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The Inhibition of Urease and Succinoxidase by Metabolic Products of *p*-Dimethylaminoazobenzene and by some Related Amines

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The inhibition of activity of a cozymase enzyme system from yeast by metabolic products of the carcinogenic azo compound *p*-dimethylaminoazobenzene has been described by Kensler, Dexter & Rhoads (1942). The metabolites isolated from the urine of rats treated with *p*-dimethylaminoazobenzene (Stevenson, Dobriner & Rhoads, 1942) were *p*-aminophenol, *N*-acetyl-*p*-aminophenol, *p*-phenylenediamine, and *N*:*N'*-diacetyl-*p*-phenylenediamine; and it was assumed that the azo linkage is split to give the primary metabolites aniline and *as*-dimethyl-*p*-phenylenediamine from which the products isolated are subsequently formed.

Inhibition of the enzyme system was observed with *p*-phenylenediamine and with *as*-dimethyl-*p*-phenylenediamine. A number of other aromatic diamines also were found to be inhibitory, and it was suggested that the inhibition is due to the oxidation products of the diamines. Attempts were made to correlate the carcinogenic action of the azo compounds with the stability of the free radical of their metabolic products. Inhibition of yeast carboxylase by these diamines was also found (Kensler, Young & Rhoads, 1942).

Potter (1942) and Potter & Dubois (1943) have extended this work to the succinoxidase system of rat liver and to urease, both of which are considered to require an intact —SH group for activity. Both enzymes are also strongly inhibited by the diamines, and the conclusion that the oxidation products are the actual inhibitors was confirmed. The fact that the activity of the succinoxidase system, in common with that of some other enzyme systems, is usually very low in tumour tissues has led to speculation that the mechanism of the

production of cancer by chemical means may be connected with the inhibitory effects of metabolic products of carcinogens on such systems (cf. Potter, 1943, 1944).

As part of an investigation designed to obtain more concrete evidence for the basis of such theories of carcinogenic action we have investigated the behaviour of the succinoxidase system of rat livers towards the metabolites of *p*-dimethylaminoazobenzene during the period of feeding this liver carcinogen.

For this purpose it was first necessary to examine more fully the conditions of inhibition of liver succinoxidase by the known metabolites of *p*-dimethylaminoazobenzene, both in control animals which were fed on normal 'stock' diets and on diets similar to those of animals under treatment for tumour production except for the omission of the *p*-dimethylaminoazobenzene.

Since it has been shown (Elson & Warren, 1944) that benzidine can be isolated from the urine of rats receiving azobenzene, and it is probable that *p*-dimethylaminoazobenzene also undergoes the 'benzidine' rearrangement *in vivo* to give as the main product of this change 2:4'-diamino-5-dimethylaminodiphenyl (I) (Jacobson & Kunz, 1898), we have included in the investigation these and related compounds.

The effect on urease of these substances and of a number of other diamines selected with the intention of obtaining more information on the conditions necessary for inhibition has also been investigated.

EXPERIMENTAL

UREASE

Urease extract. The urease preparation was obtained by shaking 0.2 g. of Arlco Jack-bean meal with 18 ml. distilled water and centrifuging. To the urease extract (0.5 ml.) were added 3 ml. of M-acetate buffer pH 5.0, the amine solution and water to 8.3 ml. After $\frac{1}{2}$ hr. at 45°, 1.7 ml. of 3% aqueous urea solution were added (total volume 10 ml.). After further incubation at 45° for 1 hr. the digest (0.1 ml.) was pipetted into a tube containing 28.9 ml. distilled water, and after mixing, 1 ml. of Nessler's reagent was added. The ammonia was then estimated colorimetrically using a Hilger 'Spekker' photoelectric absorptiometer.

(Under these conditions the total amount of ammonia liberated by the control enzyme system was usually about 10 mg.)

Action of amines. Solutions of the amines were prepared either by dissolving the hydrochloride or sulphate in water or from the free amine by dissolving in water with the aid of a little HCl. Their effect on urease activity was tested both with the freshly prepared solutions and with those allowed to stand at room temperature for a varying number of days. On standing, the formation of quinonoid oxidation products usually gave rise to coloured solutions.

The results (Table 1) show that the inhibitory activity of those amines which become coloured on standing in slightly acid solution in presence of air increases to a maximum after several days standing. Aniline and benzidine, which do not form quinonoid

Table 1. *Inhibition of urease activity by metabolic products of p-dimethylaminoazobenzene and by some related substances*

Substance	Final molarity	% inhibition					
		Freshly prepared solution	Solution after standing for				
			1 day	2 days	3 days	4 days	7 days
Aniline	1.54×10^{-3}	5	10	10	—	—	—
	0.77×10^{-3}	0	5	0	—	—	—
	0.77×10^{-4}	0	0	0	—	—	—
<i>p</i> -Aminophenol	1.84×10^{-3}	59	80	70	73	—	75
	0.92×10^{-3}	56	71	66	64	—	65
	0.92×10^{-4}	38	37	40	35	—	32
<i>p</i> -Phenylenediamine	1.10×10^{-3}	36	70	74	93	100	—
	0.55×10^{-3}	24	49	59	88	95	—
	0.55×10^{-4}	0	11	18	17	25	—
<i>o</i> -Phenylenediamine	1.10×10^{-3}	98	100	100	100	—	—
	0.55×10^{-3}	31	96	95	90	—	—
	0.55×10^{-4}	8	34	57	50	—	—
<i>m</i> -Phenylenediamine	1.10×10^{-3}	25	24	27	30	—	30
	0.55×10^{-3}	10	15	20	25	—	20
	0.55×10^{-4}	0	0	0	0	—	0
<i>as</i> -Dimethyl- <i>p</i> -phenylenediamine	0.86×10^{-3}	60	100	100	92	—	—
	0.43×10^{-3}	50	77	92	84	—	—
	0.43×10^{-4}	0	30	40	28	—	—
<i>as</i> -Diethyl- <i>p</i> -phenylenediamine	0.84×10^{-3}	30	78	100	100	100	100
	0.42×10^{-3}	20	55	71	90	100	100
	0.42×10^{-4}	0	8	20	24	44	89
<i>N:N':N'</i> -Tetramethyl- <i>p</i> -phenylenediamine	0.84×10^{-3}	91	100	100	—	100	100
	0.42×10^{-3}	77	100	100	—	100	100
	0.42×10^{-4}	38	68	46	—	54	47
Benzidine	1.10×10^{-3}	10	0	0	—	—	—
	0.55×10^{-3}	10	0	0	—	—	—
	0.55×10^{-4}	0	0	0	—	—	—
2,4'-Diamino-5-dimethylamino-diphenyl	0.67×10^{-3}	35	72	100	100	100	100
	0.34×10^{-3}	14	55	77	100	100	100
	0.34×10^{-4}	0	21	25	55	65	75
Bindschedler's green	0.70×10^{-3}	100	100	100	—	—	—
	0.35×10^{-3}	91	87	87	—	—	—
	0.35×10^{-4}	35	34	37	—	—	—

