The Fixation of Urethan Carbon Atoms in Sperm and in Resting and Rapidly Dividing Cells*

IVOR CORNMAN, HOWARD E. SKIPPER, AND JACK H. MITCHELL, JR.†

(From the Department of Anatomy, George Washington University, and the Warwick Memorial for Cancer and Allied Diseases, Washington, D.C., and the Southern Research Institute, Birmingham, Ala.)

As a result of various studies on the mechanism of action of urethan (ethyl carbamate), considerable data are available which might be used to formulate theories worthy of investigation. It is difficult, however, on reviewing the literature, to determine which of the numerous cytologic, histopathologic, and biochemical responses observed with this compound are fundamental to the anticancer activity of urethan and which are only secondary manifestations related or unrelated to its chemotherapeutic activity.

Urethan appears to be a highly specific molecule with regard to anti-leukemic action. Changes in the ester group, mono- or disubstitution at the nitrogen atom, and substitution of sulfur for the carbonyl or ethoxy oxygen, all destroy or lessen the anti-leukemic action of urethan in mice with chloroleukemia 1394 (10). There is apparently no correlation between the anti-leukemic and the leukopenic action of carbamates (10, 11), but there is a correlation between the anti-leukemic and carcinogenic activity (7–10), and there is also a correlation between the leukopenic action observed in mice (11) and the inhibition of sea-urchin egg cleavage caused by substituted carbamates (2). These facts would seem to suggest that two basic mechanisms may be involved in therapy with ethyl carbamate, one having to do with cell division and the other with differentiation processes.

Over-all tracer studies in mice have shown that the carbonyl and methylene-carbon atoms of urethan are fixed in all tissues, but to no greater extent than can be accounted for by fixation of corresponding atoms from CO_2 and ethyl alcohol, the probable *in vivo* hydrolysis products of this compound.[1]

The present experiments were designed with the thought that studies on the fate of urethan in a controlled population of rapidly dividing cells and in cells with a large proportion of desoxyribonucleoprotein (sperm) might yield clues to the role of urethan in normal and neoplastic proliferation.

Briefly, we have investigated the accumulation of urethan in resting and dividing sea-urchin eggs and also the fixation of the carbonyl and methylene carbon atoms of urethan in eggs and in sperm. Control experiments have been carried out with the *in vivo* hydrolysis products of urethan: CO_2 (as $NaHC^{14}O_3$) and methylene-labeled ethyl alcohol.

EXPERIMENTAL

Fertilized and unfertilized eggs from the sea urchin, *Tripneustes esculentus*, supplied the dividing and resting cells. These eggs normally divide at about 70 minutes after fertilization. For studies on cells with a high proportion of desoxyribonucleoprotein, sperm from a large sea cucumber were employed. The temperature of the sea water in which the eggs and sperm were suspended ranged from 25° to 27° C., with no more than 0.4° variation during the course of a single experiment.

ACCUMULATION OF URETHAN BY EGGS

Experiment No. 1.—Eggs were exposed to 20 mg. of urethan per milliliter of egg suspension (in sea water), beginning 10 minutes after fertilization. Samples were then centrifuged, quickly washed in sea water, and recentrifuged. They were then fixed in 5 ml. of saturated $HgCl_2$. The exposure durations are given in minutes and seconds in Table 1. The eggs were analyzed for their urethan content, by the procedure of Boyland and Rhoden (1). From this concentration (column 4, Table 1) and the dilution with $HgCl_2$, the concentration in the eggs at the time of fixation

* This work was supported by the American Cancer Society, upon recommendation of the Committee on Growth of the National Research Council, by the Research Fund of the Southern Research Institute, and by Mr. Ben May of Mobile, Alabama. The authors wish to express appreciation to the American Museum of Natural History for providing facilities for this work at the Lerner Marine Laboratory, Bimini, B.W.I.

† Stabilite Division, Food and Container Institute, Chicago, Illinois.

Received for publication November 6, 1950.

[1] H. E. Skipper, L. L. Bennett, Jr., C. E. Bryan, L. White, Jr., M. Newton, and L. Simpson, unpublished data.

(column 5) was calculated. Eggs left in the urethan solution at the time of the experiment did not divide. Samples removed to sea water simultaneously with fixation of the other samples did divide, and those removed and placed in urethan-free sea water even after 1 hour of exposure formed gastrulae.

