STUDIES OF THE MECHANISM OF ACTION OF URETHANE IN INITIATING PULMONARY ADENOMAS IN MICE

II. Its Relation to Nucleic Acid Synthesis*

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(Received for publication, December 20, 1956)

Urethane (ethyl carbamate) is a broad spectrum carcinogen which has been demonstrated to produce neoplasms of the lungs (1), liver (2), mesenchymal tissue (3), and skin (4) in at least two species, the mouse and the rat. Because of the simplicity of its chemical structure it has proved a particularly interesting carcinogen for those studying the mechanism of carcinogenesis by classic chemical means. Studies of the carcinogenicity (5) or mitotic poisoning effects (6) of various esters of carbamic acid chemically related to urethane, the carcinogenicity of alkylated urethanes (7), of potential urethane degradation products (8), and a wide variety of general hypnotics (9) have, however, yielded no information upon the mechanism of action of urethane other than its relation to the specificity of the molecular structure of urethane. Observations on the influence of urethane on enzymatic and other less discrete *in vitro* systems have not yet been correlated with its carcinogenic or antileukemic action though they have given rise to many interesting speculations (10). Because the general biological importance of nucleic acid to the maintenance of heritable characteristics is generally recognized and, because of the nuclear poisoning effects of urethane (11), much attention has been directed toward the carcinogen's possible influence upon nucleic acid synthesis. Recent experiments have discounted the assumption that urethane has a direct nucleotoxic action in initiating tumors (5, 6, 12). Evidence perhaps in favor of an influence upon nucleic acids, however, has been reported by Cowen (13). Since the effect of pentosenucleotides on leucocytes is the opposite of that of urethane, he tested the protective effect of these substances upon its carcinogenic action. Fewer pulmonary tumors developed in mice receiving multiple injections of these nucleotides. However, the nucleotides were largely given after urethane injections had been discontinued. In addition, the tested animals suffered severe ill effects, thereby casting considerable doubt upon the validity of the results; for it is well known that the state of the animal influences greatly the overt occurrence and growth of tumors (14).

* Supported by a grant (C-1741) from the National Cancer Institute, National Institutes of Health, United States Public Health Service.

Young, rapidly growing mice are much more responsive to standard doses of urethane than are those that are older and more slowly growing (12, 15). Since there is a recognized correlation in the rate of synthesis of desoxyribosenucleic acid and the growth rate of the body at a given time (16), it seemed worthwhile to find out to what extent the number of tumors initiated by a single injection of urethane might be influenced by simultaneous exposure of the animals to desoxyribosenucleic acid (DNA), ribosenucleic acid (RNA) their chemical components, precursors, and substances influencing their rate of synthesis.

It will be demonstrated that a single injection of a DNA hydrolysate immediately prior to exposure of mice to urethane profoundly reduces the number of tumors initiated. Aminopterin, well known to inhibit nucleic acid synthesis (17), increases the carcinogenic activity of injected urethane. This increase can be prevented by injection of a DNA hydrolysate. Of the components of nucleic acid tested the pyrimidines proved the most active inhibitors. Of the pyrimidine precursors it will be shown that orotic acid and dihydro-orotic acid also exert profound inhibitory influences upon carcinogenesis brought about through exposure of mice to urethane. Ureidosuccinic acid, a normally occurring pyrimidine precursor, (18) whose chemical structure has much resemblance to that of urethane, reduces the carcinogenic effectiveness of exposure of lung tissue *in vitro* to the intermediary elicited by injection of certain animals with urethane (19).

Materials and Methods

Materials:

Urethane (ethyl carbamate, C. P., Eimer and Amend, New York). Sodium desoxyribonucleate (Nutritional Biochemicals Corporation, Cleveland). The manufacturer used the hot alkaline extraction method of Levine (20) in its preparation from fish sperm. It is depolymerized and made up almost entirely of components the size of tetranucleotides. Aminopterin (Lederle, Pearl River). Adenine, uracil, thymine, orotic acid, oxaloacetic acid, aspartic acid, cytidylic acid, uridylic acid, cytosine, guanine, glutathione, glutamic acid, methionine, cysteine, ethionine, para-aminobenzoic acid, folic acid (Nutritional Biochemicals Corporation, Cleveland). Desoxycytidylic acid, thymidylic acid, and 5-methylcytosine (California Foundation for Biochemical Research, Pasadena). Uridine-5-phosphate (Pabst Industries, Milwaukee). Urea, nitrogen mustard (methyl bis-), (Merck, Rahway). Sodium formate (Mallinckrodt Chemical Works, New York). Asparagine (Pfanstiehl Chemical Company, Waukegan, Illinois). Acriflavin, N. F., (Abbott, Chicago). B.A.L. (2, 3,-dimercaptopropanol), (Hynson, Westcott, and Dunning, Baltimore). 4-Amino-5-imidazole carboxamide and ureidosuccinic acid synthesized by Dr. Bruce Merrifield of The Rockefeller Institute, New York, and were his generous gift. Dihydro-orotic acid was synthesized by and was the generous gift of Dr. Elliott Shaw of The Rockefeller Institute, New York.

Methods:

All chemicals, with the exception of urethane which was used as a 5 per cent solution in double distilled water, were used at the concentrations indicated in the individual experi-

ments and as solutions made isotonic with sodium chloride. The tonicity was determined by calculation from the molecular weights and, when necessary, determined or checked by the response of mouse blood cells *in vitro*. Unless contraindicated by the nature of the substance, all concentrations were used as solutions sterilized by autoclaving. The sodium desoxyribonucleate the individual nucleotides, 4-amino-5-imidazole carboxamide, and ureidosuccinic acid were sterilized by filtration through a Berkefeld V filter. The oxaloacetic acid was used immediately following preparation and not sterilized.

Mice.—Animals of the Swiss, "A," and "C" strains were used. Both the Swiss and "A" strains have a high incidence of pulmonary adenomas whereas the "C" has a low incidence. References to the specific character of the stocks may be found in a previous report (12). In all experiments the animals tested were matched as to age, weight, sex, and strain. In all *in vivo* experiments the mice received injections calculated on a per gram basis. The weights of the mice were followed over the duration of the experiments at least at weekly intervals. All mice developing intercurrent disease, such as "chuckles" in the Swiss and "C" strains or rectal prolapse in the "A" strain, were discarded. Also discarded were any animals whose weights deviated 2 gm. or more from the mean over a period of 2 consecutive weeks. All mice were kept ten to a box, with mice of tested groups within the experiment distributed in the same box. Animals were fed Purina chow supplemented with milk-soaked bread twice a week. Tap water was available at liberty. In the *in vivo* experiments the mice were killed by decapitation after the intervals indicated in individual experiments and their lungs excised. The pulmonary lobes were then separated from one another and the tumors on the surface of the lungs counted and measured as to diameter with a millimeter rule. No tumors measuring less than one-third mm. in diameter figure into the results. Intermittent microscopic sections controlled the findings. All were examined without knowledge of the group from which the individual came.

Evaluation of Results:

In Vivo Experiments.—Mice of the various intra-experimental groups were matched against one another in order of the numbers of tumors within each group that the individual animals developed and the response compared as to number of animals with tumors and number of tumors per individual, as is the standard practice. The consistency and degree of response were thus measured. In addition, results of all experiments which showed differences were treated statistically. In experiments in which equal numbers of mice survived in the compared groups, the probability of error was figured through the standard deviation of differences between matched pairs. In those experiments in which the numbers of animals were not equal the significance was calculated through the use of the "t" test (21). Only the probability value indicating that the result obtained was not due to chance is recorded herein. Due to limitations in space, the results will be presented mainly in table form and the average number of tumors compared from group to group. The results of representative experiments will be shown in chart form in which the response of individual animals in terms of tumors will be represented by black or hatched columns which refer to the group within the experiment from which the animal came. The average size of tumors of the animals of the particular groups is also shown. The inhibitory effect of an agent upon the number of tumors developing in an individual group was not considered significant if the average size of the tumors of the experimental groups was different from controls by more than 0.05 mm. in diameter. This assumed range of normal deviation in average size was determined on the basis of deviations among groups of control animals killed after a uniform interval and taken from many experiments. When the number of tumors of the experimental groups exceeded the controls, and yet the average size of tumors in the experimental group was smaller this difference must be taken as evidence indicating that the increase in number occurred despite poorer condi-