oxidation products under these conditions, show no inhibition. *m*-Phenylenediamine which again cannot form a quinonoid oxidation product shows only a very slight inhibition which does not increase on standing, but *o*-phenylenediamine which can form an *o*-quinonoid oxidation product gives considerable inhibition. The dyestuff Bindschedler's Green which represents a stabilized quinonoid form shows its maximum inhibitory action when freshly dissolved.

These results were all obtained with the amines in slightly acid solution, but in neutral or slightly alkaline solution the rate of oxidation is usually very much greater.

Thus, 2:4'-diamino-5-dimethylaminodiphenyl, one of the most powerful inhibitors, on standing at pH 7.4 showed no increase in inhibitory action during the first 2 hr. of standing, during which the purple colour of the solution reached its maximum. After 3 hr., when the purple colour had become much duller, a slight increase in inhibition was observed, and after 24 hr. the dull blackish solution gave maximum inhibition.

SUCCINOXIDASE

Since the carcinogenic action of the azo compounds is evident on the liver rather than on other organs, the effects of metabolic products and related compounds were tested on the succinoxidase system of rat livers and kidneys. The latter provides a comparison with an organ, which, though also of high succinoxidase content, is not subject to the carcinogenic action of the azo compounds.

Methods. The rats used were albinos of both sexes weighing from 100 to 180 g. The control animals were kept either on a mixed 'stock' diet of brown bread, fresh milk, oats, greens and cod-liver oil, or a diet consisting of rice or of rice with 6% casein, supplemented with greens and carrots. For the study of enzyme systems during tumour induction 0.06% of *p*-dimethylaminoazobenzene was added to the rice diets. The animals were killed by dislocating the neck and the organs were used as fresh as possible. The time which elapsed between the killing of the animal and the immersion of the reaction vessels in the water-bath for temperature equilibration was generally less than 10 min.

Measurement of activity. The experiments were carried out in the Warburg apparatus at 38°, readings being taken every 5 min. The gas phase was air, or, where slices were used, oxygen.

A 20% solution of KOH was placed in the central cup of the Warburg vessel, whenever slices were used. The addition was not found necessary with homogenates. The addition of CaCl_2 and AlCl_3 showed no effect with 40 mg. homogenate and was therefore omitted. The increase in succinoxidase activity on addition of these salts to lower concentrations of homogenate will be discussed elsewhere.

The order of addition of the constituents to the reaction vessel varied according to the problem considered. The reaction mixture consisted of: 1 ml. homogenate (= 40 mg. tissue); 0.5 ml. of 6% in 0.1 M-buffer succinate; 0.2 ml. of a

1% solution of cytochrome *c*; 0.2 ml. amine; 1 ml. 0.1 M-phosphate buffer and water to make 3 ml. In experiments with slices the volume of the homogenate was replaced by an equal volume of 0.033 M-buffer.

Enzyme systems. Homogenates of liver or kidney or slices of liver were used. The thickness of the liver slices was 0.2–0.3 mm., the area about 50 sq. mm., and the final dry weight varied from 2 to 8 mg. Homogenates were prepared in 0.033 M-phosphate buffer according to Potter & Elvehjem (1936). Experiments showed that homogenization for 2–8 min. did not alter the succinoxidase activity; a standard time of 3 min. was adopted.

Solutions. Buffer of pH 7.35 was made up from 0.1 M- KH_2PO_4 and 0.1 M- Na_2HPO_4 . (NaH_2PO_4 -NaOH buffer gave the same results.) Sodium succinate was a Ca-free 6% (w/v) solution in 0.1 M-phosphate buffer pH 7.35. Cytochrome *c* was prepared from horse heart (Keilin & Hartree, 1937, 1938), and used as a 1% solution; the Fe content of the dry preparation varied from 0.3 to 0.32%. The solutions of the amines in distilled H_2O were prepared freshly for each experiment.

RESULTS

The results are illustrated by some typical curves and by tables giving the O_2 uptake before the amine was added, and the differences in the O_2 uptake due to the presence of amine, at intervals of 0–10 and 50–60 min. after its addition. Since in the present series of experiments no differences in behaviour were found between liver and kidney the results for kidney are not presented. All data except Tables 7 and 8 refer to animals on control diets.

A. The effects of different concentrations of amines on succinoxidase activity without and with the addition of excess cytochrome *c*

In these experiments the amines were tipped into the reaction mixture 30 min. after the readings started.