Experiment No. 2.—Fertilized and unfertilized eggs were exposed to 2 mg. of urethan per milliliter of egg suspension. Exposure of fertilized eggs began 10 minutes after fertilization. The eggs were centrifuged, the sea water decanted, and the eggs frozen and kept in this condition until analyzed for urethan content (Table 2). Fertilized eggs left in the urethan-containing sea water developed more slowly than the controls but eventually formed blastulae.

TABLE 1

ACCUMULATION OF URETHAN BY FERTILIZED SEA-URCHIN EGGS*
(Initial Concentration 20 mg/ml)

Sample no.	Exposure† period	Weight of eggs (mg.)	Analyzed urethan (mg/gm eggs)	Calculated conc. in eggs at fixation‡ (mg/gm)
1	2′44″	852	3.5	25.1
2	5′22″	988	3.5	21.8
3	7′59″	700	3.3	28.9
4	10′44″	842	3.4	24.6
5	19′28″	844	4.9	35.4
6	49′15″	914	2.1	14.7
7	80′15″	727	2.9	24.0

* Urethan added 10 minutes after fertilization.

† Duration of exposure in minutes and seconds between addition of urethan and washing.

‡ The calculated concentration represents: (conc. of urethan determined by analysis) × (total wt. of eggs + fixative) ÷ (wt. of eggs).

TABLE 2

ACCUMULATION OF URETHAN BY FERTILIZED AND UNFERTILIZED SEA-URCHIN EGGS*
(Initial Concentration 2 mg/ml)

Fertilized		Unfertilized	
Exposure	mg/gm eggs	Exposure	mg/gm eggs
1′18″	1.90	1′10″	1.18
2′28″	4.60	2′23″	1.50
4′26″	2.07	4′1″	1.60
21′22″	2.21	21′12″	1.36
53′40″	1.12	56′11″	1.94

* Urethan added 10 minutes after fertilization. Exposures measured from that instant to time of freezing in minutes and seconds.

Tracer Experiments

Urethan was labeled in the carbonyl position ($H_2NC^{14}OOC_2H_5$) or the methylene position ($H_2NCOOC^{14}H_2CH_3$). Labeled bicarbonate and methylene-labeled ethyl alcohol were used as controls. The activities of each, as well as concentrations of the solutions employed, are presented in Table 3. The labeled compounds used in this study were synthesized by procedures which will be reported elsewhere.[1] All samples were assayed for carbon 14 by a gas-phase procedure already described (12), and tabulated as specific activity—i.e., μc. of activity per mole of carbon in the eggs or sperm.

Fertilized and unfertilized eggs were exposed to the four labeled compounds, then were concentrated and quick-frozen in a dry-ice acetone bath and were kept frozen until they could be assayed for fixed activity (Tables 4 and 5).

The sperm were obtained by mechanically shredding the testes of the sea cucumber and filtering the sperm through gauze. Enough sea water was added to activate half the sperm (all sperm showed activity when dispersed in a large volume of sea water). To each 50 or 100 ml. of the sperm suspension, 0.2 ml. of one of the labeled compounds was added. The samples were quick-frozen at 30 minutes and kept frozen until analyzed (Table 6).

TABLE 3

CONCENTRATIONS AND ACTIVITIES OF COMPOUNDS USED

Compound	Structure	Activity (μc/ml)	Concentration (mg/ml)
Carbonyl-labeled urethan	$O=C^{14}(OC_2H_5)(NH_2)$	6	175
Labeled sodium bicarbonate	$NaHC^{14}O_3$	10	86
Methylene-labeled urethan	$O=C(OC^{14}H_2CH_3)(NH_2)$	7	175
Methylene-labeled ethyl alcohol	$HOC^{14}H_2CH_3$	7	90

The values reported as *Free+fixed* urethan refer to specific activities of the frozen samples as such. The fixed activities were determined on aliquots subjected to repeated suspension in water (weak acid in the case of $NaHC^{14}O_3$) and vacuum drying (24 hours at 1 mm.) to remove the free compounds. Three such treatments were found to reduce the activity of exposed urchin eggs to a constant value, after which no additional activity was removed by this procedure. We have repeatedly shown that free urethan can be removed from biological specimens by vacuum distillation. In one experiment, an aliquot of each sperm sample was dialyzed against cold running water for 24 hours prior to activity assays (Table 6).

