Reaction of Urethane with Nucleic Acids *in vivo*

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1. [1-¹⁴C]Ethyl carbamate, ethyl [*carboxy*-¹⁴C]carbamate, [1-¹⁴C]ethanol and sodium hydrogen [¹⁴C]carbonate were injected intraperitoneally into C57 mice, and nucleic acids and proteins were separated from the liver and lungs with phenol as described by Kirby (1956). 2. Chromatographic analysis of the hydrolytic products of the urethane-labelled RNA showed the presence of a single radioactive compound differing in behaviour from the major pyrimidine nucleotides and purines. 3. The products from RNA labelled by [1-¹⁴C]ethyl carbamate or ethyl [*carboxy*-¹⁴C]carbamate appeared chromatographically identical but could not be detected in the RNA of mice given [1-¹⁴C]ethanol or sodium hydrogen [¹⁴C]carbonate. 4. The labelled product appeared to be the ethyl ester of cytosine-5-carboxylic acid formed by the reaction of urethane with RNA *in vivo*. 5. A direct reaction between labelled urethane or the labelled metabolite of urethane, [1-³H]-ethyl *N*-hydroxycarbamate, and RNA was not detected.

Previous work using [1-¹⁴C]ethyl carbamate and ethyl [*carboxy*-¹⁴C]carbamate for investigation of the metabolism and mechanism of action of urethane (Skipper *et al.* 1951; Berenblum, Haran-Ghera, Winnick & Winnick, 1958) showed no marked localization in the mouse tissues. Over 90% of the ¹⁴C left the body within 24 hr. but a small proportion, particularly of that from the [1-¹⁴C]-ethyl carbamate, remained. Comparison of incorporation from urethane with that from [1-¹⁴C]-ethanol and sodium hydrogen [¹⁴C]carbonate indicated that most of this residue was due to metabolic incorporation of the breakdown products of urethane into the tissues.

Although Berenblum *et al.* (1958) determined the subcellular distribution of labelled urethane they did not investigate the labelling of the nucleic acids. There was evidence, however, that urethane combines physically with nuclei and mitochondria.

In the present work, use was made of the phenol fractionation techniques developed by Kirby (1956) to determine the location of ¹⁴C in the liver and lungs of C57 mice after the intraperitoneal injection of ethyl [*carboxy*-¹⁴C]carbamate or [1-¹⁴C]ethyl carbamate.

The carcinogenic action of urethane may be due to modification of the nucleic acids, but to demonstrate a reaction *in vivo* it is necessary to isolate nucleic acids labelled by radioactive urethane and to show that a modified base has been labelled. Brookes & Lawley (1965) found that mustard gas [di-(2-chloroethyl) sulphide] preferentially alkylates

guanine, but the present results suggest that urethane attacks the pyrimidine bases of the nucleic acid.

MATERIALS AND METHODS

Animals. Male and female C57 black mice weighing 25–30 g., approx. 3 months old, were maintained on a 20% protein rat-cake diet for at least 48 hr. before death. The addition of crushed maize to the diet was avoided because it made the separation of the aqueous and interfacial layers during the subsequent fractionation of the liver and lungs difficult.

Chemicals. Cytosine, cytidine, cytidylic acid [cytidine-2'(3')-monophosphoric acid, mixed isomers], orotic acid (uracil-6-carboxylic acid), adenine and guanine were obtained from British Drug Houses Ltd., Poole, Dorset. Cytosine-5-carboxylic acid and uracil-5-carboxylic acid (iso-orotic acid) were purchased from Sigma Chemical Co., St Louis, Mo., U.S.A.; these acids and their ethyl esters (and that of orotic acid) were also made by the methods outlined by Wheeler, Johnson & Johns (1907) and Wheeler & Johns (1908). *N*⁴-Hydroxycytidine was prepared by the method of Brown & Schell (1965) and *N*⁴-ethoxycarbonyl-cytosine (kindly supplied by Dr R. Nery) by the reaction of cytosine and ethyl chloroformate. 5-Methylecytosine was obtained from Sigma Chemical Co.

