A Study of the Relationship between the Rate of Ethyl Carbamate (Urethan) Catabolism and Urethan Carcinogenesis

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SUMMARY

An enzyme system capable of catabolizing carbonyl-C^{14}-labeled ethylcarbamate to $C^{14}O_2$ was demonstrated in mouse liver, lung, and skin breis. Three weak inhibitors of this catabolic activity were found among structural analogs of urethan tested in equimolar concentration.

Liver brei high-speed supernatant solutions, obtained from 6-month-old Swiss mice, were several times as active as similar preparations from 1- and 2-week-old mice. The concentration of urethan in the blood of 13-day-old and 6-month-old mice, at various times after injection of 0.75 mg urethan/gm, was determined. The catabolic rate in adult mice was approximately 1½ times that in 13-day-old mice.

The relationship between the greater retention of urethan by young than by old mice and the greater response of younger mice to the carcinogenic action of urethan suggests that the length of time urethan remains in the body is a critical factor in determining tumor yield.

When urethan is administered to a mouse in highly carcinogenic doses, over 95 per cent is eliminated in 24 hours (7). *In vivo* studies on the rates of $C^{14}O_2$ recovery from carbonyl and methylene-labeled urethan-C^{14}, ethanol-C^{14}, and $NaHC^{14}O_3$ led Skipper *et al.* (21) to postulate the following *in vivo* reaction:

$$C_2H_5—O—\overset{\overset{O}{\|}}{C}—NH_2 \longrightarrow C_2H_5OH + CO_2 + NH_3$$

The possibility of another pathway of catabolism, leading to an intermediate carcinogenic metabolite, was explored in the studies reported previously in this series (6, 14). The problem of urethan metabolism clearly calls for further studies *in vivo*. Urethan metabolism *in vitro* seems hardly to have been explored.

The present study was undertaken to find an enzyme system capable of catabolizing urethan *in vitro* and to use this system (*a*) to search for an inhibitor of urethan catabolism which could be used *in vivo* to test the necessity of urethan breakdown for carcinogenic activity and (*b*) to compare the rate of urethan breakdown in different organs and under various physiological conditions.

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It is known that the rate of urethan catabolism is slower in tumor-bearing than in normal mice (2, 17). It was particularly of interest to determine whether there were any differences in urethan catabolic rate which could be corrected with carcinogenic activity.

In the present studies, a difference found in the *in vitro* rate of urethan catabolism by liver brei from mice of different ages led to *in vivo* experiments in which similar differences were observed. This finding, in view of the higher yield of tumors produced by urethan in younger mice (19, 20), suggested an inverse relationship between the rate of urethan catabolism and tumor production by urethan in mice.

MATERIALS AND METHODS

Materials.—Ethyl, propyl, and butyl carbamates were obtained from Eastman Organic Chemicals. Carbonyl-labeled ethyl carbamate-C^{14} (1.8 mc/mmole) was obtained from the New England Nuclear Corporation, Boston. N-hydroxyethylcarbamate, carboethoxy-L-aspartic acid, allylcarbamate, and N,N-dimethylethylcarbamate were synthesized by the Schotten-Baumann procedure by Dr. Dov Ben-Ishai of the Department of Organic Chemistry of the Weizmann Institute of Sci-

ence; β-hydroxyethylcarbamate was prepared by him from ethylene glycol and ammonia. The sulfur analogs of urethan were synthesized by Miss Aviva Lapidot, monothiourethan according to the method of Pinner (18), and xanthogenamide according to that of Debus (9).

Mice.—Swiss mice, bred in this laboratory for over 20 generations by brother-sister matings, were employed in this study under conditions previously described (4). For comparison, C3H mice, bred under the same conditions, were used in one experiment.

Assay of urethan catabolic activity in vitro.—Liver or lung breis were prepared in 4 to 12 volumes of 0.02 M phosphate buffer, pH 7.3, in a Potter-Elvehjem Teflon and glass homogenizer immersed in an ice-water bath. Homogenization at approximately 800 r.p.m. required 1 minute for liver and approximately 1½ minutes for lung. (The use of the Waring Blendor resulted in reduced activity.) The breis were centrifuged at 30,000 × *g* for 5 minutes, and the supernatant solution was freed of its fat cap and either used immediately or lyophilized. From 1.6- to 1.8-ml. aliquots of tissue brei (or boiled tissue brei as control) were placed in the incubation chamber of a 2-side-arm Warburg flask. Substrate (0.2–0.4 ml.) was placed in one side-arm, and 0.6 ml. of 1 M lactic acid in the other. On the gassing inlet stopper on the second side-arm a short rubber tube was attached. Vessels were equilibrated with shaking for 10 minutes at 37° C. in the Warburg bath, and the substrate was tipped into the flask. At the conclusion of the run the lactic acid was tipped into the incubation chamber; 1 M carbonate-free NaOH (0.6 ml.) was run through the attached rubber tube into the second side-arm, from a syringe filled with NaOH, with care taken to prevent any loss of $C^{14}O_2$. The flasks were then shaken at room temperature for complete diffusion of the $C^{14}O_2$ into the NaOH solution, which was then washed into a centrifuge tube, excess 1 M $BaCl_2$ was added; and the precipitate was washed once with H_2O and twice with 50 per cent ethanol before being plated for counting in a windowless flow Geiger counter, to a statistical error of 5 per cent or less. Self-absorption corrections were made according to the tables in Calvin *et al.* (8). Protein was determined by means of the biuret reaction (12).