Homogenates. The addition of an excess of cytochrome *c* to homogenate containing 40 mg. tissue/ml. results in an increased O_2 uptake. The extent of this increase, depending on the individual liver, is greater with livers showing low O_2 uptake without added cytochrome *c*, and varies from 5 to 100% in different livers. The characteristics of the curves varied with the concentration of the amine. Of the substances investigated (Table 2), aniline was ineffective in a concentration of 5.1×10^{-3} M, while the other amines were inhibitory and, with the exceptions of *o*- and *m*-phenylenediamine and benzidine, reacted in a similar manner. On adding the amines in concentration of 10^{-3} to 10^{-4} M in the reaction mixture, which contained an excess of cytochrome *c*, the first reaction was generally a rapid O_2 uptake, followed soon by nearly complete inhibition. This initial rapid O_2 uptake is due to the catalytic oxidation of the amines by the enzyme systems of the liver. The O_2 uptake of the amines alone at pH 7.35 at the concentrations used in the

Table 2. *Effect of metabolic products of p-dimethylaminoazobenzene and of some related substances on the succinoxidase activity of rat liver homogenate*

Substance	Final molarity	No. of estimations	Oxygen uptake before addition of amine (μ l./10 min.) (40 mg. tissue)		% difference in O_2 uptake from control after addition of amine			
			Mean	Range	First 10 min.		50-60 min	
					Mean	Range	Mean	Range
A. With addition of excess cytochrome c.								
Aniline	5.1×10^{-3}	2	41	39 to 44	+ 5	+ 5 to + 5	- 3	- 6 to 0
<i>p</i> -Aminophenol	4.2×10^{-3}	3	39	36 to 42	+23	-37 to +60	-72	-67 to -100
	4.2×10^{-4}	4	60	49 to 68	-10	- 2 to +10	-71	-49 to -88
	4.2×10^{-5}	1	64	—	-14	—	-32	—
<i>p</i> -Phenylenediamine	3.7×10^{-3}	3	52	41 to 63	+13	-13 to +38	-58	-40 to -87
	1.8×10^{-3}	3	50	32 to 61	- 4	- 9 to + 3	-57	-38 to -67
	3.7×10^{-4}	3	64	47 to 90	-20	- 4 to + 7	-69	-67 to -80
	3.7×10^{-5}	2	52	45 to 58	-21	- 2 to -40	-57	-54 to -60
<i>o</i> -Phenylenediamine	3.7×10^{-3}	2	61	46 to 75	-37	-55 to +18	-50	-47 to -52
	3.7×10^{-4}	2	61	46 to 75	-28	-28 to -28	-28	-15 to -41
<i>m</i> -Phenylenediamine	3.7×10^{-3}	3	68	52 to 77	-37	-34 to -42	-37	-31 to -40
	3.7×10^{-4}	1	52	—	-11	—	-28	—
<i>as</i> -Dimethyl- <i>p</i> -phenylenediamine	2.8×10^{-3}	2	61	56 to 66	+23	+20 to +26	-94	-92 to -96
	2.8×10^{-4}	5	54	43 to 71	-13	- 5 to +53	-60	-40 to -93
	2.8×10^{-5}	3	51	41 to 69	+28	-16 to +64	-31	- 2 to -47
	2.8×10^{-6}	1	73	—	- 8	—	+ 2	—
Tetramethyl- <i>p</i> -phenylenediamine	2.8×10^{-3}	4	67	58 to 75	+28	+ 7 to +71	-63	-50 to -71
	2.8×10^{-4}	4	64	58 to 75	+23	+16 to +33	18	-33 to 0
Benzidine	3.6×10^{-3}	3	61	43 to 82	-13	-28 to + 9	-37	-17 to -61
	7.2×10^{-3}	1	56	—	-16	—	-33	—
2,4'-Diamino-5-dimethylaminodiphenyl	2.2×10^{-3}	6	48	35 to 69	+57	+ 7 to +115	-96	-92 to -100
	2.2×10^{-4}	13	49	28 to 73	+16	-29 to +97	-87	-61 to -100
	2.2×10^{-5}	10	45	31 to 63	-19	- 6 to -33	-52	-29 to -77
	2.2×10^{-6}	3	40	30 to 47	-15	-30 to + 2	-47	-25 to -69
Bindschedler's green	2.1×10^{-3}	3	68	57 to 79	-19	-13 to -20	-71	-58 to -80
	2.1×10^{-4}	4	80	55 to 116	- 4	-41 to +75	-58	-34 to -73
	2.1×10^{-5}	1	54	—	-29	—	-22	—
B. Without addition of cytochrome c								
Aniline	5.1×10^{-3}	1	21	—	0	—	+19	—
<i>p</i> -Aminophenol	4.2×10^{-3}	3	34	36 to 42	+ 1	-22 to +20	-16	-34 to 0
	4.2×10^{-4}	4	44	34 to 55	-10	-25 to + 8	-34	-16 to -53
	4.2×10^{-5}	1	46	—	-10	—	-50	—
<i>p</i> -Phenylenediamine	3.7×10^{-3}	3	39	27 to 54	+ 4	-29 to +25	-12	- 7 to -15
	1.8×10^{-3}	1	38	—	+24	—	-23	—
	3.7×10^{-4}	3	42	28 to 59	+ 4	- 7 to +11	-33	-20 to -52
	3.7×10^{-5}	2	33	30 to 37	+26	+10 to +41	-35	-26 to -45
<i>o</i> -Phenylenediamine	3.7×10^{-3}	2	32	26 to 38	- 9	-18 to 0	-19	-37 to 0
	3.7×10^{-4}	1	11	—	0	—	0	—
<i>m</i> -Phenylenediamine	3.7×10^{-3}	2	42	26 to 58	0	- 8 to + 8	-15	- 8 to -21
	3.7×10^{-4}	1	26	—	0	—	0	—
<i>as</i> -Dimethyl- <i>p</i> -phenylenediamine	2.8×10^{-3}	2	38	33 to 43	+73	+25 to +120	-64	-56 to -72
	2.8×10^{-4}	5	44	30 to 51	- 8	-14 to +10	-14	-37 to 0
	2.8×10^{-5}	1	45	—	- 9	—	- 6	—
	2.8×10^{-6}	1	48	—	-15	—	- 6	—
Tetramethyl- <i>p</i> -phenylenediamine	2.8×10^{-3}	2	42	40 to 44	+25	+ 8 to +41	-56	-41 to -71
	2.8×10^{-4}	3	47	44 to 52	+10	-17 to +36	+ 9	-16 to +22
Benzidine	3.6×10^{-3}	3	36	32 to 40	+10	- 3 to +28	-20	-64 to + 6
2,4'-Diamino-5-dimethylaminodiphenyl	2.2×10^{-3}	5	26	18 to 42	+59	+ 9 to +132	-18	-82 to + 5
	2.2×10^{-4}	8	31	14 to 50	+16	-10 to +52	-28	-62 to +23
Bindschedler's green	2.1×10^{-3}	3	50	46 to 54	+30	-29 to +39	+ 4	-66 to +77
	2.1×10^{-4}	4	50	39 to 66	-26	-45 to +19	-17	-53 to +53
	2.1×10^{-5}	1	42	—	-14	—	0	—

enzyme experiments is negligible, and is not increased by the addition of cytochrome *c*. During the period of rapid O_2 uptake the colour of the diamines changes from pale pink-violet to dark brown or black.

