STUDIES OF THE MECHANISM OF INDUCTION OF PULMONARY ADENOMAS IN MICE

MARGARET R. WHITE
LAWRENCE BERKELEY LABORATORY

1. Introduction

The present paper is related to the frequently discussed question as to whether urethane tumorigenesis is a one stage or a multistage process. In either case, the tumorigenic process is assumed to begin with what may be called an *initial event*, a change in a single normal cell (*mutation*) resulting from a single *hit* by a tumorigenic molecule (one hit theory) or from several such hits (multihit theory). If the initial event is followed by the growth of the tumor studied, then the mechanism is described as a one stage mechanism. However, as explicitly suggested by Brues [5], the growth (of *first order mutants*) following an initial event may well be "benign" in the sense of being destined to disappear, except for the possibility of a second mutation in one of its cells creating *second order mutants*. If this second mutation in a cell of the benign growth turns into a tumor cell, then the process of tumorigenesis is called a two stage mechanism. It is easy to visualize three or four or, generally, multistage mechanisms of tumorigenesis. Naturally, there is the possibility that, with respect to some particular tumors, say pulmonary adenomas in mice, the tumorigenic process is a one stage process while, with respect to some other tumors, say pulmonary carcinomas, it is a multistage mechanism.

Some years ago a private communication from M. B. Shimkin to J. Neyman raised the question as to whether an experiment could be devised to decide whether a particular tumorigenesis, say of pulmonary adenomas in mice, is a one stage or a multistage phenomenon. The experiment contemplated was to consist of injecting mice with specified doses of urethane and counting adenomas. Briefly, the investigation by Neyman and Scott [18] resulted in the finding that, with a two stage mechanism, the fractionation of a given dose of urethane may influence the ultimate number of tumors. On the other hand, with a one stage mechanism, they concluded that the ultimate number of tumors must be independent of the time pattern in which the given fixed dose of the tumorigenic

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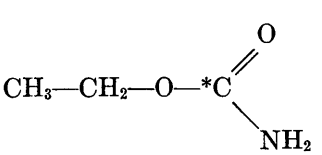
material is administered, since the *given fixed dose* should produce the same number of initial events, irrespective of the time pattern in which this material is administered.

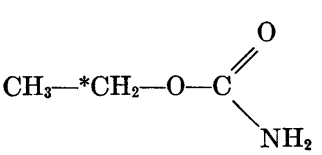
In the cited paper Neyman and Scott took it for granted that the average number of initial events is proportional to the dose of urethane in milligrams per gram of body weight of the mice (mg/g). A number of experiments performed in several laboratories [11], [21], [24], with fractionation of the total doses measured in these units (mg/g), indicated unambiguously that the presumed ultimate number of pulmonary adenomas in mice depends on the time patterns in which the same dose of urethane is administered to mice. This, then, suggested that the mechanism of this particular tumorigenesis cannot consist of just one stage. However, certain circumstances suggested doubts as to whether the average number of initial events generated by varying doses $D_1, D_2, \cdots, D_s$ of urethane is really proportional to these doses. In particular, two points of doubt emerged. One is the question whether the rate at which the urethane is catabolized into some nontumorigenic material is or is not dependent upon the dose D injected. The second point of doubt concerns the identity of the chemical entity that is actually tumorigenic: is this the intact urethane molecule or, possibly, some other molecule originating in the process of catabolism of urethane? The present study is intended to provide some information on these two particular points. The details of the background follow.

The tumor system in which pulmonary adenomas are induced in mice by administration of the carcinogen urethane (ethyl carbamate) is a useful system for quantitative studies of tumorigenesis. Urethane has been shown to produce tumors in animals other than mice and to produce a variety of types of tumors. It appears to be a true carcinogen. Pulmonary adenomas can be induced by other carcinogens and occur spontaneously in some strains of mice. They grow as small, round, white nodules which usually protrude from the surface of the lungs and are easily identifiable with the naked eye. An occasional tumor occurs deeper in the lung tissue, but the lungs of mice are very thin and somewhat translucent, so that if they are counted not too soon after urethane administration, most of the tumors are visible without the need for serial sectioning and tedious microscope work. A dose as small as $\frac{1}{16}$ mg/g induces significant numbers of tumors, and abundant tumors are induced by a dose of 1.0 mg/g.

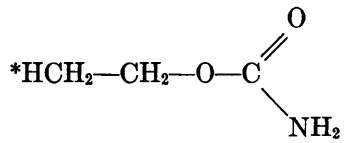
Some aspects of urethane catabolism have been investigated in other laboratories. Some of these studies, including those done in this laboratory, used urethane labeled with radionuclides. Urethane has been synthesized with ^{14}C in either of two positions in the molecule and with ^{3}H in one position as follows:



Ethyl Carbamate (carbonyl-^{14}C)



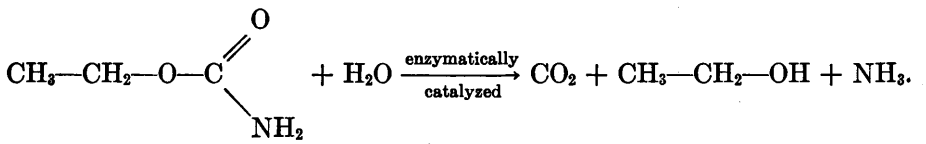
Ethyl (1-^{14}C) Carbamate



Ethyl (2-^{3}H) Carbamate

*C=^{14}C *H=^{3}H

Bryan, Skipper, and White [6] and Skipper, Bennett, Bryan, White, Newton, and Simpson [22] reported that, within 24 hours after the administration of ethyl carbamate (carbonyl-^{14}C), 90 to 95 per cent of the ^{14}C was exhaled as $^{14}CO_2$ in the breath of mice and five to ten per cent was excreted in the urine. They postulated that urethane was hydrolyzed in the body to carbon dioxide, ethyl alcohol, and ammonia, that is,



Boyland and Rhoden [4], using rats and doing chemical analyses of blood and tissues, and Berenblum, Haran-Ghera, R. Winnick, and T. Winnick [1], administering ethyl carbamate (carbonyl-^{14}C) to mice, also concluded that the intact urethane molecule disappeared from the blood and tissues of animals within 24 hours. Both Skipper and co-workers [22] and Berenblum and co-workers [1] found that when ethyl (1-^{14}C) carbamate was administered, the rate of appearance of ^{14}C in the breath was slower than when ethyl carbamate (carbonyl-^{14}C) was administered. There seemed to be no selective concentration of urethane in any of the organs analyzed by these investigators.