Enzymes. Purified uridine phosphorylase (uridine-orthophosphate ribosyltransferase) was prepared from frozen *Escherichia coli* by DEAE-cellulose chromatography (Krenitsky, Barclay & Jacquez, 1964), the cells being ruptured by sonic vibration (Paegle & Schlenk, 1952). Ribonuclease A and a suspension of alkaline phosphatase from *E. coli* in (NH₄)₂SO₄ soln. were obtained from Sigma Chemical Co., deoxyribonuclease I(D) and *Crotalus*

adamanteus venom phosphodiesterase were from Worthington Biochemical Corp., Freehold, N.J., U.S.A., and crude *Crotalus adamanteus* venom was from Koch-Light Laboratories Ltd., Colnbrook, Bucks.

Radioactive materials. [$1\text{-}^{14}\text{C}$]Ethyl carbamate (specific radioactivity 0.5–2 mc/m-mole) and ethyl [$\text{carboxy-}^{14}\text{C}$]carbamate (specific radioactivity 1–5 mc/m-mole) were obtained from the New England Nuclear Corp., Boston, Mass., U.S.A. [$1\text{-}^{14}\text{C}$]Ethanol (specific radioactivity 5–20 mc/m-mole) and $\text{NaH}^{14}\text{CO}_3$ (specific radioactivity 20–50 mc/m-mole) were supplied by The Radiochemical Centre, Amersham, Bucks. Each mouse was injected intraperitoneally with 0.1 ml. of an aqueous solution of one of these compounds, containing 0.1 mc of one of the first three compounds/ml. or 1 mc of $\text{NaH}^{14}\text{CO}_3$ /ml. The mice were given approx. 18–71 mg. of [$\text{ethyl-}1\text{-}^{14}\text{C}$]urethane/kg. or 7–36 mg. of [$\text{carboxy-}^{14}\text{C}$]urethane/kg. [$1\text{-}^3\text{H}$]Ethyl *N*-hydroxycarbamate (0.4 mc/m-mole) was made from [$1\text{-}^3\text{H}$]ethanol and kindly supplied by Dr R. Nery.

Isolation of the nucleic acids and proteins. To obtain sufficient lung tissue 40 mice, of one sex, were used for each experiment. The animals were killed 24 hr. after injection, and the liver and lungs were immediately dissected out and laid on solid CO_2 .

The isolation of the DNA, RNA and protein was achieved as described by Brookes & Lawley (1965), but the original method of Kirby (1956) was used to remove polysaccharide. The frozen livers were homogenized in at least 15 parts (v/w) of 0.5% (w/v) sodium naphthalenedisulphonate with a Waring Blender, and then stirred for 1 hr. with 15 vol. of aq. 90% (w/v) phenol to which had been added 12% (v/v) of *m*-cresol and 0.1% (w/v) of 8-hydroxyquinoline. An Ultra-Turrax homogenizer was more effective than the Waring Blender for breaking up the fibrous lungs. Some difficulty was initially encountered in the subsequent disruption of the protein–nucleic acid complexes, but this was overcome (as suggested by Dr Stanfield Rogers) by the use of freshly distilled phenol for the Kirby (1956) phenol mixture and a selected batch of sodium dodecyl sulphate.

Chromatography. Whatman no. 4 paper was used for ascending chromatography with solvent (1) methanol–conc. HCl –water (7:2:1, by vol.) or (2) ethanol–aq. NH_3 soln. (sp.gr. 0.88)–water (40:1:9, by vol.). All-glass tanks were necessary for the highly corrosive solvent (1). Whatman no. 1 paper was used for descending chromatography in solvent (3) butan-1-ol–water (43:7, v/v), (4) butan-1-ol–aq. NH_3 soln. (sp.gr. 0.88)–water (85:2:12, by vol.) or (5) butan-1-ol–acetic acid–water (2:1:1, by vol.).

The papers were dried, examined under a u.v. lamp and cut into 1 cm. strips. These strips were eluted with 0.1 *N*- HCl and E_{280} of samples of these eluates was determined.

Detection of radioactivity. The positions of the u.v.-absorbing and radioactive spots were compared by adding other samples of the eluates to bottles for a Tri-Carb liquid-scintillation spectrometer (Packard Instrument Co. Inc., Downers Grove, Ill., U.S.A.). The eluates were neutralized with NaHCO_3 soln. to prevent acid quenching of the fluorescence of the scintillation fluid. To ensure that all the radioactive material had been eluted and detected, in some cases the strips from the chromatograms were dropped directly into scintillation fluid.

