The Metabolism of Urethane and Related Compounds

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1. Urethane is metabolized in the rat, rabbit and man by a process of *N*-hydroxylation. This occurs to a smaller extent when methyl, *n*-propyl and *n*-butyl carbamates are administered to the rat and rabbit. 2. Other metabolites which have been detected in urine of animals dosed with urethane and *N*-hydroxyurethane are ethylmercapturic acid, ethylmercapturic acid sulphoxide and *N*-acetyl-*S*-carbethoxycysteine. 3. Substances which appear to be *S*-ethylglutathione and *S*-ethylglutathione sulphoxide have been detected in the bile of rats dosed with urethane or *N*-hydroxyurethane. 4. Methyl, ethyl, *n*-propyl and *n*-butyl *N*-hydroxycarbamates are excreted unchanged in the urine of rats dosed with these compounds to extents depending on the dose administered. 5. Animals dosed with methyl, ethyl, *n*-propyl or *n*-butyl carbamate or the corresponding *N*-hydroxycarbamate excrete the corresponding carbamate and *N*-hydroxycarbamate in the urine. 6. Methyl, *n*-propyl and *n*-butyl carbamates and *N*-hydroxycarbamates are excreted more slowly than are urethane and *N*-hydroxyurethane. 7. The probable role of *N*-hydroxyurethane and the processes of alkylation and carbethoxylation, and of hydroxylamine, nitroxyl and hyponitrous acid in carcinogenesis and chemotherapy with urethane, have been discussed.

Urethane (ethyl carbamate) is carcinogenic to mice (Nettleship & Henshaw, 1943), in which it induces hepatomas and lung adenomas (Law & Precerutti, 1963) and lymphomas (Doell & Carnes, 1962; Toth, Della Porta & Shubik, 1961). It does not have an appreciable leukaemogenic action on adult mice (Tannenbaum, 1961; Berenblum & Trainin, 1960; Kawamoto, Kirschbaum, Ibanez, Trentin & Taylor, 1961), but induces leukaemia by a single injection into newborn mice (Fiore-Donati, Chieco-Bianchi, de Benedictis & Maiorano, 1961), and augments leukaemogenesis by X-rays (Berenblum & Trainin, 1960, 1961), oestrogen or methylcholanthrene (Kawamoto, Ida, Kirschbaum & Taylor, 1958). The effect as initiator of skin carcinogenesis in mice (Salaman & Roe, 1953) is reduced if butyl carbamate is simultaneously administered (Garcia, 1963). Urethane is also carcinogenic to rats (Tannenbaum, Vesselinovitch, Maltoni & Stryzak-Mitchell, 1962) and hamsters (Shubik *et al.* 1961). It retards growth of the Walker rat carcinoma 256 (Haddow & Sexton, 1946), was used in the treatment of leukaemia in man (Paterson, ApThomas, Haddow & Watkinson, 1946) and is used in the treatment of multiple myelomatosis (Loge & Rundles, 1951). It produces chromosome damage in rat tissues similar to that produced by irradiation (Boyland & Koller, 1954).

These effects are apparently specific for the ethyl carbamate, since the other homologues are inactive or less active (Berenblum *et al.* 1959). These authors have also reported that *N*-hydroxyurethane has the same carcinogenic activity as urethane when tested on the skin or lung of mice.

When administered to rats, urethane was evenly distributed in tissues within a few hours, metabolized and largely excreted in the expired air as carbon dioxide, and only about 4% of the dose appeared in the urine in 24 hr. (Boyland & Rhoden, 1949; Skipper, Bryan, White & Hutchison, 1948). Beikert (1951) reported an unidentified reducing substance in the urine of rabbits dosed with urethane. The nature of some of the metabolites of urethane and related substances in the urine of rat, rabbit and man and their possible modes of action are now reported.

MATERIALS

Spectra. Visible and ultraviolet-absorption spectra were recorded with a Perkin-Elmer model 137 ultraviolet spectrophotometer, infrared-absorption spectra on a Perkin-Elmer Infracord model 137 spectrophotometer and extinctions on a Unicam SP.500 spectrophotometer.

Elementary analyses. These were by Weiler and Strauss, Oxford, England.

Animals. Animals were kept in cages designed for the separate collection of urine and faeces. Urine was collected daily under toluene in receivers immersed in solid CO₂-acetone and stored at -5°. Animals were injected intraperitoneally with methyl or ethyl carbamate as 20% (w/v) solutions in water, with *n*-propyl or *n*-butyl carbamate as 10% (w/v) solutions in arachis oil, or with methyl, ethyl, *n*-propyl or *n*-butyl *N*-hydroxycarbamate as 5% (w/v) solutions in water.

