Biochemical Pharmacology, Vol. 28, pp. 815–819.
Pergamon Press Ltd. 1979. Printed in Great Britain.

IN VITRO EFFECT OF L-TRYPTOPHAN AND ITS METABOLITES ON DIMETHYLAMINOAZOBENZENE REDUCTASE ACTIVITY OF RAT LIVER *

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(*Received* 13 *June* 1978; *accepted* 14 *August* 1978)

Abstract—The exact role of azodye reductase, a liver microsmal enzyme, and its influence on the hepatocarcinogenicity of 4-dimethylaminoazobenzene (DAB) are uncertain. The effect of DAB is depressed by many nutritional factors, including trytophan. Therefore, the effects of *o*-aminophenol and of L-tryptophan and its metabolites L-kynurenine, anthranilic acid, kynurenic acid, quinaldic acid, 3-hydroxy-DL-kynurenine, 3-hydroxyanthranilic acid, xanthurenic acid, quinolinic acid, *N*-methylnicotinamide, and *N'*-methylnicotinamide on rat liver azoreductase activity were determined, using DAB as substrate. Only 3-hydroxyanthranilic acid, 3-hydroxykynurenine and *o*-aminophenol decreased enzyme activity. The inhibition was greater if the buffered solutions (pH 7.4) of these three compounds were kept overnight before use, but the effect was prevented if these compounds were prepared in solutions of L-ascorbic acid and/or L-cysteine HCl. This observation indicates that the autoxidation products were probably responsible for inhibition of the enzyme. Further study of the oxidation products including the phenylquinoneimine formed from the oxidation of 3-hydroxyanthranilic acid in air, cinnabarinic acid, xanthommatin, 2-amino-3H-isophenoxazin-3-one, 1,9-dimethyl-2-amino-3H-phenoxazin-3-one and actinomycin D showed that all these compounds inhibited enzyme activity. A non-competitive type of inhibition was observed in the presence of cinnabarinic acid and xanthommatin. Cinnabarinic acid, xanthommatin, 2-amino-3H-isophenoxazin-3-one, 1,9-dimethyl-2-amino-3H-phenoxazin-3-one, and actinomycin D all have the same phenoxazinone ring system, suggesting that the driving factor in the inhibition of the azoreductase is the presence of the phenoxazinone chromophore. The chemical resemblance of these phenoxazinones to the coenzyme riboflavine further supports this supposition.

Although much has been learned about the metabolism of 4-dimethylaminoazobenzene (DAB), the mechanism of its carcinogenic action is still unclear. Its carcinogenicity may be decreased through various means, one of which might be to affect the enzymes involved in the metabolism of DAB. The azo group in DAB, essential for its carcinogenic action [1], is reductively cleaved by azodye reductase [2, 3] in DAB and related dyes. This enzyme is localized in the liver microsomes, requires NADPH and probably is a flavoenzyme [4, 5], since riboflavine is an essential cofactor [3, 6, 7].

Tryptophan has a variety of important metabolic pathways, but the one of greatest biological interest leads to the formation of nicotinic acid (tryptophan-niacin pathway). By this route most tryptophan metabolites including *o*-aminophenols, namely 3-hydroxyanthranilic acid (3-OHAA) and 3-hydroxykynurenine (3-OHKN), are formed [8–11]. Radioisotope studies have indicated that tryptophan and its metabolites, 3-OHAA and 3-OHKN, are involved in the biosynthesis of the phenoxazinone ring, cinnabarinic acid, and xanthommatin respectively [12–15]. Phenoxazinone structures are not uncommon in nature and have been found in many microbial metabolites (e.g. cinnabarin [16], actinomycins [17], tramesanquin [18], and questiomycin A [19, 20]). They also are the nucleus of a group of insect pigments, the ommochromes [21]. The purpose of the present study was to obtain information about the effect of tryptophan and its metabolites on DAB–azodye reductase activity, since tryptophan is known to decrease the hepatocarcinogenicity of DAB [22].

