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Effects of Amidines on Oxidases of *Escherichia coli* and of Animal Tissues

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Interest in the diamidines as antiprotozoal and antibacterial agents led us to consider their possible mechanism of action, and a study of their effects on oxidation systems offered a method of approach. Bernheim (1943, 1944) reported that certain amidines inhibited the oxidation of D-proline and of both isomers of alanine by *Escherichia coli*, but were without effect on the oxidation of glucose, succinate or pyruvate at similar concentrations.

We considered that further valuable information would possibly be revealed by studying the effects of amidines at higher concentrations, using also a wider range of substrates. Accordingly, we have carried out an investigation along these lines, with *Esch. coli* as the test organism. Propamidine (1:3-di-(4'-amidinophenoxy)propane) was mainly employed, but a number of experiments were also carried out with hexamidine (1:6-di-(4'-amidinophenoxy)hexane) and two halogenated derivatives, i.e. dibromopropamidine (1:3-di-(2'-bromo-4'-amidinophenoxy)propane) and monoiodohexamidine (1:4'-amidinophenoxy-6-(2'-iodo-4'-amidinophenoxy)-hexane). Some parallel experiments on the oxidation systems of rat tissues were included. A recent communication (Wien, Harrison & Freeman, 1948), in which some of our results were quoted, has dealt with the diamidines as bactericides.

EXPERIMENTAL

Measurement of respiration

The Warburg manometric technique was used for following O_2 uptakes at 37°. The bacterial suspension or tissue preparation was contained in the main vessel in 0.033M-phosphate buffer (for tissue preparations, phosphate-Locke medium was used), with or without the amidine salt in appropriate concentration. A 6% KOH solution (0.2 ml.) was introduced into the central tube of the vessel, and soaked into a small piece of rolled Whatman no. 41 filter paper. The substrate solution (0.2 ml.) was contained in the side arm and was tipped into the main vessel after a suitable equilibration or incubation period. The total volume of vessel contents, including those of the side arm and central tube, was always 3 ml.

Organisms

Esch. coli (National Collection of Type Cultures no. 4144) was used. The organisms were sown on trypsin digest-agar plates, and after 18-24 hr. growth the cultures were washed off the plates, using 3 ml. of distilled water for each plate. The collected suspension was centrifuged, was twice washed by resuspending in distilled water and recentrifuging, and finally diluted to 7.15 mg. dry wt./ml. by the use of Wellcome standard opacity tubes. Of this suspension 0.5 ml. was used for each vessel. It was confirmed that no appreciable change in the suspension occurred during the time occupied by the experiments. The O_2 uptake due to the organisms alone, without substrate, was usually not greater than 20 μ l./hr.; since this was small in comparison with the O_2 uptake in the presence of most substrates employed, it is permissible to regard the difference as representative of the O_2 used in the

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oxidation of the substrate. Experiments were carried out at pH values of 5.6, 6.8, 7.4 and 7.8, in view of Bernheim's (1944) emphasis on pH.

Tissue preparations

Preparations were made from the organs of rats in the minimum possible time after killing.

Liver was ground with alundum and 3-4 ml. 0.9% NaCl to a fine suspension, filtered by squeezing through gauze, and the tissue washed by diluting to 10 ml. with 0.9% NaCl. After centrifuging at high speed the deposit was suspended in 0.9% NaCl (5 ml./g. original tissue) and 0.5 ml. used in each vessel for studying the activities of the succinate, choline and cytochrome oxidase systems.

Kidneys were minced in 0.9% NaCl to give a fine suspension, which was washed by centrifuging and diluted with 0.9% NaCl (5 ml./g. original tissue). For each vessel, 0.5 ml. was used for studying D-amino-acid oxidase activity.

Brain. The whole brain was made to a uniform suspension with 3-4 ml. 0.9% NaCl in a hard-glass homogenizer, and diluted with 0.9% NaCl (6 ml. homogenate/g. original tissue). Of this 1 ml. was used for each experiment with the lactate and glucose oxidase systems.

Amidine derivatives

These were used as double isethionate salts.

RESULTS

Effects of amidines on oxidases of *Escherichia coli*

The results of a series of experiments with a number of substrates at different pH values are shown in Table 1. No initial incubation period was allowed in

after 2 hr. incubation at pH 7.8, and these effects may be compared with those observed by Bernheim (1944) with alanine and proline. With lactate, inhibitions of roughly the same order were brought about by dibromopropamidine, hexamidine and monoiodohexamidine.

Fig. 1. Effects of propamidine on Na lactate oxidation by *Esch. coli*. Bacterial suspensions at 37° with 0.01M-Na lactate. A, ○: pH 5.6. A, ×: with 12.5×10^{-5} M-propamidine, pH 5.6. B: pH 7.8. C: with 12.5×10^{-5} M-propamidine, pH 7.8.