TABLE I

Exp.	Chart No.	Test substance	Method		Mice used: Started: Strain	Started: No.	Survived: Age	Survived:	Tumor-positive mice	Total adenomas	Adenomas per mouse	Average diameter adenomas	Effect	Statistical significance
							wks.					*mm.*		
1	1	DNA	DNA/U	7 wks.—K	Sw	25	8–10	19	13	19	1	0.37	Inhibited	$P = <0.01$
			S/U			25		19	12	47	2.5	0.40		
2		DNA	DNA-DNA/U	8 wks.—K	C	20	8	14	2	2	0.14	0.42	Inhibited	$P = <0.05$
			S-S/U			20		15	5	10	0.66	0.48		
3		Aminopterin	A^1-A-A-A^2/U	7 wks.—K	Sw	20	8–10	9	8	48	5.3	0.44	Increased	
			S-S-S-S/U			20		20	12	47	2.4	0.40		
4		Aminopterin	A^1-A-A-A^2/U	8 wks.—K	Sw	25	8–10	5	5	77	15.4	0.52	Increased	
			S-S-S-S/U			25		19	18	139	7.3	0.52		
5		Aminopterin	A^1-A-A-A^2/U	8 wks.—K	Sw	25	4	16	14	44	2.75	0.45	Increased	
			S-S-S-S/U			25		19	12	27	1.4	0.50		
6		Aminopterin	A^2/U	10 wks.—K	Sw	25	8	23	23	311	13.6	0.60	O*	
			S/U			25		23	23	357	15.4	0.67		
7	2	Aminopterin	A-A^1-A-A-A^2/U	8 wks.—K	C	25	8	22	6	14	0.64	0.38	Interference	
			A-A^1-A-A/DNA^2-A^2/DNA/U			25		17	0	0	0.0	0		
			S-S-S-S/s-S/s/U			25		19	3	4	0.22	0.64		
8		Aminopterin	A^1-A-A-A/U	8 wks.—K	C	25	3–4	19	8	13	0.74	0.45	Interference	
			A^1-A-A/DNA^3-A/DNA/U			25		20	7	9	0.46	0.42		
9		Thymidylic acid	T^{4a}/U	8 wks.—K	Sw	20	8	19	16	79	4.15	0.50	O	
			S/U			20		20	18	101	5.0	0.52		
10		Thymidylic acid	T^{4b}/U	12 wks.—K	Sw	20	4–5	20	20	339	17	0.66	O	
			S/U			20		17	17	309	18	0.56		
11		Uridylic acid	Ur^{5a}-Ur^{5a}/U	8 wks.—K	C	25	3	21	10	12	0.57	0.65	O	
			S-S/U			25		22	8	10	0.46	0.50		
12		Uridylic acid	Ur^{5a}-Ur^{5a}/U	7 wks.—K	C	25	4	22	8	12	0.54	0.46	O	
			S/U			25		21	6	11	0.52	0.45		
13		Cytidylic acid	C^{6a}/U	8 wks.—K	Sw	25	5	20	19	68	3.35	0.57	Inhibited	$P = <0.05$
			S/U			25		21	17	126	6.0	0.55		

* These experiments were repeated 2–6 times, in addition to the data shown, with the same general result.

All solutions used except urethane were neutralized to pH 6.5–7 and made isotonic with sodium chloride, Those marked with a ‡ were not completely in solution but used as a suspension.

Test agent/U, injections given at same time.

Test agent—test agent, 24 hour interval.

K, killed.

U, 1 mg. urethane (5 per cent) per gram mouse subcutaneously.

S, volume of isotonic sodium chloride equivalent to test agent i.p.

DNA, 1 mg. desoxyribonucleate (5 per cent) per gm. mouse. This concentration was found isotonic for mouse red cells.

[1] 0.00025 mg. aminopterin per gm. mouse i.p. (0.0025 per cent) solution aminopterin in isotonic salt).

[2] 0.00050 mg. aminopterin per gm. mouse. i.p. (0.0025 per cent solution aminopterin in isotonic salt).

[3] 1 mg. desoxyribonucleate (5 per cent) per gm. mouse subcutaneously.

[4a] 0.2 mg. calcium thymidylate (1 per cent) per gm. mouse.

[4b] 0.1 mg. calcium thymidylate (1 per cent) per gm. mouse.

[5a] 0.1 mg. uridylic acid (2 per cent) per gm. mouse.

[5b] 0.2 mg. uridylic acid (2 per cent) per gm. mouse.

[6a] 0.1 mg. cytidylic acid (2 per cent) per gm. mouse.

TABLE I—*Continued*

Exp.	Chart No.	Test substance	Method	Mice used: Started: Strain	Started: No.	Survived: Age		Tumor-positive mice	Total adenomas	Adenomas per mouse	Average diameter adenomas	Effect	Statistical significance
						wks.					*mm.*		
14		Cytidylic acid	C[6a]/U 7 wks.—K	C	20	4	13	4	6	0.46	0.46	Inhibited	P = <0.05
			S/U		20		14	6	11	0.80	0.45		
15	3	Cytidylic acid	C[6b]-C/U 7 wks.—K	C	20	4	11	5	7	0.63	0.50	Inhibited	
			S-S/U		20		15	10	25	2.50	0.44		
16		Uridine-5-phosphate	Up[7]/U 9 wks.—K	A	25	5	24	15	31	1.3	0.54	O*	
			S/U		25		24	14	29	1.2	0.63		
17		Desoxycytidylic acid	Dc[8]/U 9 wks.—K	Sw	30	4	26	25	404	13.0	0.52	O*	
			S/U		30		27	26	345	15.0	0.52		
18		Guanine	G[9]-G/U 9 wks.—K	Sw	25	8	21	14	55	2.62	0.40	O*	
			S-S/U		25		20	16	58	2.80	0.38		
19	4	Adenine	Ad[10]-Ad/U 7 wks.—K	Sw	25	3	23	19	112	4.91	0.48	Increased*	P = <0.01
			S-S/U		25		24	16	54	2.25	0.43		
20		4-amino-5-imidazole carboxamide	4a[11]/U 7 wks.—K	Sw	25	8	22	15	31	1.4	0.40	Increased*	P = <0.01
			S/U		25		20	7	14	0.7	0.49		
21		2-6-diaminopurine	2,6[12]-2,6-2,6-2,6-2,6/U 8 wks.—K	C	30	3	21	11	16	0.76	0.50	O*	
			S-S-S-S-S/U		30		26	13	17	0.65	0.40		
22		Thymine	Th[13]/U 7 wks.—K	Sw	70	3	49	23	56	1.1	0.40	Inhibited	P = <0.01
			S/U		70		51	32	90	1.8	0.43		
23	5	Thymine uracil	Th[14]/U		30		27	19	60	2.2	0.50	Inhibited	
			Ua[15]/U 8 wks.—K	Sw	30	5	24	14	64	2.66	0.50	No sig. dif.*	P = <0.01
			S/U		30		27	19	81	3.0	0.50		
24		5-methylcytosine	Mc[16]/U 8 wks.—K	Sw	25	4	24	10	26	1.1	0.50	Dubious dif.*	Not sig.
			S/U		25		25	16	38	1.6	0.48		
25		Cytosine	Cy[17]/U 9 wks.—K	Sw	25	4	15	11	48	3.2	0.53	O	
			S/U		25		19	14	63	3.3	0.57		
26		Cytosine	Cy[17]/U 7 wks.—K	Sw	30		27	19	93	3.44	0.47	O*	
			S/U		30	4	26	23	88	3.38	0.40		

[6b] 0.2 mg. cytidylic acid (2 per cent) per gm. mouse.
[7] 0.1 mg. uridine-5-phosphate (1 per cent) per gm. mouse.
[8] 0.2 mg. desoxycytidylic acid (2 per cent) per gm. mouse.
[9] 0.2 mg. guanine‡ (2 per cent) per gm. mouse.
[10] 0.2 mg. adenine‡ (2 per cent) per gm. mouse.
[11] 0.15 mg. 4-amino-5-imidazole carboxamide (0.75 per cent) per gm. mouse.
[12] 0.1 mg. 2,6 diaminopurine (0.5 per cent) per gm. mouse.
[13] 0.2 mg. thymine‡ (2 per cent) per gm. mouse.
[14] 0.4 mg. thymine‡ (2 per cent) per gm. mouse.
[15] 0.4 mg. uracil‡ (2 per cent) per gm. mouse.
[16] 0.4 mg. 5 methylcytosine (2 per cent) per gm. mouse.
[17] 0.4 mg. cytosine (2 per cent) per gm. mouse.

TABLE I—*Concluded*

Exp.	Chart No.	Test substance	Method	Mice used: Started: Strain	Started: No.	Survived: Age	Survived: No.	Tumor-positive mice	Total adenomas	Adenomas per mouse	Average diameter adenomas	Effect	Statistical significance
						wks.					*mm.*		
27		Orotic acid	O^{18}-O/U	C	25		17	5	9	0.53	0.47	Inhibited*	$P = <0.05$
			S-S/U 8 wks.—K		25	7	21	10	20	0.91	0.52		
28	6	Orotic acid	O^{18}-O/$\frac{U}{2}$	C	25	7	13	0	0	0	0.40	Inhibited	$P = <0.05$
			S-S/$\frac{U}{2}$ 7 wks.—K		25		19	6	9	0.45			
29	7	Orotic acid	O^{19}/U-29 $\frac{}{2}$	C	25	5	14	2	2	0.14	0.50	Inhibited*	$P = <0.01$
			$\frac{O^{20}U}{2\ 2}$		25		19	4	7	0.37	0.69		
			$\frac{O^{21}U}{4\ 2}$ 11 wks.—K		25		20	7	8	0.40	0.56		
			S/$\frac{U}{2}$		25		21	8	11	0.52	0.57		
30		Dihydro-orotic acid	D^{22}/O	Sw	25	5	21	9	17	0.8	0.52	Inhibited*	$P = <0.05$
			S/O 8 wks.—K		25		21	12	32	1.53	0.51		
31		Ureido-succinic-acid	US^{23}/U	Sw	25	4	23	12	31	1.35	0.43	O	
			S/U 7 wks.—K		25		22	11	29	1.32	0.37		
32		Ureido-succinic acid	Us^{23}-Us/U	C	25	4	22	6	7	0.33	0.50	Dubious inhibition*	Not sig.
			S-S/U 8 wks.—K		25		18	7	12	0.66	0.46		
Results of Experiments 33 and 34 are included in the text.													
35		Asparagine	As^{24}/U	A	25	5	21	18	53	2.5	0.57	Inhibited*	$<P = 0.05$
			S/U 10 wks.—K		25		22	20	76	3.5	0.52		
36		Aspartic acid	Ap^{25}/U	A	25	5	23	12	37	1.6	0.48	O*	
			S/U 8 wks.—K		25		24	14	30	1.25	0.54		
37		Urea	Ue^{26}/U	A	25	5	21	8	16	0.76	0.45	O*	
			S/U 8 wks.—K		25		19	9	15	0.78	0.45		
38		Oxalo-acetic acid	Ox^{27}/U	Sw	25	4	13	9	49	3.77	0.62	Increased*	$<P = 0.05$
			S/U 8 wks.—K		25		16	14	31	1.93	0.70		
39		Na formate	F^{28}/U	A	25	8	22	21	81	3.7	0.56	O*	
			S/U 10 wks.—K		25		21	19	63	3.0	0.56		

[18] 0.1 mg. orotic acid‡ (1 per cent) per gm. mouse.
[19] 0.075 mg. orotic acid (0.25 per cent) per gm. mouse.
[20] 0.0375 mg. orotic acid (0.125 per cent) per gm. mouse.
[21] 0.0188 mg. orotic acid (0.063 per cent) per gm. mouse.
[22] 0.1 mg. dihydroorotic acid (1 per cent) per gm. mouse.
[23] 0.2 mg. ureidosuccinic acid (1 per cent) per gm. mouse.
[24] 0.3 mg. asparagine (3 per cent) per gm. mouse.
[25] 0.2 mg. aspartic acid (1 per cent) per gm. mouse.
[26] 0.6 mg. urea (2 per cent) per gm. mouse.
[27] 0.5 mg. oxaloacetic acid (2.5 per cent) per gm. mouse.
[28] 0.4 mg. sodium formate (2 per cent) per gm. mouse.

tions for growth. The relation between the size and number of tumors of various sorts and the conditions of body growth have been well shown by Tannenbaum (14).