Only small amounts of ^{14}C were present and a careful check was therefore made for ^3H contamination or variation

in the quenching of the scintillation fluid by use of the channels-ratio facility on the Packard instrument.

Assay of radioactive tissue components. To prevent precipitation, RNA and DNA solutions were first incubated with ribonuclease or deoxyribonuclease respectively, and proteins were dissolved in tetraethylammonium hydroxide before addition to the scintillation fluid, as described by Roberts & Warwick (1966). The scintillation fluid containing protein samples was kept in the dark for 10–12 hr. before counting to ensure the decay of the long-lived phosphorescence of alkaline scintillation fluid exposed to light.

Nucleic acid hydrolysis. RNA was hydrolysed to the purine bases and pyrimidine nucleotides, and DNA was partially hydrolysed, by treatment with *N*- HCl for 1 hr. at 100° (Smith & Markham, 1950). Hydrolysis by formic acid in sealed ampoules for 1 hr. at 180° (Wyatt, 1951) was followed by evaporation of the formic acid in a vacuum desiccator containing NaOH pellets. The effect of treatment with 20% (v/v) H_2SO_4 for 2 hr. at 170° in sealed ampoules on the ^{14}C labelling of the RNA was also determined.

DNA was converted into nucleotides by treatment with deoxyribonuclease, followed by *Crotalus adamanteus* venom phosphodiesterase. Nucleic acid (5 mg.) was dissolved in 1 ml. of 0.01 *M*- NH_4HCO_3 soln., pH 7, and an equal volume of 0.015 *M*- MgCl_2 was added. The mixture was incubated at 37° for 1 hr. with 0.015 ml. of deoxyribonuclease solution (concn. 5 mg./ml.), the pH was raised to 8.5 with aq. NH_3 soln., and incubation was continued for a further 2 hr. with 0.025 ml. of a solution containing phosphodiesterase (5 mg./ml.). This mixture was chromatographed on paper, or chromatographed on a column (10 cm. $\times$ 1.5 cm.) of Whatman PDE100F2 experimental fast-flow DEAE-cellulose developed with 500 ml. of a 0.01–0.2 *M*- NH_4HCO_3 gradient.

Further treatment of the RNA hydrochloric acid hydrolysate before chromatography. (a) Periodate oxidation. Tankó, Zsindely & Berencsi (1967) showed that treatment with periodate followed by lysine, under carefully controlled conditions, liberates bases from mononucleotides. Hydrochloric acid hydrolysates of labelled RNA treated in this manner contained no specific compounds that could be detected on chromatograms.

(b) Hydrolysis by alkaline phosphatase and nucleosidase. The labelled RNA was hydrolysed in *N*- HCl and the mixture neutralized by the addition of aq. NH_3 soln. and removal of the excess on a steam bath. To 0.1 ml. of the solution (containing approx. 5 mg. of RNA) were added 0.4 ml. of 0.01 *M*-tris–acetate buffer, pH 8, and 0.1 ml. of the *E. coli* alkaline phosphatase suspension. Incubation for 2 hr. at 37° was followed by the addition of 0.5 ml. of 0.1 *M*-sodium phosphate buffer, pH 8, containing approx. 1 uridine-splitting unit of nucleosidase (Krenitsky *et al.* 1964), and incubation for another 15 hr. The incubated material was then chromatographed.

(c) Hydrolysis by crude snake (*Crotalus adamanteus*) venom. This venom contains phosphodiesterase and 5'-nucleosidase. Hydrochloric acid hydrolysate of RNA (containing about 5 mg. in 0.1 ml.) was neutralized and incubated overnight at 37° with 0.4 ml. of 0.01 *M*-tris–acetate buffer, pH 8, containing 1 mg. of venom. The product was examined by paper chromatography.

(d) Reaction with hydrazine. This reagent reacts with pyrimidines to form pyrazoles or pyrazolones and urea. A

method based on that of Baron & Brown (1955) was used. RNA (about 5mg./ml.) was dissolved in *N*-HCl and hydrolysed by heating it on a steam bath for 1 hr. The hydrolysate was cooled and neutralized with *N*-NaOH, and the mixture was made 50% (v/v) with respect to hydrazine hydrate. This mixture was heated for a further 1 hr. at 70–75° and compared chromatographically with known pyrimidines treated with hydrazine, the position of the pyrazolone and pyrazole derivatives being detected by examination under a u.v. lamp followed by spraying with FeCl₃ soln. (15%, w/v, in 10%, v/v, HCl) or Ehrlich's reagent (Dihlmann, 1953). Ethyl acetate did not extract pyrazolone or pyrazole derivatives containing a carboxyl group from the reaction mixture.