As the concentration of the inhibitors is progressively lowered, the initial increase in O_2 uptake

Fig. 1. Effect of the addition of different concentrations of 2:4'-diamino-5-dimethylaminodiphenyl on the succinoxidase activity of homogenates from the same liver with and without addition of cytochrome *c*. 40 mg. tissue per vessel. Amine tipped at $\uparrow$. Curve *A* = cytochrome *c* added. Curve *B* = no cytochrome *c* added. (1) $\circ-\circ$ = control. (2) $\bullet-\bullet$ = 2.2×10^{-4} M-amine. (3) $\Delta-\Delta$ = 2.2×10^{-5} M-amine. (4) $\times-\times$ = 2.2×10^{-6} M-amine.

is diminished and with concentrations of 10^{-5} – 10^{-6} M it can no longer be observed, but a gradual slowing down of the O_2 uptake occurs. The minimum concentration of the strongest inhibitor which is effective is about 10^{-6} M, but the effectiveness in low concentrations depends also on the individual rat liver tested. The range of variation in different estimations upon rats receiving the diets used is shown in the tables and no explanation of these individual variations is offered at present, but further experiments on this point are in progress.

Benzidine, and *o*- and *m*-phenylenediamine which are considerably less inhibitory than the *p*-compound, are effective in concentrations of 10^{-3} – 10^{-4} M and showed no initial increase in O_2 uptake.

When no cytochrome *c* was added to the homogenates the effects of the amines were not uniform. The oxidation and the inhibitory activity of the amines were greatly increased by the addition of cytochrome *c* (Fig. 1). In some livers the addition of an inhibitory amine in concentrations as low as 2.2×10^{-6} M to 40 mg. of liver produced a decrease in the O_2 uptake when an excess of cytochrome *c* was

Fig. 2. Effect of the addition of *as*-dimethyl-*p*-phenylenediamine on the succinoxidase activity of homogenates from the same liver with and without addition of cytochrome *c*. 40 mg. tissue per vessel. Amine tipped at $\uparrow$. Curve *A* = cytochrome *c* added. Curve *B* = no cytochrome *c* added. (1) $\circ-\circ$ = control. (2) $\bullet-\bullet$ = 2.8×10^{-4} M-amine.

added, but no decrease of the O_2 uptake could be observed in absence of added cytochrome *c* even with 100 times higher amine concentrations (Fig. 1). A slight increase in the O_2 uptake was often observed throughout the experimental period of over 1 hr. In other experiments, however, also without additional cytochrome *c*, the addition of 10^{-4} M amine resulted in an initial increase and subsequent inhibition (Fig. 2).

The succinoxidase system of the livers of rats which had been treated with *p*-dimethylaminoazobenzene showed the same general behaviour towards the inhibitors as that of the control animals (Table 3).

Table 3. *Effect of metabolic products of p-dimethylaminoazobenzene and of some related substances on the succinoxidase activity of the livers of rats fed p-dimethylaminoazobenzene. (Figures in brackets are results obtained without addition of excess cytochrome c)*

Substance	Days of feeding p-dimethyl- aminoazo- benzene	Final molarity	O ₂ uptake before addition of amine (μ L/10 min.) (40 mg. tissue)		% difference in O ₂ uptake from control after addition of amine			
					First 10 min.		50-60 min.	
<i>p</i> -Aminophenol	91	4.2×10^{-3}	37	(16)	+ 9	(+18)	-68	(+12)
	112	4.2×10^{-3}	21	(18)	+95	(+14)	-94	(+33)
	112	4.2×10^{-4}	21	(18)	-11	(-29)	-100	(-42)
<i>p</i> -Phenylenediamine	91	3.7×10^{-3}	42	(31)	-13	(-21)	-59	(-48)
<i>N:N'</i> -Dimethyl- <i>p</i> -phenylene- diamine	107	2.8×10^{-3}	42	(22)	+40	(+91)	-95	(-40)
	120	2.8×10^{-3}	46	—	+40	—	-88	—
	127	2.8×10^{-3}	26	(17)	+96	(+4)	-100	(-27)
	155*	2.8×10^{-3}	54	(32)	-22	(-6)	-60	(-27)
	21	2.8×10^{-4}	54	(36)	-4	(+20)	-45	(+3)
	88	2.8×10^{-4}	59	(35)	-27	(-17)	-73	(-40)
	100*	2.8×10^{-4}	52	(32)	+7	(+19)	-100	(+5)
	120	2.8×10^{-4}	46	(33)	+9	(+14)	-84	(-44)
	129	2.8×10^{-4}	36	(25)	+58	(+72)	-100	(-36)
	151	2.8×10^{-4}	54	(35)	-5	(0)	-77	(-29)
	265	2.8×10^{-4}	81	(41)	-23	(+12)	-82	(-45)
	100*	2.8×10^{-5}	52	(32)	+16	(+19)	-23	(+5)
	120	2.8×10^{-5}	46	—	0	—	-59	—
	127	2.8×10^{-5}	26	—	-33	—	-30	—
	155	2.8×10^{-5}	57	—	-11	—	-17	—
<i>N:N':N':N'</i> -Tetramethyl- <i>p</i> -phenylenediamine	73	2.8×10^{-3}	34	(24)	-12	(+67)	-80	(-30)
	73	2.8×10^{-4}	34	(24)	+15	(+35)	+37	(-27)
Benzidine	98	3.6×10^{-3}	42	(26)	-13	(-13)	-36	(-17)
	101	3.6×10^{-3}	37	—	-13	—	-68	—
2,4'-Diamino-5-dimethyl- aminodiphenyl	48	2.2×10^{-3}	43	(35)	+53	(+28)	-98	(-90)
	7	2.2×10^{-4}	41	(39)	-25	(-12)	-63	(-29)
	34	2.2×10^{-4}	45	(36)	+7	(+14)	-98	(-75)
	48	2.2×10^{-4}	43	(35)	0	(+3)	-95	(-88)
	111*	2.2×10^{-4}	71	(38)	-26	(0)	-75	(-57)
	136*	2.2×10^{-4}	68	(38)	-31	(-5)	-91	(-34)
	178*	2.2×10^{-4}	63	(42)	-18	(+17)	-91	(-31)
	289	2.2×10^{-4}	53	(37)	+9	(+65)	-85	(-55)
	353	2.2×10^{-4}	61	(49)	-21	(+1)	-85	(-22)
	461	2.2×10^{-4}	53	(38)	0	(-6)	-98	(-84)
	7	2.2×10^{-5}	41	—	-20	—	-47	—
	289	2.2×10^{-5}	53	(37)	+20	(+51)	-42	(+6)
	353	2.2×10^{-5}	61	—	-30	—	-94	—

