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Enzymic demethylation of a carcinogenic tryptophan metabolite, 8-methoxykynurenic acid

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8-METHOXYKYNURENIC acid is a urinary metabolite of tryptophan in humans,[1] swine[2] and monkeys.[3] Bryan *et al.*[4,5] reported that it was carcinogenic for the mouse bladder when implanted in this organ in cholesterol pellets. Systemic administration of 8-methoxykynurenic acid has also been shown to produce a significant incidence of malignant tumors of the mouse lymphoreticular system.[6] Recently, Lower *et al.*[7] investigated the fate of 8-methoxykynurenic acid *in vivo* to find out whether this compound need be metabolized to display carcinogenic activity, but its demethylation was not examined. The present paper describes some properties of this demethylating system.

Male Donryu strain rats (150–200 g) were used in all experiments. The liver or kidney was immediately removed and homogenized with 4 vol. of isotonic KCl. Microsomes were separated from the soluble fraction by centrifuging the 24,000 *g* supernatant at 100,000 *g* for 60 min. The 24,000 *g* supernatant fraction consisting of the microsomes plus soluble fraction was used mainly in this study.

Demethylase activity was determined by estimating the amount of xanthurenic acid and formaldehyde formed. The assay medium contained 15 μmoles of 8-methoxykynurenic acid, 50 μmoles of $MgCl_2$, 50 μmoles of nicotinamide, 2·4 μmoles of NADPH, 0·5 m-mole of potassium phosphate buffer, pH 7·5 and 24,000 *g* supernatant obtained from 800 mg of liver or kidney in a total volume of 8 ml. Enzyme was omitted in the blank. The incubation was carried out at 37° for 1 h with shaking. Formaldehyde was assayed with the Nash reagent.[8] Known amounts of formaldehyde carried through the incubation and assay procedures served as standards. To estimate the amount of xanthurenic acid, the reaction was stopped by adding 0·5 ml of 50% trichloroacetic acid and the mixture was filtered. Seven ml of the filtrate was adjusted to pH 8 with a 4 N KOH and applied to a column (1 × 5 cm) of Dowex 1 (formate form), which was washed with 50 ml of distilled water, 30 ml of 2 N HCOOH, 30 ml of 4 N HCOOH and eluted with 70 ml of 6 N HCOOH. The eluate with 6 N HCOOH was evaporated in vacuo and the residue was dissolved in a small volume of aqueous ammonia. The total volume was spotted on a thin layer plate (0·5 mm thick) of Avicel SF cellulose powder (Merck) and the chromatogram was developed with a mixture of ethylacetate–isopropanol–28% aqueous ammonia (9:6:4 by vol.). The region corresponding to xanthurenic acid was scrapped from the plate with a razor blade into a glass tube and extracted with 50 ml of methanol. The extract was evaporated to dryness and the residue was dissolved in 4·0 ml of 0·1 M potassium phosphate buffer, pH 7·5; the optical density at 342 nm was measured against that of the blank. For calculation of the amount of xanthurenic acid formed, the molecular extinction coefficient was taken as 6500. Known amounts of xanthurenic acid in 0·1 M potassium phosphate buffer, pH 8 were simultaneously subjected to column and thin layer chromatography.

8-Methoxykynurenic acid was incubated with the 24,000 *g* supernatant fraction obtained from 800 mg of rat liver and the reaction product was analyzed as described above. An additional spot with yellow fluorescence under ammonia gas appeared on a thin layer chromatogram besides the light blue spot of 8-methoxykynurenic acid. The product was chromatographically identical with authentic xanthurenic acid [cf. reference 2, Table 2]. If the supernatant was boiled or the reaction stopped at zero time xanthurenic acid was not detected. Negligible amounts of xanthurenic acid were formed if the microsomal or soluble fractions were used alone and if the 24,000 *g* supernatant fraction was dialyzed against 0·005 M potassium phosphate buffer, pH 7·5, no xanthurenic acid was formed. When the dialyzed microsomal and

TABLE 1. ESTIMATION OF REACTION PRODUCTS

Enzyme source	Xanthurenic acid formed (μmole/hr)	Formaldehyde formed (μmole/hr)
Liver	0·55	0·58
Kidney	0·34	0·32

The 24,000 *g* supernatant fraction obtained from 800 mg of rat liver or kidney, was incubated at 37° for 1 hr with the assay medium containing 15 μmoles of 8-methoxykynurenic acid, 50 μmoles of $MgCl_2$, 50 μmoles of nicotinamide, 2·4 μmoles of NADPH and 0·5 mmole of potassium phosphate buffer, pH 7·5 in a final volume of 8 ml. Values are the mean of four experiments.