Control experiments. Ethyl [*carboxy*-¹⁴C]carbamate (0.025mc) was added to the sodium naphthalenedisulphonate solution used during the homogenization of about 25g. of normal mouse liver. The tissue was then fractionated in the normal manner. Ethyl [*carboxy*-¹⁴C]carbamate (0.025mc) was also added to unlabelled liver RNA (2mg. in 1 ml.) just before HCl hydrolysis and the radioactivity on the subsequent chromatograms was compared with that of urethane treated alone in the same way.

Experiments in vitro. [1-¹⁴C]Ethyl carbamate or ethyl [*carboxy*-¹⁴C]carbamate (0.01 mc of either) or 0.5 mc of [1-³H]ethyl *N*-hydroxycarbamate (0.4 mc/m-mole) was added to 100mg. of normal liver RNA dissolved in 5ml. of 0.2M-tris-acetate buffer, pH 6. After incubation for 1 hr. at 37° the RNA was precipitated by making the mixture 70% (v/v) with respect to ethanol. The RNA was then freed from excess of urethane by redissolving and reprecipitating it twice, and the radioactivity of the washed RNA was determined.

Protein. The proteins ('cytoplasmic' and 'nuclear') isolated from the livers of mice given labelled urethane were also heated at 100° in *N*-HCl for 1 hr. and chromatographed on paper.

RESULTS

Table 1 shows the labelling associated with the liver and lung proteins precipitated from the phenol used in the first extraction ('cytoplasmic'), with the proteins liberated from the interfacial

layer ('nuclear') and with the RNA and DNA 24 hr. after injection of either of the two labelled urethanes, labelled sodium hydrogen carbonate or labelled ethanol. All four compounds labelled the proteins and RNA but only the urethanes definitely labelled the DNA.

For chromatography the labelled RNA was hydrolysed with *N*-hydrochloric acid for 1 hr. at 100°; this treatment caused little loss of radioactive material. The radioactive component in DNA and RNA labelled by either urethane was found, however, to be unstable to more vigorous hydrolysis; after heating in formic acid at 180° for 1 hr. and evaporation over sodium hydroxide pellets in a vacuum desiccator most of the ¹⁴C was lost. The radioactivity was also greatly decreased by heating the nucleic acids in 20% (v/v) sulphuric acid in sealed ampoules at 170° for 2 hr. before placing them in the desiccator.

Chromatography of the urethane-labelled liver RNA hydrochloric acid hydrolysate gave a single radioactive spot associated with, but differing slightly from, the normal pyrimidine nucleotides in four solvents, but in solvent (2) ¹⁴C was located close to adenine (Table 2 and Fig. 1). The chromatographic mobilities of the radioactive materials in the RNA labelled by ethyl [*carboxy*-¹⁴C]carbamate and by [1-¹⁴C]ethyl carbamate were identical (Table 2). About 80% of the ¹⁴C from the urethane-labelled RNA was found in this single spot, but no definite radioactive region could be detected on chromatograms of the hydrochloric acid hydrolysates of RNA labelled by [1-¹⁴C]ethanol or sodium hydrogen [¹⁴C]carbonate developed with solvent (1). However, this may be partly due to incorporation causing the small amount of ¹⁴C to be divided between the four or five (including ribose) spots of the normal components of RNA.