Chromatography. Whatman no. 1 chromatography paper (unless otherwise stated) was employed for descending development in the following solvent systems: (a) butan-1-ol-propan-1-ol-aq. 2 *N*-NH₃ (2:1:1, by vol.); (b) butan-1-ol-acetic acid-water (12:3:5, by vol.). For thin-layer chromatography, glass plates were coated with films of silica gel G (E. Merck A.-G., Darmstadt, West Germany) of 0.25 mm. thickness and the chromatograms were developed in (c) acetone-light petroleum (b.p. 40-60°) (3:7, v/v), or (d) chloroform-acetone-light petroleum (b.p. 40-60°) (2:1:7, by vol.), or (e) ethanol-benzene-aq. 50% (v/v) acetic acid (7:1:1, by vol.). For the detection of compounds on chromatograms the following reagents were used: (i) ammoniacal aq. 2% AgNO₃; (ii) 0.1 *M*-K₂Cr₂O₇-acetic acid (1:1, v/v) followed by 0.1 *M*-AgNO₃ (Knight & Young, 1958); (iii) platinum iodide (Toennies & Kolb, 1951); (iv) *p*-dimethylaminocinnamaldehyde (2 g. in 100 ml. of 6 *N*-HCl and 100 ml. of ethanol) (Harley-Mason & Archer, 1958); (v) 0.05% (w/v) ninhydrin in acetone, followed by heating the chromatograms at 85-90° for 2-10 min.; (vi) NaI-HCl-starch (Thompson, Arnold & Morris, 1963b); (vii) 1% (w/v) FeCl₃ in aq. 50% ethanol, (viii) 1% (w/v) sodium aminopropylsulfide in water containing 0.1% (w/v) of MgCl₂·6H₂O. A Chromatolite lamp (Hanovia Ltd.) was used as a source of u.v. light.

Carbamates and *N*-hydroxycarbamates gave red spots with reagent (iv). *N*-Hydroxycarbamates gave purple

colours with (vii) and reddish spots with (viii). *N*-Hydroxyurethane, its *N*- and *O*-monosubstituted and *NO*-disubstituted derivatives gave dark spots with (i) after 5-10 min., 20-40 min. and 1-2 hr. respectively. *N*- and *O*-Substituted *N*-hydroxycarbamates gave mauve colours with (viii). *S*-Substituted cysteines and glutathiones gave red to bluish-purple spots with (v) and yellow to brown spots with (ii), and sulphides and sulfoxides grey to brown spots on a red background with (iii). Sulfoxides also gave brown spots with (vi). The *R_F* values of the compounds are given in Table 1.

Chemicals. Light petroleum was the fraction b.p. 60-80°, unless otherwise stated. Hydroxylamine hydrochloride (reagent grade) and ethyl carbamate were obtained from Hopkin and Williams Ltd., Chadwell Heath, Essex; methyl carbamate, sodium aminopropylsulfide and methyl, ethyl and *n*-butyl chloroformates were from British Drug Houses Ltd., Poole, Dorset; *n*-propyl and *n*-butyl carbamates were from Eastman Kodak Ltd., Rochester, N.Y., U.S.A., and *n*-propyl chloroformate was from L. Light and Co. Ltd., Colnbrook, Bucks.

Alkyl *N*-hydroxycarbamates and derivatives. Methyl, ethyl, *n*-propyl and *n*-butyl *N*-hydroxycarbamates were prepared by the reactions of the corresponding alkyl chloroformates with hydroxylamine in the presence of Na₂CO₃ and ethyl *O*-acetyl-, ethyl *NO*-diacetyl- and ethyl *O*-carbethoxy-*N*-hydroxy-carbamates by the acetylation or carbethoxylation of *N*-hydroxyurethane, as described by Boyland & Nery (1964b). In the preparation of *N*-hydroxyurethane, the crude oil obtained after evaporation of the ethereal extract distilled at 82-84° (0.6 mm.). In another preparation, the crude mixture was neutralized with 6 *N*-HCl at 0°, evaporated to dryness *in vacuo*, the residue extracted with ethanol, the ethanol extract dried (MgSO₄) and the solvent evaporated off *in vacuo*. Attempted distillation of this residual oil at 0.6 mm. caused an explosive decomposition when the

Table 1. *R_F* values of reference compounds and their occurrence as metabolites of urethane and *N*-hydroxyurethane in the urine of the rat and rabbit

For details of preparations etc., see text. +, Detected; —, not detected; *, probably produced by the spontaneous rearrangement of the *N*-isomer.

Compound	Solvent system	...	<i>R_F</i>				Occurrence as metabolite of			
							Urethane		<i>N</i> -Hydroxyurethane	
			(a)	(b)	(c)	(d)	Rat	Rabbit	Rat	Rabbit
Urethane	—	—	—	—	0.35	0.11	+	+	+	+
<i>N</i> -Hydroxyurethane	—	—	—	0.79	0.23	0.04	+	+	+	+
<i>O</i> -Acetyl- <i>N</i> -hydroxyurethane*	—	—	—	—	0.45	0.22	+	+	+	+
<i>N</i> -Acetyl- <i>N</i> -hydroxyurethane	—	—	—	—	0.48	0.24	+	+	+	+
<i>NO</i> -Diacetyl- <i>N</i> -hydroxyurethane	—	—	—	—	0.59	0.46	—	—	—	—
<i>O</i> -Carbethoxy- <i>N</i> -hydroxyurethane	—	—	—	—	0.50	0.26	—	—	—	—
<i>NO</i> -Dicarbethoxy- <i>N</i> -hydroxyurethane	—	—	—	—	0.61	0.48	—	—	—	—
<i>S</i> -Ethylcysteine	0.27	0.50	—	—	—	—	—	—	+	—
<i>S</i> -Ethylcysteine <i>S</i> -oxide	0.10	0.26	—	—	—	—	—	—	—	—
<i>N</i> -Acetyl- <i>S</i> -ethylcysteine	0.33	0.82	—	—	—	—	+	+	+	+
<i>N</i> -Acetyl- <i>S</i> -ethylcysteine <i>S</i> -oxide	0.29	0.74	—	—	—	—	—	—	+	+
<i>S</i> -Carbethoxycysteine	0.25	0.57	—	—	—	—	—	—	—	—
<i>N</i> -Carbethoxycysteine	0.30	0.53	—	—	—	—	—	—	—	—
<i>NS</i> -Dicarbethoxycysteine	0.37	0.83	—	—	—	—	—	—	—	—
<i>N</i> -Acetyl- <i>S</i> -carbethoxycysteine	0.35	0.75	—	—	—	—	+	+	+	+

bath temperature was 80–85°. The feasibility of distillation as a means of purification of this compound appears to depend upon the method of its preparation and the degree of its purity.