MATERIALS AND METHODS

L-Tryptophan, L-kynurenine sulfate, anthranilic acid, kynurenic acid, quinaldic acid, 3-hydroxy-DL-kynurenine, 3-hydroxyanthranilic acid, xanthurenic acid, quinolinic acid, *N*-methylnicotinamide, *N'*-methylnicotinamide and L-cysteine·HCl were purchased from the Sigma Chemical Co., St. Louis, MO. *o*-Aminophenol was obtained from the Eastman Kodak Co., Rochester, NY. L-Ascorbic acid was obtained from ICN Pharmaceutical, Inc., Cleveland, OH. Cinnabarinic acid (2-amino-3H-phenoxazin-3-one, 1,9-dicarboxylic acid) was a gift from Prof. A. Butenandt, Max-Planck-Institut, Munich, Germany. The phenylquinoneimine formed from the air oxidation of 3-OHAA and the 2-amino-3H-phenoxazin-3-ones of 3-OHKN (xanthommatin) and 2-amino-3-hydroxytoluene were gifts from Prof. R. R. Brown, Madison, WI. Actinomycin D was obtained from CalBiochem, San Diego, CA. 4-Dimethylaminoazobenzene (DAB) was purchased from K & K Laboratories, Inc., NY, and was purified as described by Miller and Miller [23].

All tryptophan metabolites, *o*-aminophenol, cinnabarinic acid, and xanthommatin were dissolved in 0.1 M potassium phosphate buffer, pH 7.4, just before

* Part of this work was presented at the meeting of the American Association for Cancer Research, Washington, DC, April 5–8, 1978.

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use. Some experiments were carried out after keeping the solutions of tryptophan metabolites overnight at 4°. DAB, 2-amino-3H-isophenoxazin-3-one, 1,9-dimethyl-2-amino-3H-phenoxazin-3-one and actinomycin D were dissolved in absolute ethanol.

Young male Fischer rats (Charles River Breeding Laboratories, Inc., Wilmington, MA) were used (weight range 75–100 g). The animals were fed Wayne Lab Blox. The animals had free access to food and water until they were killed by decapitation for the enzyme assay. The livers were removed immediately and chilled in ice. Homogenates (10%, w/v) were prepared in distilled water. The DAB–azodye reductase activity (substrate: DAB) was measured, in the presence of an NADPH generating system, according to procedures described by Mueller and Miller [2] and Conney *et al.* [24]. The amount of azodye reduced was calculated as the difference between the added amount and the amount remaining at the end of the assay. The enzyme activity was expressed as nmoles DAB reduced/min/g of liver. The results were evaluated by Student's *t*-test.

An *in vivo* study on the effect of tryptophan was conducted by feeding a group of male Fischer rats, 39-days-old at the start, a control diet(s) to which 1% tryptophan or 1% tryptophan plus vitamin B_6 had been added. After 31 days on these diets the animals were killed and the azoreductase activity was estimated according to the procedures mentioned previously.

RESULTS

Table 1 shows the effects of L-tryptophan, its metabolites and *o*-aminophenol on DAB–azodye reductase activity. Of the various compounds used only 3-OHAA, 3-OHKN and *o*-aminophenol inhibited the enzyme activity. If fresh solutions of 3-OHAA and 3-OHKN were added to the incubation medium, the per cent inhibition of the azoreductase was 51 and 5 per cent, respectively (Table 1). The per cent inhibition was increased if the inhibitor solution was left in air for a period of time. Significant inhibition up to 100 per cent was obtained if the solutions of 3-OHAA, 3-OHKN and *o*-aminophenol were kept overnight before use (Table 2). This confirmed that the autoxidation [12–15] of these compounds was responsible for the inhibition rather than the compounds themselves. The difference in the per cent inhibition in the presence of the same concentration of 3-OHAA and 3-OHKN, 51 and 5 per cent, respectively (Table 1), could be explained from calculation of the energies of the highest filled orbital of 3-OHAA and 3-OHKN. This indicated that the 3-OHAA molecule is a better electrón donor than 3-OHKN [25]. However, when the same concentrations of these three compounds were prepared in buffered solutions of L-ascorbic acid and/or L-cysteine·HCl and kept overnight before use, no inhibition was observed in the presence of these antioxidants (Table 2).

