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# Selective stimulation by urethan of hepatic microsomal aniline hydroxylation

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URETHAN (ethyl carbamate) is a relatively simple aliphatic amide with multipotential as a carcinogen.[1] It exerts its hepatocarcinogenic action in newborn rodents and in 70 per cent hepatectomized adult male mice.[2,12] Evidence is available to suggest that urethan must be metabolized to exert its carcinogenic action.[13] Furthermore, urethan has been shown to decrease the hexobarbital sleeping time of normal animals,[14] suggesting an interaction with the microsomal mixed function oxidase. After administration of urethan *in vivo* to rats, liver microsomes were found to have a 2-fold elevation of aniline hydroxylase activity, without significant change in either the rate of aminopyrine demethylation, the activity of NADPH-cytochrome c reductase, or the levels of cytochrome $b_5$ and P-450.[15] Mixed function oxidase activities of residual liver of partially hepatectomized animals treated with urethan were either unaffected or increased to a lesser degree than that observed in sham-operated animals.[15]

To investigate these phenomena further, the effects of urethan on the induction of mixed function oxidase activity have been studied in a system *in vitro* consisting of liver slices in iso-osmotic medium as previously described.[16]

Slices of rat liver (approximately 100 mg wet weight) were added within 10 min of killing of the animal to 5 mM aniline and 2 mM NADPH in 3 ml of iso-osmotic medium containing varying concentrations of urethan. After 20 min of incubation, the reaction was terminated by addition of 1·5 ml of 20% trichloroacetic acid or 0·2 ml of 1 M acetate buffer, pH 5·0. Aniline hydroxylase has a pH optimum of about 8·0,[17] and almost no *p*-aminophenol was generated at pH 5·0. Where indicated, 100 units of β-glucuronidase (type 10, Sigma Chemical Co., St. Louis, Mo.) was added for an additional 30 min before termination of the reaction with trichloroacetic acid. *p*-Aminophenol production from aniline and formaldehyde production from aminopyrine were measured as previously described.[18]

When added to liver slices from normal untreated rats at a concentration of 10 mM, urethan was without effect on the *N*-demethylation of aminopyrine, but caused up to a 60 per cent enhancement of aniline hydroxylase activity (Table 1). When liver slices from 70 per cent partially hepatectomized adult male rats were used 12–16 hr after the operation, no significant increase in aniline hydroxylase activity was produced by the carcinogen (Table 1, $P < 0{\cdot}1$). This action of urethan could not be demonstrated with liver homogenates, microsomes (Table 2), nor with mitochondria. The extent of enhancement by urethan of aniline hydroxylase activity in liver slices was dependent upon the concentration of carcinogen in the medium as is shown in Fig. 1: maximum stimulation occurred above a concentration of 4 mM.

TABLE 1. EFFECT OF URETHAN ON LIVER SLICE AMINOPYRINE DEMETHYLASE AND ANILINE HYDROXYLASE ACTIVITIES IN NORMAL AND REGENERATING RAT LIVER*

| | Urethan (10 mM) | Normal (nmoles/g liver/min) | Regenerating† (nmoles/g liver/min) |
|---|---|---|---|
| Aminopyrine demethylase‡ | – | 484 ± 67 | |
| | + | 468 ± 74 | |
| Aniline hydroxylase§ | – | 4·6 ± 0·7 | 3·7 ± 0·22 |
| | + | 7·2 ± 0·9\| | 4·2 ± 0·21 |

* Liver slices of about 100 mg wet weight were used; values are means ± standard errors for at least six experiments.

† Adult male rats were subjected to 70 per cent partial hepatectomy in the evening and were killed 12–16 hr later. Values are for four experiments.

‡ Formaldehyde was measured.

§ *p*-Aminophenol was measured.

\| $P < 0{\cdot}05$.

The enhanced activity of aniline hydroxylase produced by urethan did not appear to be due to the synthesis of new enzyme, since incubation of liver slices in iso-osmotic medium containing actinomycin D (1 μg/ml) for up to 1 hr was without effect on the urethan-induced increase in enzyme activity. Similarly, incubation of slices in medium containing 20 μg of puromycin/ml did not prevent the effect. Stimulation of enzyme activity required the presence of carcinogen. Thus, after preincubation of slices for 30 min in

TABLE 2. EFFECT OF URETHAN ON ANILINE HYDROXYLASE ACTIVITY OF HOMOGENATES AND MICROSOMES*

| | Homogenate† | Microsomes‡ |
|---|---|---|
| No urethan | 4·8 | 0·41 |
| Urethan (10 mM) | 5·2 | 0·42 |

* The values are the means of three separate experiments.

† Liver homogenate equivalent to 250 mg of liver/3 ml was used. Activity expressed as is nmoles *p*-aminophenol/min/g liver.