* Tumour-free part of liver of tumour-bearing animals.

Slices. When tissue slices were used as the source of the enzyme system, the amines gave on the whole the same results as they did with homogenates to which cytochrome *c* had been added. The addition of cytochrome *c* to slices had generally no augmenting effect on the O₂ uptake of the succinoxidase system, nor any effect on the inhibitory activity of the amines. The inhibition, as compared with that of the homogenate, starts somewhat later and usually does not reach 100% in a 1 hr. period (Fig. 3). When comparing the succinoxidase activity per unit weight of liver in the form of homogenate with that of slices it should be borne in mind that

the homogenate does not take up O₂ in the absence of succinate, while slices do so, and that dry weight refers in these experiments to initial dry weight in the case of homogenates and to final dry weight in the case of slices.

B. *The effects of the amine on the enzyme system when added before the succinate*

The effect of a delayed addition of succinate was tested by adding the inhibitor to the reaction mixture in the main vessel not containing succinate and by tipping the succinate from the side-bulb 20-30 min. after readings started. In the control

experiments, the side-bulb of the vessel contained the same amount of amine as was tipped into the main vessel in which the inhibition was followed. Thus the control includes the negligible O_2 uptake due to the oxidation of the amine alone. By this procedure, with added cytochrome *c*, not only was no initial increase observed with inhibitor concentrations of 10^{-3} – 10^{-4} M, but there was almost 100 %

be observed which ceased on the addition of the succinate.

Fig. 3. Effect of the addition of *as*-dimethyl-*p*-phenylenediamine on the succinoxidase activity of homogenate and slices from the same liver. Excess of cytochrome *c* added. Amine tipped at $\uparrow$. Curve A = 40 mg. homogenate per vessel. Curve B = Slices of liver. O_2 uptake calc./mg. final dry weight. (1) $\circ$ — $\circ$ = control. (2) $\bullet$ — $\bullet$ = 2.8×10^{-3} M-amine.

inhibition when the succinate was added. It seems that the 30–50 min. which elapse between the making up of the reaction mixture, including the amine, and the readings after addition of succinate, were sufficient to oxidise the amines and to inhibit the succinoxidase system completely (Table 4, Fig. 4). Even with substances which are known to be oxidized by the cytochrome oxidase system, e.g. *p*-phenylenediamine, in concentrations of 3.7×10^{-3} to 3.7×10^{-4} M, only a small O_2 consumption could

Fig. 4. Effect of a variation in the order of adding the components to the homogenate from the same liver on the inhibition produced by different concentrations of 2,4'-diamino-5-dimethylaminodiphenyl. 40 mg. tissue per vessel. Cytochrome *c* added. Curve A = Amine tipped to the complete succinoxidase system at $\uparrow$. Curve B = Succinate tipped to the reaction mixture containing homogenate and different concentrations of the amines at $\uparrow$. (1) $\circ$ — $\circ$ = control. (2) $\square$ — $\square$ = 2.2×10^{-3} M-amine. (3) $\bullet$ — $\bullet$ = 2.2×10^{-4} M-amine. (4) $\triangle$ — $\triangle$ = 2.2×10^{-5} M-amine. (5) $\times$ — $\times$ = 2.2×10^{-6} M-amine.

Table 4. *Effect of metabolic products of p-dimethylaminoazobenzene and some related compounds on the succinoxidase activity of rat liver homogenate when added before the succinate. Excess cytochrome c added in all experiments*