This observation suggested a study of the effect of the products formed from the oxidation of 3-OHAA, 3-OHKN, and *o*-aminophenol and some other related phenoxazinone structures on the enzyme activity. Table 3 presents the effects on the enzyme of increasing the concentration of the product formed from the air oxidation of 3-OHAA and the 2-amino-3H-phenoxazin-3-ones of 3-OHAA, 3-OHKN, *o*-aminophenol, 2-amino-3-hydroxytoluene and actinomycin D. All the compounds inhibited the enzyme activity. The percentage of inhibition increased when the concentration of the inhibitors in the incubation medium increased from 5×10^{-5} M to 4×10^{-4} M. Although only cinnabarinic acid inhibited the enzyme activity to an appreciable extent at 5×10^{-5} M, complete inhibition occurred in the presence of 4×10^{-4} M of the air oxidation product of 3-OHAA, cinnabarinic acid and xanthommatin (Table 3). Less inhibition was obtained in the presence of 2-amino-3H-isophenoxazin-3-one, 1,9-dimethyl-2-amino-3H-phenoxazin-3-one and actinomycin D at 4×10^{-4} M (25, 40 and 74 per cent, respectively).

Lineweaver–Burk plots of $[I/V]$ vs $[I/\text{DAB}]$ in the presence of cinnabarinic acid and xanthommatin revealed that the inhibition was of the non-competitive type [26] (Figs. 1 and 2).

Table 4 shows results of the *in vivo* effect of tryptophan on axoreductase activity. As can be seen, tryptophan had no effect on the azoreductase activity even in the presence of vitamin B_6.

Table 1. Effects of L-tryptophan and metabolites on DAB–azoreductase activity

Compound*	Final concn (mM)	(nmoles DAB reduced†/min/g liver)	Inhibition (%)
0.0		202 ± 7.2	
L-Tryptophan	1.67	210 ± 6.6	
L-Kynurenine sulfate	1.67	212 ± 5.9	
Anthranilic acid	1.67	199 ± 6.3	
Kynurenic acid	1.67	199 ± 5.2	
Quinaldic acid	1.67	197 ± 6.5	
3-Hydroxy-DL-kynurenine	1.67	191 ± 5.6	5
3-Hydroxyanthranilic acid	1.67	99 ± 4.9	51
Xanthurenic acid	1.67	202 ± 5.2	
Quinolinic acid	1.67	202 ± 6.0	
N-methylnicotinamide	1.67	219 ± 5.8	
N'-methylnicotinamide	1.67	200 ± 4.7	
o-Aminophenol‡	1.67	172 ± 4.6	15

* All the compounds were freshly prepared in 0.1 M potassium phosphate buffer, pH 7.4, before addition to the incubation medium.

† Each value is the mean ± S. E. M. of four individual determinations.

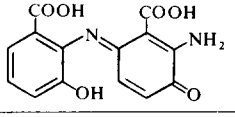
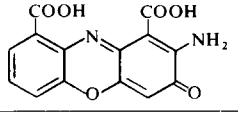
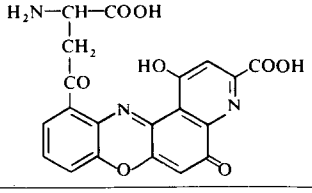
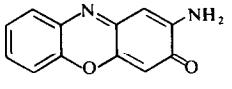
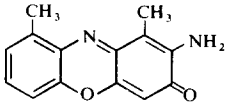
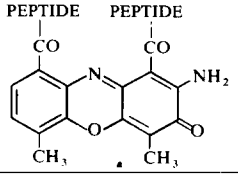
‡ Not a tryptophan metabolite.

