Studies with Tryptophan Metabolites *in vitro*

KYNURENINE METABOLISM IN LIVER HOMOGENATES OF NORMAL AND *SCHISTOSOMA MANSONI*-INFESTED MICE

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The conversion of kynurenine into kynurenic acid and anthranilic acid in both normal and *Schistosoma mansoni*-infested mouse liver was investigated. It was found that in the *S. mansoni*-infested mouse liver there is probably a deficiency of pyridoxal phosphate that resulted in an inhibition of kynurenine transaminase and a low production of kynurenic acid. Deoxypyridoxine and its phosphorylated derivative inhibited kynurenine transaminase in the normal liver in a pattern qualitatively similar to that observed with infested liver. The lowered concentration of pyridoxal phosphate in the infested liver is discussed in view of the possibility of two combined mechanisms: (a) an antimetabolite being secreted by the infesting worms or present in its eggs that partially inhibited the phosphorylation of pyridoxal, and (b) concentration of pyridoxal phosphate by the worms, resulting in a lowered concentration of the cofactor in the host tissue.

The association between vesical schistosomiasis and cancer of the bladder has been amply demonstrated (Krager, 1963), but the exact nature of this association is still unknown. Studies in this and other Laboratories have indicated that increased quantities of certain metabolites of tryptophan are produced during schistosomal infestation (Abdel-Tawab, Kelada, Abdel-Daim & Makhyoun, 1966; Abdul-Fadl & Khalafallah, 1961; Khalafallah & Abdul-Fadl, 1964). Since some of these metabolites are carcinogenic (Boyland & Watson, 1956; Allen, Boyland, Dukes, Horning & Watson, 1957; Bryan, Brown & Price, 1964; Bryan, Morris & Brown, 1965), the possibility of a causal relationship between the increase in the production of the carcinogenic metabolites and the induction of bladder tumours attracted the attention of many investigators and was the subject of a number of recent studies (Boyland, 1963; Wallace & White, 1963; Alifano, Papa, Tancredi, Elicio & Quagliariello, 1964; Price & Brown, 1962). However, the exact mechanism by which schistosomal infestation induces such changes in tryptophan metabolism is still obscure.

Most of the known clinical conditions inducing abnormal tryptophan metabolism affect the formyl-kynurenine pathway in a manner suggesting

functional pyridoxine deficiency (Price, 1961; Price & Brown, 1960). This is generally manifested by the urinary excretion of elevated amounts of certain tryptophan metabolites such as kynurenine, 3-hydroxykynurenine and their acetyl derivatives, kynurenic acid or xanthurenic acid or both, depending on the species (Williams & Wiegand, 1960), after the ingestion of a loading dose of tryptophan. Such a pattern is found in bilharziasis, particularly if accompanied with bladder cancer (Abdel-Tawab *et al.* 1966). This disordered metabolic pattern can generally be corrected by supplementing the diet with pyridoxine (Abdel-Tawab *et al.* 1966; Price & Brown, 1962; Price, 1961), which supports the view that there is a functional pyridoxine deficiency under these conditions.

Most of the studies carried out in this field, however, were based on the analysis of urine samples collected from bilharzial patients with or without bladder tumours. The use of humans under these conditions makes it difficult to eliminate factors such as the social stratum, dietary habits, sex, age, multiplicity of parasitic infestation and other variables that greatly affect the results obtained. Therefore it is uncertain whether the observed changes in tryptophan metabolism, generally attributable to pyridoxine deficiency, are directly associated with the schistosomal infestation alone. Studies with animals would eliminate these variables and would permit the use

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of their livers for a closer examination of the enzyme systems involved.

In the present investigation liver homogenates of both normal and *Schistosoma mansoni*-infested mice were used to study the metabolism of kynurenine, which is the central metabolite in the formyl-kynurenine pathway of tryptophan metabolism, and its conversion into kynurenic acid and anthranilic acid.