The anesthetic effect of urethane can be taken as an indicator of the concentration and rate of metabolism of the agent (22). Some of the nucleic acid derivatives in high doses influence the degree and duration of the anesthetic effect of urethane. Even though it had previously been found that the neoplastic response of individual animals was related to the amount of urethane given rather than the peak concentration or duration of exposure (12) the anesthetic influence of urethane on experimental and control groups was always noted. The dose levels of the derivatives of nucleic acid dealt with herein produced no differences between experimental and control groups in anesthetic effects. Another possible variable concerns the known correlation between growth rate of animals at the time of injection with urethane and their response in terms of tumors (12, 15). It might be thought that the inhibitory effects of some of these agents might be through a non-specific inhibition of growth rate at the time of injection with urethane. It has been demonstrated by Bullough (23) that the mitotic rates of various tissues, amongst them the skin, at a given time varies in the same direction though the individual tissue rates may be of a differing intensity. It seemed therefore reasonable to use the mitotic rate in the skin of individual animals as an index of the relative growth rate of their bodies at a given moment of time. All the substances found to reduce the number of tumors initiated by urethane were tested in this relation. Tests were made 5½ hours after injection of the individual substances in the same amount per gram used in these experiments. The number of mitoses occurring in 1 cm. ear epithelium, an aggregate length made up of several 6 micron sections of the biopsy specimen, was taken as an index of the growth rate of the animal as a whole (23). Among groups of animals of ten mice each, matched as to weight, age, and sex, no decrease in mitotic rate in the ear epithelium was found on comparison of groups injected with the agents under test with animals receiving an equal volume of isotonic saline.

Experiments in Vitro.—The techniques used in the experiments in which the exposure of the lung tissue to the carcinogen was carried out *in vitro* will be described in detail further on.

Influence of DNA, Its Rate of Synthesis, Certain of Its Constituents, and Certain of Their Precursors upon the Carcinogenic Effect of Urethane

Studies with radioactive isotopes have demonstrated that over 99 per cent of the urethane given in a single dose of 1.5 mg. or less per gm. mouse is excreted within 24 hours (24). Also it has been shown that lung tissue implanted 24 hours after injection of the hosts with urethane develops no growths though exposure of mice to urethane after implantation results in the occurrence of many tumors in the implanted tissue (25). The brief period of oncogenic activity of urethane injected in a single dose in an amount which readily produces lung tumors (26) would appear to provide opportunity to test whether various metabolites or substances would modify its oncogenic effectiveness. If so they might interfere at certain known metabolic loci and thereby cast light upon the mechanism of the carcinogenic action of urethane.

Findings in Experiments 1 through 39 (Table I, Charts 1 through 8).—The substances tested are listed in Table I in the general order of the size and complexity of their molecules and in relation to their estimated position in the pathway of nucleic acid synthesis. The experiments were done in this order and the methods used were essentially identical in them all. The dose

levels chosen were, with the exception of aminopterin, well within the level tolerated by the animals. Because of this similarity it has seemed sufficient to outline the individual experiments in table form rather than describe each in detail.

All the pertinent data are included in Table I except the relative responsiveness of the individual animals. This is shown in representative accompanying charts (1 through 8). No test substance was given after injection of the animals with urethane because only the initiatory phase of carcinogenesis was to be studied (24, 12, 25). The experimental and control animals were similarly maintained after injection to insure that the subsequent growth of the neoplasms would take place under the same conditions. The facts sought are concerned with direction of effect rather than quantitative comparison and only such trends will be noted in the text description.

In Experiments 1 and 2 (Table I, Chart 1) it is evident that the DNA hydrolysate had a profound inhibitory influence upon the carcinogenic activity of urethane. The weight curve of the individual animals of Experiment 1 (Chart 1) makes clear that the brief exposure to this dose of the DNA hydrolysate was without manifest non-specific toxic effect which might have influenced the rate of growth of the neoplasms and henceforth their manifest number. The number of tumors in the DNA-treated animals was too few to allow their size to be taken as a measure of relative growth rate in these experiments.

Nucleic acid hydrolysates are well known to have a broad spectrum of biological activities not necessarily related to nucleic acid metabolism. Consequently, aminopterin, which inhibits desoxynucleic acid synthesis, was used in an attempt to clarify to some extent the meaning of the DNA inhibition. Several days of treatment of mice with aminopterin are necessary for inhibition of nucleic acid synthesis at dose levels which allow survival of a considerable proportion of the animals (17). In Experiments 3, 4, 5, 7, and 8, this procedure was carried out. Death of so large a proportion of animals (Table I) seemed an indication that enough of the antagonist was being given to have the desired effect. However, too few animals survived the aminopterin treatment for proper statistical study of individual experiments; in consequence, repeated experiments were necessary. Despite the fact that the animals were sickly for almost a week after aminopterin and urethane treatment, it is evident that the pretreatment with aminopterin resulted in an increase in adenomas brought about by urethane injection. Comparison of the average size of tumors in the various experimental and control groups indicates that this increase in tumors occurred despite the fact that the tumors were growing less well and, accordingly, fewer would have a chance to reach a discernible size in the time interval allowed. In contrast to the potentiating effect of aminopterin when given under conditions known to inhibit DNA synthesis (17), no effect, except a slight reduction in the number of tumors appearing, was seen when only immediate pretreatment with this agent was used (Experiment 6, Table I). Such a short treatment

schedule with aminopterin at this dose level has been found to exert no influence on nucleic acid synthesis (17). The slight reduction in the number of tumors in this experiment could be accounted for by the reduction in their

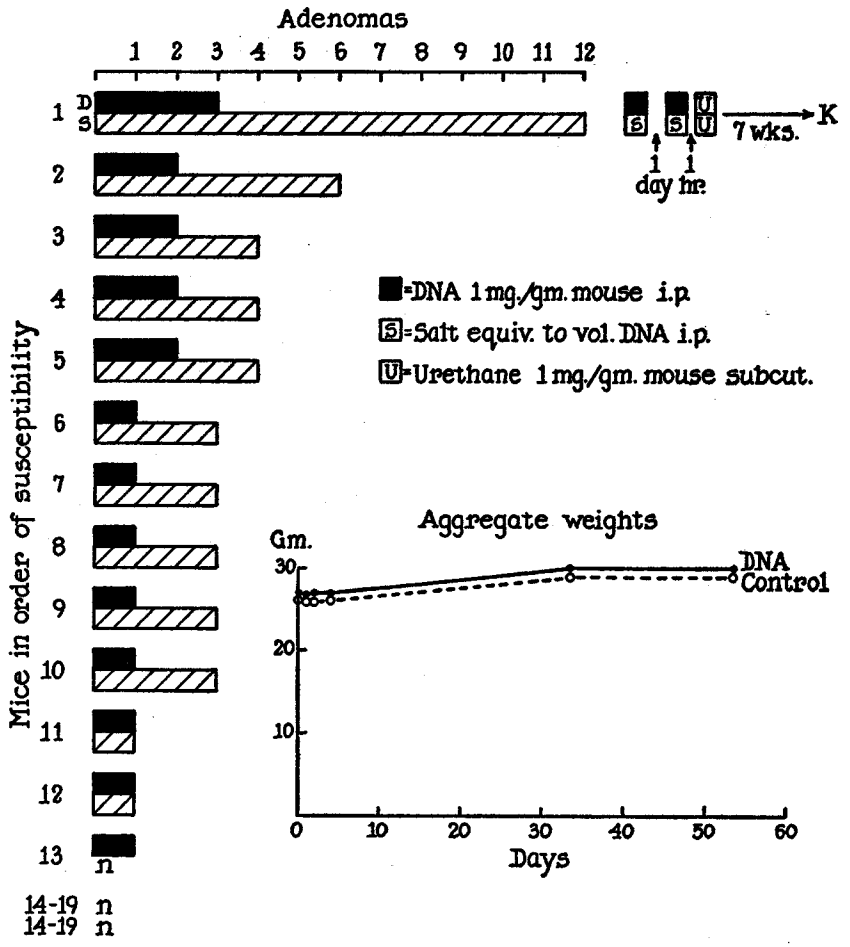


CHART 1. The inhibitory influence of injection of a DNA hydrolysate upon the initiation of adenomas by exposure of mice to urethane. (Experiment 1).

growth rate because of non-specific toxic effects of the drug. That the growth rate of the tumors of the aminopterin-treated mice was less than controls was indicated by the smaller average size of adenomas in the experimental group.