Table 2. Effects of L-ascorbic acid and L-cysteine·HCl on the inhibition of DAB–azoreductase activity by the oxidation products of 3-hydroxyanthranilic acid, 3-hydroxykynurenine and *o*-aminophenol

Compound*	Final concn (mM)	DAB reduced† (nmoles/min/g liver)	Inhibition (%)
0.0		207 ± 6.2	
L-Ascorbic acid	1.67	206 ± 6.0	
L-Cysteine·HCl	1.67	216 ± 5.9	
3-Hydroxyanthranilic acid	1.67	0.0	100
3-Hydroxyanthranilic acid + L-ascorbic acid	1.67	207 ± 5.1	
3-Hydroxyanthranilic acid + L-cysteine·HCl	1.67	214 ± 4.9	
0.0		190 ± 5.1	
L-Ascorbic acid	1.67	191 ± 4.5	
L-Cysteine·HCl	1.67	199 ± 5.0	
3-Hydroxykynurenine	1.67	0.0	100
3-Hydroxykynurenine + L-ascorbic acid	1.67	190 ± 6.1	
3-Hydroxykynurenine + L-cysteine·HCl	1.67	197 ± 4.5	
0.0		196 ± 5.2	
L-Ascorbic Acid	1.67	190 ± 5.0	
L-Cysteine·HCl	1.67	200 ± 5.5	
o-Aminophenol	1.67	124 ± 4.9	37
o-Aminophenol + L-ascorbic acid	1.67	191 ± 4.8	
o-Aminophenol + L-cysteine·HCl	1.67	199 ± 4.5	

* All solutions were prepared in 0.1 M potassium phosphate buffer, pH 7.4, and left overnight before use.
† Each value is the mean ± S. E. M. of four individual determinations.

Table 3. Effects of increasing the concentration of the oxidation products of 3-OHAA, 3-OHKN, *o*-aminophenol and 2-amino-3-hydroxytoluene and actinomycin D on the DAB–azoreductase activity

Compound*	Chemical structure	Final concn (M)	DAB reduced† (nmoles/min/g liver)	Inhibition (%)
Phenylquinoneimine formed from the air-oxidation of 3-OHAA		0	181 ± 4.9	
		5×10^{-5}	184 ± 4.8	
		1×10^{-4}	119 ± 4.2	34
		4×10^{-4}	0.0	100
Cinnabarinic acid		0	209 ± 5.1	
		5×10^{-5}	86 ± 3.8	59
		1×10^{-4}	46 ± 4.0	78
		4×10^{-4}	0.0	100
Xanthommatin		0	178 ± 5.0	
		5×10^{-5}	158 ± 4.9	11
		1×10^{-4}	46 ± 3.5	74
		4×10^{-4}	0.0	100
2-Amino-3H-iso-phenoxazin-3-one		0	196 ± 5.0	
		5×10^{-5}	191 ± 4.7	
		1×10^{-4}	194 ± 4.6	
		4×10^{-4}	147 ± 4.8	25
1,9-Dimethyl-2-amino-3H-phenoxazin-3-one		0	185 ±5.5	
		5×10^{-5}	183 ± 6.0	
		1×10^{-4}	136 ± 4.1	27
		4×10^{-4}	112 ± 4.5	40
Actinomycin D		0	171 ±6.1	
		5×10^{-5}	175 ± 6.3	
		1×10^{-4}	137 ± 5.7	20
		4×10^{-4}	44 ± 4.9	74

* The oxidation products of 3-OHAA and 3-OHKN were dissolved in 0.1 M potassium phosphate buffer, pH 7.4; 2-amino-3H-iso-phenoxazin-3-one, 1,9-dimethyl-2-amino-3H-phenoxazin-3-one and actinomycin D were dissolved in absolute ethanol.
† Each value is the mean ± S. E. M. of five individual determinations.