RESULTS

Within 2 minutes, fertilized eggs accumulated a higher concentration of urethan than was present in the surrounding medium. This occurred at a concentration which prevented division (20 mg/ml: Column 5, Table 1), or at a lower dose which merely retarded development (2 mg/ml: Column 2, Table 2).

Only a small proportion of the urethan which entered fertilized or unfertilized eggs (Table 4, *Free+fixed*) was not removed by vacuum distillation. The amount of urethan carbon which was fixed by the cells was no greater than the carbon fixed from alcohol or bicarbonate (*Fixed corrected*, Table 4). Fixation was about equal for carbonyl and methylene urethan carbons and bicarbonate, and the ratio was unaltered with longer or shorter exposure (Table 5). Fixation of ethanol carbon was higher than the other three but declined slightly with prolonged exposure.

In sea-cucumber sperm the ratio of fixation of urethan carbon to alcohol or bicarbonate carbon was higher than in the eggs, and in most instances the sperm fixed more of the urethan carbon than carbon from alcohol or bicarbonate. This was true when fixation was tested by vacuum distillation (Table 6, Exp. No. 1) or by dialysis (Exp. No. 2). Extraction of desoxyribonucleic acid (DNA) from the sperm resulted in loss of fixed carbon, with the DNA retaining more of the ethanol or bicarbonate carbons than urethan carbons.

DISCUSSION

It must be emphasized at the onset of this discussion that the experiments reported herein are exploratory in nature and were designed to obtain qualitative evidence of the relationship between the reaction of parts of the urethan molecule with the whole resting and dividing cell and with a living substance (sperm) which is predominantly

TABLE 4

FIXATION OF CARBON 14 FROM LABELED COMPOUNDS BY FERTILIZED AND UNFERTILIZED SEA-URCHIN EGGS

	Unfertilized Eggs*				Fertilized Eggs†			
	Activity added	Specific activity‡			Activity added	Specific activity‡		
Compound	(μc/50 ml)	Free+fixed	Fixed	Fixed corrected	(μc/50 ml)	Free+fixed	Fixed	Fixed corrected
Carbonyl-labeled urethan	0.6	2.67	0.014	0.023	0.24	0.68	0.007	0.012
Sodium bicarbonate	1.0		0.035	0.035	0.40	0.17	0.010	0.010
Methylene-labeled urethan	0.7	2.84	0.010	0.014	0.28	1.09	0.010	0.014
Methylene-labeled ethyl alcohol	0.7	1.66	0.090	0.129	0.28	0.90	0.050	0.072

* Exposed for 29 minutes.

† Exposed for 35 minutes beginning 31 minutes after fertilization.

‡ Specific activities in μc/mole of egg carbon. Corrected for the differences in total activity added to eggs.

TABLE 5

THE SPECIFIC ACTIVITIES OF FERTILIZED SEA-URCHIN EGGS AT VARIOUS PERIODS AFTER EXPOSURE TO CERTAIN C^{14}-LABELED COMPOUNDS*

	Activity added	Amount added	Specific activity fixed† exposure period (minutes)				Fixed corrected‡
Compound	(μc/50 ml)	(mg/50 ml)	(11)	(30)	(48)	(73)	(30 min.)
Carbonyl-labeled urethan	0.6	17.5	0.03	0.04	0.04	0.07	0.07
Sodium bicarbonate	1.0	8.6	0.03	0.03	0.05	0.09	0.03
Methylene-labeled urethan	0.7	17.5	0.02	0.05	0.03	0.05	0.07
Methylene-labeled ethyl alcohol	0.7	9.0	0.15	0.14	0.10	0.07	0.20

* Exposure begun at 6½ minutes after fertilization.

† Specific activity in μc/mole of egg carbon.

‡ Corrected for the differences in total activity added to eggs.