Treatment of the hydrochloric acid hydrolysate

Table 1. *Labelling of mouse liver and lung tissue fractions 24 hr. after intraperitoneal injection of ¹⁴C-containing compounds*

Labelling compound	Radioactivity (counts/min./mg.)							
	Liver				Lung			
	Cytoplasmic protein	Nuclear protein	RNA	DNA	Cytoplasmic protein	Nuclear protein	RNA	DNA
[1- ¹⁴ C]Ethyl carbamate	330	320	500	410	210	160	410	320
Ethyl [<i>carboxy</i> - ¹⁴ C]carbamate	200	190	600	320	80	80	130	100
[1- ¹⁴ C]Ethanol	400	350	430	12	320	330	230	14
NaH ¹⁴ CO ₃	630	730	200	8	180	140	160	10

The radioactivity is expressed as counts/min./mg. corrected to 100% efficiency. When NaH¹⁴CO₃ was used ten times as much ¹⁴C was given (see the text). The values given are the mean of two separate experiments; variation was within 20%. Each sample contained 1.25mg. of protein or nucleic acid. The overall counting efficiency, with quenching of the scintillation fluid by the sample solutions, was 70–80%.

Table 2. R_F values of some purine and pyrimidine derivatives on paper chromatograms

Composition of the solvents and other details are given in the text.

Compound	Further treatment Solvent ...	R_F				
		(1)	(2)	(3)	(4)	(5)
Cytosine	None	0.54	0.51	0.12	0.17	0.38
Cytidine	None	0.56	0.46	0.08	0.09	0.33
Cytidylic acid	None	0.67	0.10	0	0	0.20
Cytidylic acid	Hydrazine	0.62	0.65	0.39	0.38	0.66
5-Methyleytosine	None	0.65	0.54	0.23	0.22	0.44
Cytosine-5-carboxylic acid	None	0.44	0.31	0.01	0.10	0.0
Cytosine-5-carboxylic acid	Hydrazine	0.44	0.25	0.01	0.02	0
Ethyl cytosine-5-carboxylate	None	0.65	0.54	0.56	0.32	0.70
Ethyl cytosine-5-carboxylate	Hydrazine	{ 0.33 0.61 }	0.24	0	0	0.60
N ⁴ -Ethoxycarbonylcytosine	None	0.74	0.59	0.20	0.20	0.76
N ⁴ -Hydroxycytidine	None	0.75	0.58	0.27	0.25	0.51
Uracil	None	0.66	0.58	0.30	0.30	0.58
Uridine	None	0.70	0.57	0.13	0.20	0.46
Uridylic acid	None	0.83	0.13	0.10	0	0.32
Uridylic acid	Hydrazine	0.81	0.57	0.16	0.15	0.74
Uracil-6-carboxylic acid (orotic acid)	None	0.58	0.41	0.12	0.03	0.39
Uracil-6-carboxylic acid (orotic acid)	Hydrazine	0.77	0.20	0.03	0.01	0.60
Ethyl uracil-6-carboxylate	None	0.85	0.52	0.40	0.36	0.78
Uracil-5-carboxylic acid (iso-orotic acid)	None	0.54	0.12	0.27	0	0.43
Uracil-5-carboxylic acid (iso-orotic acid)	Hydrazine	0.78	0.52	0	0	0.73
				0.24	0.24	
Ethyl uracil-5-carboxylate	None	0.73	0.49	0.49	0.12	0.70
Ethyl uracil-5-carboxylate	Hydrazine	0.57	0.30	0.01	0.05	0.35
Adenine	None	0.43	0.53	0.11	0.27	0.35
Guanine	None	0.25	0.30	0.04	0.05	0.26

Radioactive spots from liver RNA hydrolysed with HCl

Labelling urethane	Further treatment Solvent ...	R_F				
		(1)	(2)	(3)	(4)	(5)
Ethyl [<i>carboxy</i> - ¹⁴ C]carbamate	None	0.79	0.55	0.05	0.05	0.21
Ethyl [<i>carboxy</i> - ¹⁴ C]carbamate	Phosphatase	{ 0.78 0.65*		0 0.17*		
Ethyl [<i>carboxy</i> - ¹⁴ C]carbamate	Hydrazine	†	0.22	0.05	0.05	0.58
[1- ¹⁴ C]Ethyl carbamate	None	0.80	0.54	0.04	0.07	0.21

* Two spots: the other value is due to unchanged material.

† Not detectable.

of urethane-labelled liver RNA with alkaline phosphatase moved the position of the radioactive spots from the nucleotide to the nucleoside region of paper chromatograms developed in solvents (1) and (3) (Table 2), but the addition of nucleosidase after the phosphatase had no further effect.