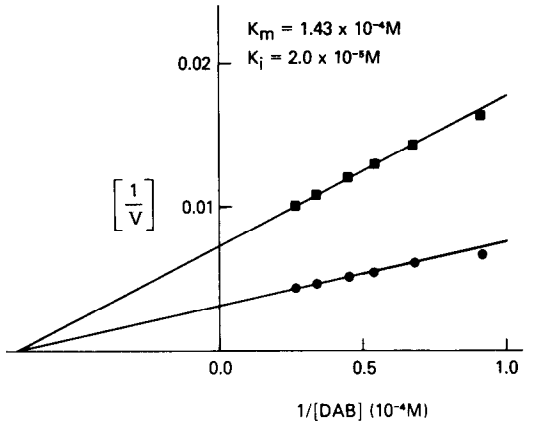


Fig. 1. Effect of cinnabarinic acid on the activity of DAB–azodye reductase as a function of DAB concentration. The assays were carried out as described in Materials and Methods. The velocities are expressed as nmoles of DAB reduced/min/g of liver. Key: (●——●) enzyme alone; (■——■) enzyme with 2.78 × 10^{-5} M cinnabarinic acid.

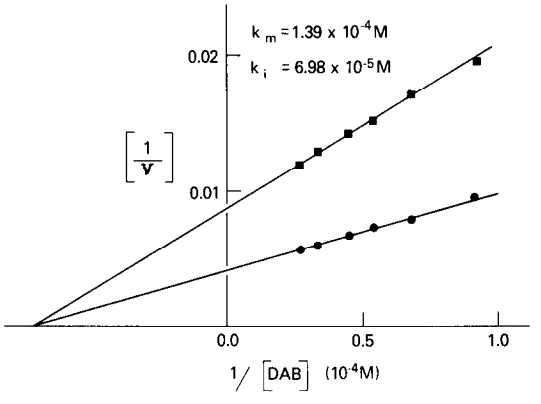


Fig. 2. Effect of xanthommatin on the activity of DAB-azodye reductase as a function of DAB concentration. The assays were carried out as described in Materials and Methods. The velocities are expressed as nmoles of DAB reduced/min/g of liver. Key: (●——●) enzyme alone; and (■——■) enzyme with 8 × 10^{-5} M xanthommatin.

Table 4. Effect of tryptophan feeding on azoreductase activity*

Group	DAB reduced† (nmoles/min/g liver)
Control	207 ± 10.2
1% Tryptophan	204 ± 9.3
1% Tryptophan + B_6	196 ± 11.1

* Male Fischer rats, 39-days-old, were fed the various diets for 31 days and then were killed for enzyme assay. Control diet was Wayne meal; the second group had 1% tryptophan added to Wayne meal; the third had 1% tryptophan and 50 mg vitamin B_6 added/kg of diet.

† Mean ± S. E. M. of ten animals in each group.

DISCUSSION

This *in vitro* study on the effects of *o*-aminophenol and of tryptophan and its metabolites on DAB–azodye reductase activity revealed that only 3-OHAA, 3-OHKN and *o*-aminophenol inhibited enzyme activity (Table 1). Furthermore, the increase in inhibition observed after keeping the solutions of these compounds overnight suggested that the autoxidation products of these *o*-aminophenols, rather than the compounds themselves, were responsible (Table 1 and 2). Previous studies have shown that some tryptophan metabolites, namely 3-OHAA and 3-OHKN, are involved in the biosynthesis of the phenoxazine ring [12–15]. There are several reports on the chemistry and formation of the phenoxazinone compounds, but the actual mechanism of the synthesis of this chromophore is not clear. Butenandt *et al.* [14] and Butenandt [21] suggested that xanthommatin and related pigments arise from 3-OHKN by oxidative condensation in a manner similar to chemical oxidation. In the oxidation dimerization of 3-OHAA, an *o*-quinoneimine is believed to be a transitional intermediate [27]. As suggested by Morgan *et al.* [28] and Subba Rao and Vaidyanathan [29], the highly reactive transient, *o*-quinoneimine, can condense in two ways, namely with another molecule of *o*-quinoneimine to form a phenylquinoneimine (the main product of the air oxidation of 3-OHAA) or with a molecule of 3-OHAA to form cinnabarinic acid. The ease of condensation for these *o*-aminophenols is due undoubtedly to the presence of two acceptors, C = C — C = NH and C = C — C = O, and the two donor groups —NH_2 and —OH [30]. Subba Rao and Vaidyanathan [30] suggested the same mechanism for the formation of 2-amino-3H-isophenoxazin-3-one from *o*-aminophenol. Inhibition of DAB azodye reductase by the autoxidation products was prevented when these *o*-aminophenols were prepared in buffered solutions of L-ascorbic acid and/or L-cysteine·HCl (Table 2), compounds which are known to prevent the oxidation of *o*-aminophenols [31, 32]. Schlegel *et al.* [32] concluded that 3-OHAA is not a carcinogen but that its oxidation product is. The finding that the phenoxazine dimer of 3-OHAA produced a significant incidence of bladder tumors after implantation in experimental animals lends support to this view [33].