MATERIALS AND METHODS

Animals. Adult albino mice weighing 15–20 g. fed *ad libitum* on a specially prepared diet containing all the necessary factors were used. Exposure of mice to cercariae of *S. mansoni* was started 60 days before the experiments (Oliver & Stirewalt, 1952). The infested animals were used only after producing eggs for at least 1 week.

Materials. Kynurenine sulphate, kynurenic acid, pyridoxal hydrochloride and deoxypyridoxine hydrochloride were purchased from Sigma Chemical Co. (St Louis, Mo., U.S.A.). ATP was supplied by Ormonoterapia Richter Ltd. (Milan, Italy) and α -oxoglutarate by L. Light and Co. Ltd. (Colnbrook, Bucks.). Anthranilic acid was prepared and tested for purity in this Laboratory. Pyridoxal phosphate and deoxypyridoxine phosphate were also prepared in this Laboratory by the method described by Beiler & Martin (1947). In some experiments high-quality commercial pyridoxal phosphate (grade 'A'; Calbiochem, Los Angeles, Calif., U.S.A.) was used. The results obtained with either sample of pyridoxal phosphate were indistinguishable. Redistilled water from an all-glass still was used to make solutions.

Preparation of the homogenates. The mice were killed by exsanguination after stunning by a blow on the head. The fresh livers were quickly removed and placed in ice-cold 0.25 M-sucrose solution. Tissue homogenates (10%, wet wt./vol.) were prepared in the sucrose solution in a Potter-Elvehjem homogenizer.

For the removal of the worms from the livers infested with *S. mansoni*, the perfusion technique described by Radke, Berrios-Duran & Moran (1957) was used. The complete removal of the worms was checked by pressing the livers between two glass plates and examination under the dissecting microscope. In some experiments, the infested livers were homogenized without the removal of the infesting worms.

Incubations. Reaction mixtures (final volume 4 ml.) were incubated in 25 ml. Erlenmeyer flasks shaken in a water bath kept at 37° with air as the gas phase. At the end of the incubation, 1 ml. of 16% (w/v) trichloroacetic acid was added to each flask and the mixture transferred to centrifuge tubes with 1 ml. of distilled water. The precipitate was removed by centrifugation and the supernatants were frozen until analysed. Flasks were run in duplicate and a zero-time flask was included in each set of experiments.

The concentrations of the different materials, when present in the incubation medium, unless otherwise stated, were: DL-kynurenine sulphate (5.0 μ M), pyridoxal hydrochloride (40.0 μ g.), deoxypyridoxine hydrochloride (40.0 μ g.), ATP (5 mm), CaCl_2 (5 mm), MgSO_4 (1 mm), potassium phosphate buffer, pH 7.4 (50 mm), α -oxoglutarate (30.0 μ M), pyridoxal phosphate (40.0 μ g.), deoxypyridoxine

phosphate (40.0 μ g.) and 10% whole-liver homogenate (2.0 ml.).

Quantitative estimation of metabolites. Kynurenine, anthranilic acid and kynurenic acid were determined by the method of Miller, Tauchida & Adelberg (1953). Kynurenine and anthranilic acid were also determined by the method of Mason & Berg (1952). Student's *t* test was performed on the data and the probability values (*P*) are included in Table 1; *P* values less than 0.05 were considered significant.

RESULTS

The albino rat has been used for most of the studies on the metabolism of tryptophan in animals, but, since this animal is an unsuitable host for *S. mansoni* (Stirewalt, Kuntz & Evans, 1951), the albino mouse was used in the present work. However, the kinetics of the production of kynurenic acid and anthranilic acid from kynurenine were studied in the livers of both species (Fig. 1). It is clear that the production of the two metabolites from kynurenine in both species is very similar.

After 3 hr. of incubation, the concentrations of both metabolites reached a plateau and the utilization of kynurenine was nearly maximal. All the subsequent incubations were thus carried out for 3 hr. Under these conditions the maximum amount of kynurenine utilized was never more than 50% of that present at the start of incubation, indicating that the amount of kynurenine present in the incubation medium was not rate-limiting.