Urethan determination on whole blood.—Mice were given injections intraperitoneally of 0.75 mg urethan/gm as a 5 per cent solution in distilled water containing approximately 2 μc urethan-C^{14}/ml. The "alkali labile" method (5) was applied to 0.5-ml. samples of heparinized blood obtained from the orbital sinus of adult mice. Blood from 13-day-old mice was pooled to obtain two or more 0.5-ml. samples. Confidence intervals (95 per cent) were calculated from a table of "*t*" values (10).

RESULTS

A preliminary survey of urethan catabolic activity in mouse organs was made by incubating mouse blood or breis of liver, lung, or skin with carbonyl-labeled urethan-C^{14}. The production of $C^{14}O_2$ was greatest in liver samples; lung, skin, and blood showed decreasing activity in that order. The supernatant solution from centrifugation of liver brei at 30,000 × *g* for 5 minutes showed catabolic activity which was destroyed by boiling for 1 minute. Similar boiled enzyme preparations were used as controls in all subsequent incubations.

Since the highest activity was found in liver, it was chosen for further study. A comparison of activities of liver brei supernatant solutions at varying substrate concentrations showed that 0.02 M urethan (a concentration which is found in animals after urethan injection) was above the limiting substrate concentration, and that 0.1 M urethan had no inhibitory effect on urethan catabolism. Therefore, for subsequent incubations, 0.02 M urethan was used. In the presence of excess enzyme the reaction proceeded at a maximal rate for 10 minutes, and the addition of Mg^{++} to 4×10^{-3} M, adenosine triphosphate to 5×10^{-3} M, or boiled liver brei to 40 mg wet weight/ml had no effect on the system.

The structural analogs of ethyl carbamate shown in Table 1 were tested in equimolar concentration as possible inhibitors of its catabolism. Butyl carbamate, monothiourethan, and β-hydroxyethylcarbamate showed equally weak inhibitory activity. The relationship between concentration of liver brei supernatant solution and its catabolic activity in releasing $C^{14}O_2$ from carbonyl-labeled urethan-C^{14} was tested and found to be linear up to a concentration of 1.1 mg protein/ml.

The pH optimum for this system was investigated (as shown in Chart 1) with the following precautions. Since nonenzymatic breakdown of urethan occurs in alkali, appropriate boiled enzyme blank values were subtracted at each pH tested. The effects of the buffers (11) used to cover the pH range tested (phosphate, Tris, glycine-NaOH) were compared at a common pH value, and no specific buffer effects were found. The maximum catabolic rate was found in the region of pH 9.

In order that the activities of liver and lung of mice at different ages could be compared under approximately physiological conditions, subsequent incubations were carried out at pH 7.3.

Table 2 shows the results of this comparison. The activity in liver was higher than in lung, but, while the specific activity of lung brei was essentially the same in mice of different ages, the activity in liver was several times higher in 6-month-old than in 1- or 2-week-old mice.

An *in vivo* experiment for determining the urethan content in blood of mice of different ages at varying times after urethan injection, which was suggested by this difference in catabolic rates, is presented in Chart 2. It can be seen that 13-day-old mice retained a comparable amount of urethan almost twice as long as 6-month-old mice. The *in vivo* rates of catabolism calculated from these curves are presented in Table 2 for comparison with the much higher rates obtained *in vitro*. That the source of the "alkali labile" $C^{14}O_2$ determined was volatile, and therefore presumably free urethan, was confirmed by determinations of the amount of nonvolatile C^{14} in blood obtained 5 hours after urethan injection, from both 13-day-old and 6-month-old animals. These values corresponded to less than 0.02 mg. urethan ml.