Kaye [14], using ethyl carbamate (carbonyl-^{14}C), found that two-week-old Swiss mice catabolized urethane more slowly than those six months old, and that C3H mice catabolized it more rapidly than did Swiss mice. Mirvish, Cividalli, and Berenblum [16], by chemical analysis of livers for urethane content, found that adult Swiss mice catabolized urethane at approximately ten times the rate of newborn Swiss mice. Cividalli and co-workers [7], by blood analysis for ^{14}C from ethyl carbamate (carbonyl-^{14}C), found that there was a rapid increase in the rate of catabolism of urethane in SWR mice as age increased from 1 to 30 days, and that newborn mice of five other strains also eliminated it slowly as compared to adult SWR mice. The rates of urethane elimination in these five strains did not seem well correlated with the differences in susceptibility of the adult animals of these strains to tumor induction.

Skipper and co-workers [22] inferred from their data that the rate of hydrolysis of urethane decreased with time after administration, whereas Kaye [14] stated that urethane was catabolized at a constant rate. After administration of ethyl carbamate (carbonyl-^{14}C), Grogan, Lane, Liebelt, and Smith [10] measured the concentration of ^{14}C in blood and liver of intact mice and of partially hepatec-

tomized mice that were sacrificed at 1, 2, 4, 8, or 24 hours after urethane administration. They reported that the disappearance of urethane from blood and liver was approximately linear for 8 hours and appeared to be more rapid in the next 16 hours.

As stated above, it is not known whether urethane or some metabolite of urethane is the active carcinogen. N-hydroxy urethane was thought to be a possible carcinogenic metabolite of urethane since it is chemically closely related and is carcinogenic. Studies by Mirvish [15] and Biota, Mirvish, and Berenblum [2] suggested that the carcinogenicity of N-hydroxy urethane is due to its conversion to urethane rather than the reverse. Nery [17] proposed that urethane is metabolically activated *in vivo* and that an intermediary metabolite which can be formed from either urethane or N-hydroxy urethane is probably the proximate carcinogen.

None of the cited experiments investigated the possible differences in results from administration of various dosages. The various investigators used different dosages and different strains and ages of animals. In order to investigate the relationship of dose to tumor induction, it is important to know whether internal exposure of the animals to the molecule (and thus risk of producing initial events in the cell) is proportional to the administered dose over a fairly wide dosage range in animals of the same strain, sex, and age. We know of no direct means of determining internal exposure to the intact urethane molecule, but we can assert that the exposure cannot exceed the lesser of the two values of exposure calculated from the body retention times of the ethyl moiety and the carbonyl moiety. If the tumor yield correlates closely with the smaller of these calculated internal exposures, it would seem likely that the intact molecule is responsible for the tumorigenic process. If, on the other hand, the tumor yield is more nearly proportionate to the larger exposure value, it is likely that some metabolite of urethane that contains the corresponding fraction of the molecule is the key to the tumorigenic process. We therefore undertook studies on the rate of catabolism of urethane given in various dosages and labeled with ^{14}C in either the ethyl or carbonyl position.

2. Materials and methods

Female A/Jax mice were obtained from Jackson Laboratory when they were three to four weeks old. The mice were numbered with metal ear tags and randomized into dosage categories by use of tables of random numbers. They were housed ten to a large plastic cage, with wood shavings for bedding, but no two animals of the same dose category were housed together. They were fed Simonsen's white diet to which terramycin was added during milling with the aim of keeping them as disease free as possible. Their water was chlorinated and HCl was added to pH 2.5 in order to discourage *Pseudomonas aeruginosa* infections. The mice were ten to eleven weeks old and their mean weight was 20.3 g

(range, 15 to 26 g) when urethane was administered. They were sacrificed for tumor counts exactly 24 weeks after urethane administration. Tumors were counted as previously described [25]. Untreated control animals were housed with the experimental animals. One hundred and thirty controls and 259 urethane treated animals were sacrificed for tumor counts.

Ethyl carbamate (carbonyl-^{14}C) and ethyl (1-^{14}C) carbamate were obtained from Schwarz Bioresearch. Nonradioactive urethane was obtained from Eastman Organic Chemicals. Injection solutions combining radioactive and nonradioactive urethane to the desired total urethane concentrations were made up in sterile distilled water such that each solution contained 1 μCi/ml. Measurements of the exact radioactivity in each solution were made by diluting aliquots with scintillation solution and counting in a Nuclear-Chicago Mark I scintillation counter. The scintillation solution used for these tests and for counting urine and feces samples consisted of 12.5 g PFO, 0.31 g POPOP, and 125 g naphthalene diluted to 1 liter with *p*-dioxane. The urethane concentration of the injection solutions was adjusted to a set of values ranging from 1.25 to 14.00 per cent. The mice were injected with 0.01 ml of the appropriate solution per gram of body weight. Thus, for example, to administer a dose of 0.125 mg/g to a 20 g mouse, we injected 0.20 ml of 1.25 per cent solution of urethane, which contained 0.2 μCi of ^{14}C.