Substance	Final molarity	No. of experiments	Mean O ₂ uptake of the control (with succinate but without addition of amine) (μ l./10 min.) (40 mg. tissue)	Mean % difference in O ₂ uptake from control after addition of amine and subsequent addition of succinate	
				First 10 min.	50-60 min.
Aniline	5.1×10^{-3}	2	58	+ 4	+ 3
<i>p</i> -Aminophenol	4.1×10^{-3}	1	39	- 78	- 78
	4.1×10^{-4}	5	53	- 72	- 72
	4.1×10^{-5}	2	51	0	0
<i>p</i> -Phenylenediamine	3.7×10^{-3}	3	52	- 53	- 70
	1.8×10^{-3}	2	43	- 96	- 96
	3.7×10^{-4}	3	63	- 77	- 77
	1.8×10^{-4}	1	54	- 37	- 34
	3.7×10^{-5}	2	51	- 30	- 36
<i>o</i> -Phenylenediamine	3.7×10^{-3}	2	60	- 41	- 52
	3.7×10^{-4}	2	29	- 24	- 21
<i>m</i> -Phenylenediamine	3.7×10^{-3}	3	68	- 14	- 9
	3.7×10^{-4}	1	52	- 20	- 20
<i>as</i> -Dimethyl- <i>p</i> -phenylenediamine	2.2×10^{-3}	3	57	- 96	- 100
	2.2×10^{-4}	5	52	- 100	- 100
	2.2×10^{-5}	3	56	- 13	- 27
	2.2×10^{-6}	2	64	- 1	- 10
<i>N:N':N':N'</i> -Tetramethyl- <i>p</i> -phenylenediamine	2.8×10^{-3}	3	42	- 75	- 83
	2.8×10^{-4}	2	38	- 95	- 100
Benzidine	3.6×10^{-3}	3	63	- 45	- 52
	3.6×10^{-4}	2	64	- 4	- 9
2,4'-Diamino-5-dimethylamino-diphenyl	2.2×10^{-3}	2	45	- 98	- 100
	2.2×10^{-4}	6	50	- 94	- 100
	2.2×10^{-5}	7	53	- 25	- 31
	2.2×10^{-6}	3	47	- 11	- 13
Bindschedler's green	2.1×10^{-3}	2	66	- 97	- 100
	2.1×10^{-4}	2	66	- 94	- 89
	2.1×10^{-5}	1	56	- 25	- 31

Table 5. *Comparison of the effect of some amines on the succinoxidase activity of liver homogenate with and without added cytochrome c when added before and after the succinate*

(Results on one horizontal line, and those bracketed, are from one animal)

Substance	Final molarity	% difference in O ₂ uptake from control							
		Cytochrome c added				No cytochrome c added			
		Amine tipped		Succinate tipped		Amine tipped		Succinate tipped	
		0-10 min.	50-60 min.	0-10 min.	50-60 min.	0-10 min.	50-60 min.	0-10 min.	50-60 min.
<i>p</i> -Aminophenol	4.2×10^{-4}	- 22	- 76	- 81	- 81	- 8	- 53	- 63	- 72
	4.2×10^{-4}	+ 10	- 56	- 65	- 65	- 7.5	- 50	- 23	- 23
	4.2×10^{-5}	- 30	- 40	0	0	—	—	—	—
	4.2×10^{-4}	- 2	- 88	- 89	- 91	- 13	- 16	- 10	- 10
<i>p</i> -Phenylenediamine	3.7×10^{-4}	- 4	- 60	- 64	- 67	0	- 20	- 25	- 21
	3.7×10^{-4}	- 22	- 72	- 93	- 93	0	0	0	0
<i>as</i> -Dimethyl- <i>p</i> -phenylenediamine	2.2×10^{-3}	+ 26	- 92	- 100	- 100	+ 120	- 42	- 100	- 100
	2.2×10^{-4}	- 26	- 66	- 91	- 91	+ 10	0	- 83	- 83
2,4'-Diamino-5-dimethylamino-diphenyl	2.2×10^{-4}	- 22	- 72	- 86	- 86	- 10	- 10	- 82	- 82
	2.2×10^{-4}	- 29	- 68	- 93	- 95	- 10	- 53	- 87	- 81
	2.2×10^{-5}	- 46	- 59	- 39	- 45	- 15	- 26	- 7	- 26

With concentrations of 10^{-5} – 10^{-6} M of the inhibitors, added before the succinate was tipped into the reaction mixture, there were only slight inhibitions, which were of about the same order of magnitude as those obtained in experiments where the amines were tipped (Table 5). In experiments on the same homogenate the higher (10^{-3} – 10^{-4} M) concentration of the amines when added before the succinate showed a nearly complete inhibition with or without addition of cytochrome *c*. With lower concentrations (10^{-5} M) of the amine they showed similar degrees of inhibition whether the amine was added before the succinate, or during the enzyme reaction.

(2) Inhibition experiments on tissues with low oxidative capacity were included since the mechanism of inhibition is presumably connected with oxidation of the amines by enzyme systems. The amount of succinoxidase varies widely in different tissues (Elliott & Greig, 1938; Schneider & Potter, 1943; Rosenthal & Drabkin, 1944). At the lower end of the scale are placed lung, spleen, intestinal mucosa, testis, and tumours of diverse origin. The tissues with low succinoxidase content investigated were: spleen, testis, Walker rat sarcoma and hepatic rat tumours induced by oral administration of *p*-dimethylaminoazobenzene and of 2-acetamidofluorene. With tumour tissue care was taken to

Table 6. *Effect of exposure to air on the inhibitory activity of some diamines*

Substance	(Cytochrome <i>c</i> added)							
	Inhibition % 1 hr. after adding the compound							
	Made up freshly	After standing (days)						
		1	2	4	5	14	20	21
<i>p</i> -Phenylenediamine, 1.8×10^{-3} M	67.0	44	—	—	—	—	—	—
	56.5	—	—	40	—	—	—	—
	57.0	—	—	—	—	—	45	—
	40.0	—	—	—	—	—	—	28
% decrease in inhibitory activity		34.3	—	29.5	—	—	21	30
<i>as</i> -Dimethyl- <i>p</i> -phenylenediamine, 2.2×10^{-4} M	100	93.4	—	—	—	—	—	—
	53	—	—	44.5	—	—	—	—
	70	—	—	—	—	0	—	—
% decrease in inhibitory activity		6.6	—	16.0	—	100	—	—
2,4'-Diamino-5-dimethylaminodiphenyl, 2.2×10^{-4} M	85.2	65.3	—	—	—	—	—	—
	68.2	—	31	—	—	—	—	—
	89.0	—	—	—	44	—	—	—
	100.0	—	—	—	—	—	34.5	—
	100.0	—	—	—	—	—	—	0
% decrease in inhibitory activity		23.4	54	—	50.4	—	65	100

C. *The effects of oxidation of the amine solutions on the inhibitory activities*

In this experiment the effect of the oxidation of the amines on their inhibitory power was followed (1) by exposing the solutions of the amines to air, and (2) by the effect on tissues with low oxidative capacity.