It is possible that the influences of aminopterin upon the carcinogenic activity of urethane might be through mechanisms other than through the effect upon

nucleic acid synthesis.[1] Consequently, an attempt was made to determine whether the DNA hydrolysate might eliminate the aminopterin effect. In

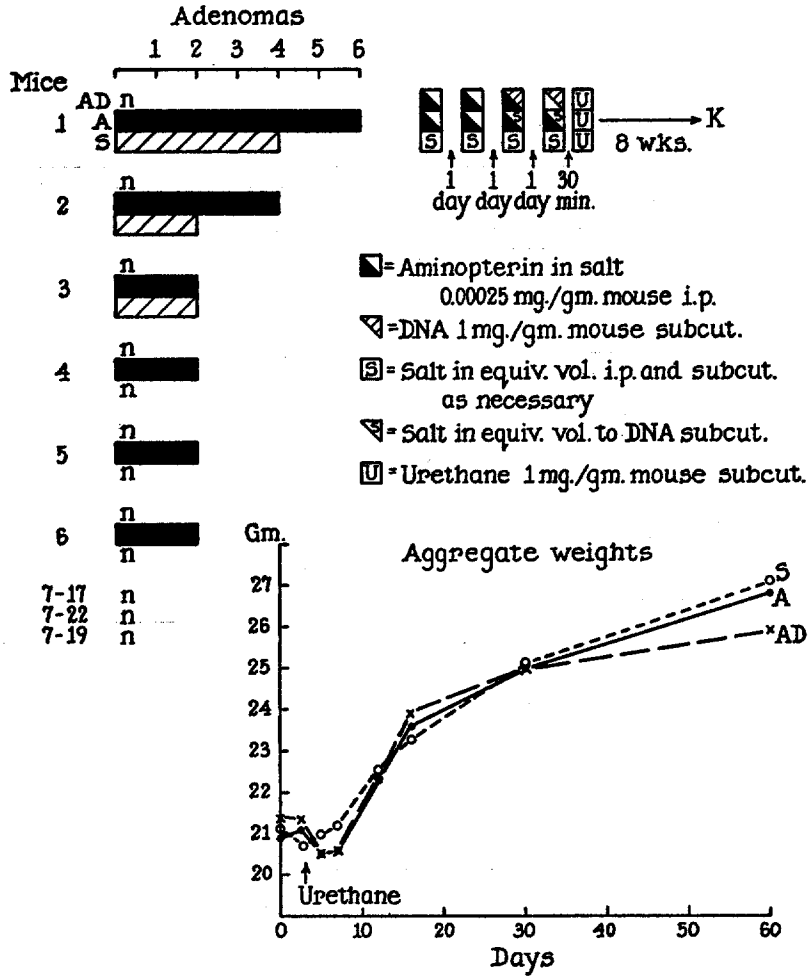


CHART 2. The potentiating effect of pretreatment of animals with aminopterin upon the initiation of adenomas by exposure of mice to urethane and its blockage by injection of a DNA hydrolysate. (Experiment 7).

Experiments 7 and 8 (Table I and Chart 2) it is clear that injection of DNA into mice pretreated with aminopterin greatly reduces the adenomatous re-

[1] Unpublished experiments have indicated that the aminopterin effect is not mediated through its side action on the choline-methionine system which results in fatty livers (See Petering, *Physiol. Rev.*, 1952, **32**, 197). Deficiencies in the same pathway can be produced by starvation and the resulting fatty livers can be prevented by methionine. Methionine,

sponse of the mice to urethane. Hence, it seemed more likly that the interference of initiation of adenomas with urethane by DNA was metabolically specific.

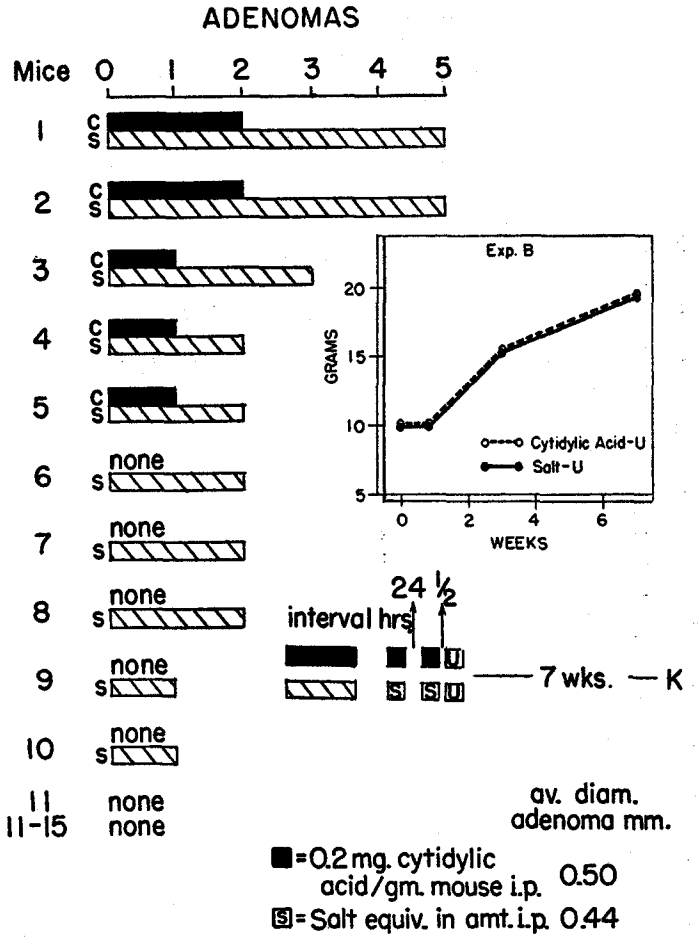


CHART 3. The inhibitory influence of cytidylic acid. (Experiment 15).

For this reason, various constituents of nucleic acid were put to test. Of the pyrimidine nucleotides tested (Experiments 9 through 17) only cytidylic acid yielded any effect upon the response in terms of number of tumors brought about by urethane (Experiments 13 to 15, Chart 3). Like DNA, cytidylic acid

rather than exerting an inhibitory influence on urethane carcinogenesis in fasted animals as would be expected should aminopterin be exerting its influence through producing fatty livers, actually greatly increases the number of adenomas initiated.

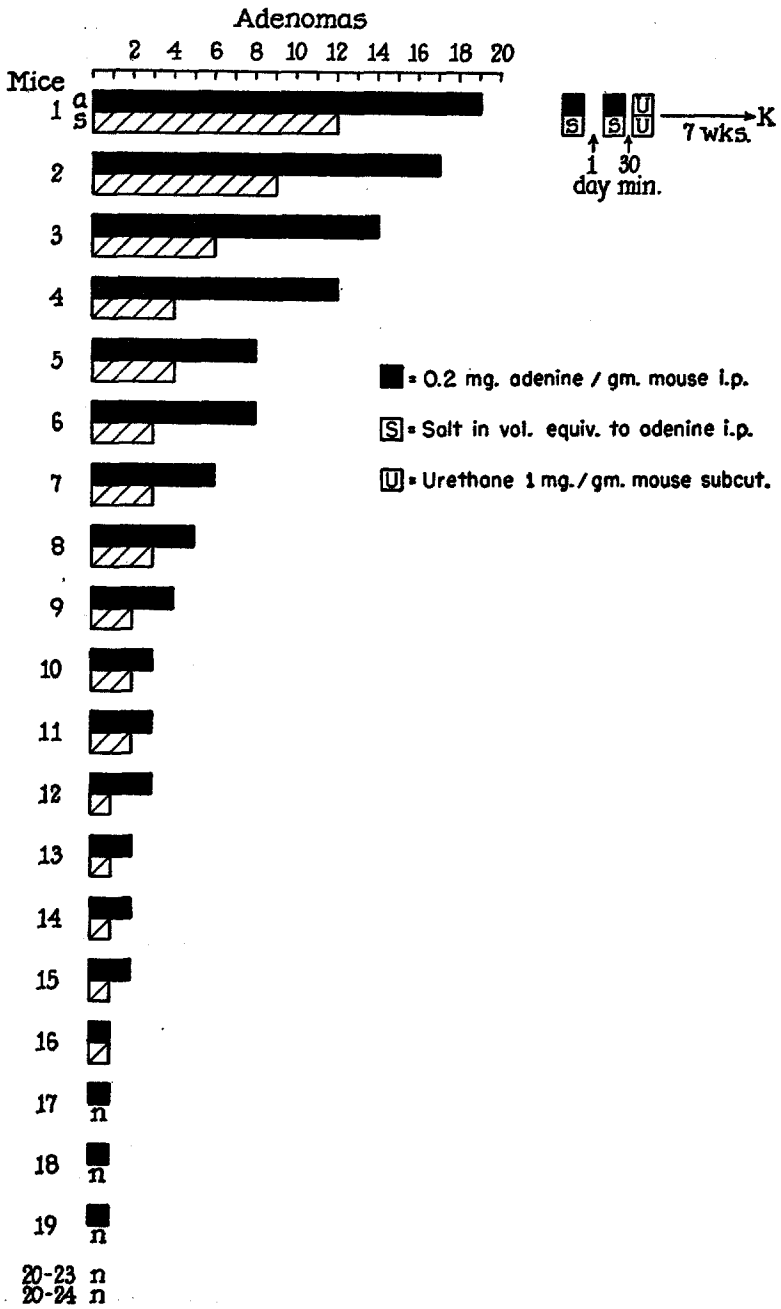


CHART 4. The potentiating influence of adenine. (Experiment 19).

reduced the number of adenomas initiated. Two dimensional paper chromtog-a raphy of the cytidylic acid in a butanol: urea and an isoamyl alcohol: phosphate solvent (27) showed only one substance, that at the *Rf* of cytidylic acid.

Because of the results with adenine (Experiment 19, Chart 4) which increased the carcinogenic effect of urethane the various purine nucleotides were not examined. 4-amino-5-imidazolecarboxamide, a precursor to adenine (28) also potentiated the carcinogenic effect of urethane (Experiment 20). Guanine was without effect, but, in view of its extremely low solubility, little significance can be attached to this result. 2,6-diaminopurine which interferes in purine synthesis and influences the lethal effects of urethane in bacterial systems (29) proved without influence upon the carcinogenic activity of urethane (Experiment 21).