A solution of methyl *N*-hydroxycarbamate (0.45 g.) in benzene (15 ml.) containing phenyl isocyanate (0.65 g.), after 16 hr. at 23°, was filtered, the filtrate evaporated *in vacuo*, and the residual semi-solid recrystallized from light petroleum to yield colourless needles of *phenylcarbamoyl-methyl N-hydroxycarbamate* (0.6 g.), m.p. 129° (Found: C, 51.7; H, 5.0; N, 13.7. $C_9H_{10}N_2O_4$ requires C, 51.4; H, 4.8; N, 13.3%). Similarly prepared from the *N*-hydroxycarbamates and equimolar amounts of phenyl isocyanate in benzene were: (i) *phenylcarbamoyl-ethyl N-hydroxycarbamate hemihydrate*, m.p. 82°, as colourless plates from benzene–light petroleum (Found: C, 51.2; H, 5.7; N, 11.9. $C_{10}H_{12}N_2O_4 \cdot \frac{1}{2}H_2O$ requires C, 51.5; H, 5.6; N, 12.0%), which gave *phenylcarbamoyl-ethyl N-hydroxycarbamate* after drying over P_2O_5 at 60° (15 mm.) for 1 hr., m.p. 83°, as colourless plates (Found: C, 54.0; H, 5.4; N, 12.2. $C_{10}H_{12}N_2O_4$ requires C, 53.6; H, 5.4; N, 12.5%); (ii) *phenylcarbamoyl-n-propyl N-hydroxycarbamate*, m.p. 110°, as colourless plates from benzene–light petroleum (Found: C, 55.3; H, 6.2; N, 12.1. $C_{11}H_{14}N_2O_4$ requires C, 55.45; H, 5.9; N, 11.8%); (iii) *phenylcarbamoyl-n-butyl N-hydroxycarbamate*, m.p. 118°, as colourless plates from light petroleum (Found: C, 57.4; H, 6.2; N, 11.1. $C_{12}H_{16}N_2O_4$ requires C, 57.1; H, 6.4; N, 11.1%). Yields of recrystallized materials were 80–85%.

Infrared spectra. *N*-Hydroxyurethane showed bands at 3300 (N–H stretching), 2995, 1745 (C=O stretching), 1490, 1450, 1382, 1340, 1263 (C–O stretching), 1171, 1120 (C–O stretching), 1060, 1030 and 775 cm^{-1} . The band at 3300 cm^{-1} was also present in the monoacetyl, monocarbethoxy and phenylcarbamoyl derivatives, indicating that these compounds possessed the general structure $R \cdot O \cdot CO \cdot NH \cdot O \cdot R^1$, where R^1 was the substituent group; it was absent from *NO*-diacetyl-*N*-hydroxyurethane. No attempt was made to determine the contribution of the tautomeric hydroxamic acid form, $R \cdot O \cdot C(OH) : N \cdot O \cdot R^1$, to the structure of the *N*-hydroxycarbamates or their mono-*O*-substituted derivatives.

Solutions of the alkyl *N*-hydroxycarbamates in *n*-NaOH or 5% sodium ethoxide in ethanol formed the corresponding alkyl carbonates and, on standing, Na_2CO_3 . A solution of *N*-hydroxyurethane (0.2 g.) and *N*-acetylcysteine (0.3 g.) in 5% (w/v) $NaHCO_3$ (5 ml.), after 40 hr. at 23°, contained small amounts of *N*-acetyl-*S*-carbethoxy- and *N*-acetyl-*S*-ethyl-cysteine detected by paper chromatography in solvents (a) and (b). Cysteine hydrochloride (0.3 g.), under similar conditions, formed *S*-carbethoxy- and *S*-ethyl-cysteine. When urethane was incubated with acetylcysteine or cysteine no *S*-carbethoxy and *S*-ethyl derivatives appeared to be formed.

S-Ethylcysteine and *S*-ethylcysteine sulfoxide were prepared as described by Roberts & Warwick (1958). Ethyl mercapturic acid and its dicyclohexylammonium salt were prepared as described by Thompson, Barnsley & Young (1963a).