Heating the neutralized hydrochloric acid hydrolysate of liver RNA, labelled *in vivo* by ethyl [*carboxy*-¹⁴C]carbamate, with hydrazine hydrate altered the R_F values of the ¹⁴C-containing spot in some of the solvents used (Table 2). No radioactivity could be detected when solvent (1) was used to develop the chromatogram.

Chromatography of hydrolysates of lung RNA, labelled by [1-¹⁴C]ethyl carbamate, in solvent (1) or (2) showed the presence of a radioactive component similar to that found in liver RNA. The limited amount of material available prevented the use of other solvents. The ¹⁴C content of lung RNA labelled by ethyl [*carboxy*-¹⁴C]carbamate was only one-third of that labelled by [1-¹⁴C]ethyl carbamate, and could not be clearly detected on the paper chromatograms.

After partial hydrolysis of urethane-labelled liver DNA with hydrochloric acid and chromatography in solvents (1) or (2), the radioactivity remained

Fig. 1. Eluates with 0.1N-HCl from paper chromatograms of HCl hydrolysates of RNA labelled *in vivo* by ethyl [carboxy- ^{14}C]carbamate. (a) Chromatogram run in solvent (1), methanol-conc. HCl-water (7:2:1, by vol.); (b) run in solvent (2), ethanol-aq. NH_3 soln. (sp.gr. 0.88)-water (40:1:9, by vol.). The radioactive peaks are shown hatched.

associated with the apurinic acid residue on the origins. Enzymic hydrolysis with deoxyribonuclease and phosphodiesterase followed by chromatography on paper or on DEAE-cellulose columns also did not separate the ^{14}C from the undegraded DNA residue.

Cytoplasmic and nuclear proteins labelled by urethane did not give any detectable radioactive spots on the chromatograms when treated in the same way as nucleic acid.

The experiments with RNA *in vitro* did not give any detectable radioactive reaction products of normal RNA and labelled urethane or hydroxy-urethane.

Orotic acid, uracil-5-carboxylic acid and cytosine-5-carboxylic acid were found, by chromatography, to be stable in N-hydrochloric acid at 100° for 1 hr. Under these conditions about 70–80% of the ethyl esters of these compounds were hydrolysed to the acids, but in 0.1N-hydrochloric acid only 10–20% hydrolysis occurred. Orotic acid was stable to formic acid at 180° for 1 hr., but the pyrimidine-5-carboxylic acids were converted into the bases. N^4 -Ethoxycarbonylcytosine was completely hydrolysed to uracil and a little cytosine in less than 10 min. at 100° in N-hydrochloric acid, but N^4 -hydroxycytidine was stable for at least 1 hr.

The R_F values of the pyrazolone and pyrazole derivatives obtained by the reaction of pyrimidines with hydrazine are shown in Table 2. Ferric chloride solution was the better reagent for the detection of these pyrazolones and pyrazoles on paper, as Ehrlich's reagent gave only faint colours.

The pyrazole obtained from the ethyl ester of cytosine-5-carboxylic acid was unstable to development in the strongly acid solvent (1); two brown streaky spots appeared when the chromatogram was sprayed with ferric chloride solution instead of the usual single discrete blue-green spot.

DISCUSSION

These results indicate that after the injection of [^{14}C]urethane a labelled pyrimidine-5-carboxylic acid was present in RNA. This was not due to the incorporation of metabolic breakdown products of urethane into a normal constituent since labelled ethanol or sodium hydrogen carbonate did not give the same result.

The same labelled compound was formed with both [^{14}C]ethyl carbamate and ethyl [carboxy- ^{14}C]carbamate, showing that the ethoxycarbonyl group had combined with the nucleic acids. Partial hydrolysis of urethane-labelled DNA with hydrochloric acid liberated the purines but left the ^{14}C associated with the apurinic acid residue. The chromatographic behaviour of the radioactive component in hydrochloric acid hydrolysates of urethane-labelled liver RNA suggested a pyrimidine nucleotide, and this view was supported by the apparent formation of a nucleoside after treatment with alkaline phosphatase. The resistance of the suspected nucleoside to attack by a nucleosidase may be due to enzyme specificity.