Among the pyrimidine bases only thymine reduced the number of adenomas produced by the single exposure of animals to urethane (Experiments 22 and 23). Uracil, cytosine, and 5-methyl cytosine were ineffective in the dose range tested (Experiments 23 to 26). The average size of adenomas in animals receiving the single dose of thymine prior to urethane was so close to that of the control groups that it was clear that no non-specific toxic effect mediated the reduction in the number of tumors in the thymine-treated animals by influencing their growth. The lack of any such toxic effect was also confirmed by the weight curves (Chart 5).

The activity of thymine made it appear worthwhile to find whether the precursors to the pyrimidines might influence the carcinogenic activity of urethane. For this reason, studies were made of the action of orotic acid (30, 31). The results of Experiments 27 to 29, (Table I and Chart 6) made plain that not only did orotic acid profoundly modify the responsiveness of the animals to urethane but that upon varying the dose of either urethane (Experiments 27 and 28) or orotic acid (Experiment 29) the two substances acted much like competitive antagonists of one another. In Experiment 29, all animals were fasted 24 hours prior to exposure, to eliminate dietary pyrimidines. Experiments with dihydro-orotic acid (Experiment 30), the precursor to orotic acid, (32, 18), also revealed an inhibition of the carcinogenic activity of urethane. Ureidosuccinic acid, a metabolic precursor of dihydro-orotic acid (32) on the other hand, had no significant activity at the dose level used (Experiments 31 and 32, Table I). It seems quite possible, however, that it may have been metabolized before the exposure of the mice to urethane had elicited the carcinogenic substance responsible for the initiation of the pulmonary tumors (19), since it has been demonstrated that urethane itself is not the immediate carcinogen for the pulmonary cells but rather elicits or is transformed to the carcinogen after injection in the several species tested (19). This seems the more likely in view of the results of *in vitro* studies which follow.

It seemed possible that the metabolic effect produced by the intermediary

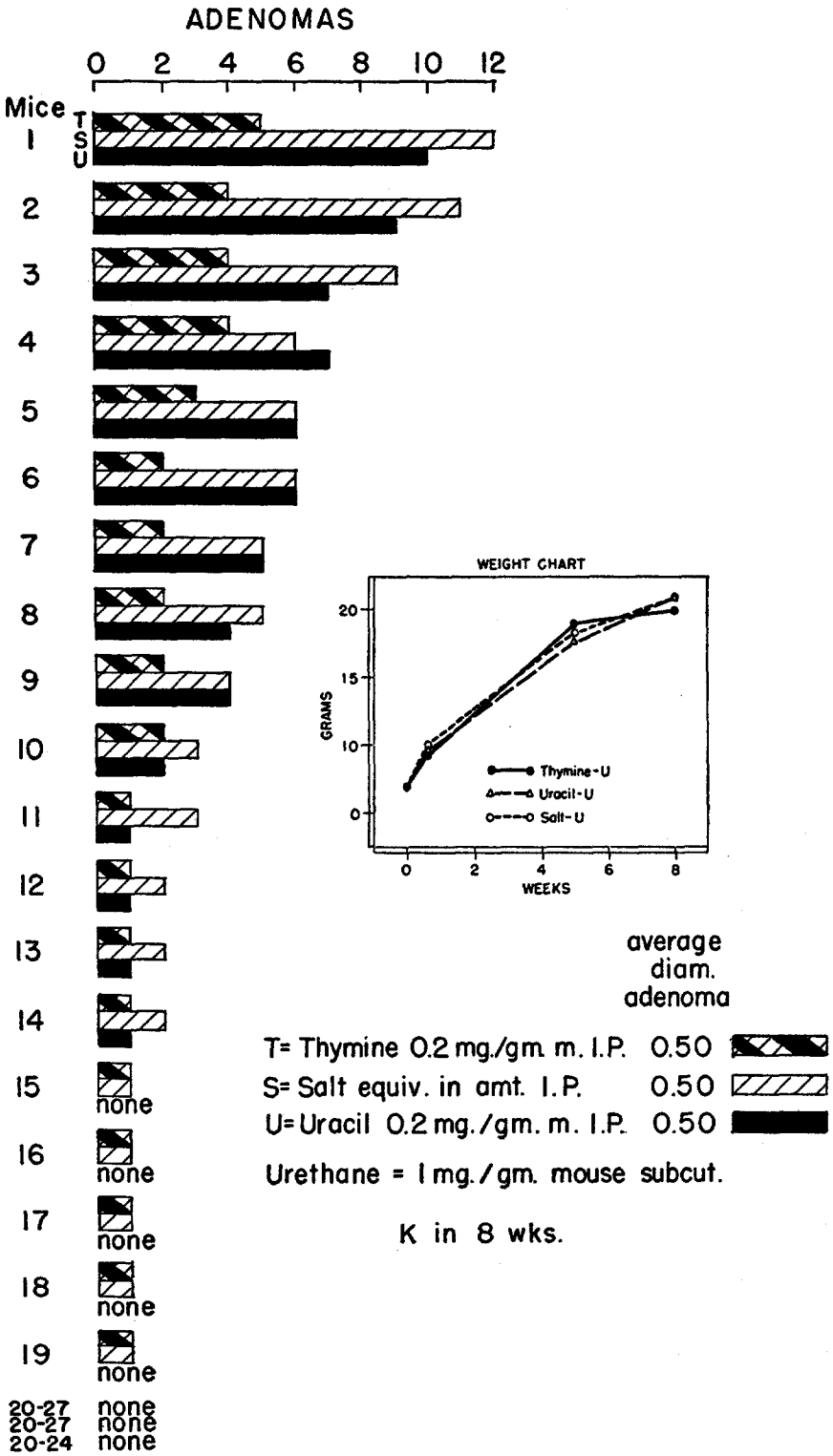


CHART 5. The inhibitory influence of thymine and the absence of effect of uracil when given immediately prior to injection of animals with urethane. (Experiment 23).

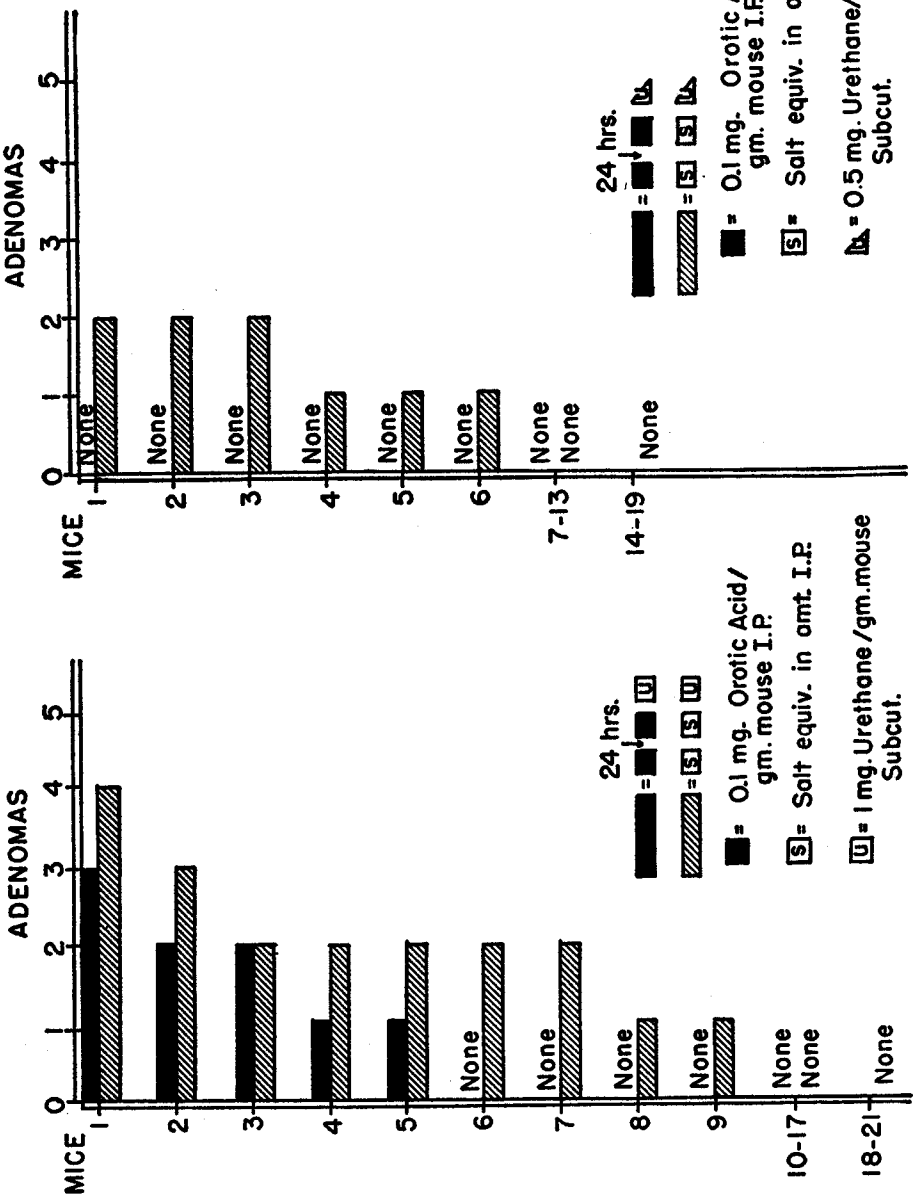


CHART 6. The inhibitory influence of orotic acid and its increasing effectiveness when asmaller dose of urethane is used. (Experiments 27 and 28).

carcinogen might be modified through the use of additional substances, should the reaction occur with sufficient rapidity and in an *in vitro* system rather than after implantation of the exposed tissues.