Table 2 gives the results of a series of experiments in which the amidines were incubated with the bacterial suspension at pH 7.8 for 2 hr. at 37° before the addition of substrate; Q_{O_2} values (μ l. O_2 absorbed/mg. dry wt. organisms/hr.) are included. Inhibitions by particular amidines on different substrates can be compared. Oxidation of all the substrates (malate, succinate and glutamate in addition to

Table 1. Effects of amidines on oxidations by *Escherichia coli*

(Washed suspensions of bacteria, 37°; substrate added at the same time as amidine.)

Substrate (0.01M)	Amidine (12.5×10^{-5} M)	Inhibitions (%)							
		pH 5.6		pH 6.8		pH 7.4		pH 7.8	
		In 1st hr.	In 2nd hr.	In 1st hr.	In 2nd hr.	In 1st hr.	In 2nd hr.	In 1st hr.	In 2nd hr.
Na lactate	Propamidine	8	0	23	35	38	57	39	78
Na lactate	Hexamidine	0	0	30	51	39	61	63	76
Na lactate	Monoiodohexamidine	25	24	45	52	66	84	73	90
Na pyruvate	Propamidine	0	0	0	19	30	32	33	21
Glucose	Propamidine	0	0	0	0	31	54	28	65
Na acetate	Propamidine	0	2	0	12	6	45	40	78
D-Alanine	Propamidine	15	44	25	50	21	59	53	73

these experiments, beyond that required for thermal equilibration. Fig. 1 illustrates the variations of oxygen uptake with time, in the case of lactate, and the manner in which this is influenced by propamidine at pH values of 5.6 and 7.8; similar variations with time were observed with the other substrates. The systems governing the oxidations of lactate, glucose, pyruvate, acetate and D-alanine were all substantially inhibited. The inhibitions were maximal

those mentioned in Table 1) were inhibited by concentrations of amidines of the order of 12.5×10^{-5} M or less. Lactate was affected to approximately the same degree as alanine; both these systems were sensitive to concentrations of propamidine as low as 1.25×10^{-5} M. The halogenated derivatives inhibited oxygen uptake slightly more than the parent compounds, and hexamidine rather more than propamidine, but the differences were small.

Table 2. *Effects of amidines on oxidation systems of Escherichia coli*

(Washed suspensions of bacteria, incubated 2 hr. with amidine before tipping in substrate solution; reaction at 37°, pH 7.8.)

Substrate (0.01 M)	Amidine, concentration (M × 10 ⁻⁵)	Q _{O₂} Control without amidine	Q _{O₂} With amidine	Inhibition by amidine (%)
D-Alanine	Propamidine, 1.25	30.5	20.4	33
D-Alanine	Propamidine, 12.5	30.5	6.2	80
D-Alanine	Hexamidine, 12.5	33.2	2.0	94
D-Alanine	Dibromopropamidine, 12.5	33.2	4.2	87
Na lactate	Propamidine, 1.25	94.0	57.6	39
Na lactate	Propamidine, 12.5	94.0	30.0	68
Na lactate	Hexamidine, 12.5	94.6	22.4	76
Na lactate	Dibromopropamidine, 12.5	55.8	9.2	84
Na lactate	Monoiodohexamidine, 12.5	69.0	6.2	91
Glucose	Propamidine, 12.5	31.6	13.2	58
Glucose	Dibromopropamidine, 12.5	62.4	9.2	86
Glucose	Monoiodohexamidine, 12.5	77.0	0.6	99
Na malate	Propamidine, 12.5	26.2	7.2	72
Na malate	Hexamidine, 12.5	35.2	4.0	89
Na malate	Dibromopropamidine, 12.5	13.4	1.6	88
Na malate	Monoiodohexamidine, 12.5	39.6	3.0	92
Na acetate	Propamidine, 12.5	64.2	9.2	86
Na succinate	Propamidine, 12.5	34.6	20.0	42
Na glutamate	Propamidine, 12.5	14.6	4.4	69
Na pyruvate	Propamidine, 12.5	74.8	23.8	68