(1) Solutions of each substance were made up either in distilled water, or in 0.1 or 0.033M-phosphate buffer and tested for inhibitory activity after varying periods of exposure to air and to light. Table 5 shows that the inhibitory activity decreased on standing. 2,4'-Diamino-5-dimethylaminodiphenyl and *as*-dimethyl-*p*-phenylenediamine became completely non-inhibitory when kept in slightly alkaline (pH 7.35) solutions. *p*-Phenylenediamine, on the other hand, was still distinctly inhibitory even after 22 days. A decrease in activity was shown by all solutions, but in varying degrees.

exclude necrotic material or surrounding tissue. The O₂ uptake of these tissues was generally not inhibited by amines which proved strongly inhibitory towards organs with high succinoxidase content such as liver or kidney (Tables 7 and 8).

DISCUSSION

Whilst Kensler, Dexter & Rhoads (1942) suggest that the enzyme-inhibitory action of aromatic diamines is correlated with their free radical stability, Potter (1944) associates their toxicity to enzymes rather with their autoxidizability. The present results confirm the view that an intermediate oxidation product, which is probably of a quinonoid structure, is responsible for their enzyme-inhibitory properties.

In the case of urease, oxidation of the amines must first be brought about (e.g. by exposure to air) before maximum inhibitory activity is observed. When the amines are allowed to stand in

Table 7. *Effect of the addition of some diamines on the succinoxidase activity of rat tissues with low oxidative capacity*

Organ	Substance	Final molarity	O ₂ uptake before addition of amine (μl./10 min.) (40 mg. tissue)	% difference from control after addition of amine (50–60 min.)
Spleen	<i>as</i> -Dimethyl- <i>p</i> -phenylenediamine	2.2×10^{-3}	2	+100
		2.2×10^{-3}	4	+50
		2.2×10^{-3}	3	+41
		2.2×10^{-3}	7	+57
	<i>N:N:N':N'</i> -Tetramethyl- <i>p</i> -phenylenediamine	2.0×10^{-3}	6	+75
	2:4'-Diamino-5-dimethylaminodiphenyl	2.2×10^{-4}	6	0
		2.2×10^{-4}	6	0
Testis	<i>as</i> -Dimethyl- <i>p</i> -phenylenediamine	2.2×10^{-3}	8	+12
		2.2×10^{-3}	9	+11
		2.2×10^{-3}	22	-46
	<i>N:N:N':N'</i> -Tetramethyl- <i>p</i> -phenylenediamine	2.0×10^{-3}	7	+80
	2:4'-Diamino-5-dimethylaminodiphenyl	2.2×10^{-3}	8	+61
		2.2×10^{-3}	9	+15

Table 8. *Effect of the addition of some diamines on the succinoxidase activity of tumour tissues*

Tumour	Compound tested	Final molarity	Mean O ₂ uptake μl./g. wet wt./hr.	% dry weight	Homogenate		Slices	
					<i>Q</i> _{homogenate* succinate}	% change in O ₂ uptake (50–60 min after diamine)	<i>Q</i> _{slices succinate} †	% change in O ₂ uptake (50–60 min. after diamine)
Walker sarcoma: 8-day transplant	2:4'-Diamino-5-dimethylamino-	2.2×10^{-4}	850	15.4	5.5	Nil	15.4	-38
12-day transplant	diphenyl	2.2×10^{-4}	450	16.8	2.7	Nil	8.5	Nil
Tumour of liver of rat fed with acetamidofluorene for 368 days	2:4'-Diamino-5-dimethylamino-diphenyl	2.2×10^{-4}	1200	14.5	8.3	Nil	13.2	11
Liver of same animal	Ditto	2.2×10^{-4}	10,000	24.5	41.0	-69	45.5	-68
Tumour of liver of rat fed with acetamidofluorene for 363 days	2:4'-Diamino-5-dimethylamino-diphenyl	2.2×10^{-4}	4450	17.4	25.6	-65	30.0	-42.5
Liver of same animal	Ditto	2.2×10^{-4}	9600	25.4	38.0	-65	36.5	-36.0
Tumour of liver of rat fed with dimethylamino-azobenzene for 18 months	<i>p</i> -Phenylenediamine	1.8×10^{-3}	1880	14.8	12.7	Nil	—	—
Liver of same animal	Ditto	1.8×10^{-3}	4500	26.3	17.6	Nil	—	—
Tumour of liver of rat fed with dimethylamino-azobenzene for 18 months	<i>p</i> -Phenylenediamine	1.8×10^{-3}	1150	7.9	14.6	Nil	24.5	10
Liver of same animal	Ditto	1.8×10^{-3}	6600	26.7	24.7	-48	49.0	-39.5

* *Q*_{homogenate, succinate}, succinic oxidase activity, μl. O₂/mg. dry wt./hr., calc. on the initial dry wt. of the homogenate.† *Q*_{slices, succinate}, succinic oxidase activity, μl. O₂/mg. dry wt./hr., calc. on the final dry wt. of the slices.

acid solution (pH 3-4) in contact with air, their inhibitory activity increases gradually, and several days' standing is often necessary before maximum inhibition is obtained (Table 1). The complete succinoxidase system, however, is capable of bringing about such a rapid oxidation of the amines that maximum inhibitory activity is achieved during the course of the experiment (1½ hr.). Thus the inhibition shown by freshly prepared solutions does not increase on allowing the solutions to stand in contact with air, as it does in the case of urease, but gradually decreases as the available amine in the solution is removed by its complete oxidation to black insoluble products.