General Method in Vitro Experiments.—

Rabbits are among the species which, following injection with urethane, produce *a* carcinogenic intermediate acting on the pulmonary cells (19). Serum from this species taken 5 hours after intraperitoneal injection of the animals with 1.5 mg. urethane per gm. rabbit was used to supply the *in vitro* carcinogen as in previous experiments (19). Mouse lung tissue was obtained from feta mice late in gestation by the methods described by Rous and Smith (33) or from neonatal animals. Precautions for asepsis were used throughout. The lung tissue was hashed into tiny fragments between knife blades while suspended in Ringer's solution, then was separated, and divided into the number of lots necessary for the individual experiments. Each lot was suspended in the intermediate containing rabbit serum with and without addition of the metabolite under test in each experiment. Exposure was at 37°C. for the period indicated. Following exposure, the lung fragments were transferred to Ringer's solution, rinsed with 10 cc. and resuspended in fresh Ringer's. These suspensions were then implanted in the posterior thigh muscles of mice of the same strain as that supplying the tissue. In all instances material from the experimental and control groups were implanted in opposite legs of the same host. After an interval of 8 weeks the implants were excised and examined for tumors in serial section.

In the evaluation of the results it is evident that, since what is being compared is the relative response of the lung tissue exposed to the carcinogen *in vitro*, the volume of tissue surviving, rather than the number of mice implanted, is the critical factor. The amount of tissue surviving was determined histologically, all the tissue being cut serially at 6 μ and the aggregate amount of lung tissue measured exclusive of extraneous tissue and cysts. In studies involving a similar *in vitro* method but with ultraviolet radiation (34, 35) or nitrogen mustard (36) as the carcinogen all trends yet found have proved reproducible with every attempt. Nevertheless a 100 per cent difference in the response of compared tissues is used as indicating a significant difference. Only the results of the *in vitro* tests made with ureidosuccinic acid and in a single instance with uridine-5-phosphate will be reported at this time.

Experiment 33.—The lungs from 12 one week old "A" mice were excised and hashed into small pieces as described above and divided into three parts. One part was added to 1 cc. posturethane rabbit serum—Ringer's mixture (0.9 cc. serum and 0.1 cc. Ringer's); one part to a mixture containing 0.9 cc. serum plus 0.1 cc. 2 per cent ureidosuccinic acid at pH 7; and the remaining part was added to 0.9 cc. serum containing 0.1 cc. 2 per cent uridine-5-phosphate at pH 7. All were exposed simultaneously with intermittent agitation for 1 hour at 37–38°C. in a water bath. Afterwards the exposed tissues of each lot were washed with 10 cc. and followed by 5 cc. of Ringer's solution. Each was suspended in 2 cc. Ringer's and implanted in 0.2 cc. amounts in alternately opposite posterior thighs of 15 normal young adult "A" strain mice. After an interval of 2 months the hosts were killed, and the implants were excised and examined in serial section for adenomas.

The tissue exposed in this experiment to carcinogenic serum plus Ringer's yielded five adenomas; that exposed to the same serum containing uridine-5-phosphate yielded three adenomas. None were found in the tissues exposed to the same carcinogenic serum but containing 0.2 per cent ureidosuccinic acid. The tumors were scattered singly among the 30 implants of the 15 hosts used.

No difference was noticeable in the relative amount of surviving lung tissue from the three exposed lots.

The inhibitory influence of ureidosuccinic acid upon the carcinogenic effect of the urethane intermediate was confirmed in the following experiment:

Experiment 34.—This experiment was similar to Experiment 33 with the following exceptions: only ureidosuccinic acid was tested; the final concentration used was 0.1 per cent; 20 "A" strain fetuses from three pregnant does late in gestation supplied the lung tissue under test; and 18 normal young adult "A" mice were recipients of the tissue. Again the implants were examined 2 months after exposure and implantation.

The tissue exposed to the serum-Ringer's mixture developed five adenomas. That exposed to the serum–ureidosuccinic acid mixture developed only one. Except in one instance in which one implant had two tumors the adenomas were scattered singly among the 36 implants carried by the 18 hosts. No differences were noted in the amounts or general character of the implanted tissue when compared microscopically.

Uridine-5-phosphate has not been subsequently tested at this writing.

Studies with Small Molecular Precursors

Since urethane itself is not the immediate carcinogen for the lung but rather elicits or is transformed into the active carcinogen (19) it seemed worthwhile to find if any of the small molecules constituting basic building blocks of nucleic acid would influence its carcinogenic activity. Aspartic acid (18), urea (18), oxaloacetic acid (18), and formate (17, 16) appear to be among the more important of these. Only the results of representative experiments are shown in Table I, Experiments 36 to 39. Aspartic acid, urea, and formate were without any detectable effect upon the number of tumors initiated. Oxaloacetic acid, however, considerably increased the number of tumors appearing. This finding was repeated in three additional experiments. As oxaloacetic acid is chemically highly reactive, it seemed possible that urethane might be combining directly with it. Experiments in which the oxaloacetic acid and urethane were mixed, incubated at 37°C. for 2 hours and then injected together, showed no greater activity to induce tumors than when the two substances were injected separately. The results of these experiments are not shown in the table.

In view of the inactivity of aspartic acid, tests were made with asparagine as this might be a more active form in the pathway of synthesis of ureidosuccinic and orotic acid. Upon testing, asparagine was found to distinctly inhibit the carcinogenic activity of injected urethane (Experiment 37).

Negative Results with Metabolically Active Substances Which in Full-Fed Mice Are Unrelated to DNA Synthesis (Experiments 40 through 49, Table II)

In the evaluation of the specificity of any series of tests such as the above described the possibility that similar effects might occur with a great variety

TABLE II

The lack of effect of a variety of metabolically active substances not closely related to nucleic acid synthesis upon urethane carcinogenesis. The substances tested were injected in each instance immediately prior to urethane. The method of test is identical with that used in the experiments listed in Table I.

Experiment	Test substance	Method	Surviving mice	Adenomas per mouse
40	Glutathione	Glu[1]/U	25	14
		S/U	25	14
41	BAL	BAL[2]/U	18	2
		S/U	18	2
42	Glutamic acid	GA[3]/U	18	2
		S/U	19	2.4
43	Methionine	M[4]/U	28	2.5
		S/U	34	2.9
44	Cysteine	Cy[5]/U	23	1.2
		S/U	20	1.3
45	Ethionine	E[6]/U	15	0.66
		S/U	19	0.68
46	Folic acid	F[7]/U	24	3.5
		S/U	23	3.2
47	Acriflavin	Ac[8]/U	22	0.5
		S/U	23	0.5
48	Nitrogen mustard	NH[10]/U	21	2.1
		S/U	24	2.0
49	Para-aminobenzoic acid	Pb[9]/U	16	13
		S/U	16	10

The / line separating the test substance from urethane indicates the test substance was given immediately prior to urethane injection.

The test substance was given intraperitoneally in the amount indicated immediately prior to subcutaneous injection of 1 mg. urethane per gm. mouse used as a 5 per cent solution in double distilled water.

The amount of the test substances used are as follows:—

[1] Glutathione, 1.3 mg./gm. mouse.

[2] British antileuicite, 0.012 mg./gm. mouse.

[3] Glutamic acid, 1 mg./gm. mouse.

[4] Methionine, 1 mg./gm. mouse.

[5] Cysteine, 0.9 gm./gm. mouse.

[6] Ethionine, 0.5 mg./gm. mouse.

[7] Folic acid, 0.05 mg./gm. mouse.

[8] Acriflavin, 0.025 mg./gm. mouse.

[9] Para-aminobenzoic acid, 0.06 mg./gm. mouse.

[10] Nitrogen mustard, 0.001 mg./gm. mouse.

All solutions were isotonic and neutralized to pH 7.

of metabolic substances is obvious. In studies directed toward finding other intermediary pathways influencing the activity of injected urethane, wholly negative results have so far been obtained. The method of test was similar to that of the preceding *in vivo* experiments. The individual substances tested include those listed in Table II. In this table the substance used, the amount given, the number of surviving mice, and the average number of tumors in the experimental and control groups are shown. All were used in amounts approaching the amount maximally tolerated by mice. In none of these instances was a significant influence demonstrated. Other substances which have proved without effect, but which have only been tested on one occasion, include potassium cyanate, choline, and histidine.

DISCUSSION

The fact is clear that the exposure of mice to nucleic acid hydrolysates, certain of the components of nucleic acid, and substances influencing nucleic acid synthesis, such as aminopterin and pyrimidine and purine precursors, simultaneously with urethane profoundly modifies the effectiveness of the carcinogen. The absence of effect of biologically active substances not particularly related to nucleic acid synthesis (Table II) speaks for the general chemical specificity of the results. In view of our limited knowledge of nucleic acids and their synthesis, the crudeness of the test methods, and the complexities of the problem under study it is not possible to interpret the meaning of the results except in a very general way. However, when the experimental findings are examined in relationship to one another and in relation to known facts concerning the intermediary metabolism of nucleic acid it would appear that certain definitive conclusions may be drawn.

