coefficient.

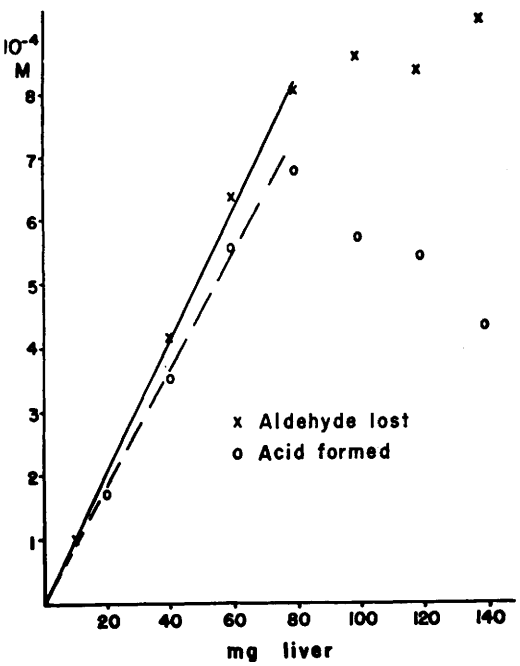

FIG. 2. Influence of enzyme concentration on disappearance of salicylaldehyde and formation of salicylic acid. Abscissa: enzyme concentration in mg of fresh liver per 3 ml of reaction mixture. Ordinate: aldehyde lost or acid formed in 10 min. The initial concentration of salicylaldehyde was 10^{-3} M in 3 ml.

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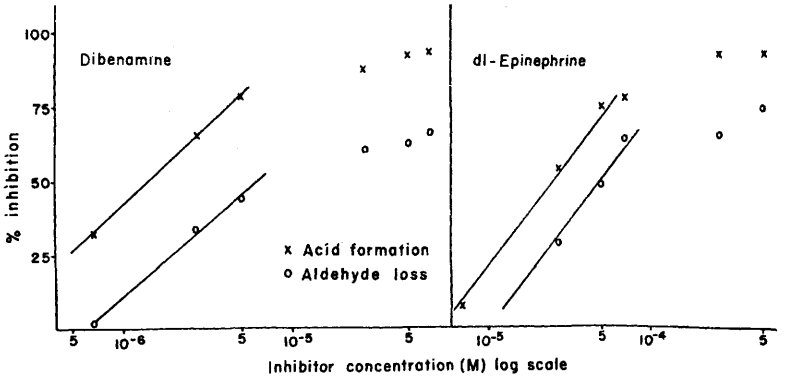

FIG. 3. Inhibition of aldehyde oxidation by Dibenamine (left) and epinephrine (right). Abscissa: molar concentration of inhibitor (log scale); ordinate: % inhibition of aldehyde loss (○) and acid formation (×).

cell debris was separated by centrifugation and the clear supernatant was made up with the buffer to correspond to 200 mg of fresh liver per ml. The reaction was started by adding the substrate to the enzyme solution. The total volume of the reaction mixture was 3 ml containing 0.4 ml of the enzyme solution, 1 μM of substrate and whatever inhibitor was being tested. The final buffer concentration was 0.067 M. In some experiments the enzyme and substrate concentrations were varied. After 10 minutes' incubation at 37.5°C the reaction was stopped by addition of 7 ml of 15% trichloracetic acid. The solution was left standing for at least one hour and filtered on hard paper (Whatman No. 50). When inhibitors were used, these were incubated with the enzyme solution for 15 minutes before addition of the substrate. The filtered solutions were read on the Beckman spectrophotometer at 303 and 325 mμ against a blank containing all the reagents except the substrate. From these readings the concentrations of salicylaldehyde and salicylic acid were calculated on the basis of the extinction coefficients given above. Since the aldehyde absorbs also at 303 mμ, a correction was made by means of its molar extinction coefficient at 300 mμ (1250). No saligenin formation could be detected in any of the experiments. Results are expressed in μM of aldehyde lost and acid formed during the reaction. All amines, unless otherwise stated, were used as hydrochlorides.†

Results. Fig. 2 shows that reaction velocity increases linearly with enzyme concentration up to about 80 mg of liver. It is seen that all the aldehyde destroyed is not converted into acid. The fate of this fraction of salicylaldehyde is unknown. It is likely that the oxidation goes beyond the stage of salicylic acid. This probably also occurs with high concentrations of enzyme.