The radioactive component of urethane-labelled RNA was stable to N-hydrochloric acid for 1 hr. at

100° but not to formic acid at 180°, whereas *N*⁴-ethoxycarbonylcytosine was completely converted into uracil (and a little cytosine) after 10 min. in hydrochloric acid at 100°. On the other hand, orotic acid (uracil-6-carboxylic acid) was stable to the formic acid treatment. Iso-orotic acid (uracil-5-carboxylic acid) and cytosine-5-carboxylic acid formed uracil and cytosine respectively on heating with formic acid but were resistant to hydrolysis by hydrochloric acid. The *carboxy*-¹⁴C-labelled RNA lost radioactivity when heated in 20% (v/v) sulphuric acid at 170° for 2 hr. Wheeler *et al.* (1907) found that orotic acid was not altered by this treatment but iso-orotic acid was quantitatively converted into uracil.

The ethyl esters of these 5-carboxylic acids were partially hydrolysed by hydrochloric acid whereas the labelling of RNA from mice given [1-¹⁴C]ethyl carbamate was stable to this treatment. The ester of the 5-carboxylic acid nucleotide, however, should be more stable to acid hydrolysis than the ester of the free base as the pyrimidine in the nucleotide would have the keto structure.

The reaction with hydrazine was also in agreement with the product being a pyrimidine-5-carboxylic acid derivative. Hydrazine replaces the urea moiety of pyrimidines or their nucleotides and would be expected to react with the ethyl ester of cytosine-5-carboxylic acid as shown in Scheme 1. The chromatographic behaviour of this reaction product was similar to that of the radioactive component from hydrochloric acid hydrolysates of ethyl [*carboxy*-¹⁴C]carbamate-labelled RNA treated with hydrazine.

Urethane itself did not appear to react with RNA *in vitro* under physiological conditions but this was not unexpected in view of other evidence that a

metabolite is responsible for the carcinogenic action of urethane. The carcinogenic action of urethane occurs at sites remote from that of application (Haddow, 1963; Liebelt, Yoshida & Gray, 1961; Trainin, Precerutti & Law, 1964). It is inactive in test systems in which metabolism of foreign compounds does not proceed (see Nery, 1968).

A possible reaction of the urethane metabolite *N*-hydroxyurethane could be as shown in Scheme 2, by analogy with the reversible reaction of hydroxylamine with the C-5-C-6 double bond of cytosine (Brown & Schell, 1965; Verwoerd, Zillig & Kohlhaage, 1963), but this direct reaction was not detected. Nery (1968) has suggested that the *S*-ethoxycarbonyl derivatives formed *in vivo* by urethane and *N*-hydroxyurethane (Boyland & Nery, 1965) are due to the reaction of metabolically generated ethoxycarbonyl radicals. The chemical oxidation of *N*-hydroxyurethane also forms these radicals (Boyland & Nery, 1966) and the carcinogen *N*-methyl-*N*-nitrosourethane *S*-ethoxycarbonylates cysteine *in vitro* (Schoental & Rive, 1965). Whether the formation of the ethyl ester of cytosine-5-carboxylic acid plays a part in carcinogenesis is unknown, but Berenblum *et al.* (1959), after testing urethane derivatives for skin initiating action and lung carcinogenesis, suggested that the carcinogenic activity was dependent on the intact ethoxycarbonyl group of urethane.

[1-¹⁴C]Ethyl carbamate and ethyl [*carboxy*-¹⁴C]carbamate labelled the liver fractions to a similar extent, but the former gave better labelling of the lung components (Table 1). Comparison with the results obtained with [1-¹⁴C]ethanol and sodium hydrogen [¹⁴C]carbonate showed that this difference was mainly due to metabolic incorporation of the ¹⁴C, but the results with the two labelled

Scheme 1.

Scheme 2.

urethanes were noteworthy, as in the adult mouse the lungs were more susceptible than the liver to the carcinogenic action of urethane (see Mirvish, 1969).

Unlike the alkylating agent studies by Brookes & Lawley (1965) urethane did not appear to label the purines. The total labelling of the nucleic acid was small, however, the radioactive spot formed on the chromatograms giving counts only two to three times that of the background, and any other labelled components containing significantly less ^{14}C would not have been detected. The extent of the reaction of urethane with RNA was only approximately one urethane molecule/ 7×10^3 – 6×10^4 base residues.

Farber *et al.* (1967) found that administration of labelled ethionine (a compound that induces liver tumours in rats) preferentially labelled the RNA, in particular the soluble RNA, of the liver, but the radioactive components formed were not finally identified.

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