TABLE 6

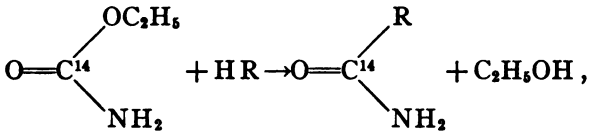
FIXATION OF CARBON 14 FROM LABELED COMPOUNDS BY ACTIVATED SEA CUCUMBER SPERM*

	Experiment No. 1			Experiment No. 2			
	Activity added	Amount added	Specific activities vacuum-treated	Activity added	Amount added	Specific activities	
Compound	(μc/50 ml)	(mg/50 ml)		(μc/50 ml)	(mg/50 ml)	Vacuum-treated	Dialyzed
Carbonyl-labeled urethan	0.6	17.5	0.73	1.2	35.0	0.18	0.022
Sodium bicarbonate	1.0	8.6	0.08	2.0	17.3	0.02	0.008
Methylene-labeled urethan	0.7	17.5	0.46	1.4	35.0	0.24	0.099
Methylene-labeled ethyl alcohol	0.7	9.0	0.14	1.4	18.1	0.21	0.019

* Exposure 30 minutes. Specific activity in μc/mole of sperm carbon, corrected for differences in total activity added.

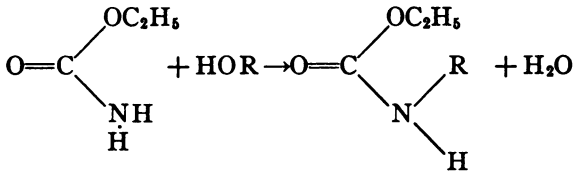
genetically active material, i.e., desoxyribonucleoprotein. Despite fluctuations in the data which cannot be explained at this early stage, definite trends are discernible which shed light on the mechanism of action of urethan.

In the tracer experiments, the fixed atoms are presumably, although not necessarily, combined chemically with some cell component. If the carbonyl-carbon atom of urethan reacted extensively with active groups of amino acids important in cell division as suggested by Dustin (4),



it is possible that our experiments would have demonstrated a high degree of carbonyl-carbon fixation as compared to the methylene-carbon atoms.

If urethan *per se* were adsorbed to cell fractions or reacted through the amide group as follows:



then one might expect to find both the carbonyl and the methylene-carbon atoms fixed to a greater extent than corresponding atoms from *in vivo* hydrolysis products of this molecule, CO_2, and ethyl alcohol.

Our present results would seem to indicate that unfertilized (resting) and fertilized (dividing) sea-urchin eggs fix both the carbonyl and the methylene atoms of urethan to a lesser extent than the methylene carbon of ethyl alcohol. The labeled carbon from $NaHC^{14}O_3$ was fixed (probably by entering the normal metabolic pathways) at a rate somewhat similar to that of the above-mentioned urethan carbon atoms.

The fixed urethan represents only a small portion of the urethan accumulated in the cell. The present observations suggest that the removable urethan is the fraction responsible for inhibition of mitosis, since on washing the eggs quickly recover. It possibly follows that fixing of urethan carbon in these eggs might be accounted for along paths having little to do with cleavage. At least, ethanol is eliminated as the route by which urethan affects cell division. For, although ethanol penetrates in comparable amounts (cf. *Free+fixed*, Table 4) and contributes more fixed activity in the eggs, 10 times as much ethanol as urethan is required to retard division of Tripneustes eggs (Cornman, unpublished data).

At this point in the argument, however, it is well to recall that the doses used in the tracer experiments were a tenth as high as the levels needed to retard cleavage. One may suspect that the fate of urethan in eggs will change as the physiology of the egg is altered by the narcotic. Even within the division-inhibiting range, the dose-effect relationships of sea-urchin eggs are different at low and high concentrations (5). Accordingly, it may prove worth-while to investigate the effect of urethan itself on the fixation of labeled urethan.

Klotz *et al.* (6), in a mass law analysis of the binding ability of bovine albumin, have shown that urethan is capable of displacing anions from a protein complex. This reaction was observed within a period of minutes, insofar as displacement effects were involved, and is additional evidence of the fixation of at least a part of the urethan molecule to protein.

Perhaps the most interesting portion of this study is the section of the results presented in Table 6 which indicate that sperm fix more urethan carbon in proportion to bicarbonate or ethanol carbon than do eggs. In this relationship the urethan methylene carbon is the more consistent, being fixed in greater amounts than the ethanol methylene carbons by sperm, and in lesser amounts than the ethanol by eggs. This same relationship was true in sperm dialyzed for 24 hours, despite the greater loss of radioactivity which may well represent the removal of dialyzable urethan fixation products. This preferential fixation of urethan carbon in cells with a high proportion of desoxyribonucleoprotein, when considered in the light of mutagenic, carcinogenic, and anti-cancer activity of this compound, is, to say the least, suggestive. Cowen (3) has reported an inhibition of urethan carcinogenesis by pentose nucleotides.