The observation that only the *o*-aminophenols with a free position *para* to the phenolic group (*o*-aminophenol, 3-OHAA and 3-OHKN) which can undergo oxidative dimerization [27] affected azodye reductase activity is additional evidence for the importance of the phenoxazinone ring structure in inhibiting the enzymic activity.

The inhibition of the DAB–azodye reductase by cinnabarinic acid, a tryptophan metabolite [34] and by xanthommatin gives further evidence that these oxidation products of 3-OHAA and 3-OHKN are responsible for the inhibition, rather than the compounds themselves. It was also deemed important to determine the type of inhibition in the presence of these phenoxazinones. As can be seen from Figs. 1 and 2, the inhibition was of the non-competitive type. The non-competitive mechanism by which cinnabarinic acid and xanthommatin inhibited the azodye reductase may be attributed to the presence of several active groups in their chemical structures. These groups may help in the binding of

these phenoxazinones to a different region on the enzyme (not the active site). This binding may affect the configuration of the enzyme and cause conformation changes on the active site of the enzyme. Since increasing the concentration of DAB did not counteract these compounds, the inhibitory effect of cinnabarinic acid and xanthommatin is probably brought about by an irreversible non-competitive mechanism. Inhibition patterns which are apparently non-competitive are frequently encountered with irreversible inhibitors since the decrease in V_{max} simply reflects the fact that some enzyme has been removed from the system. In analogous fashion, the inhibition of the enzyme in the presence of 2-amino-3H-isophenoxazin-3-one, 1,9-dimethyl-2-amino-3H-phenoxazin-3-one and actinomycin D, and the fact that all the compounds have the same phenoxazinone ring suggest that the driving factor in the inhibition of the azodye reductase activity is the presence of the phenoxazinone chromophore in the incubation medium.

In vivo, it appears that tryptophan even in the presence of additional vitamin B_6 had no effect on the azoreductase activity of the liver. The discrepancy between *in vivo* and *in vitro* procedures might be explained by the fact that tryptophan is essential for protein synthesis and for maintaining nitrogen balances and, therefore, may be used rapidly. Another explanation might be that tryptophan may not always go through the nicotinic acid pathway since tryptophan has several metabolic pathways *in vivo*.

Thus, the degree of inhibition of azoreductase activity appears to depend on the structures of the compounds and the substitution at the 1- and 9-positions of the phenoxazinone ring. The resemblance between these phenoxazinones and the coenzyme, riboflavine, indicates that the phenoxazinones may inhibit the azoreductase by displacing the coenzyme from an active site.

Acknowledgements—The authors are very grateful to Professor A. Butenandt and Dr. W. Schafer for their generous gift of cinnabarinic acid, and to Professor R. R. Brown for his generous gift of the air oxidation product of 3-OHAA, xanthommatin and 1,9-dimethyl-2-amino-3H-phenoxazin-3-one. We are indebted to F. Williams and C. Derflinger for assistance in various aspects of these studies.

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