To compare the rate of urethan catabolism in adult mice of different strains, determinations were made of the urethan content in the blood of adult

TABLE 1

INHIBITION OF URETHAN CATABOLISM*

Compound	Formula	Per cent inhibition
Ethylcarbamate	$H_2N-\overset{\overset{O}{\|}}{C}-O-C_2H_5$	
Propylcarbamate	$H_2N-\overset{\overset{O}{\|}}{C}-O-C_3H_7$	1
Butylcarbamate	$H_2N-\overset{\overset{O}{\|}}{C}-O-C_4H_9$	18
Monothiourethan	$H_2N-\overset{\overset{O}{\|}}{C}-S-C_2H_5$	16
Xanthogenamide	$H_2N-\overset{\overset{S}{\|}}{C}-O-C_2H_5$	4
β-Hydroxyethylcarbamate	$H_2N-\overset{\overset{O}{\|}}{C}-O-C_2H_4-OH$	16
N-Hydroxyethylcarbamate	$HO-N(H)-\overset{\overset{O}{\|}}{C}-O-C_2H_5$	0
Carboethoxy aspartic acid	$HOOC-\underset{\underset{H}{\|}}{C}-CH_2COOH$ with $N(H)-\overset{\overset{O}{\|}}{C}-O-C_2H_5$	0
N,N-Dimethylethylcarbamate	$H_3C-N(H_3C)-\overset{\overset{O}{\|}}{C}-O-C_2H_5$	0
Allylcarbamate	$H_2N-\overset{\overset{O}{\|}}{C}-CH_2-CH_2=CH_2$	0

* All compounds were tested at a concentration of 0.02 M in a system containing 0.02 M ethylcarbamate, with liver brei supernatant solution from 6-month-old mice.

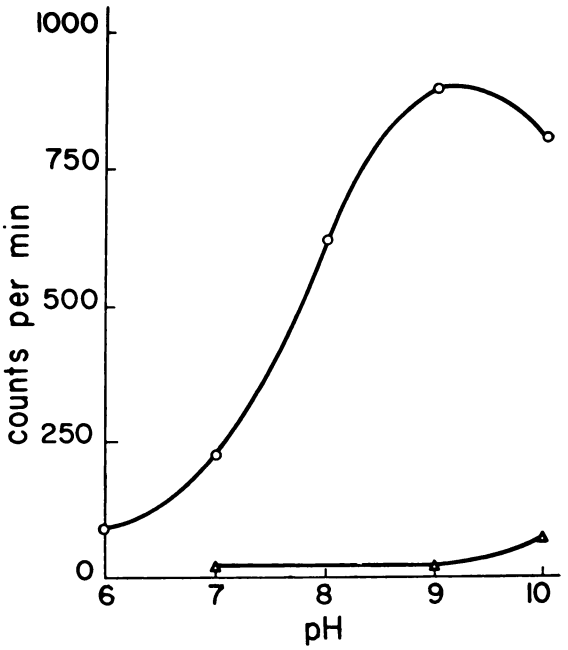

CHART 1.—Relationship between pH of incubation mixture and urethan catabolic activity of mouse liver brei supernatant solution, 4.8 mg. lyophilized solution per flask.
Circles = activity corrected for boiled enzyme blank values.
Triangles = boiled enzyme blank values.

TABLE 2

URETHAN CATABOLIC ACTIVITY IN MICE OF DIFFERENT AGES

Age of mice	Specific activity (mμmoles/hr/mg wet weight)		
	Whole mouse *in vivo*	Liver *in vitro*	Lung *in vitro*
1 week		2.8	0.8
2 weeks	0.69	4.8	3.0
6 months	0.97	36.1	3.4

C3H mice at varying times after urethan injection. The rate of catabolism of urethan (1.66 μmoles/hr/gm) was significantly higher than in Swiss adult mice (0.97 μmoles/hr/gm). For example, the urethan content in the blood, 7½ hours after urethan injection, was 0.32 mg/ml (±0.13 mg/ml 95 per cent confidence interval) in adult Swiss mice, while in C3H mice this value was only 0.08 mg/ml (±0.05 mg/ml).

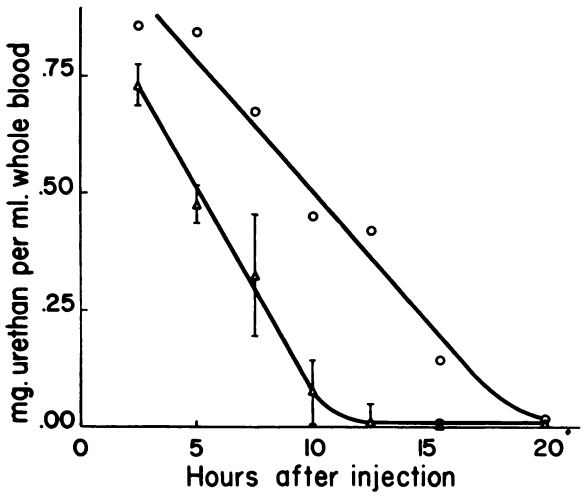

CHART 2.—Urethan catabolism in 13-day-old and in 6-month-old Swiss mice injected with 0.75 mg urethan/gm.
Circles = 2-week-old mice.
Triangles = 6-month-old mice.
Vertical lines indicate 95 per cent confidence intervals.

