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THE INHIBITION OF PROTEOLYTIC ACTIVITY BY ETHYL CARBAMATE

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SUMMARY

A study was made of the effects of ethyl carbamate (urethan) in low concentrations on the clotting of blood plasma and on the activity of chymotrypsin. An inhibitory effect was found on the clotting of dilute sheep plasma with urethan concentrations in the range 20–150 mM. The action of crystalline α chymotrypsin on *N*-acetyl-L-tyrosine ethyl ester was inhibited by concentrations as low as 8 to 60 mM. These are urethan concentrations normally found in the organs of animals which have received the usual anesthetic, antimitotic or carcinogenic doses of urethan (1 mg/g of body weight). Most reported inhibitions of enzyme activity due to urethan have referred to much higher concentrations.

The non-carcinogenic homolog of urethan, *n*-propyl carbamate, was found to inhibit chymotrypsin activity to the same degree as urethan. This suggests that the antiproteolytic activity of ethyl carbamate is not specifically related to its carcinogenic potency.

INTRODUCTION

In the course of studies on the mechanism of ethyl carbamate (urethan) carcinogenesis it became of interest to investigate the enzyme system which catabolizes urethan[1]. A possible mechanism for the breakdown of urethan may involve an esterase. Urethan is an inhibitor of both cholinesterase[2,3] and pseudocholinesterase[2] and has been reported to inhibit crude preparations of some proteolytic enzymes[3]. Recently, SCHUEL[5] found that crystalline preparations of chymotrypsin or trypsin could be used to reverse, in part, the antimitotic action of urethan on the cleavage of eggs obtained from the marine annelid worm *Chaetopterus pergamentaceous*. We investigated *in vitro* the effect of urethan on proteolytic enzyme systems not previously studied which are capable of utilizing esters as substrates, in order to obtain information from these model systems on possible intracellular sites of action of urethan. The possible relevance to carcinogenesis of any sites discovered could be tested by comparing the effects of ethyl carbamate with those of one of its non-carcinogenic homologs, *e.g.* *n*-propyl carbamate .

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MATERIALS AND METHODS

Determination of plasma clotting time

Sheep plasma was prepared by collection of blood from the jugular vein in polypropylene centrifuge tubes containing one tenth volume of 4% sodium citrate. The blood was centrifuged at 2–4° at the highest speed of the Servall SS-1 centrifuge (30 000 × *g*) for 5 min and the plasma poured off into plastic tubes which were stored in the deep-freeze at approx. −30°. Assays were performed by a slight modification of the method of SWOAP AND KUIZINGA[6]. Diluted plasma (either 20 or 25% plasma in 0.154 *M* NaCl) in 0.5-ml portions and 0.5 ml of urethan solution or of 0.154 *M* NaCl were pipetted into 12 × 75 mm acid-washed Pyrex test tubes. The system was activated by the addition of 50 μl of 0.1 M $CaCl_2$. The tubes were capped with Parafilm, mixed by inversion, and incubated in a 37° water bath. The end point used was the formation of a complete gel (which was determined by inverting the tube). Control and experimental determinations were performed simultaneously.

Determination of chymotrypsin activity

Crystalline *α*-chymotrypsin obtained from the Worthington Biochemical Corporation, Freehold, N. J. (U.S.A.) was assayed spectrophotometrically[7] according to a modification of the method of SCHWERT AND TAKENAKA[8]. Readings were taken on a Beckman DU spectrophotometer at 1-min intervals in cells maintained at constant temperature by means of water circulation coils surrounding the cell compartment. Incubations were carried out in a total volume of 3.2 ml of 0.05 M sodium phosphate buffer at pH 7.0 containing $2.3 \cdot 10^{-4}$ M *N*-acetyl-L-tyrosine ethyl ester. The enzyme was used either immediately after being dissolved in 10^{-3} M HCl or after storage in a deep-freeze at approx. −30°. (The fresh enzyme had slightly higher activity than the frozen and thawed samples.) Ethyl carbamate, *n*-propyl carbamate and control determinations were run simultaneously. The *n*-propyl carbamate was maintained in solution at 1 M concentration by being kept at 37° prior to dilution in the incubation mixture.

RESULTS

Inhibition of plasma clotting

Since sharp and convenient end points of firm clots were obtained at final plasma concentrations of 10 and 12.5%, with average clotting times of 3.2 and 4.4 minutes respectively, these dilutions were used in the assay system.

The inhibition of dilute sheep plasma clotting by urethan is shown in Fig. 1. From 24 to 48% inhibition was obtained with final urethan concentrations ranging from 20 to 150 mM.