The profound inhibition of the initiation of adenomas effected by simultaneous injection of DNA with urethane is striking (Experiments 1 and 2, Chart 1). An almost equal inhibitory influence was obtained in preliminary experiments not included in this report when an RNA hydrolysate was used with urethane. In contrast to DNA however the injection of the ribosenucleic acid hydrolysate made the animals ill for the 1st week and the reduction in number of tumors could be ascribed in considerable degree to an over-all influence on the health of the animals and reduced growth of the tumors (14). Because of this RNA hydrolysates were not subsequently used though the components of RNA like DNA were tested in the experiments which followed. As the effects of either of these substances might be through mechanisms other than an influence upon nucleic acid synthesis, the results of the experiments in which animals were pretreated with aminopterin under conditions known to inhibit nucleic acid synthesis (17) (Experiments 3 to 5) are particularly pertinent. The potentiation of the carcinogenic effect of urethane occurred despite the fact that the aminopterin treated animals did poorly, as shown by their

weight curves (Chart 2), and that their adenomas grew at a slower rate (Table I). Both these conditions alone would yield results tending toward an over-all reduction in number of tumors (14). The lack of any effect of aminopterin when given over too short a period to influence nucleic acid synthesis by the time of urethane injection (17) and the prevention of the potentiating effect of aminopterin by injection of the animals with DNA (Experiments 7 and 8) (Chart 2) provide evidence pointing toward the chemical specificity of both aminopterin and DNA. Biologic parallels to the prevention of an aminopterin effect with DNA have been demonstrated both in the reversal of the aminopterin inhibition of growth in the chick embryo by DNA (38) and in the dilution of the incorporation of isotopically tagged formate into DNA by thymidine, a path of synthesis known to be blocked by aminopterin (39). That there is a relationship between the synthesis of nucleic acid and the mechanism of carcinogenesis brought about through exposure of mice to urethane seems clear. The results of Experiments 9 to 39, in which the various components of both DNA and RNA were put to test add weight to this conclusion.

The interpretation of the results of the experiments in which various nucleic acid components and precursors were used is conditioned by the methods of testing and by many important variables. A foremost possibility relates to whether the action of various metabolites tested is at the level of formation of the carcinogenically active intermediate derived from or elicited by urethane (19) or at the level of the action of the carcinogen upon the pulmonary cells. Since ureidosuccinic acid (Experiments 33 and 34) was demonstrated to have *in vitro* influences upon the carcinogenic effect of the active intermediary substance, it appears that this substance at least and, in all probability, other more fully formed components of nucleic acid exert their influences at the level of the pulmonary cells. Another possibility relates to whether the inhibitory action of any of these substances is simply related to an over-all inhibition of growth or whether they produce their effect without influencing the growth rate of the animals. As described none of the substances, which when given with urethane resulted in a reduction in the number of tumors initiated, were found to reduce the mitotic rate of ear epithelium of animals injected with similar amounts of these substances and biopsied 5 hours later. It is evident that the possible activity of any of these agents depends upon its being present at the level of the pulmonary cells in the proper concentration and at the proper time to block or by-pass any interference or imbalance in the synthetic chain set off by the carcinogen. Many of these molecules are degraded at rapid rates (37). For these reasons the negative experiments *in vivo* cannot be regarded in any sense as conclusive tests. This is particularly pertinent in relation to the activity or lack of activity of the nucleotides tested. Though thymidylic acid (40), uridylic acid (41), cytidylic acid (42), desoxycytidylic acid (43), and

uridine-5-phosphate (44) have all been shown to be incorporated into the DNA of mammals upon injection, only cytidylic acid (Experiments 13 to 15, Chart 3 exerted any *in vivo* influence upon the effectiveness of the carcinogen. On the other hand ureidosuccinic acid, inactive *in vivo*, exerted an inhibitory action in the *in vitro* tests (Experiments 33 and 34). The *in vivo* effect of cytidylic acid proved of special interest in view of the opposite and potentiating influence of adenine (Experiment 19) and its precursor, 4-amino-5-imidazole carboxamide (28) (Experiment 20), upon the carcinogenic activity of urethane. This is particularly the case since an antagonism has been demonstrated between adenosine and cytidine in mutant strains of *Neurospora* (45). It is noteworthy that isotopically labelled cytidylic acid contributes both to RNA and DNA pyrimidines (46). That desoxycytidylic acid was without effect upon the adenomatous response is perhaps a parallel to its lack of activity in diluting the incorporation of desoxyuridine into thymidine in bone marrow or Ehrlich ascites tumor cell systems (40). Bearing as well on these other negative tests there is considerable reason to believe that there is a large variation in the uptake of individual nucleic acid components by somatic cells of different sorts (37). The disparity however in the results obtained with these mononucleotides as compared with the tetranucleotide exposure (DNA) is striking and suggests that certain nucleotide combinations arising from the *in vivo*, presumably enzymatic (46), hydrolysis of the tetranucleotides might account for the profound inhibitory effect of DNA.

Because of the potentiating effect of adenine and the absence of effect of 2,6-diamino purine (Experiment 21) when given under conditions known to interfere in purine synthesis (47), the purine nucleotides were not tested.

Among the pyrimidine bases the activity of thymine (Experiments 22 and 23, Chart 5) was striking. This result seems the more important since the thymine used was prepared synthetically rather than derived from nucleic acid and, hence, could not possibly be contaminated with other nucleic acid components which could conceivably be present in small amounts in pyrimidine bases and in nucleotides of natural origin. It seems particularly significant that of the pyrimidines only thymine has been shown to be incorporated specifically into DNA (46) Uracil, on the other hand, known to be incorporated into RNA (37, 46) exerted no influence on the carcinogenic actions of urethane. It has also been demonstrated by Fink and Fink (48) that supplementary thymine diminishes the incidence of spontaneous pulmonary adenomas in mice. It is perhaps noteworthy that cytosines 5-methyl equivalent to thymine, 5-methyl cytosine, exerted an inhibitory influence in the same direction as thymine. Though this difference (Experiment 24) did not prove statistically significant under the method of test it is considered to indicate a trend and fits with the fact that thymine is one of the degradation products (37) of 5-methyl-cytosine.

The inhibitory action of thymine raised the possibility that the primary site of action of the carcinogen might be at the level of the pyrimidine precursors.

Of the various precursors and components of nucleic acid which have been traced by isotopic technics into the formed nucleic acid molecule, orotic acid has proved to be one of the substances most effectively and universally in-

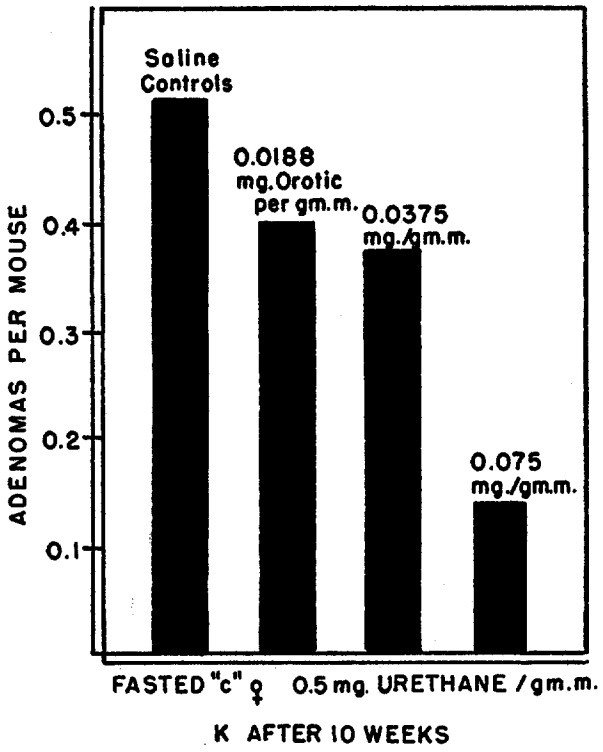
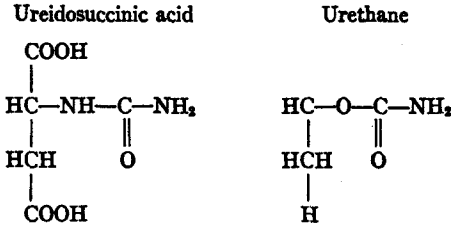


CHART 7. The inhibitory influence of orotic acid and its increasing effectiveness in this respect as larger amounts are used in fasted animals receiving constant amounts of urethane. (Experiment 29).

corporated in nucleic acid synthesis. It has been found exclusively in the pyrimidines. The studies describing these findings have been recently reviewed (37). It appears of special importance therefore that, of the various agents tested for influence upon urethane carcinogenesis, orotic acid proved by far the most active *in vivo* inhibitor (Experiments 27 to 29). That its action is suggestive of competitive inhibition may prove especially pertinent (Experiments 28 and 29, Charts 6 and 7). Nevertheless, the inhibitory activity of dihydro-orotic acid (Experiment 30) suggests that the point of interference of the carcinogen lies among the precursors of orotic acid since dihydro-orotic acid is its precursor

(32, 18). The inhibition of the *in vitro* activity of the carcinogen by ureidosuccinic acid, the precursor to dihydro-orotic acid (32), adds further weight to this view (Experiments 33 and 34). With ureidosuccinic acid it is plain that the time of exposure and perhaps the concentration play a critical role in the action of metabolites upon the carcinogenic process, it having no effect *in vivo* (Experiments 31 and 32) and yet distinct *in vitro* inhibitory influences (Experiments 33 and 34). In addition, it must be pointed out that, despite our knowledge of the incorporation of ureidosuccinic acid into nucleic acid pyrimidines *in vitro*, it has yet to be shown that when injected into intact animals this substance enters into the pathway of nucleic acid synthesis (46). Although its *in vitro* inhibitory activity (Experiments 33 and 34) does not necessarily indicate that its immediate action under the conditions of test were mediated through its relation to nucleic acid, this interpretation seems the more likely in view of the other findings. That an effect was brought about at all indicates that part of the metabolic sequence of carcinogenesis may be studied through these *in vitro* means.