N-Acetyl-*S*-carbethoxycysteine. Ethyl chloroformate (2.5 g.) was added dropwise to a stirred solution of *N*-acetylcysteine (3 g.) and $NaHCO_3$ (6 g.) in water (50 ml.). Stirring was continued until a negative nitroprusside test (about 45 min.) for free thiol was obtained and then for a further

hour. The mixture was acidified to pH 4.0 with acetic acid, extracted with chloroform (4 × 30 ml.), the combined chloroform extracts were washed with water (20 ml.), dried (Na_2SO_4), and evaporated *in vacuo* to yield a colourless residual oil, which did not crystallize, but appeared to be chromatographically homogeneous, gave positive reactions with reagents (ii) and (iii) and negative reactions with ninhydrin or nitroprusside. After heating with 4 *N*-HCl at 100° for 1 hr., it formed *S*-carbethoxycysteine, which gave a positive ninhydrin reaction (with reagent v) and a negative nitroprusside reaction. With diazomethane in methanol-ether, it gave an oil, b.p. 154–156° (0.5 mm.), which solidified and gave the *methyl ester* as fine colourless needles from light petroleum, m.p. 50–51° (Found: C, 43.5; H, 6.3; N, 5.6; S, 12.6; mol. wt., 236. $C_9H_{15}NO_5S$ requires C, 43.4; H, 6.1; N, 5.6; S, 12.9%; mol. wt., 249). On heating with 4 *N*-HCl at 100° for 1 hr., this compound gave *S*-carbethoxycysteine. After 24 hr. in 2 *N*-NaOH at 23°, it gave ethylmercapturic acid, *N*-acetylcysteine and *NN'*-diacetylcysteine.

The following compounds were obtained as chromatographically homogeneous specimens, but were not further characterized: (i) *N*-Acetyl-*N*-hydroxyurethane, a minor component of the products of acetylation of *N*-hydroxyurethane with acetic anhydride or of the carbethoxylation (with ethyl chloroformate) of acetohydroxamic acid, was eluted with ether from silica gel scraped off the appropriate R_F region after development of thin-layer chromatograms in solvent system (c). It rearranged to the *O*-isomer on standing at room temperature, and was slowly hydrolysed in water to *N*-hydroxyurethane. The *O*-isomer, by contrast, did not rearrange, was slowly hydrolysed by warm water, and readily so by cold aq. 2 *N*- NH_3 soln., *N*-NaOH or *N*-HCl, to yield *N*-hydroxyurethane. (ii) *S*-Carbethoxycysteine. Ethyl chloroformate (1 g.) was added dropwise over 20 min. to a rapidly stirred solution of cysteine hydrochloride (1.5 g.) in water (60 ml.) containing $NaHCO_3$ (3 g.). After 4 hr., the resulting solution was adjusted to pH 3.0 with 4 *N*-HCl, extracted with chloroform (3 × 25 ml.), the combined chloroform extracts were washed with water (2 × 15 ml.), dried (Na_2SO_4), and evaporated *in vacuo* to yield the title compound as a colourless gum. Elution with chloroform of the appropriate strips cut from chromatograms (3 MM chromatography paper) developed in solvent system (b), followed by evaporation of the solvent *in vacuo*, afforded chromatographically pure *S*-carbethoxycysteine. This compound gave a positive ninhydrin but a negative nitroprusside reaction. (iii) *NS*-Dicarbethoxycysteine was similarly obtained as a colourless gum from the reaction of ethyl chloroformate (2.2 g.) with cysteine hydrochloride (1.5 g.) in water (50 ml.) containing $NaHCO_3$ (3.6 g.). (iv) *NN'*-Dicarbethoxycysteine was similarly obtained from the reaction of ethyl chloroformate (1.2 g.) with cysteine (1.2 g.) in aq. $NaHCO_3$. (v) *N*-Carbethoxycysteine was obtained as a colourless gum by decomposition of the lead salt with H_2S after reduction of (iv) with zinc dust–HCl in a manner similar to that described for the preparation of *N*-acetylcysteine (Pirie & Hele, 1933). It gave a positive nitroprusside and a negative ninhydrin reaction.

On heating under reflux with 5% (w/v) KOH in 90% ethanol, compounds (i)–(v), and all the other carbalkoxy derivatives (e.g. the *N*-hydroxycarbamates), deposited K_2CO_3 . On heating at 100° with 6 *N*-HCl, ethylmercapturic acid sulfoxide gave *S*-ethylcysteine and *S*-ethylcysteine sulfoxide; with thioglycolic acid, it gave ethylmercapturic

acid (cf. Thompson & Young, 1960). On heating with 6 *N*-HCl at 100° for 2 hr., *N*-carbethoxycysteine gave cysteine, and *N*-acetyl-*S*-carbethoxy- and *NS*-dicarbethoxy-cysteine gave *S*-carbethoxycysteine and other unidentified products. In alkaline media, *S*-carbethoxycysteine rearranged to *N*-carbethoxycysteine (cf. Schoental & Rive, 1963).

Examination of urine. Urine of animals and patients dosed with urethane or *N*-hydroxyurethane was treated as follows; treatment was controlled by comparison with normal urine collected before dosing. Urine was adjusted to pH 7.0 with acetic acid and extracted continuously with ether for 16 hr. The ether extract was dried (Na_2SO_4), evaporated *in vacuo* at 20°, and the residual gum examined for urethane, *N*-hydroxyurethane and other neutral components. Part of the aq. layer (aq. fraction 1, Scheme 1) was set aside for enzymic hydrolysis experiments and the rest adjusted to pH 2.0 with 10 *N*-HCl and extracted with chloroform until no more products were extracted. The combined chloroform extract, after drying (Na_2SO_4), was evaporated *in vacuo* and the residual gum examined for the presence of ethylmercapturic acid, ethylmercapturic acid sulphoxide, *N*-acetyl-*S*-carbethoxycysteine and other chloroform-soluble acidic components. The aq. layer (aq. fraction 2, Scheme 1) was stirred with charcoal (5–10 g.), the charcoal filtered off, washed with water until washings were neutral, stirred with methanol containing 5% (v/v) of aq. NH_3 (sp.gr. 0.88), filtered off, the filtrate evaporated *in vacuo*, and the residue examined for the presence of *S*-ethylcysteine and other metabolites. The pH of the combined aq. filtrate and washings (aq. fraction 3, Scheme 1) was adjusted to 4.0 with 4 *N*-NaOH and the glucuronosides were precipitated as lead salts and regenerated with H_2S (cf. Smith & Williams, 1949).