Each of the 70 experimental groups was comprised of four animals. When a group was treated, the mice were injected as rapidly as possible (usually less than a minute between the first and fourth injections) and quickly put into the metabolism cage. The cages were of plastic with raised wire screen bottoms which allowed most of the urine and feces to fall through to the cage floor. Food and water were available. Air from a tank of compressed air, aged to reduce its natural radioactivity, was passed through a calibrated flowmeter and into the metabolism cages at a rate of approximately 300 cc/min. The air, which now included the expired breath of the animals, flowed from the metabolism cage through a U-tube filled with water absorber (Drierite) and into a 250 cc ionization chamber. The charge collected in the ionization chamber because of ionization caused by radioactive decay of ^{14}C was measured with a vibrating reed electrometer [23]. The potential in millivolts produced by this charge was recorded every 20 seconds on a 12 channel Leeds and Northrup recorder. The air leaving the ionization chamber passed through soda lime to remove the radioactive CO_2, through a second flowmeter to monitor for leaks in the system, and into a wet test meter. Readings were taken from the wet test meter periodically to accurately measure the air flow. The radioactivity in the breath of the animals was followed in this manner for approximately 24 hours in most experiments and for 48 hours in a few experiments. Three sets of the above described equipment were used and each was standardized with gas containing trace amounts of $^{14}CO_2$. The concentration of $^{14}CO_2$ in the gas was determined by the method of Jeffay and Alvarez [12]: measured volumes of gas were passed through fritted

glass dispersion tubes into CO_2 absorber solutions; aliquots of the absorber solution were diluted with scintillation fluid and counted. A calibration curve relating millivolts recorded to the amount of ^{14}C in the ionization chamber was then plotted.

After the mice were removed, at the end of a run, urine and feces were quantitatively washed from the metabolism chamber, diluted to an exact volume, homogenized with a magnetic stirrer, and centrifuged, and an aliquot was added to scintillation fluid for counting. Duplicate samples were taken and each was counted at least twice.

The areas under the curves relating ^{14}C in the breath to time after administration of urethane were measured with a Bendix Data Digitizer in order to obtain the time integral of internal exposure. Mathematical and computer methods for handling these data were worked out by Claude Guillier of Neyman and Scott's group and are described in a companion paper [9]. The end result of these calculations is a value called *milligram-hours per gram weight of mouse* (mg-hrs/g), a measure of the apparent internal exposure of the animal to that part of the molecule in which the ^{14}C atom was located. These calculations assume that at any instant the amount of unrecovered ^{14}C is still in the animal. There was no reason to believe that the experimental procedure allowed loss of any of the ^{14}C eliminated by the animals. However, if some of the difference between injected and recovered ^{14}C was due to experimental error, the internal exposure calculations would lead to values higher than the true values.

3. Results

Urethane acts as an anesthetic at a dosage of about 1 mg/g. The animals used in these experiments showed slight grogginess at dosages of 0.5 and 0.75 mg/g. Dosage of 1.0 mg/g produced unconsciousness for an hour or two; 1.2 mg/g, four to six hours; and 1.4 mg/g, eight or more hours. In animals that received 1.2 mg/g, 3 of 40 did not survive the anesthesia, and in those receiving 1.4 mg/g, 14 of 40 did not survive. During the 24 week holding period, there were a few deaths in other dosage groups from causes apparently unrelated to urethane administration. Though there were five experiments performed at each dosage, some of these are not included in the data because of the deaths.

The rates of catabolism of urethane were obtained using Guillier's calculations [9] of urethane exhaled/g mouse (based on either carbon label) over small time intervals to obtain rates at particular times after the injection of urethane.

Figure 1 shows the rates of catabolism of various doses of urethane as computed from the rates at which the carbonyl carbon is eliminated in the breath of the animals. With a dose of 0.125 mg/g, the rate of catabolism drops off very rapidly after three hours. When 0.25 mg/g is administered, the rate reaches a peak at two to three hours, decreases slightly for the next four hours and then a rapid decrease begins. When the dose is 0.5 to 1.2 mg/g, the rate gradually rises for eight to nine hours and then, after an interval which increases with dose,

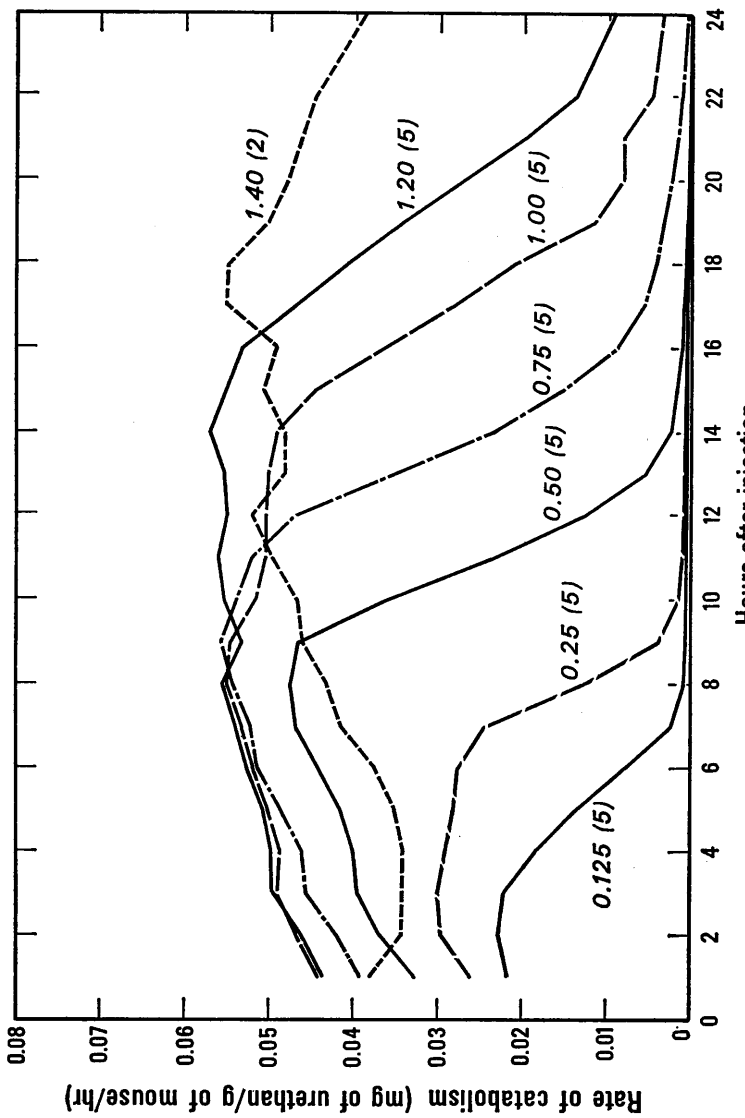


FIGURE 1

Rate of catabolism of urethane as measured with ethyl carbamate (carbonyl-^{14}C). Each curve is labeled with the injected dose in mg/g. Numbers in parentheses are the numbers of experiments used to obtain the curves.