SUMMARY

Fertilized eggs of *Tripneustes esculentus*, when exposed to concentrations of urethan which reversibly inhibited division or to lower concentrations which retarded division, rapidly accumulated urethan in concentrations equal to or exceeding the surrounding concentration. Unfertilized eggs accumulated urethan more slowly and did not exceed the surrounding concentration.

Most of the urethan accumulated in the eggs was in a free or loosely bound state. Cells inhibited by this free urethan began to divide again, once the urethan was removed by washing.

Using tracer technics, it was shown that fertilized and unfertilized eggs fixed only a very

small portion of the carbonyl and methylene-carbon atoms from their accumulated urethan; no more than might be accounted for by fixation of carbon from urethan hydrolysis products, CO_2 and ethyl alcohol.

Sea-cucumber sperm fixed more of the urethan carbon in proportion to carbon from labeled bicarbonate or ethanol than did eggs.

It is suggested that urethan is accumulated within the dividing cell and exerts its effect on mitosis while free or loosely bound within the cell. However, the specific fixation of carbon from two groups in the urethan molecule to sperm (high in desoxyribonucleoprotein) suggests that this compound combines with nuclear material. This affinity for nucleoproteins may be related to the well-known mutagenic, carcinogenic, and anti-cancer activity of urethan, since such processes are often considered to be associated with nucleoprotein metabolism.

REFERENCES

1. BOYLAND, E., and RHODEN, E. The Distribution of Urethane in Animal Tissues, as Determined by a Microdiffusion Method, and the Effect of Urethane Treatment on Enzymes. Biochem. J., **44**:528–31, 1949.
2. CORNMAN, I. Inhibition of Sea-Urchin Egg Cleavage by a Series of Substituted Carbamates. J. Nat. Cancer Inst., **10**:1123–38, 1950.
3. COWEN, P. N. Inhibition of the Carcinogenic Properties of Urethane by Pentose Nucleotides. Brit. J. Cancer, **3**:94–97, 1949.
4. DUSTIN, P., JR. The Cytological Action of Ethylcarbamate (Urethane) and Other Carbamic Esters in Normal and Leukaemic Mice, and in Rabbits. Brit. J. Cancer, **1**:48–59, 1947.
5. FISHER, K. C., and HENRY, R. J. The Effects of Urethane and Chloral Hydrate on Oxygen Consumption and Cell Division in the Egg of the Sea Urchin, *Arbacia punctulata*. J. Gen. Physiol., **27**:469–81, 1944.
6. KLOTZ, I. M.; TRIWUSH, H.; and WALKER, F. M. The Binding of Organic Ions by Proteins. Competition Phenomena and Denaturation Effects. J. Am. Chem. Soc., **70**:2935–41, 1948.
7. LARSEN, C. D. Evaluation of the Carcinogenicity of a Series of Esters of Carbamic Acid. J. Nat. Cancer Inst., **8**:99–101, 1947.
8. ———. Studies of Pulmonary Tumor Induction in Mice by Derivatives of Carbamic Acid. Cancer Research, **7**:726, 1947.
9. ———. Pulmonary Tumor Induction with Alkylated Urethans. J. Nat. Cancer Inst., **9**:35–37, 1948.
10. SKIPPER, H. E., and BRYAN, C. E. Carbamates in the Chemotherapy of Leukemia. III. The Relationship between Chemical Structure and Anti-Leukemic Action of a Series of Urethan Derivatives. J. Nat. Cancer Inst., **9**:391–97, 1949.
11. SKIPPER, H. E.; BRYAN, C. E.; RISER, W. H., JR.; WELTY, M.; and STELZENMULLER, A. Carbamates in the Chemotherapy of Leukemia. II. The Relationship between Chemical Structure, Leukopenic Action, and Acute Toxicity of a Group of Urethan Derivatives. J. Nat. Cancer Inst., **9**:77–88, 1948.
12. SKIPPER, H. E.; BRYAN, C. E.; WHITE, L., JR.; and HUTCHISON, O. S. Techniques for *in vivo* Tracer Studies with C^{14}. J. Biol. Chem., **173**:371–81, 1948.