The effect on kynurenine metabolism of the perfusion technique used to remove the worms from the infested livers was investigated on normal liver with or without the addition of pyridoxal phosphate. The results obtained with normal perfused livers were indistinguishable statistically from those obtained with unperfused normal livers (Table 1).

The production of kynurenic acid and anthranilic acid from kynurenine was studied in liver homogenates of both normal and *S. mansoni*-infested, worm-free, mouse livers with the results shown in Table 2. It is clear from Expts. 1, 2 and 3 (Table 2) that the production of kynurenic acid is significantly inhibited in the infested mouse liver as compared with normal. This was also observed after incubation for 1 hr. and 2 hr. The inhibition mainly affects the kynurenine transaminase system leading to the synthesis of kynurenic acid, with no significant effect on kynureninase leading to the synthesis of anthranilic acid. Ca^{2+} and Mg^{2+} ions and/or pyridoxal and ATP, in the concentrations needed to synthesize pyridoxal phosphate (Gunsalus & Razzel, 1955), had hardly any effect in counteracting the inhibition of kynurenine transaminase in the infested liver (Expts. 2 and 3, Table 2). Only after the addition of pyridoxal phosphate (Expt. 4, Table 2) was the infested liver able to synthesize kynurenic acid to the extent of the normal liver,

Fig. 1. Kinetics of the production of kynurenic acid and anthranilic acid from kynurenine in the liver homogenates of the rat (a) and the mouse (b). The incubation mixture (4ml.) contained 10% whole-liver homogenate (2ml.), kynurenine (5 μ M), pyridoxal hydrochloride (40 μ g.), ATP (5mM), CaCl_2 (5mM), MgSO_4 (1mM) and α -oxoglutarate (30 μ M) in 50mM-potassium phosphate buffer, pH 7.4. The values given are the means of four to eight experiments expressed as μ moles/g. of liver. $\circ$, Kynurenic acid; $\blacktriangle$, anthranilic acid; $\blacksquare$, kynurenine utilized.

Table 1. *Effect of perfusion on kynurenine metabolism in normal mouse liver*

Incubations were carried out for 3hr. at 37° in a total volume of 4ml. containing 10% whole-liver homogenate (2ml.), CaCl_2 (5mM), MgSO_4 (1mM), α -oxoglutarate (30 μ M) and DL-kynurenine sulphate (5.0 μ M). The results are calculated as μ moles/g. of liver and are the means of four to eight experiments.

Addition	Normal, perfused		Normal, not perfused	
	Kynurenic acid	Anthranilic acid	Kynurenic acid	Anthranilic acid
None	1.33	2.45	1.2	2.17
Pyridoxal phosphate (40 μ g.)	4.54	10.7	3.86	9.28

though the kynurenine transaminase system of the latter was greatly stimulated. It therefore seems that pyridoxal phosphate insufficiency is most probably the factor responsible for the observed inhibition to the kynurenine transaminase system in the infested liver, since it is the coenzyme needed for that transamination reaction. The relative inhibition of both enzyme systems is best illustrated by the ratio of the amount of anthranilic acid to the amount of kynurenic acid produced. For the normal liver this ratio is always below 3. For the infested liver, however, the anthranilic acid/kynurenic acid ratio is much higher than 3, which clearly illustrates the greater inhibition encountered on the production of kynurenic acid as compared with that of anthranilic acid. It is noteworthy that in the presence of pyridoxal phosphate the an-

thranilic acid/kynurenic acid ratio of both infested and normal liver is again below 3 (Expt. 4, Table 2). This indicates that pyridoxal phosphate stimulated both enzyme systems in normal liver to nearly the same relative extent. In the infested liver, however, greater stimulation was encountered with the originally inhibited kynurenine transaminase system.