the rate rapidly declines. When 1.4 mg/g is given, there is a slight decline in rate which persists for three hours, and the rate stays lower than that for doses of 0.5 to 1.2 mg/g for at least nine hours. A possible explanation of the variations in rates of elimination at these doses is that the normal enzyme system responsible for converting the carbonyl radical to CO_2 is saturated at the blood level of urethane produced by a dose between 0.25 and 0.5 mg/g, that doses between 0.5 and 1.2 mg/g stimulate additional production of enzyne to a new, higher level which then becomes saturated, and that doses of 1.4 mg/g temporarily partially poison the enzyme producing system. Slowed respiration, circulation, and metabolism, due to deep anesthesia, probably also contribute to the relatively low rate of catabolism at this high dosage. The maximum rate of catabolism as measured with the carbonyl tracer is approximately 0.056 mg/g/hr.

Figure 2 shows the rates of catabolism of various dosages of urethane as measured by the rates at which the ethyl carbon is eliminated. The situation here is somewhat more complicated since ethyl alcohol, into which this part of the urethane molecule supposedly is metabolized [22], is more slowly hydrolyzed and degraded to CO_2 than is the carbonyl radical. Thus, this part of the molecule probably circulates longer in the blood, and so more of it is likely to enter the normal metabolic pathways than is the CO_2 from the carbonyl carbon. The maximum rate of catabolism as measured with the ethyl carbon is approximately 0.045 mg/g/hr.

In some cases, the data used to obtain Figures 1 and 2 differ from those used in the other figures and tables. If the animals lived more than 48 hours after the end of the metabolism experiment, they were used in these figures. If they died before the time for sacrifice, they were not used in the other figures and the tables.

In the experiments with lower doses (0.125 and 0.25 mg/g), in which it was possible to roughly estimate the long lived component at the end of the curve (not shown), it was found that the amount of ^{14}C from the ethyl labeled urethane which entered this component was about twice the corresponding portion of the carbonyl labeled urethane.

Table I tabulates the results of the measurements for integrated internal exposure at 24 hours, along with the numbers of tumors induced. The relative errors of the means are reasonably small for the integrated internal exposure; for induced tumors, they are larger. The mean number of tumors in control animals was 0.41 (S.E. = 0.08).

In Figure 3, two estimates of integrated internal exposure, based on the different carbon labels, are plotted against the injected doses. The relationships are obviously curvilinear; that is, as injected dose increases, the integrated internal exposure of the animal to urethane (or its breakdown products) increases more than proportionately.

In Figure 4a, tumors in animals injected with carbonyl labeled urethane are plotted against injected dose; in Figure 4b, they are plotted against internal

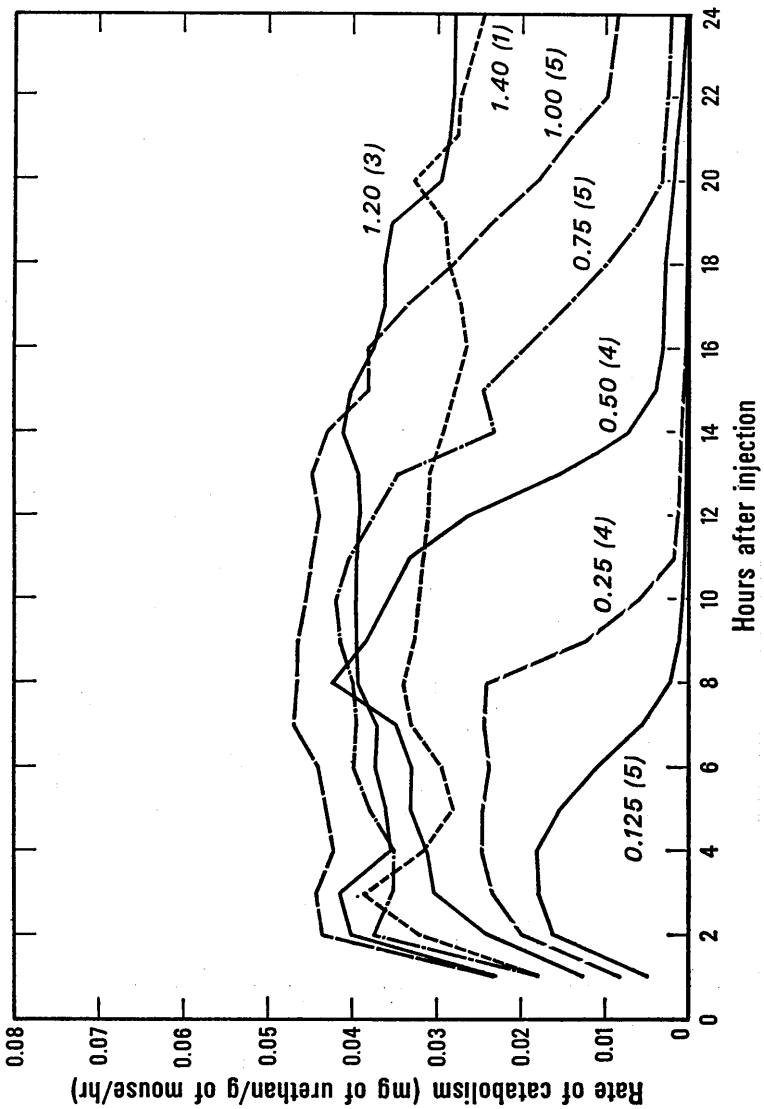


FIGURE 2

Rate of catabolism of urethane as measured with ethyl (1-^{14}C) carbamate. Each curve is labeled with the injected dose in mg/g. Numbers in parentheses are the numbers of experiments used to obtain the curves.

exposure. (The marked dip in the curve at a dose of 0.5 mg/g is probably due to random biological variability, since we have not seen this effect in other experiments and it did not occur in the animals used in the experiments in which the ^{14}C was in the ethyl group.) Figures 5a and 5b show the corresponding relationships when the label was in the ethyl carbon. With either carbon label, the tumor induction *versus* internal exposure comes nearer to being a linear relationship (up to a dose of 1.0 mg/g) than does tumor induction *versus* injected dose. On the basis of these data, it appears that internal exposure estimated from measurements with the label in the ethyl group has a better correlation with tumor induction than does that estimated by use of a label in the carbonyl group.