DISCUSSION

The demonstration of an enzyme system capable of catabolizing urethan *in vitro* provided a method of searching for an inhibitor of urethan catabolism which could be used *in vivo*. Structural analogs of urethan, therefore, were tested as inhibitors of urethan catabolism. The concentration used corresponded to a physiologically tolerated level of urethan. The finding of three weak inhibitors of this concentration raises the possibility of more effective *in vitro* inhibitory action at higher inhibitor:substrate ratios. However, it does not seem to offer an effective means of *in vivo* inhibition.

Rogers (19, 20) reported a greater susceptibility of younger mice to lung tumor production by urethan injection (also see Chart 3) and concluded that "the natural proliferative activity of the alveolar cells during youth plays a major part in the formation of the tumors." In the present study it was shown that older mice catabolized urethan more rapidly (Chart 2), probably owing to the increased catabolic activity in the liver of older mice (Table 2). Therefore, it seems likely that the length of time urethan remains in the body is a critical factor in determining the tumor yield. This would be analogous to the action of carcinogenic hydrocarbons (3).

It is interesting to note that a dose of 77 μg urethan/gm, in 2-week-old mice, produced a significant increase in the number of lung tumors found 20 weeks later (6), though it can be calculated that this dose was completely broken down in 2 hours. It therefore exerted its effects within a very short time interval. It has also been shown by transplantation studies (16) that the carcinogenic exposure of lung tissue in mice given injections of urethan is effective only during the first day following injection—the time when urethan is presumed to be present in the body. These observations raise the interesting possibility that urethan might be acting only as an initiator in the lung (19) (as in the case of the skin), with promotion occurring spontaneously (3).

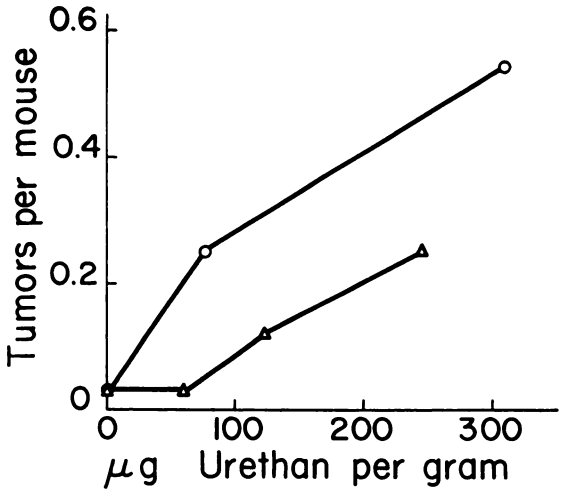

CHART 3.—Lung tumor production by a single injection of urethan into Swiss mice of different ages.
Circles = 13–15-day-old mice.
Triangles = 47–55-day-old mice.
Data from Berenblum, Kaye, and Trainin (6).

A constant rate of catabolism of urethan, as found in these studies, has also been observed in the rabbit (1), in which the rate of catabolism is much slower. In mice, Skipper (21) presents data showing a constant rate of $C^{14}O_2$ production from carbonyl-labeled urethan, but later in the same paper he assumes a rate of catabolism decreasing with time. The constant rate of metabolism *in vivo* is in accord with the observation that the *in vitro* catabolic system is already saturated with substrate at levels normally found in mouse plasma following urethan injection. The constant rate of urethan

catabolism means that a given total dose of urethan requires the same number of hours for total breakdown irrespective of how the dose is divided. Therefore, previous studies attempting to assess the influence of total dose and length of time urethan remains in the body were only able to show that peak concentration of urethan was not the determining factor (13). Within a time period measured in hours or a few days, dividing the dose of urethan had no effect on the tumor yield (19).

That the catabolic rate may be one of the genetically controlled factors important in comparisons between different mouse strains as well as a physiological factor within the same strain is indicated by the more rapid rate of catabolism in adult C3H mice than in Swiss adult mice, C3H mice being less susceptible to lung carcinogenesis than Swiss mice (15). It is also of interest that the *in vivo* rate of urethan metabolism has been found to be slower in tumor-bearing mice than in normal mice (2, 17).

A constant rate of breakdown of urethan may indicate that a system involved in its breakdown is working at constant capacity. If within a cell this system is largely taken up with urethan breakdown, perhaps other cell processes in which this system is normally involved might be damaged, and the length of time of this diversion of, or interference with, normal function might be proportional to the carcinogenic action of urethan.

It would, therefore, be important to characterize the enzyme system which breaks down urethan and to find out what possible derangements of a cell's normal functioning an interference with this system is likely to cause. Perhaps from such studies we may derive some better ideas of where to look within the cell in order to discover the carcinogenic effects of urethan.

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