The production of kynurenic acid and anthranilic acid from kynurenine was further investigated in the normal mouse liver. The maximum activity of kynurenine transaminase and kynureninase, as shown by the amounts produced of kynurenic acid and anthranilic acid respectively, is only encountered in the presence of pyridoxal phosphate, which is the natural coenzyme for both systems (Expts. 9 and 10, Table 3). Addition of deoxypyridoxine hydrochloride to the system containing Ca^{2+} , Mg^{2+} , ATP and pyridoxal hydrochloride inhibited kynurenine transaminase, as shown by the lowered production of kynurenic acid, but apparently stimulated kynureninase activity (compare Expt. 2 with Expt. 1, Table 3). The latter effect might be due to the kynurenine transaminase blockage by deoxypyridoxine with the accumulation of kynurenine and its degradation by the uninhibited kynureninase. However, when deoxypyridoxine was added to the system lacking pyridoxal hydrochloride (Expt. 3, Table 3), greater inhibition of kynurenine transaminase was produced. At the same time, anthranilic acid production was less than would be expected if substrate-induced stimulation of kynureninase had occurred. This suggests that kynureninase was also inhibited under these conditions. Inhibition of the latter system was also produced when deoxypyridoxine

Table 2. *Kynurenine metabolism in normal and S. mansoni-infested mouse liver*

Incubations were carried out for 3 hr. at 37° in a total volume of 4 ml. containing 10% whole-liver homogenate (2 ml.). When present, the final concentrations were: pyridoxal hydrochloride (40 µg.), ATP (5 mM), CaCl₂ (5 mM), MgSO₄ (1 mM), pyridoxal phosphate (40 µg.), α-oxoglutarate (30 µM) and DL-kynurenine sulphate (5.0 µM). The results are calculated as µmoles/g. of liver and are the means of four to eight experiments. N, Normal liver; I, infested liver freed from the infesting worms.

Expt. no.	Additions to the system					Metabolite determined				Anthranilic acid	
	Pyridoxal	ATP	CaCl ₂	MgSO ₄	Pyridoxal phosphate	Kynurenic acid		Anthranilic acid		Kynurenic acid ratio	
						N	I	N	I	N	I
1	+	+	+	+	—	2.22	0.05 (P 0.01*)	3.80	3.33 (P 0.2)	1.71	66.7
2	—	—	+	+	—	1.30	0.03 (P 0.01*)	3.12	3.30 (P 0.2)	2.38	110.0
3	+	—	+	+	—	1.95	0.40 (P 0.01*)	3.53	6.00 (P 0.1)	1.81	15.0
4	—	—	—	—	+	4.63	4.03 (P 0.50)	11.03	11.13 (P 0.1–0.5)	2.38	2.75

* Difference statistically significant.

Table 3. *Effect of deoxypyridoxine on kynurenine metabolism by normal mouse liver homogenates*

Incubations were carried out for 3 hr. at 37° in a total volume of 4 ml. containing 10% whole-liver homogenate (2 ml.). When present, the final concentrations were: pyridoxal hydrochloride (40 µg.), deoxypyridoxine (40 µg.), deoxypyridoxine phosphate (40 µg.), ATP (5 mM), CaCl₂ (5 mM), MgSO₄ (1 mM), pyridoxal phosphate (40 µg.), α-oxoglutarate (30 µM) and DL-kynurenine sulphate (5.0 µM). The results are calculated as µmoles/g. of liver and are the means of four to eight experiments.

Expt. no.	Additions to the system					Metabolite determined				
	Pyridoxal	ATP	CaCl ₂	MgSO ₄	Deoxypyridoxine	Deoxypyridoxine phosphate	Pyridoxal phosphate	Kynurenic acid	Anthranilic acid	
1	+	+	+	+	—	—	—	2.22	3.80	
2	+	+	+	+	+	—	—	1.00	5.00	
3	—	+	+	+	+	—	—	0.05	2.00	
4	—	—	—	—	—	+	—	0.36	2.30	
5	—	—	+	+	—	+	—	0.48	2.33	
6	+	+	—	—	—	+	—	0.81	3.25	
7	+	±	+	+	—	+	—	0.66	3.40	
8	—	—	—	—	—	+	+	2.10	6.70	
9	—	—	—	—	—	—	+	4.63	11.03	
10	+	+	+	+	—	—	+	4.41	13.15	