TABLE I

INTERNAL EXPOSURE AND INDUCED TUMORS

n = number of experiments, with four mice per experiment, that were continued to completion. One hundred and thirty control animals had mean tumors/animal of 0.41 ± 0.08. No measurements for integrated internal exposure could be obtained for the injected dose of 1.40 mg/g in the ethyl (1-^{14}C) carbamate case due to the death of at least one animal during each experiment. Tumors were counted on the nine survivors and the mean and standard error were 31.1 and 3.27 respectively.

Injected dose	Ethyl carbamate (carbonyl-^{14}C)					Ethyl (1-^{14}C) carbamate				
		Internal exposure (mg-hrs/g) (24 hrs)		Tumors per mouse			Internal exposure (mg-hrs/g) (24 hrs)		Tumors per mouse	
	n	Mean	S.E.	Mean	S.E.	n	Mean	S.E.	Mean	S.E.
0.125	4	0.60	0.01	2.45	0.40	5	1.07	0.02	2.45	0.35
0.25	4	1.64	0.09	6.44	0.44	4	2.32	0.06	6.12	0.85
0.50	4	4.14	0.13	10.13	1.34	4	5.24	0.16	14.31	2.08
0.75	4	6.97	0.18	24.87	0.76	5	9.09	0.14	23.10	3.10
1.00	5	11.03	0.47	34.15	1.38	4	12.92	0.29	32.56	2.63
1.20	5	14.78	0.47	38.00	2.11	2	18.64	0.32	39.00	1.22
1.40	2	21.66	1.36	36.75	1.00	—	—	—	—	—

In these experiments, as well as in another in which doses greater than 1.0 mg/g were administered [26], there appears to be a real change in the relationship of dosage to tumor induction at about 0.75 to 1.0 mg/g. Fewer tumors than would be expected occur with doses above 1.0 mg/g. This effect is in the opposite direction to that which would be predicted by the internal exposure curves.

Table II shows the amounts of the ^{14}C administered in the labeled compounds which were recovered within the first 24 hours after the administration of urethane. The percentage measured in the breath was higher for the carbonyl label than for the ethyl label. In the urine and feces, this situation was reversed.

For most dosages, one experiment was performed in which the run was continued for 48 hours rather than being stopped at approximately 24 hours.

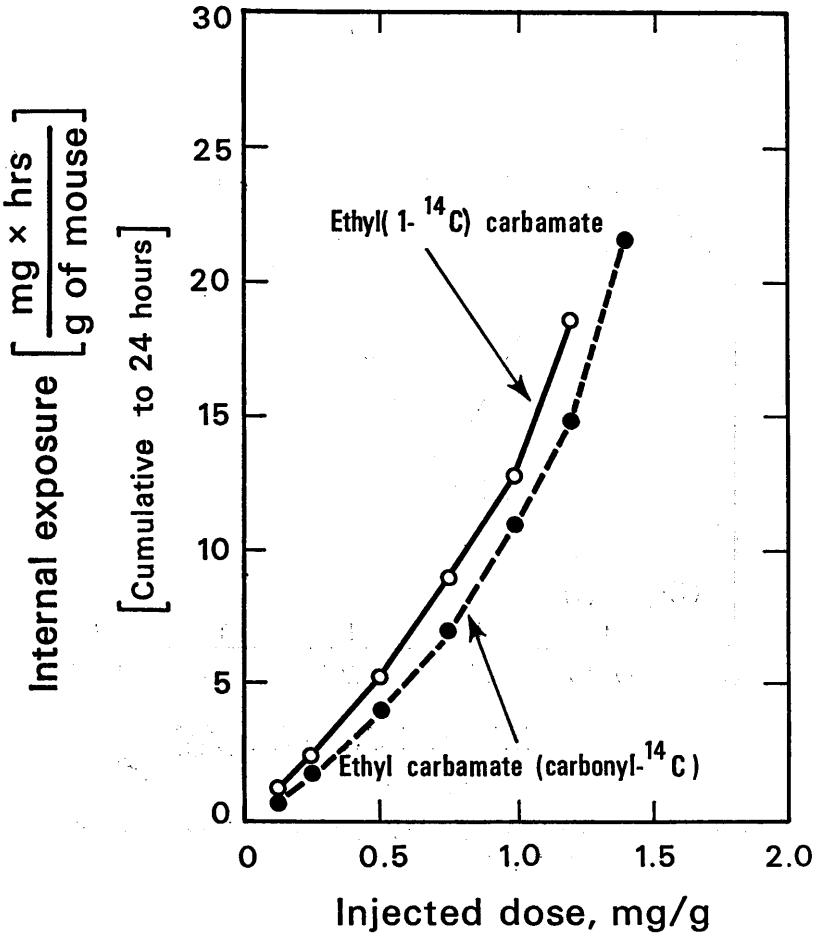


FIGURE 3

Internal exposure *versus* injected dose as measured with ^{14}C label in two positions in the urethane molecule.