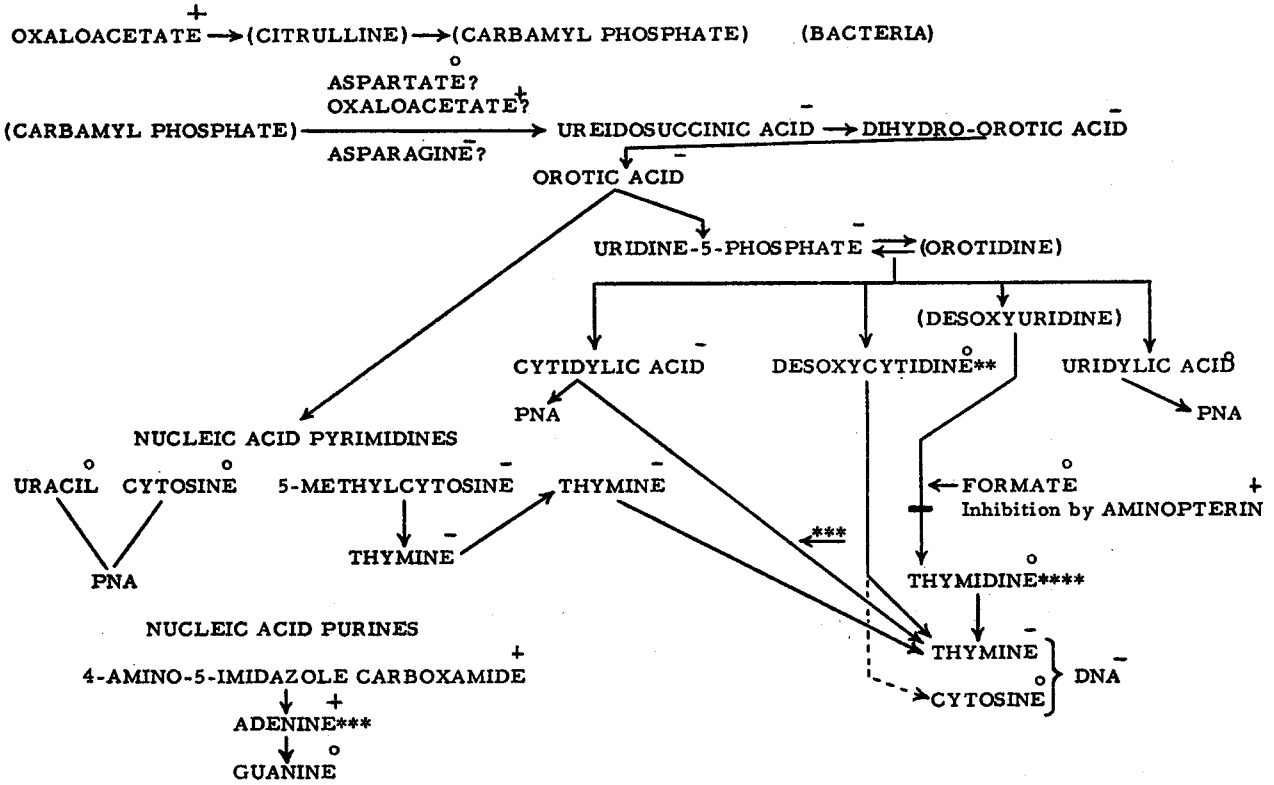
The significance of the *in vitro* activity of ureidosuccinic acid has perhaps more specific relations. Among the foremost of these is its remarkable similarity in molecular structure to urethane:



This similarity has further interest when it is considered that oxaloacetic acid which has been shown to be a precursor to ureidosuccinic acid (18) potentiates the *in vivo* carcinogenic activity of urethane (Experiment 38). The well known interrelation between oxaloacetic acid, aspartic acid, Krebs's cycle, and the incorporation of the carbamyl group of citrulline into pyrimidines through ureidosuccinic acid appears pertinent (see review, reference 37) to the interpretation of this result. This is particularly the case since an intermediary substance, and not urethane itself, is the active carcinogen at the level of the lung (19). Might urethane, for example, be interfering with or diluting the utilization of carbamyl groups ($NH_2—\overset{\overset{O}{\|}}{C}—R$) in the synthesis of ureidosuccinic acid and thereby producing the carcinogenic intermediate interfering in the synthesis of nucleic acid pyrimidines? The evidence in this report is consistent with such a view.

When taken in relation to one another the above facts make it evident that the mechanism of action of urethane in initiating these pulmonary tumors is closely associated with nucleic acid synthesis. Whether RNA or DNA is particularly involved is however much less clear. The potentiating or inhibitory activities of the various substances used are all consistent with an influence on DNA synthesis. This, however, is not the case for RNA. Aminopterin, for example, is known to interfere in both adenine and thymine synthesis (46). As adenine itself markedly potentiates the carcinogenic effect of urethane (Experiment 19, Chart 4) an interference in its synthesis would scarcely be expected to also potentiate, as was the effect produced by aminopterin (Experiments 3, 4, 5, 7, and 8). As it is through an interference in the production of adenine that aminopterin interferes in RNA synthesis (37) one would conclude that an effect on RNA is not the influence of urethane which is being inhibited by components of the injected DNA hydrolysate (Experiments 1, 2, 7, and 8). It is then much more likely that the effect of aminopterin on urethane carcinogenesis is through its interference in the synthesis of thymine. The inhibitory activity of thymine itself (Experiments 22 and 23) is also inconsistent with an influence of urethane on RNA as thymine has been found by isotope tracer methods to be incorporated specifically in DNA and not degraded or modified in a way so as to become part of RNA (46). The lack of effect of uracil, when like thymine given only at the time of urethane injection (Experiment 23) adds further weight to the view that the influence of urethane is on DNA synthesis for uracil is known to be incorporated into RNA upon injection but not DNA (46). Moreover (in preliminary experiments not included herein) when uracil is given the day prior to urethane as well as at the same time and thus has an opportunity to be degraded or modified and used in resynthesis of other nucleic acid pyrimidines (37) it has an inhibitory influence. The potentiating effect of aminopterin seems best explained by a decrease in the *de novo* synthesis of DNA thymine produced by aminopterin and a consequent increase in uptake of exogenously provided thymine and perhaps thymine-like substances. Such effects are well known in experiments in which isotopically tagged purines are used (49). On the basis of these findings it would appear likely that the action of urethane is related to DNA rather than RNA synthesis. But what sort of an effect is the carcinogen exerting. Conceivably the carcinogen could be an atypical pyrimidine ultimately incorporated in nucleic acid and henceforth resynthesized and passed along from cell to cell. On the other hand, its action could be merely the result of temporary interference in the synthesis of certain nucleic acid components, the latter possibly ultimately modifying the final structure of the nucleic acid molecule through a change in distribution of components at the time of synthesis. As pointed out above, the potentiation of urethane carcinogenesis by aminopterin (Experiments 3 to 5) could be considered a result of a diminution in the diluting effect of otherwise normally

CHART 8. Test agents and known pathway of nucleic acid synthesis.*



+, increased number of tumors initiated by urethane. −, decreased number of tumors initiated by urethane. 0, no effect under test conditions.

* Components and general path of DNA synthesis in mammals taken from literature (37, 46).

** Desoxycytidylic acid was used in experiments in this paper.

*** Antagonism between adenosine and cytidine shown in *Neurospora*.

**** Calcium thymidylate was used in experiments in this paper.

synthesized thymine (39, 37) upon the incorporation of any new atypical pyrimidine into the DNA of the cells undergoing neoplastic change. This is perhaps the case also with adenine (Experiment 21) a known antagonist of cytidylic acid—a precursor of DNA thymine (46) (Chart 8). Furthermore, if the carcinogenic effect were simply a non-specific reduction of available thymine at the time of synthesis of DNA, one might expect aminopterin itself to be carcinogenic. This has not been the case in repeated experiments (from unpublished data). On the other hand, injections of mice with a hydrolysate of adenoma DNA yielded no tumors in a single test. However, if this postulated atypical pyrimidine is synthesized within the pulmonary cell—as would seem likely to be the case because of the *in vitro* inhibitory effect of ureidosuccinic acid (Experiments 33 and 34)—it might be that following *in vivo* injection, the atypical pyrimidine is degraded before reaching the lung. That certain pyrimidines are rapidly degraded is well established (37). Future *in vitro* tests may cast light on this point. Whatever the fact may be it is evident that the sequence of biochemical syntheses in the pathway to the ultimate carcinogenic effect may be blocked or diluted by substances active at the respective levels in nucleic acid synthesis of ureidosuccinic acid, dihydro-orotic acid, orotic acid, cytidylic acid, and at the level of synthesis of DNA thymine (Chart 8). Though it seems likely that the carcinogenic influence of urethane is exerted through modifying DNA rather than RNA synthesis, lack of evidence as to the specific relation at this time precludes anything other than the suggestion that the ultimate carcinogenic effect is nuclear. Nevertheless, this view is consistent with facts previously reported concerning the initiation of pulmonary adenomas with ultraviolet radiation (34, 35) and nitrogen mustard (36).

SUMMARY

The process of carcinogenesis following exposure of mice to urethane is demonstrated in the present work to be intimately related to nucleic acid synthesis. Injection of animals with a DNA hydrolysate immediately prior to a single exposure of the animals to urethane markedly reduced the number of pulmonary adenomas initiated. Aminopterin, known to interfere in nucleic acid synthesis (46), potentiated the carcinogenic action of urethane and this potentiation was blocked by injection of a DNA hydrolysate.

Of the components and precursors of nucleic acids the pyrimidine series seemed especially concerned. Alterations in the utilization of oxaloacetate, ureidosuccinic acid, dihydro-orotic acid, orotic acid, cytidylic acid, and thymine appeared to be critical steps in the oncogenic process, following upon the primary disorder of cellular metabolism initiated by the carcinogen. All these substances except oxaloacetate profoundly reduced the number of tumors initiated by urethane. Oxaloacetate potentiated the carcinogenic effect.

When these results are viewed together and in relation to known facts concerning nucleic acid synthesis they provide evidence suggesting that the point

of action of the carcinogen is in the pathway of nucleic acid synthesis below orotic acid and perhaps at the level of ureidosuccinic acid.

The potentiating influence of adenine, 4-amino-5-imidazole carboxamide, and aminopterin, the lack of effect of uracil, and the inhibitory influence of thymine together suggest that DNA rather than RNA is the nucleic acid critical to the oncogenic response of mice to urethane.

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