METHODS

Determination of carbamates. The method, which is a modification of that described by Devaux, Mesnard & Cren

(1963), is based on the fact that ethanolic KOH converts carbamates into cyanate, which gives a blue cobalt complex. The method finally employed was as follows. The urine sample containing carbamate was neutralized with N-HCl or N-KOH , extracted with 2 equal volumes of ether, the combined ethereal extracts were dried (Na_2SO_4) at 0° , filtered and the residue was washed through with 20 ml. of ice-cold ether, the ether fraction evaporated to dryness at 20° (15 mm.) in a rotary film evaporator and the residue dissolved in ethanol (6 ml.). The rest of the procedure also applies to the direct estimation of carbamates dissolved in ethanol, as follows: portions (2 ml.) of the ethanolic solution were treated with 5.6% (w/v) KOH in aq. 90% ethanol (0.5 ml.), mixed, heated at 70° for 1 hr. in stoppered tubes, cooled in ice-water, and treated with acetic acid (0.075 ml.) and ethanolic 1% (w/v) CoCl_2 (0.1 ml.); the total volume was made up to 4 ml. with ethanol, and the extinction determined at $610 \text{ m}\mu$ against a blank made up similarly but containing no carbamate.

When methyl, ethyl, *n*-propyl and *n*-butyl carbamates were dissolved in ethanol and determined in this way, they gave solutions having molar extinction coefficients (calculated on the concentrations of the final solutions) 45.0, 49.8, 51.5 and 49.7. Compared with ϵ 88.0 for potassium cyanate, these figures indicate the extent to which the carbamates were converted into cyanate under the conditions employed. Since no higher values were obtained on longer heating, it is probable that about half of the carbamate present was decomposed by some other route, e.g. to yield NH_3 , CO_2 and an alcohol. When added to normal rat or human urine the four carbamates were recovered to the extent of 20, 43, 57 and 65% respectively; these figures reflect the partition coefficients of the carbamates in urine-ether. Urea and *N*-hydroxyurea in ethanol, estimated as above, gave solutions having ϵ 0.84 and 48.6 respectively. Methyl, ethyl, *n*-propyl, *n*-butyl and ethyl *O*-acetyl-*N*-hydroxycarbamates did not yield cyanate with alkali and so did not interfere.

Determination of N-hydroxycarbamates. N-Hydroxycarbamates, like the N-arylhydroxylamines (Boyland &

Scheme 1. Fractionation of metabolites from the urine. Experimental details are given in the text.

Nery, 1964a), give coloured pentacyanoferroate complexes (I) in the presence of Mg^{2+} ions:

The methods used for their determination in water and urine solutions were those described by Boyland & Nery (1964b). Urine samples were first acidified to pH 4 with acetic acid, shaken with charcoal, filtered and the filtrate was adjusted to pH 7.5 with *N*-NaOH before the reagents were added. When doubtful results were obtained, i.e. when the concentration of the *N*-hydroxycarbamate in urine was too low, the neutralized filtrate was evaporated to dryness *in vacuo* at 35–40°, the residue dissolved in ethanol, filtered, the filtrate evaporated *in vacuo*, the residue dissolved in a small volume of water and the determination carried out on 3.5 ml. portions. The *N*-hydroxycarbamates added to normal rat or human urine were quantitatively recovered after such treatment. For the determination in ether extracts of large volumes of urine, the ether was removed by evaporation in a rotary film evaporator at 15° (15 mm.), the residue dissolved in a suitable volume of water and the determination carried out on 3.5 ml. portions.

The method was used to determine the stability of solutions of *N*-hydroxyurethane at 22°. It was stable at pH 6.5–7.5, less stable in alkali than in acid, and more stable in water than in urine. In urine at pH 7.0, *N*-hydroxyurethane decomposed to the extent of 4 and 34% after 16 and 56 hr. at 22° respectively; in water, the corresponding figures were 0.4 and 7%.

Determination of ethylmercapturic acid. The method used was essentially that described by Maw (1956) and modified by Thompson *et al.* (1963a) and depends on the colour given by the ethylmercapturic acid–sodium nitroprusside complex in acid solution. Under the conditions employed, ethylmercapturic acid and *S*-ethylcysteine gave red solutions (λ_{max} , 518 $m\mu$) having molar extinction coefficients (calculated on the concentrations of the final solutions) 57.8 and 30.4 respectively. The corresponding sulphoxides or *N*-acetyl-*S*-carbethoxycysteine did not interfere. Ethylmercapturic acid could not be determined directly in urine solutions, but only after extraction. The urine containing ethylmercapturic acid was acidified to pH 2.0 with 6 *N*-HCl and extracted twice with equal volumes of chloroform. The combined chloroform extracts were evaporated to dryness *in vacuo*, the residue was dissolved in water (6 ml.) and portions (2.5 ml.) were treated with 0.25 ml. of 1.34% (w/v) sodium nitroprusside and 0.5 ml. of 5 *N*-NaOH. After 20 min. the solutions were cooled in ice-water, 1 ml. of 88% phosphoric acid was added, the contents were mixed and the extinctions determined at 518 $m\mu$ in a 1 cm. cell against a blank made up similarly from an equal volume of normal urine. The recovery of ethylmercapturic acid added to urine, under these conditions, was 27–30%.