Table 3. *Effects of propamidine on tissue oxidation (37° and pH 7.6)*

Source of enzyme	Substrate, concentration (M)	Propamidine (× 12.5 × 10 ⁻⁵ M)	Period of in- cubation of enzyme and amidine (hr.)	Duration* of ex- peri- ment (hr.)	O ₂ uptake (μl.)	Q _{O₂}	Inhibition (%)
Rat-liver extract	Nil	0	2	1	2.7	0.06	—
	Na succinate, 0.03	0	2	1	333	8.32	—
	Na succinate, 0.03	1	2	1	313	7.82	6
	Choline chloride, 0.03	0	2	1	77	1.92	—
	Choline chloride, 0.03	1	2	1	23	0.58	73
	Choline chloride, 0.03	0	Nil	1	184	4.60	—
	Choline chloride, 0.03	1	Nil	1	48	1.20	74
	p-Phenylenediamine, 0.5	0	2	1	209	3.22	—
	p-Phenylenediamine, 0.5	1	2	1	233	5.84	0
Minced rat kidney	Nil	0	Nil	1	38	0.96	—
	D-Alanine, 0.013	0	Nil	1	169	4.22	—
	D-Alanine, 0.013	1	Nil	1	167	4.18	<1
Brain homogenate	Nil	0	1	2	66	0.50	—
	Na lactate, 0.017	0	1	2	165	1.23	—
	Na lactate, 0.017	1	1	2	149	1.11	10
	Glucose, 0.013	0	1	2	153	1.14	—
	Glucose, 0.013	1	1	2	131	0.97	14

* After tipping in substrate.

Effects of amidines on tissue oxidases

Effects of propamidine on some oxidation systems of rat tissues are shown in Table 3; the concentrations of the foregoing experiments were used. No significant effects were exerted on oxidation of glucose and lactate by rat brain; the succinate and cytochrome oxidases (the latter represented by the oxidation of p-phenylenediamine) and the D-amino-acid oxidase activity of kidney were likewise unaffected. The only

positive effect noted was that on choline oxidase, which was appreciably inhibited by 12.5 × 10⁻⁵ M-propamidine.

DISCUSSION

The observed effects of the amidines on the oxidases of *Esch. coli* are of interest with reference to the anti-bacterial actions of these compounds. Although no definite conclusions regarding the mode of inhibition of bacterial growth may be drawn from results of

enzyme studies on only one organism, the ability of amidines to exert toxic effects on different oxidation systems is a factor to be taken into account, especially in view of the fact that a high pH value favours inhibition of growth (Elson, 1945) as well as of respiration. The connexion is not apparently a simple one, since the differences in bacteriostatic power between halogenated derivatives and parent compounds are not paralleled by the same differences in anti-oxidase activity. To give an example, the concentrations required to produce bacteriostasis for *Esch. coli* are $11.8 \times 10^{-5} \text{ M}$ for propamidine and $0.57 \times 10^{-5} \text{ M}$ for dibromopropamidine (Wien *et al.* 1948): the corresponding figures for inhibition of oxidation were 80 and 87 % for alanine, and 68 and 84 % for lactate, using $12.5 \times 10^{-5} \text{ M}$ concentrations of the amidines. It cannot, therefore, be claimed that the anti-oxidase effects reflect the therapeutic efficacy of the compounds.

Regarding the mechanism of the inhibitory effect on respiration, it may be that the amidines exert effects on the particular dehydrogenases concerned with the different substrates, but the fact that so many systems are involved would appear to render this unlikely. It is more likely that an inhibitory effect is exerted on a respiratory mediator common to the various systems. One interesting possibility is that the system governing the direct transfer of molecular oxygen may be depressed; this would partly be supported by the absence of effect on the lactate oxidation system of brain, a cozymase-linked system, and by the slight effects of the amidines in inhibiting the growth of anaerobic organisms. It is conceivable that the cytochrome system peculiar to

Esch. coli may be inhibited; the absence of an effect on the cytochrome oxidase of liver does not conflict with this possibility, in view of the different cytochrome compositions of the two types of cell (Keilin & Harpley, 1941). It was not possible to pursue the point.

Finally, experiments on tissue oxidation revealed an apparently specific inhibition of the choline oxidase of liver. This point is of interest in connexion with the chronic toxicity of the amidines, which produce characteristic fatty changes in the liver. In view of the part played by choline metabolism in liver function, especially in regard to fatty changes, the inhibitory effect of propamidine on choline oxidase *in vitro* may well have some bearing on the degenerative changes produced in the liver *in vivo*.

SUMMARY

1. The respiration of washed cells of *Escherichia coli* in presence of various substrates was strongly inhibited by certain diamidines in concentrations of the order of $1.25\text{--}12.5 \times 10^{-5} \text{ M}$.

2. There was no simple relationship between the bacteriostatic potencies of the amidines and their effects on respiration.

3. Propamidine had no effect on several tissue oxidation systems, but inhibited the choline oxidase of liver.

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The Quantitative Determination of Barbiturates in Tissues by Ultraviolet Absorption Spectrophotometry

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The purpose of this paper is to describe a method for the estimation of very small amounts of barbiturates in mammalian tissues. The problem arose in connexion with work on the intermediary meta-

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bolism of the drugs, when it was found that the methods previously described were inapplicable for various reasons which will be discussed.

The basis for the various spectrophotometric procedures is the fact that barbituric acid and its derivatives of pharmacological importance, including