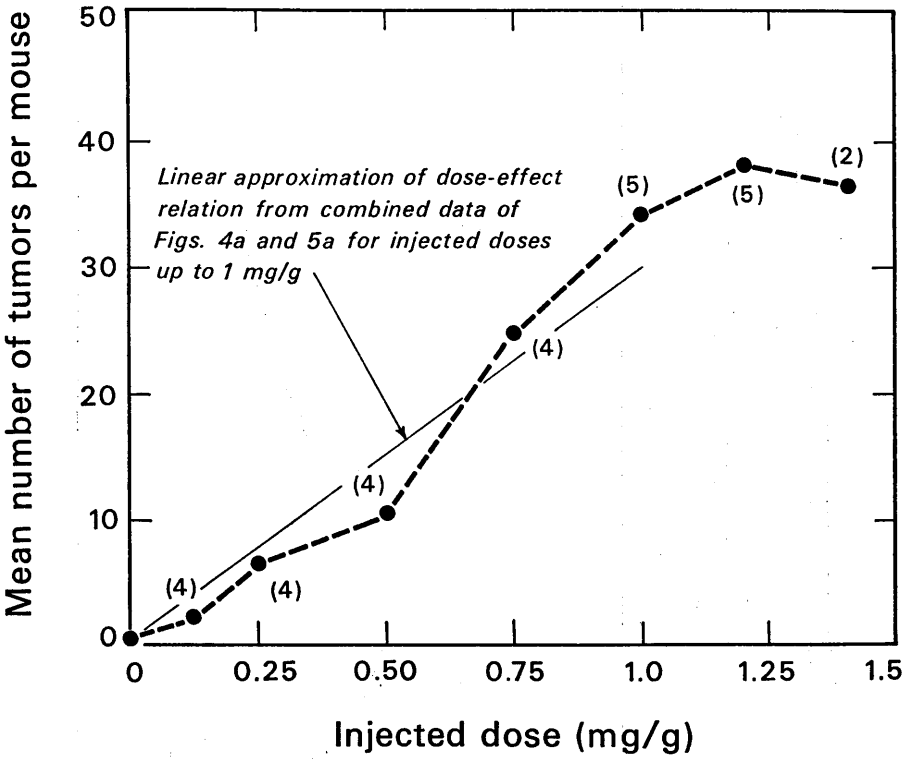


FIGURE 4a

Tumor response related to injected dose in mice treated with ethyl carbamate (carbonyl-^{14}C).

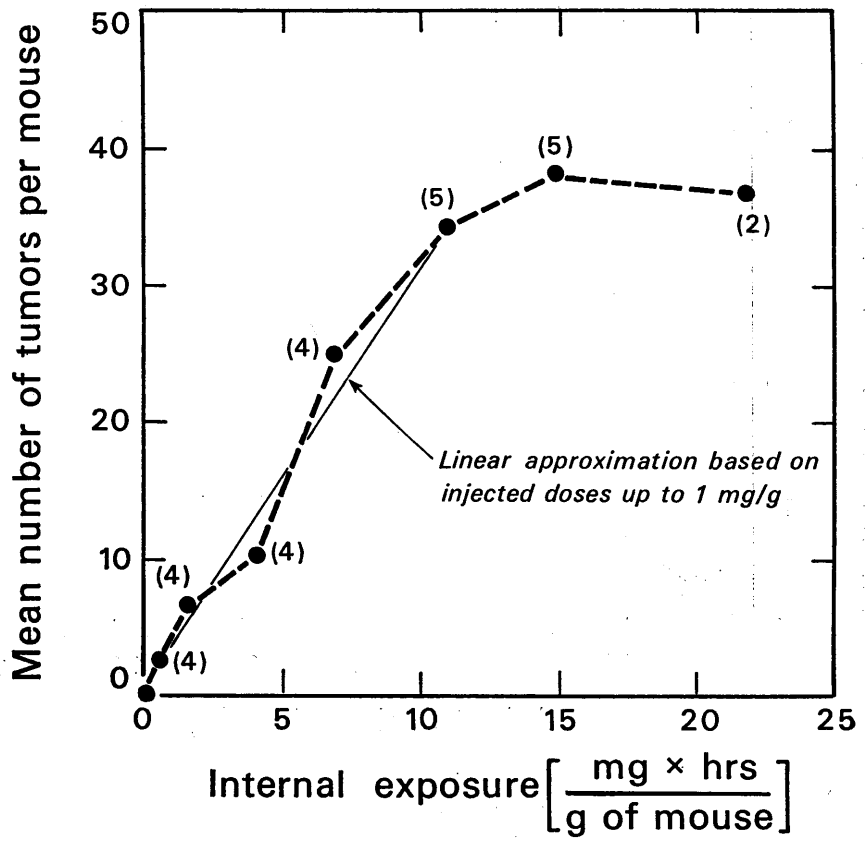


FIGURE 4b

Tumor response related to internal exposure as measured with ethyl carbamate (carbonyl-^{14}C).

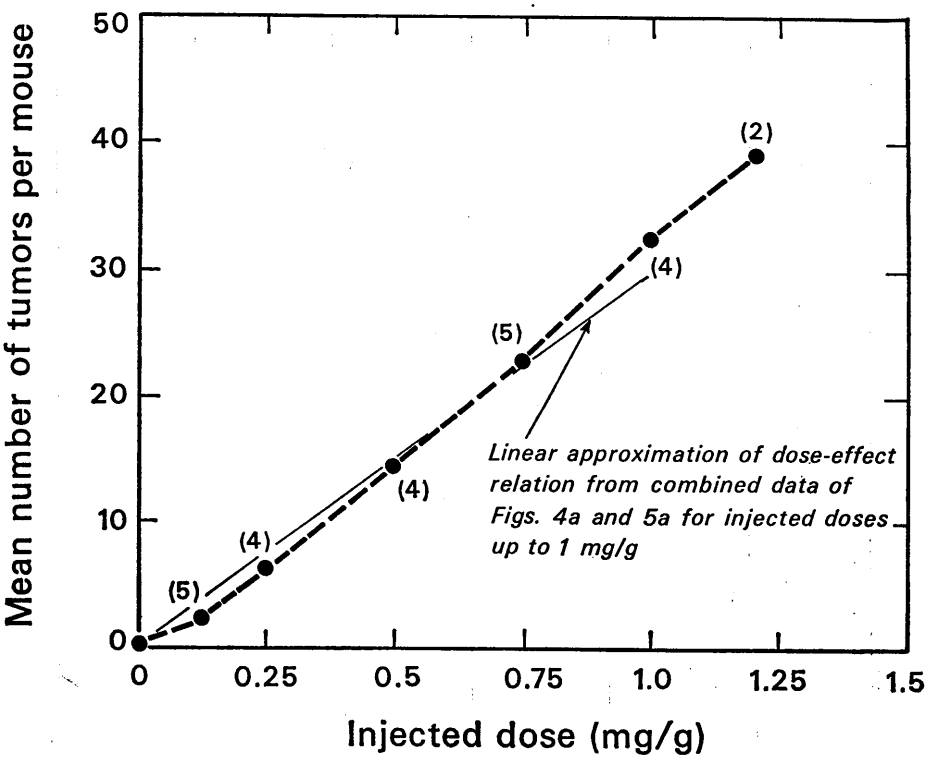


FIGURE 5a

Tumor response related to injected dose in mice treated with ethyl (1-^{14}C) carbamate.