When the oxidation of succinate in liver homogenate is measured without the addition of excess cytochrome *c* it is not necessarily the succinic dehydrogenase content of the tissue that is determined, since the cytochrome *c* content may be a limiting factor. This was apparently the case in the experiments where addition of cytochrome *c* increased the O_2 uptake considerably. The O_2 uptake of the succinoxidase system in homogenized tissue to which no cytochrome *c* has been added may, however, be a truer estimate of the activity of that system as it would exist in the intact tissue. Obviously a deficiency of cytochrome *c* *in vivo* cannot become manifest when estimations of the O_2 uptake are made in the presence of an excess of cytochrome *c*. Frequently the addition of cytochrome *c* causes only a small increase in O_2 uptake of the homogenate, while the inhibitory activity of the amines may be 100 times increased. This disproportion of the effects of cytochrome *c* on the O_2 uptake of the succinoxidase system and on its inhibition by the amines seems to indicate either that the cytochrome oxidase system has a selectively greater affinity for the succinic dehydrogenase system, or that the cytochrome *c* is not available in the same way for the oxidation of the amine when bound to fragments of the cells as when it is dissolved freely in the reaction mixture.

The curves of oxygen uptake obtained when inhibitory amines are added to the succinoxidase system combine the effect of amine oxidation and the subsequent inhibition of the enzyme system. The first reaction is mostly apparent at the start; thus high concentrations of amines appear to act at first as accelerators instead of inhibitors. The higher the concentration of the amines, the greater is the apparent acceleration at the beginning of the experiment.

The inhibitions obtained on systems containing excess cytochrome *c* by (1) adding the inhibitor to the reaction mixture at the time of the preparation of the mixture and the succinate 30-40 min. later, and by (2) adding the inhibitor during the reaction, were in most cases the same, provided comparison

of the curves of O_2 uptake is made 1 hr. after the oxidation of the inhibitory amine has started. The oxidation of the amine starts in the first case when the reaction mixture is made up, and in the second case when the inhibitor is tipped into the complete enzymesystem 30 min. after the readings started. The latter curve is therefore shifted by this time interval.

The inhibitory activity of the amines towards the succinoxidase system decreases with the degree to which the oxidation proceeds in their aqueous solutions exposed to air, as judged by the change of colour from colourless to pink-violet and further to black. The amounts of completely oxidized and thus inactive products increase, and only the remaining unchanged substances are oxidized in the liver homogenate and act by their intermediate oxidation products. It seems therefore that *as*-dimethyl-*p*-phenylene diamine and 2:4'-diamino-5-dimethylaminodiphenyl become completely oxidized on standing. The oxidation of *p*-phenylenediamine, however, reaches an equilibrium with a relatively small amount of oxidized substance, whereafter its inhibitory capacity for the succinoxidase system remains unchanged.

With tissues of low oxidative capacity, whose cytochrome oxidase content is also low, the addition of the amines, even when an excess of cytochrome *c* is added, does not result in any rapid oxidation of the amines, nor in an inhibition of the succinoxidase system. This result is obtained on tissues which normally have a low O_2 uptake, such as spleen or testis, and on tissues arising from organs (liver) normally showing a high O_2 uptake but impaired in their oxidative capacity through pathological changes. Tumours of the liver, similarly to tumours of other origin, were found deficient in oxidative capacity, and they were generally not influenced by diamines. Thus the lack of inhibition by compounds which were found to be strongly inhibitory in experiments with liver or kidney is not characteristic of any one type of tissue. It depends entirely on the low oxidative capacity of the latter, e.g. one sample of testis with a comparatively high succinoxidase content showed a definite inhibition with 2:4'-diamino-5-dimethylaminodiphenyl. One tumour induced by 2-acetamidofluorene, a cholangioma which metastasized widely in the omentum, showed a succinoxidase content only 20-30% lower than that of the apparently unchanged part of the liver of the same animal; both values were within the normal range of liver tissue. The O_2 uptake of this tumour and of the liver tissue was inhibited by the addition of 2.2×10^{-4} M-2:4'-diamino-5-dimethylaminodiphenyl.

SUMMARY

1. The conditions under which certain amines related to the metabolites of the carcinogenic substance *p*-dimethylaminoazobenzene inhibit urease

and the succinoxidase activity of homogenates and slices of rat tissues have been investigated.

2. Their inhibitory action on urease increases to a maximum on allowing their solutions to oxidize by exposure to air, thus indicating an intermediate oxidation product as the actual inhibitor. Aniline and benzidine, which are not easily oxidized by exposure to air, are non-inhibitory. All the *p*-diamines tested are strongly inhibitory. *m*-Phenylenediamine shows only slight inhibitory activity.

3. In general those amines which inhibit urease also inhibit succinoxidase, but with this enzyme system the oxidation of the amines by the cytochrome oxidase component is so rapid that maximum inhibitory activity is obtained with freshly prepared solutions, and on exposure of the solutions to air a decrease in activity occurs.

4. In the succinoxidase system with addition of cytochrome *c* the higher concentrations of the inhibitory amines (10^{-3} – 10^{-4} M) generally give initially an increase in O_2 uptake followed by complete inhibition of the enzyme activity. With lower concentrations (10^{-5} – 10^{-6} M) only a gradually increasing inhibitory effect is observed. Without addition of cytochrome *c* results are less uniform in that the higher concentrations cause inhibition only

in some of the livers; the lower concentrations are often inactive.

5. The succinoxidase of the liver of animals which had been fed *p*-dimethylaminoazobenzene, even in tumour-free parts of the liver of tumour-bearing animals, showed the same general behaviour towards the amines as that of control animals.

6. The initial increase in O_2 uptake is due to the catalytic oxidation of the amines by the tissues, their oxidation in absence of tissue being negligible.

7. When the amines are added to the enzyme 20–30 min. before the succinate their complete inhibitory activity is observed immediately on addition of the succinate.

8. On tissues of low oxidative capacity no inhibitory effect on the O_2 uptake in presence of succinate is produced by amines which inhibit tissues of high succinoxidase content.

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The Calcium and Nitrogen Content of Human Bone Tissue Cleaned by Micro-dissection

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The object of the work here reported was to obtain as accurate a figure as possible for the proportion of calcium salt to organic (collagenous) matrix in human bone substance. We have been unable to find any satisfactory series of figures in the literature, one of the chief sources of error being the

inclusion of variable amounts of the soft tissue within the bone.

The material we have studied is mainly normal human bone with a view to establishing criteria for use in a further study of pathological bone; one group of pathological bone (sequestra) is also included.