Determination of *N*-acetyl-*S*-carbethoxycysteine. The estimation of this compound was based on the observation (on chromatographic evidence) that it was the only observed metabolite, in addition to ethylmercapturic acid, which was capable of yielding a steam-distillable thiol from acid solution after alkaline hydrolysis. Like the *S*-ethyl and *S*-phenyl esters (Otto & Rossing, 1886) of ethyl thio-

carbonate, which gave diethyl and ethyl phenyl sulphides respectively on alkaline hydrolysis, *N*-acetyl-*S*-carbethoxycysteine yielded small amounts of ethylmercapturic acid and, probably, some ethyl thiocarbonate; the latter, on acidification, is known to give carbon oxysulphide, which is hydrolysed to H_2S and CO_2 . When a solution of *N*-acetyl-*S*-carbethoxycysteine in urine was subjected to alkaline hydrolysis, acidified and steam-distilled, 15–20% of the theoretical amount of thiol compounds appeared in the steam-distillate. This may indicate that the major products of alkaline degradation are ethanol, CO_2 and *N*-acetyl-cysteine. The recovery of ethylmercapturic acid was 30–33%.

The method used was as follows. The urine sample (100 ml.) was treated with 10 *N*-NaOH (30 ml.), heated at 100° for 1 hr., cooled, treated with 10 *N*-HCl (33 ml.), steam-distilled into 4% (w/v) salicylsulphonic acid (10 ml.) until the distillate no longer reduced iodine, and the distillate titrated at 0° with 1 *M*- KIO_3 in the presence of 1% (w/v) sodium starch glycolate (0.5 ml.) and 5% (w/v) KI (5 ml.). A blank value obtained by similar treatment of normal urine (100 ml.) was subtracted. The approximate amount of *N*-acetyl-*S*-carbethoxycysteine (*A*) was calculated from the relationship $A = 5(B - 0.3C)$, where *B* was total SH (as determined by the iodometric titration) and *C* was the total ethylmercapturic acid (as determined by the nitroprusside method), all three values (*A*, *B* and *C*) being expressed as percentages of the total dose. When ethylmercapturic acid (2 mg.) and *N*-acetyl-*S*-carbethoxycysteine (5 mg.) were added to normal rat urine (100 ml.) and each component was determined by the methods described, the recovery values obtained were 95 and 84% respectively.

RESULTS

Metabolism of urethane in the rat, rabbit and man

The neutral fraction (Scheme 1) from the urine of 16 female rats which received five daily doses of 1 g. of urethane/kg. (total dose, 16.6 g.), from the urine of two rabbits which received six daily doses of 1 g. of urethane/kg. (total dose, 27.5 g.), and from the urine of two male patients who received two daily doses of 2 g. of urethane (total dose, 8 g.), showed the presence, by thin-layer chromatography in solvent systems (c) and (d), of urethane, *N*-hydroxyurethane and a mixture of *N*- and *O*-acetyl-*N*-hydroxyurethane. Two-dimensional thin-layer chromatography (first run in solvent *d*, followed by spraying with 2 *N*-ammonia and running again in solvent *c* after 1 hr.) showed that the spots corresponding to *N*- and *O*-acetyl-*N*-hydroxyurethane gave *N*-hydroxyurethane. The acidic fraction (Scheme 1) contained ethylmercapturic acid and *N*-acetyl-*S*-carbethoxycysteine. The material obtained by elution of strips cut off the appropriate regions, after hydrolysis with 6 *N*-hydrochloric acid at 100° for 2 hr., showed the presence of compounds which were indistinguishable from *S*-ethylcysteine and *S*-carbethoxycysteine. No metabolite was detected in the methanol–ammonia fraction (Scheme 1) of the urine of the rat, rabbit and man.

Glucuronide fraction (Scheme 1). This yielded a gum, which gave a naphtharesorcinol reaction, but attempts to fractionate or to prepare a crystalline tetra-*O*-acetyl methyl ester by the use of acetic anhydride and diazomethane were unsuccessful. Hydrolysis of the gum with bacterial β -glucuronidase at pH 5.5 for 16 hr. at 37°, followed by ether extraction, yielded neither urethane nor *N*-hydroxyurethane. Samples (200 ml.) of the aq. fraction 1 (Scheme 1) were freed from ether under reduced pressure, adjusted to pH 5.5 with acetic acid, diluted with 0.2M-acetate buffer (pH 5.5, 50 ml.) and incubated with ox-liver (Ketodase, 2 ml.) or bacterial β -glucuronidase (0.2g.), or with Taka-diasatase (0.2 g.) containing glucosaccharo-1 $\rightarrow$ 4-lactone (0.5 g.) at 37° for 16 hr. The incubated mixtures were extracted with ether (4 $\times$ 50 ml.), the ether extracts dried (sodium sulphate), evaporated *in vacuo* and the urethane and *N*-hydroxyurethane contents of the residual gums quantitatively determined and compared with those of 200 ml. urine samples treated similarly, but containing no enzyme. No increase was observed. Glucuronides or ethereal sulphates of urethane or *N*-hydroxyurethane are probably not metabolites of urethane. Tsukamoto, Yoshimura & Tatsumi (1963) were also unable to find urethane glucuronide in the urine of rabbits dosed with urethane.