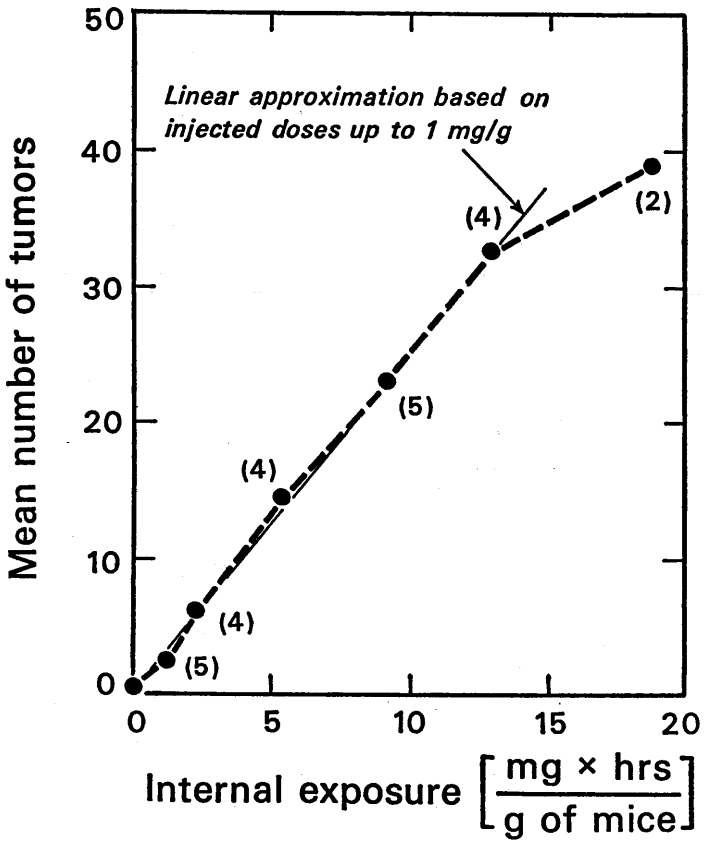


FIGURE 5b

Tumor response related to internal exposure as measured with ethyl (1-^{14}C) carbamate.

TABLE II

RECOVERY OF INJECTED ^{14}C AT 24 HOURS

n = number of experiments, with four mice per experiment, that continued to completion. At least one animal died in each of the experiments with administered dose 1.40 mg/g in the ethyl (1-^{14}C) carbamate case. Therefore no data are available for this point.

Administered dose (mg/g)	Per cent of ^{14}C recovered at 24 hours: Ethyl carbamate (carbonyl-^{14}C)					Ethyl (1-^{14}C) carbamate				
		Breath		Urine and feces			Breath		Urine and feces	
	n	Mean	S.E.	Mean	S.E.	n	Mean	S.E.	Mean	S.E.
0.125	4	90.6	1.0	1.75	0.07	5	76.6	0.5	3.23	0.19
0.25	4	87.3	1.1	1.67	0.24	4	77.9	1.0	2.87	0.24
0.50	4	87.2	0.7	2.05	0.25	4	80.6	1.3	3.63	0.16
0.75	4	89.8	1.2	2.09	0.35	5	76.5	0.8	3.70	0.12
1.00	5	86.1	1.7	2.01	0.12	4	78.5	1.6	3.66	0.18
1.20	5	85.5	0.5	2.55	0.38	2	66.6	2.2	3.62	0.22
1.40	2	73.1	6.4	3.06	0.39	—	—	—	—	—

Table III lists the internal exposures and the percentage recovery of ^{14}C for the labeled compounds calculated for both 24 and 48 hours. Comparison of these values indicates that the integrated internal exposure to the carbonyl carbon in the second 24 hours is 19 to 32 per cent of that in the first 24 hours; and for the ethyl carbon, the corresponding range is 26 to 54 per cent. Since these measurements are for only one experiment, they are subject to considerable error. These data do, however, indicate that there is considerable exposure to urethane or its metabolic products after the first 24 hour period.

No analysis of tissues for ^{14}C content was performed. It was therefore not possible to determine how much of the difference between ^{14}C in the administered dose and that recovered was due to experimental error and how much to retention by the animals. The 48-hour experiments indicate that there certainly are long lived components into which these carbon atoms enter. Further experiments will be necessary to assess more accurately the magnitude of the long lived components.

4. Discussion

The rate at which urethane is catabolized is neither exponential nor constant as has been proposed by other investigators [14], [22]. The system is more complicated than can be explained by either of these simple descriptions and is dependent on the size of the dose. Administered doses of 0.125 and 0.25 mg/g appear not to saturate the system responsible for breaking off the carbonyl group from the molecule. Doses of 0.50 mg/g and above do appear to saturate this system initially and, since the rate of catabolism continues to rise for some hours, probably stimulate production of the enzyme [13] responsible for this process.

Since the ethyl carbon must go through at least two steps before it appears

TABLE III

COMPARISON OF VALUES OBTAINED AT 24 HOURS WITH THOSE OBTAINED AT 48 HOURS AFTER URETHANE ADMINISTRATION

These data pertain to one experiment at each dosage.

The figures for the per cent recovery of injected ^{14}C are for ^{14}C in breath plus that in urine and feces.

No experiment was continued for 48 hours in the ethyl carbamate (carbonyl-^{14}C) case for injected dose 0.25 mg/g.