In order to determine whether the clotting time of mouse blood could be prolonged by previous urethan administration, adult female C57BL/6 mice were given intraperitoneal injections of 1.5 mg urethan/g body weight (as a 10% solution in deionized water). Control mice were injected with equivalent volumes of 0.154 M NaCl solution. Blood was taken 1 h after the injections by bleeding from the orbital sinus. Timing was begun when 0.5 ml was collected in a 12-mm heavy walled conical

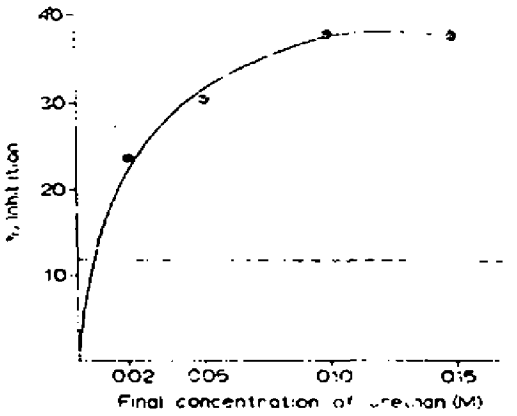

Fig. 1. Inhibition of sheep plasma clotting by urethan. Incubation mixtures contained 10 or 12.5% citrated sheep plasma and either urethan or 0.154 M NaCl. A total volume of 1 ml was activated with 50 μl of 0.1 M $CaCl_2$ and incubated at 37° until the formation of a complete gel (tested by inversion of the tube). The dashed line indicates the extreme of the range of control values. The experimental points represent averages of determinations at the two dilutions of plasma used.

Pyrex centrifuge tube. Tubes were kept at room temperature (approx. 23°) and were tipped every 15 sec. Each test was performed separately. The average clotting time of blood from control mice was 3 min 00 sec and of blood from urethan-treated mice 2 min 50 sec, showing no inhibition by urethan of whole blood coagulation.

Inhibition of chymotrypsin activity

In tests carried out at a final urethan concentration of $6.25 \cdot 10^{-2}$ M and a temperature of 30°, inhibition of chymotrypsin activity was demonstrated. Fig. 2 shows this inhibition at two concentrations of chymotrypsin.

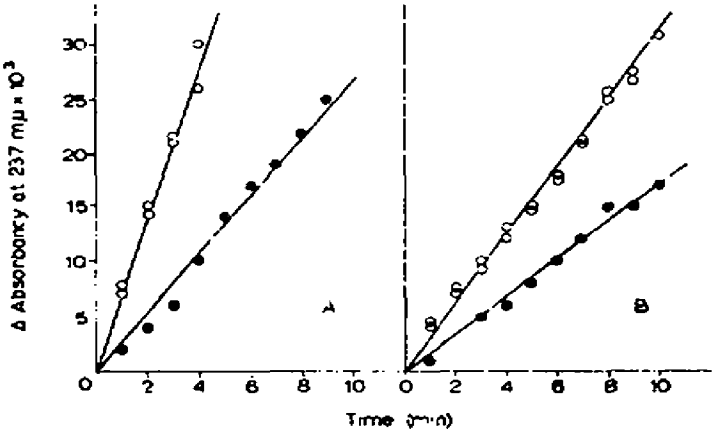

Fig. 2. Inhibition of chymotrypsin activity by urethan. ○—○, controls (overlapping circles indicate duplicate readings); ●—●, incubations with $6.25 \cdot 10^{-2}$ M urethan. A, 2.65 μg enzyme added; B, 1.33 μg enzyme added. Incubation at 30° in a total volume of 3.2 ml containing $2.3 \cdot 10^{-4}$ *N*-acetyl-L-tyrosine ethyl ester and 0.05 M sodium phosphate buffer at pH 7.0.

To approximate more closely to physiological conditions, the incubation temperature was raised to 37° and lower concentrations of urethan were tested. From the initial slopes of the activity curves (drawn as in Fig. 2) the inhibition of chymotrypsin activity was calculated and plotted as a function of the final urethan concentration. From 14 to 38% inhibition was found at concentrations of urethan ranging from 10 to 60 mM (Fig. 3).

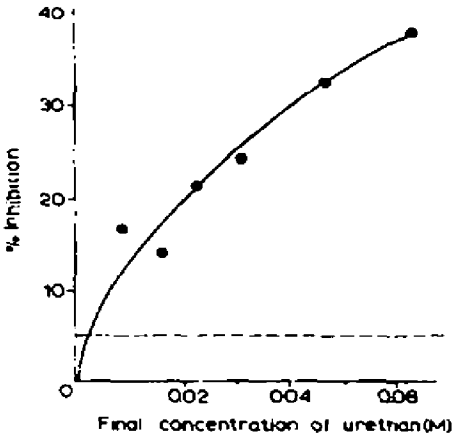

Fig. 3. Inhibition of chymotrypsin activity at 37° as a function of urethan concentration. Incubation mixture as in Fig. 2A. The dashed line indicates the extreme of the range of control values.