Inhibition experiments were performed with 80 mg of liver extract. As seen in Fig. 3, the aldehyde loss cannot be entirely inhibited, no matter how high the concentration of inhibitor. Inhibition is therefore best expressed in terms of acid formation. Fractional inhibition is linearly related to the log of inhibitor concentration and the concentration of inhibitor required to cause 50% inhibition of acid formation is read from curves like those shown in Fig. 3.

Inhibition of the oxidation of salicylaldehyde by epinephrine and related amines is shown in Table I. The range of concentrations which cause 50% inhibition goes from 10^{-6} M to 10^{-3} M. One adrenergic amine, ephedrine, was found inactive. No inhibition was observed with the following aliphatic and aromatic amines: methylamine, diethylamine,

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TABLE I. Inhibition of Aldehyde Oxidation by Adrenergic Amines.

Compound	50% inhibition (M conc.)
Epinine	1.6×10^{-5}
Adrenalone	$7.3 \times$ "
l-Epinephrine	1.4×10^{-5}
dl-Epinephrine	$2.4 \times$ "
Adrenochrome	$2.5 \times$ "
d-Epinephrine	$3.7 \times$ "
m-Synephrine	$4 \times$ "
5-Hydroxy-tryptamine creatinine sulfate (Serotonin)	$4.3 \times$ "
dl-Norepinephrine	$5 \times$ "
Ethylnorepinephrine	$5 \times$ "
Tryptamine	$6 \times$ "
p-Synephrine	1.3×10^{-4}
Tyramine	$1.4 \times$ "
p-Hydroxy-N-methylbenzedrine (Veritol)	$1.5 \times$ "
Isopropylnorepinephrine	10^{-3}

ethanolamine, benzylamine, methylmandelamine, 2-hydroxybenzylamine, histamine, acetylcholine and thyroxine. Two aromatic amino-acids, tyrosine and phenylalanine, were also inactive. Two deaminated derivatives of adrenergic amines, benzoylcarbinol and 3,4-dihydroxybenzoylcarbinol (derived from epinephrine) inhibited aldehyde oxidation in the concentration range of 10^{-4} M. The latter compounds were synthesized at this Institute by Mr. B. W. Turnquest.

Table II shows that oxidation of salicylaldehyde to salicylic acid is also inhibited by adrenergic blocking agents. All drugs of proved adrenolytic activity, with the notable exception of yohimbine, were found inhibitory for the reaction. The most potent inhibitors were Dibenamine and its oxyisopropyl derivative Dibenzyline. The ergot alkaloids and Priscol were less active followed by the benzodioxane derivative, 933F, and the weak adrenolytic agent diethylaminoethanol. Phenylmethylpiperazine and a few β-haloalkylamine derivatives were inactive at the concentration of 10^{-3} M; all have weak adrenolytic activity. Nitrogen mustard itself, which belongs to the same chemical series and which has no adrenolytic action, was found inactive on aldehyde oxidation.

Among the large number of other compounds tested, two groups deserve special mention: the aldehydes and antihistamine drugs. Three aliphatic aldehydes inhibited the oxidation of salicylaldehyde at varying concentrations: crotonaldehyde (3.2×10^{-5} M), butyraldehyde (4×10^{-5} M) and acetaldehyde (2.7×10^{-4} M). Formaldehyde and two aromatic compounds, benzaldehyde and anisaldehyde, were inactive.

Four antihistamine drugs were tested. Neoantergan (2-[2-dimethylaminoethyl(*p*-methoxybenzyl)amino]pyridine) was inactive but the other 3 proved potent inhibitors: Phenergan (10-(2-Dimethylamino-1-propyl)phenothiazine) at 7×10^{-6} M, Pyribenzamine (2-[Benzyl (2-Dimethylaminoethyl)] aminopyridine) at 2.3×10^{-5} M and Antergan (N',N'-Dimethyl - N - phenyl - N - benzylethylenediamine) at 3×10^{-5} M.