phosphate was added to the incubation mixture (Expt. 4, Table 3). Ca²⁺ and Mg²⁺ and/or pyridoxal and ATP were not able to counteract the inhibition produced by deoxypyridoxine phosphate on both enzyme systems, except for a slight increase in activity when pyridoxal hydrochloride and ATP were present, presumably owing to the synthesis of pyridoxal phosphate. A greater increase in activity occurred when authentic pyridoxal phosphate was added (Expt. 8, Table 3). In this case the effect was greater on the kynureninase enzyme system, which might indicate a lower requirement for pyridoxal phosphate of that enzyme compared with kynurenine transaminase, since it was inhibited last and recovered first.

The effect of deoxypyridoxine phosphate on the

two enzyme systems of the normal liver seems, at least qualitatively, to be similar to the effect of *S. mansoni* infestation. In the experiments shown in Table 4 increasing concentrations of deoxypyridoxine phosphate were added to the incubation mixture. Results obtained with the infested, worm-free, liver without deoxypyridoxine phosphate are also included in Table 4 (Expt. 6). The values obtained with the latter are very similar to those with normal liver supplemented with deoxypyridoxine phosphate. This supports the hypothesis that the infested liver is probably lacking in active pyridoxal (cf. Table 2). This could be due either to a lack of the vitamin or to inhibition of its phosphorylation, and is discussed below.

Homogenates of worm-infested livers of mice,

Table 4. *Comparison of the effects of deoxypyridoxine phosphate and S. mansoni infestation on kynurenine metabolism by mouse liver*

Incubations were carried out for 3 hr. at 37° in a total volume of 4 ml. containing 10% whole-liver homogenate (2 ml.), α -oxoglutarate (30 μ M) and DL-kynurenine sulphate (5.0 μ M). The results are expressed as μ moles/g. of liver and are the means of four to eight experiments.

Expt. no.	Infestation with <i>S. mansoni</i>	Deoxypyridoxine phosphate (μ g./ml.)	Metabolite determined	
			Kynurenic acid	Anthranilic acid
1	—	2	1.2	3.12
2	—	4	1.2	3.10
3	—	6	0.93	3.00
4	—	8	0.33	2.20
5	—	10	0.36	2.30
6	+	—	0.10	2.05

Table 5. *Effect of S. mansoni worms on kynurenine metabolism by homogenates of S. mansoni-infested mouse livers*

Incubations were carried out for 3 hr. at 37° in a total volume of 4 ml. containing 10% whole-liver homogenate (2 ml.), pyridoxal hydrochloride (40 μ g.), CaCl_2 (5 mM), MgSO_4 (1 mM), α -oxoglutarate (30 μ M) and DL-kynurenine sulphate (5.0 μ M). The results are calculated as μ moles/g. of liver. I, Infested liver freed from the infesting worms; IW, infested liver from which the worms were not removed.

Expt. no.	Additions to system			Metabolite determined				Anthranilic acid	
				Kynurenic acid		Anthranilic acid		Kynurenic acid ratio	
	Pyridoxal	CaCl_2	MgSO_4	I	IW	I	IW	I	IW
1	—	+	+	0.03	0.75	3.30	4.50	110.0	6.0
2	+	+	+	0.40	2.00	6.00	6.5	15.0	3.3

made without removal of the infesting worms, metabolized kynurenine in a pattern closer to normal than the corresponding livers from which the worms were removed (Table 5). The amounts of kynurenic acid produced in the livers from which the worms were not removed were higher than the corresponding values obtained with worm-free livers. Apparently the worms are responsible for the difference. This is much more evident when the anthranilic acid/kynurenic acid ratio in both livers is examined. The ratio for worm-containing homogenates is much lower than that for the worm-free homogenates and is much closer to the ratios obtained with normal liver (cf. Table 3). Obviously the endogenous coenzyme of the worms is responsible for the partial correction of the inhibition of kynurenine transaminase in the infested liver. The effect of pyridoxal (Expt. 2, Table 5) under these conditions is not clear and could be an extension of its effects mentioned above (Table 3).