One animal died in the experiment with injected dose 1.40 mg/g in the ethyl (1-^{14}C) carbamate case, so no data are available.

Injected dose	Internal exposure (mg-hrs/g)						Per cent recovery of injected ^{14}C					
	Ethyl carbamate (carbonyl-^{14}C)			Ethyl (1-^{14}C) carbamate			Ethyl carbamate (carbonyl-^{14}C)			Ethyl (1-^{14}C) carbamate		
	24 hr	48 hr	Ratio of 48 to 24	24 hr	48 hr	Ratio of 48 to 24	24 hr	48 hr	Difference	24 hr	48 hr	Difference
0.125	0.61	0.79	1.31	1.11	1.71	1.54	92.8	94.7	1.9	77.9	81.2	3.3
0.25	—	—	—	2.44	3.64	1.49	—	—	—	78.4	81.8	3.4
0.50	4.31	5.67	1.32	5.30	7.12	1.34	88.0	89.3	1.3	82.8	86.5	3.7
0.75	7.42	8.80	1.19	9.42	13.05	1.39	91.0	94.1	3.1	77.6	81.7	4.1
1.00	12.46	14.88	1.19	13.00	16.33	1.26	87.9	91.3	3.4	83.0	87.9	4.9
1.20	15.21	19.08	1.25	18.99	26.07	1.37	86.0	86.8	0.8	67.2	78.2	11.0
1.40	23.02	27.74	1.21	—	—	—	68.7	89.4	20.7	—	—	—

in the breath as CO_2, the curves for its catabolism are more complicated than those for the carbonyl carbon. The initial rise in the rate is more prolonged, and the "plateau" is flatter and somewhat more prolonged, indicating a slower and hence probably more complicated catabolism process. No attempt has been made in these studies to analyze the curves into their various components except for some rough measurements on the tail (not shown) of the curves of animals given low dosages. Further studies on individual animals are planned. Such curves should be easier to analyze than those obtained from each run in this investigation, since each is a composite of the breath of four animals. The more prolonged stay in the animals of the ethyl moiety of urethane as compared with the carbonyl moiety is in agreement with findings of Skipper and co-workers [22] and Berenblum and co-workers [1].

Internal exposure of the animals to the urethane molecule, based on the length of stay of either the carbonyl or ethyl carbon, is not linearly related to administered dose. At doses of 1.0 mg/g and below, the internal exposure values appear to have a more linear relationship to tumor induction than does administered dose. The fact that small doses give disproportionately less internal exposure than large ones may be the explanation for the phenomenon, found in earlier experiments, of the induction of fewer tumors with fractionated doses as compared with the corresponding single dose; that is, the risk of inducing an initial event in the cell is smaller if a total dose is divided into fractions.

The internal exposure based on the persistence of the ethyl carbon in the body tentatively appears to be a better fit to tumor incidence than does that based on the carbonyl carbon. If this holds true in more extensive experiments, it may mean that the ethyl part of the molecule is more intimately involved in the process responsible for tumorigenic action. This would, however, not necessarily rule out the possibility that the intact molecule is necessary for the primary reaction with tissue components. Once reacted, the carbonyl carbon might be hydrolyzed from a larger molecule, leaving the ethyl part of the molecule attached.

Some experiments performed in other laboratories on the binding of the ^{14}C from labeled urethane to cellular constituents are of interest with regard to our findings on integrated internal exposure. Boyland and Williams [3] found ^{14}C in RNA and DNA of liver and lungs after giving either ethyl (1-^{14}C) carbamate or ethyl carbamate (carbonyl-^{14}C). The liver fractions were labeled equally well regardless of which carbon of urethane was labeled; however, ethyl (1-^{14}C) carbamate was more efficient in labeling lung nucleic acids than was ethyl carbamate (carbonyl-^{14}C). They state that this was probably mainly due to metabolic incorporation of ^{14}C released in the catabolism of urethane, but that the results were noteworthy in view of the fact that urethane is more carcinogenic for lungs than liver. Grogan and co-workers [10], working with partially hepatectomized mice, found no significant labeling of either DNA or RNA when they gave ethyl carbamate (carbonyl-^{14}C). Prodi, Rocchi, and Grilli [19] gave rats tracer doses of ethyl (1-^{14}C) carbamate, ethyl carbamate (carbonyl-^{14}C), or

ethyl (2-^{3}H) carbamate. They found ^{14}C from ethyl (1-^{14}C) carbamate in DNA, RNA, cytoplasmic proteins, and nuclear proteins of liver, spleen, lung, kidney, and skin. Essentially the same components were labeled when ethyl (2-^{3}H) carbamate was used, except that skin and kidney were not analyzed in this case. Negligible activity was found in the organs of animals to which ethyl carbamate (carbonyl-^{14}C) had been administered. They concluded from their experiments that there is true binding of the ethyl moiety of the urethane molecule to RNA and DNA rather than metabolic utilization of the ethyl alcohol resulting from hydrolysis of urethane. Thus, the preponderance of evidence is that the ethyl moiety is more permanently fixed in the tissues.

Acute deaths in the groups receiving 1.2 and 1.4 mg/g indicate that these doses are definitely in the toxic range. There may be competing risks here in that the less vigorous animals, which did not survive these high doses, may also have been the least resistant of the group to tumor induction. Another possibility is that, in the animals that did survive these doses, there may be considerable cell death as compared to lower doses, and this factor may be involved in the lower than expected tumor incidence. There may also be some cell death, though at a diminished level, in the animals receiving smaller doses. This may explain the increased cell proliferation, as measured by the incorporation of thymidine, without increased cellularity seen in several laboratories [8], [20], [26], that is, proliferation to replace dead or dying cells.

In summary, with regard to the two questions posed in the introduction, it appears that the rate at which urethane is catabolized is dependent upon dose size, and that the number of initial events in the cell (first order mutants) might not be strictly proportional to administered dose. The question of the identity of the proximal carcinogen is still unanswered, but the data presented here seem to indicate that the ethyl moiety may be more intimately involved with the tumorigenic activity than is the carbonyl moiety.

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