Salicylaldehyde oxidation is inhibited by NaCN (3.8×10^{-6} M). No inhibition was observed with iodoacetic acid, *p*-chloromercuribenzoic acid, ethylenediamine tetraacetate, ascorbic acid, sulfanilamide and two isonicotinic acid derivatives which inhibit monoaminoxidase (isonicotinylhydrazine) and diaminoxidase (1-isonicotinyl-2-isopropylhydra-

TABLE II. Inhibition of Aldehyde Oxidation by Adrenergic Blocking Agents.

Compound	50% inhibition (M conc.)
N-(2-chloroethyl)dibenzylamine (Dibenamine)	1.4×10^{-6}
N-(2-chloroethyl)phenoxyisopropyl-N-benzylamine (Dibenzyline, SKF 688A)	$1.8 \times$ "
N-ethyl-N(2-bromoethyl)-1-napthalenemethylamine (SY 28)	$6.7 \times$ "
N-ethyl-N(2 chloroethyl)benzhydrylamine (SY 2)	1.2×10^{-5}
Hydergine*	$2.5 \times$ "
N-methyl-N(2-chloro-2-phenylethyl)benzylamine (Lilly 08353)	$2.7 \times$ "
2-Benzyl-2-imidazoline (Priscol)	10^{-4}
Dihydroergotamine methanesulfonate	$2 \times$ "
2-(1-Piperidylmethyl)-1,4-benzodioxan (Benodaine, 933 F)	$5 \times$ "
Diethylaminoethanol	1.8×10^{-3}

* Mixture of equal parts of dihydro-ergocornine, -ergokryptine and -ergocrystine methanesulfonates. Molar concentration calculated on basis of mean molecular wt.

zine)(2). The reaction is not influenced by the various B vitamins, xanthine and its derivatives (adenine, guanine, allantoin, theobromine) and the following alkaloids: atropine, eserine, morphine, pilocarpine, strychnine. Other inactive compounds were: barbital, phenobarbital, thiourea, procaine, percaine, tetraethylammonium bromide, coramine, hesperidin and polyoxyethylene sorbitan monolaureate (Tween 20).

In view of the fact that both adrenergic amines and agents blocking adrenergic action inhibited aldehyde oxidation, it seemed important to determine whether these 2 groups of substances had the same type of inhibitory action. When reaction velocity was plotted against enzyme concentration the slope of the line obtained was not influenced by the concentration of inhibitor in the case of Dibenamine. With epinephrine, however, this slope was found to be a function of inhibitor concentration. According to Ackermann and Potter(3), this means that Dibenamine combines irreversibly with the enzyme while epinephrine causes only a reversible inactivation. By using other tests based on the influence of substrate concentration on reaction velocity (4), it was possible to show that inhibition by epinephrine and also by acetaldehyde is of a competitive nature.

Discussion. Three enzymes have been described which can oxidize aldehydes in the liver. The first, aldehyde mutase, converts aldehydes into an equimolecular mixture of the corresponding acid and alcohol(5). The enzyme dealt with in the present study does not produce any salicylalcohol and differs from aldehyde mutase by the fact that it is not inhibited by iodoacetic acid(5). Xanthine oxidase which also oxidizes aldehydes is different from the enzyme considered in this paper in that it is inhibited by ascorbic acid (6). Moreover, liver extract, as prepared in the present study, does not oxidize xanthine and xanthine oxidase prepared from milk is not inhibited by Dibenamine.

Liver aldehyde oxidase, as described by Gordon *et al.*(7) and studied by Knox(8) is very similar to the enzyme described above. Its relative affinity for salicylaldehyde and crotonaldehyde is compatible with the results just mentioned. Some minor differences are probably due to the differences in technics used. The results show that there are present in the liver extracts at least 2 enzymes capable of oxidizing salicylaldehyde. Only one of these is inhibited by the compounds investigated.