In order to determine if this inhibition was as specifically limited to the ethyl ester of carbamic acid as is the carcinogenicity of the carbamate homologous series[9,10], parallel determinations of the inhibitory capacity of ethyl and *n*-propyl carbamates were carried out. In all tests *n*-propyl carbamate and ethyl carbamate showed similar inhibitory activities, as may be seen from Fig. 4.

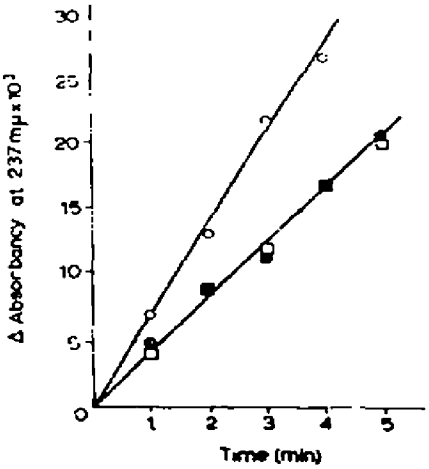

Fig. 4. Inhibition of chymotrypsin activity at 37° by ethyl-and *n*-propylcarbamates. ○—○, control; ●—●, ethyl carbamate; □—□, *n*-propyl carbamate. Incubation mixture as in Fig. 2A.

The possibility that urethan could act as a substrate for chymotrypsin or trypsin, though unlikely from considerations of the specificities of these enzymes[11], was ruled out by direct test. α-Chymotrypsin and crystalline lyophilized trypsin were assayed[1] at a final concentration of 75 μg/ml and at pH 7.4 and 9.3 for their ability to catabolize [*carbonyl*-^{14}C]urethan. Both enzymes proved completely inactive on 0.02 M urethan as substrate.

DISCUSSION

Although the search for inhibition of specific enzyme systems to explain urethan's mechanism of action[12,13] has been pursued for many years, BOYLAND AND WILLIAMS-ASHMAN[13] concluded after a survey of the literature to 1951 that "any significant enzyme inhibition with concentrations of urethan comparable with those attained in the tissues of animals treated with tumor inhibitory doses of this substance has yet to be demonstrated". In almost all the experiments surveyed by BOYLAND AND WILLIAMS-ASHMAN, and those published to date, enzyme inhibition takes place at concentrations of urethan twenty to fifty times those found in blood or other organs after administrations of 1 mg urethan/g of body weight (equivalent to 11 mM, which is an anesthetic, antimitotic and carcinogenic dose). The present experiments demonstrate that both the clotting of dilute blood plasma and chymotrypsin acting *in vitro* are inhibited by concentrations of urethan within the range of carcinogenic doses.

The anticlotting activity of urethan in a dilute plasma system as opposed to lack of any effect *in vivo* may be explained by the great excess of clotting factors in whole blood or plasma[14] (*e.g.* a 200–300-fold excess of prothrombin) which makes quantitative evaluation of antiproteolytic activity extremely difficult. In view of this and the complication that other processes besides proteolysis are involved in plasma clotting, inhibition of chymotrypsin, an enzyme which shows high activity on native protein substrates provided a more amenable model for a possible *in vivo* action of urethan on intracellular proteinases such as the cathepsins.

The similar inhibitory effects of ethyl carbamate and the almost noncarcinogenic[9,10] *n*-propyl carbamate on chymotrypsin activity, would indicate that this inhibition is probably not related to urethan carcinogenesis. However, it is conceivable that inhibition of proteolytic activity plays a role in the blockage of cell division produced by the urethan series, if a proteolytic mobilization stage is necessary for the formation or maintenance of the mitotic spindle or cleavage furrow[15].

In this connection, the finding by SCHUEL that urethan inhibition of the cleavage of *Chaetopterus pergamentaceous* eggs may be partially reversed by application of 0.1–0.001% chymotrypsin or trypsin[6,16], among other agents, is of great interest.

Since urethan can act at the stage of cleavage furrow formation and cause regression of the furrow once formed as well as destruction of the mitotic spindle[6,17], it may interact with a labile intracellular sol-gel equilibrium, as discussed by ANDERSON[18]. Assuming urethan to act by virtue of its antiproteolytic activity in such a system, one may speculate that a continual supply of newly formed structural components, provided by a continual low level proteolytic activity, is necessary for the maintenance as well as the formation of the spindle and/or cleavage furrow.

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