A direct assay of pyridoxal phosphate in both normal and infested livers with or without worms was also carried out by the method described by Sherman (1954). The results are shown in Table 6.

The amount of active pyridoxal contained in the infested liver still having the infesting worms is

Table 6. *Pyridoxal phosphate assay of normal and S. mansoni-infested mouse liver*

The results are the means of four to 12 experiments.

Expt. no.	Condition	Pyridoxal phosphate content (μ g./g. of liver)
1	Normal liver	83
2	Normal liver, perfused	74
3	Perfused infested liver (no worms)	49
4	Perfused infested liver (with worms)	112

higher than that contained in either the normal liver or the infested liver from which the worms were removed. Each worm is thus estimated to contain 2–3 μ g. of pyridoxal phosphate (from the observation that infested livers contained 20–30 worms and an excess of 60 μ g. of active pyridoxal/g.).

DISCUSSION

It is evident from the present study that there exists functional pyridoxine deficiency in the livers

of mice infested with *S. mansoni*. The effect of this deficiency is manifested by an inhibition of the kynurenine transaminase system with the resultant low synthesis of kynurenic acid. The other enzyme system utilizing kynurenine, namely kynureninase, also requires pyridoxal phosphate as coenzyme. However, there is no evidence of inhibition to that system, since the quantities of anthranilic acid recovered were very close to normal. This difference in the response of the two enzymes to pyridoxal phosphate deficiency was shown earlier by Price, Brown & Larson (1957), Price & Brown (1960), Price (1961) and Yess, Price, Brown, Swan & Linkswiler (1964), using pyridoxine antimetabolites. The difference in response of different pyridoxine-dependent enzymes towards pyridoxine antimetabolites and pyridoxal phosphate deficiency has been noted previously (for review see Holtz & Palm, 1964).

The assumed functional pyridoxine deficiency present in the livers of mice infested with *S. mansoni* worms must be related to the presence of these parasites. It might be due to an antimetabolite(s) secreted by the worms in the liver or present in the eggs contained therein or to depletion of the vitamin from the host liver by the infesting worms (Table 5). The similarity, at least qualitatively, between the effect of worm infestation and deoxypyridoxine supplementation has been described (Table 4). In both cases pyridoxal hydrochloride and ATP were not able to relieve the inhibition of kynurenine transaminase (Expt. 1, Table 2, and Expt. 7, Table 3) under conditions that would otherwise lead to the synthesis of pyridoxal phosphate. The site of inhibition therefore seems to lie at least partially in the phosphorylation of pyridoxal. In fact deoxypyridoxine, a pyridoxine antimetabolite, was shown to inhibit the phosphorylation of pyridoxine and pyridoxal by phosphokinase (Hurwitz, 1952, 1955; Snell, 1958). It would therefore seem that there is an antimetabolite of pyridoxine and/or an agent that inhibits primarily the phosphorylation of pyridoxal present in the livers of the *S. mansoni*-infested mice. This would result in decreased concentrations of pyridoxal phosphate, with the resultant inhibition of kynurenine transaminase.

The concentrations of active pyridoxal obtained by direct assay in both normal and infested livers with or without worms support the hypothesis that the worms concentrate pyridoxal phosphate. It is thus possible that both secretion of an antimetabolite inhibiting the phosphorylation of pyridoxal and concentration of pyridoxal phosphate by the infesting worms may be occurring at the same time.

It is noteworthy that, in the present study, deoxypyridoxine caused a relatively greater in-

hibition of kynurenine transaminase than of kynureninase, since this antimetabolite inhibited kynureninase in preference to kynurenine transaminase in other species (for review see Holtz & Palm, 1964). This difference is not extraordinary, since species differences in the response of enzymes to antimetabolites are widely known (Daniel, 1961). It is emphasized that infestation with *S. mansoni*, which paralleled the effects of deoxypyridoxine in all respects, also inhibited kynurenine transaminase to a greater extent than kynureninase in the mice used in the present work.

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