The most important question raised by the results of the present work concerns the possible role of aldehyde oxidase in the pharmacological actions of its inhibitors. It has been assumed that adrenergic blocking agents acted by inhibiting some hypothetical mechanism essential for the action of adrenergic substances(9). It was seen that aldehyde oxidation is inhibited by both adrenergic and adrenolytic agents. The fact that inhibition by adrenergic amines is reversible and competitive suggests that the aldehydes formed from them by the action of monoaminoxidase have a greater affinity for aldehyde oxidase than salicylaldehyde. This possibility, however, is difficult to investigate because of the extreme instability of these aldehydes.

There is a fairly good parallelism between adrenergic blocking activity and inhibition of aldehyde oxidase, but it is weakened by the failure of the potent adrenolytic drug, yohimbine, to inhibit the enzyme. The latter fact may be due to some metabolic change that yohimbine has to undergo before exerting its blocking action.

Inhibition caused by some antihistamine drugs leaves open the possibility of aldehyde oxidase also taking part in their pharmacological action. This, however, can perhaps also be explained by the well known overlap between adrenolytic and histaminolytic actions. It was shown that the action of aldehyde oxidase is reversible(8); under anaerobic conditions, the enzyme reduces acids to aldehydes. The possibility is now being studied that this action is somehow related to the pharmacological properties of the inhibitors. This and other investigations may show whether the facts reported in this paper are coincidental or are associated with the mechanism by which adrenergic compounds exert their action.

Summary. A method based on the ultraviolet spectrum of salicylaldehyde and sali-

cylic acid is presented for the study of liver aldehyde oxidase. By the use of this method it was observed that aldehyde oxidase is inhibited by epinephrine and other adrenergic amines as well as by adrenergic blocking agents. It was observed that inhibition by the first group of substances is reversible and competitive while inhibition by adrenolytic drugs is irreversible and non-competitive.

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Ionization Constants of Basic Dyes in Relation to Gastric Secretion.* (20286)

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Since Ingraham and Visscher(1) have shown that basic dyes are secreted by the stomach, a study was undertaken to see whether or not there was a relation between the ionization constants of the dyes and their ability to be secreted by the stomach. Ray and Jung(2) have determined the ionization constants of basic dyes from titration data in unbuffered 50% alcoholic solutions, and attempted to correlate them with their tendency to be secreted by the stomach. However, Ray and Peters(3) noticed certain discrepancies in their data. In the hope of finding an explanation for these discrepancies, Woislawski (4) redetermined the ionization constants of the dyes, which Ray and Jung have used, by titration in aqueous medium. He also noticed discrepancies between his values and those found by Ray and Jung.

The present study was undertaken because it was hoped that the determination of the ionization constants of the dyes by a different method(5), namely, from spectrophotometric data in buffered 50% alcoholic solutions, might shed some light on these discrepancies.

Method. A stock solution was prepared by dissolving 1 x 10^{-4} M of the dyes in 95% alcohol. To 10 ml of the stock solution in a 100 ml volumetric flask was at first added either 50 ml N HCl, N/10 HCl, phthalate buffer pH 3.0, acetate buffer pH 5.0, phosphate buffer pH 6.7, borax-HCl buffer pH 8.2, N/10 NaOH or N NaOH, which was then diluted to the mark with 95% alcohol. The final concentration of the dyes was thus 1 x 10^{-5} M. In both the buffered and unbuffered solutions the pH shifted toward the alkaline region upon dilution with alcohol. The absorption spectra were measured using a Beckman Model DU spectrophotometer. Spectra were measured from 400-700 mμ at 24 $\pm$ C. For the determination of pH a Beckman pH meter (model G) with glass electrode 1190T and calomel electrode 1170 was used. When the pH rose above 10 a Beckman glass electrode 1190E was used. Because the pKa cannot be converted to pK_B with any degree of accuracy in 50% alcoholic solutions, only pKa values are given in this paper. The pKa of the dyes was determined by the formula of Flexser *et al.*(6), $pKa = pH_m + \log E_B\text{-}E_m/$

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† Deceased April 16, 1953. Paper prepared for publication by F. E. Ray.