Quantitative determination of urethane, N-hydroxyurethane, acetyl-N-hydroxyurethane, ethylmercapturic acid and N-acetyl-S-carbethoxycysteine. *N*-Hydroxyurethane was determined as follows. The urine sample (100 ml.) was acidified at 0° with acetic acid to pH 4.0, shaken with charcoal (1 g.), filtered, and the charcoal washed with water (10 ml.); the filtrate and washing were combined, neutralized with *N*-sodium hydrogen carbonate, and evaporated at 40° *in vacuo*; the residue was extracted with ethanol (20 ml.), the ethanolic extract filtered, evaporated *in vacuo*, the residue dissolved in water (8 ml.) and determinations were carried out

as described in the Methods section. Acetyl-*N*-hydroxyurethane was similarly determined after treatment of urine samples (100 ml.) with 4*N*-hydrochloric acid (11 ml.) for 16 hr. at 4° and estimation of the *N*-hydroxyurethane content against a blank made up similarly from normal urine (100 ml.) to which the determined amount of *N*-hydroxyurethane had been previously added. Further confirmation of the results were obtained by applying a measured volume of the ethanolic extract as streaks on thin-layer plates, developing the chromatograms in solvent (c), eluting with ethanol the material scraped off the appropriate regions and estimating the hydroxyurethane and acetyl-*N*-hydroxyurethane content of the residues obtained after evaporation *in vacuo* of the eluates. Urethane, ethylmercapturic acid and *N*-acetyl-*S*-carbethoxycysteine were determined as described (above) on 100 ml. urine samples. The results obtained are given in Table 2.

Metabolism of N-hydroxyurethane in the rat and rabbit

The neutral fraction (Scheme 1) from the urine of 20 female rats receiving seven daily doses (0.4 g./kg.) of *N*-hydroxyurethane (total dose, 15 g.), after concentration *in vacuo*, was applied as streaks on glass plates coated with silica gel of 0.50 mm. thickness, developed in solvent (c), and the band corresponding to *N*-hydroxyurethane scraped off, eluted with ether, concentrated *in vacuo*, the residue rechromatographed in solvent (d) and the elution process repeated. The ethereal eluate was dried (sodium sulphate), treated with phenyl isocyanate (0.4 ml.), evaporated *in vacuo* after 20 min. at 22°, and the residual gum crystallized from benzene-light petroleum (b.p. 60–80°) to yield phenyl-carbamoyl-*N*-hydroxyurethane (0.365 g.) as colourless plates; the m.p., mixed m.p. and infrared spectrum were identical with those of an authentic specimen. The fractions corresponding to *N*- and

Table 2. *Metabolites of urethane and N-hydroxyurethane in the urine of the rat, rabbit and man*

Figures in parentheses are mean values.

Compound given	Species	Dose (g./kg.)	Metabolites* occurring in 24-hr. urine (% of dose)		
			Acetyl- <i>N</i> - hydroxyurethane	Ethylmercapturic acid	<i>N</i> -Acetyl- <i>S</i> - carbethoxycysteine
Urethane	Rat	1.0	0.1, 0.1, 0.2 (0.14)	0.09, 0.1, 0.2 (0.13)	1.5, 1.8, 2.1 (1.8)
	Rabbit	1.0	0.1, 0.2, 0.3 (0.2)	0.05, 0.2, 0.3 (0.2)	1.8, 2.2, 2.2 (2.1)
	Man	2 g./adult male	0.5, 0.6 (0.6)	0.06, 0.2 (0.13)	0.2, 1.5 (0.9)
<i>N</i> -Hydroxyurethane	Rat	0.4	8.5, 9.6, 11.0 (9.7)	0.4, 0.7, 0.7 (0.6)	1.7, 2.0, 2.0 (1.9)
	Rabbit	0.4	9.0, 15, 20 (14.7)	0.2, 0.3, 0.3 (0.3)	1.8, 2.2, 2.7 (2.2)

* The amounts of urethane and *N*-hydroxyurethane excreted are given in Table 3.

O-acetyl-*N*-hydroxyurethane, obtained similarly, were hydrolysed with cold *N*-hydrochloric acid and aq. *N*-ammonia soln. to yield *N*-hydroxyurethane in varying amounts.

The fraction eluted off charcoal (Scheme 1), by chromatography in solvent systems (a), (b) and (e), contained a substance indistinguishable from *S*-ethylcysteine.