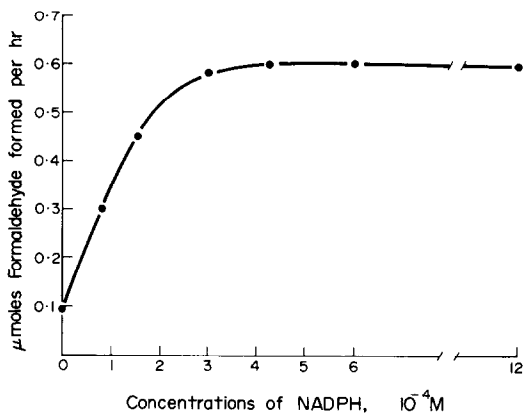

FIG. 1. Effect of NADPH on the demethylation activity. The 24,000 *g* supernatant fraction obtained from 800 mg of rat liver was incubated for 1 hr at 37° with the assay medium described in Table 1 except that the NADPH concentration was varied. Each point represents the mean of three determinations.

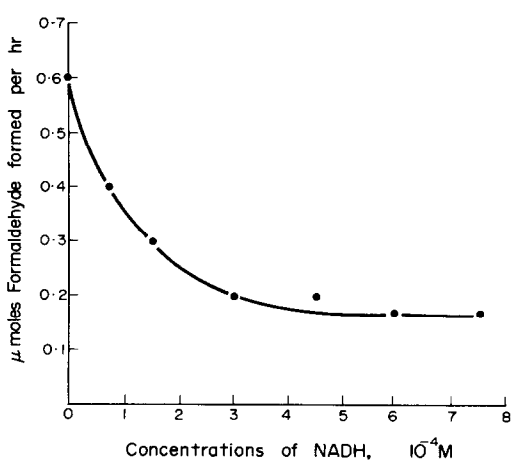

FIG. 2. The influence of NADH on the enzyme activity of the 24,000 *g* supernatant fraction of rat liver. The assay medium described in Table 1 was used except that the NADH concentration was varied as indicated. Each point is the average of three determinations.

undialyzed soluble fraction were combined, xanthurenic acid was produced. This suggests that certain low molecular weight compound in the soluble fraction were involved on the demethylation.

A balance study of the reaction products formed in the enzymic demethylation of 8-methoxykynurenic acid was made after incubation with the 24,000 *g* supernatant fraction obtained from 800 mg of rat liver or kidney, as noted in Table 1. About 1 mole of formaldehyde was generated for each mole of xanthurenic acid formed. The demethylation activity was also found in rat kidney, but was lower than in the liver. Until now, an *O*-demethylating enzyme system has not been detected in the kidney of any species. Incubation in air was effective, but there was negligible enzyme activity under anaerobic conditions.

Figures 1 and 2 show the pyridine nucleotide requirements for the demethylation activity. As shown in Fig. 1, NADPH produced detectable activity at 0·075 mM and full activity at 0·3 mM. NADH concentrations of 0·08 mM and 0·3 mM in the presence of 0·3 mM NADPH resulted in about 30 per cent and 60 per cent decrease in demethylation activity, respectively (Fig. 2). These results suggest that the demethylation of 8-methoxykynurenic acid is regulated, in part, by the concentration ratio of intracellular pyridine nucleotides. Gaylor *et al.*[9] reported that the properties of the sterol-demethylating system are similar to the properties of the stearic acid-desaturating system of liver.[10] They showed that the efficacy with NADH on the sterol demethylation is about 10 to 15 per cent greater than with NADPH.[9] In the present study, NADH could not replace NADPH. Therefore, it was suggested that the demethylation of 8-methoxykynurenic acid may be catalyzed by a different enzyme system from the sterol-demethylating system.[9,11]

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