‡ Three mg of liver microsomes was added to 3 ml of assay medium. Activity is expressed as nmoles *p*-aminophenol/min/mg microsomes.

iso-osmotic medium containing 10 mM urethan at either 37 or at 0 , aniline hydroxylase activity was stimulated only when the slices were assayed in medium containing urethan.

The product of aniline hydroxylation, *p*-aminophenol, is conjugated by UDP-glucuronyl transferase of the liver endoplasmic reticulum. Since urethan only exerted its effects in liver slices, the possibility existed that it was either affecting breakdown of the conjugate by lysosomal $\beta$-glucuronidase, or the conjugation of *p*-aminophenol itself; since urethan is known to inhibit mitochondrial function,[19] the increase in aniline hydroxylation conceivably could have been due to interference with energy production necessary for generation of UDP-glucuronic acid (or other high energy conjugates). This possibility was ruled out by assaying for total *p*-aminophenol after metabolism of aniline (Fig. 2). Liver slice pairs incubated in the presence of urethan showed an increase in both the amount of free *p*-aminophenol and in total *p*-aminophenol production. About 20 per cent of the total *p*-aminophenol formed was found as the glucuronide.

Administration of urethan to rats at a level of 1·0 g/kg (approximately 14 mM) has been shown to cause near maximal elevation of hepatic microsomal aniline hydroxylase activity by 24 hr (assayed *in vitro* in the absence of urethan), without any apparent effect on aminopyrine demethylase activity.[15] In contrast, however, addition *in vitro* of the carcinogen to liver slices did not result in enhanced activity of aniline hydroxylase, unless urethan was present during the enzymatic assay. The mechanism of enhancement of aniline hydroxylase activity is at present unknown, but it appears that new enzyme synthesis is not involved. Furthermore, the increase in aniline hydroxylase activity has been shown not to be related to the

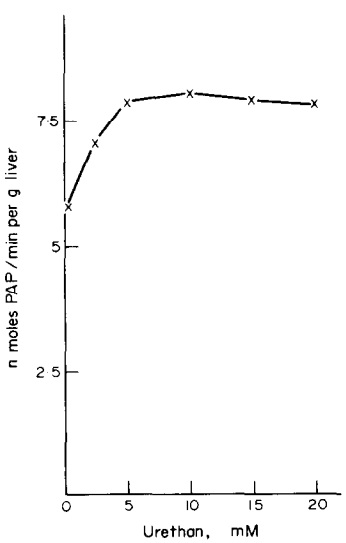


FIG. 1. Concentration dependence of urethan stimulation of aniline hydroxylase activity. Liver slices (about 100 mg wet weight) were incubated as described in the text with the indicated concentrations of urethan. PAP, *p*-aminophenol.

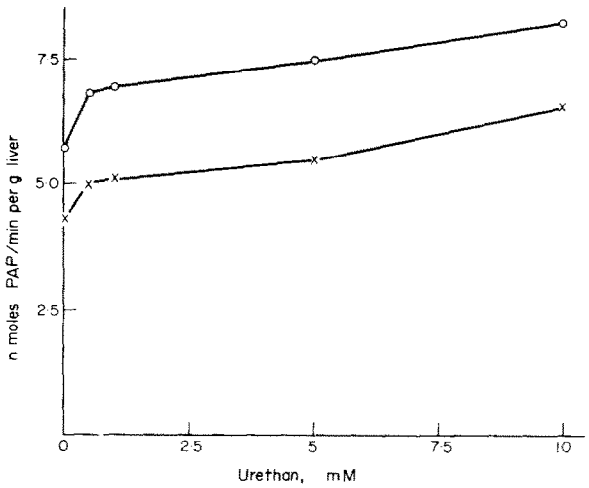


FIG. 2. Conjugation of *p*-aminophenol (PAP) to form a glucuronide, and lack of influence of conjugation on stimulation of aniline hydroxylase activity by urethan. Conditions are as in Fig. 1. Assay for conjugated product is described in the text; ○ = total *p*-aminophenol, × = unconjugated *p*-aminophenol.

extent of glucuronidation of the product *p*-aminophenol. The urethan effect on aniline hydroxylation differs from the stimulatory action of ethyl isocyanide[20] and acetone,[21,22] both of which have been shown to act directly on the microsomes.

A possible mechanism which must be considered is that urethan blocks an alternate pathway in the metabolism of aniline, one utilizing some component of the microsomal mixed function oxidase. Competition for NADPH or aniline by an alternate route would not appear to be the answer, since both substrates were present in large excess. Of interest was the observation that urethan did not significantly increase aniline hydroxylase activity in liver slices from partially hepatectomized rats, a tissue preparing for DNA synthesis and cell replication. This finding was in agreement with our earlier report that urethan administered *in vivo* did not appreciably elevate aniline hydroxylase activity in microsomes of partially hepatectomized rats.[15]

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