The gum obtained after evaporation *in vacuo* of the chloroform from the acidic fraction (Scheme 1) was applied as streaks on sheets of 3MM chromatography paper. Strips corresponding to the R_F region of *S*-ethylmercapturic acid were cut off the chromatograms after development in solvent (b), eluted with chloroform, the chloroform extract was dried (sodium sulphate), concentrated *in vacuo* to about 10 ml., extracted with 5% (w/v) sodium carbonate (3×15 ml.), the aqueous extract acidified to pH 2 with hydrochloric acid and extracted with chloroform (4×20 ml.); the chloroform extracts were dried (sodium sulphate), evaporated to dryness *in vacuo*, the residual gum was dissolved in methanol (2 ml.), applied as streaks on glass plates coated with silica gel of 0.5 mm. thickness, the chromatograms were developed in solvent system (e), and the band corresponding to the R_F region of ethylmercapturic acid (R_F 0.65–0.70) was scraped off and eluted with chloroform (15 ml.). The gum obtained after drying (sodium sulphate) and evaporating the chloroform extract was dissolved in acetone (5 ml.), neutralized with dicyclohexylamine, evaporated *in vacuo*, the residue washed with cold acetone (5 ml.), dissolved in the minimum volume of warm ethanol and diluted with warm acetone until the solution became cloudy. On cooling, the solution deposited a solid (5 mg.), which did not melt below 300° and was discarded. Similar treatment of the filtrate with acetone yielded a second crop (15 mg.), m.p. 206°, which was not identified, and a third crop (2 mg.) of dicyclohexylammonium *S*-ethylmercapturate, identical in its m.p., mixed m.p. and chromatographic behaviour with an authentic specimen.

A sample of the gum obtained after evaporation *in vacuo* of the acidic fraction (Scheme 1) was applied as streaks on 3MM chromatography paper, strips were cut off (R_F 0.75–0.83) the chromatograms developed in solvent (b) and eluted with chloroform. Similar hydrolytic and chromatographic experiments to those described for the corresponding fraction of urine from rats and rabbits dosed with urethane indicated the presence of ethylmercapturic acid, ethylmercapturic acid sulphoxide and *N*-acetyl-*S*-carbethoxycysteine as metabolites of *N*-hydroxyurethane.

Glucuronide fraction (Scheme 1). Similar results were obtained to those described for urines from animals dosed with urethane.

Quantitative determination of urethane, N-hydroxy-

urethane, acetyl-N-hydroxyurethane, ethyl mercapturic acid and N-acetyl-S-carbethoxycysteine. *N*-Hydroxyurethane was determined directly on filtrates (3.5 ml.) of urine samples (10 ml.) which had been decolorized with charcoal (see the Methods section). The other metabolites were determined as is described for urethane metabolites. The results obtained are given in Table 2.

Bile of the rat after dosing with urethane or N-hydroxyurethane

Urethane. Two male rats (approx. body wt. 250 g.) with bile fistulae (Boyland, Ramsay & Sims, 1961) were given four daily intraperitoneal injections of 0.1 g. of urethane dissolved in 1 ml. of water, and the bile was collected twice daily and stored under toluene at -5° . The bile was freed from toluene by aspiration at the water pump, acidified with acetic acid to pH 4.0, centrifuged, the supernatant stirred with charcoal (5 g.), the charcoal filtered off, washed with water (500 ml.), eluted with methanol (100 ml.) containing aq. ammonia soln. (sp.gr. 0.88, 5 ml.), the eluate concentrated *in vacuo* and the residual gum examined by paper chromatography in butan-1-ol-acetic acid-water (2:1:1, by vol.) and also in solvents (a) and (b). A substance indistinguishable from *S*-ethylglutathione (Roberts & Warwick, 1958), which gave *S*-ethylcysteine, glutamic acid and glycine on hydrolysis with hydrogen bromide (sp.gr. 1.7) at 100° for 2 hr., was obtained.

N-Hydroxyurethane. Examination of the bile of rats dosed with *N*-hydroxyurethane revealed the presence of a glutathione derivative which, after hydrolysis with hydrogen bromide (sp.gr. 1.7) at 100° for 2.5 hr., yielded *S*-ethylcysteine sulphoxide, *S*-ethylcysteine, glutamic acid and glycine. It was probably *S*-ethylglutathione sulphoxide since it was also detectable with reagents (iii), (v) and (vi), but not with reagent (ii).

Interconversion of carbamates and N-hydroxycarbamates as a general phenomenon in the rat, rabbit and man

Adult female rats (approx. body wt. 200 g.) were given single doses of 0.3–1.0 g. of carbamate/kg. or 0.02–0.4 g. of *N*-hydroxycarbamate/kg., intraperitoneally; rabbits (approx. body wt. 2 kg.) were given 1 g. of urethane/kg. or 0.2 g. of *N*-hydroxyurethane/kg. by intraperitoneal injection; four adult male patients were given 2 g. of urethane each by mouth. The urine was collected during the first and second 24 hr. periods separately, except for patients, whose urines were collected for the first 24 hr. period only. Each urine sample was decolorized with charcoal. For carbamate urines, one-half was

Table 3. *Interconversion of carbamates and N-hydroxycarbamates in the animal body*

Figures in parentheses are mean values.

		Metabolites in urine (% of dose)				
		R.O.CO.NH ₂		R.O.CO.NH.OH		
Compound given	Species	Dose (g./kg.)	First day	Second day	First day	Second day
(A) Carbamates (R.O.CO.NH ₂)						
R = Methyl	Rat	1.0	1.5, 1.5, 1.8, 4.6, 4.6, 5.8 (3.3)	—, —, 4.4, 4.6, 5.3, 5.4 (4.9)	0.004, 0.008, 0.008, 0.01, 0.01, 0.01 (0.008)	—, —, 0.002, 0.01, 0.20 (0.06)
Ethyl	Rat	1.0	0.8, 0.9, 1.0, 1.8, 1.9, 2.2, 2.2, 2.8 (1.7)	—, —, —, 0.2, 0.2, 0.2, 0.9, 0.9 (0.5)	0.06, 0.06, 0.07, 0.08, 0.08, 0.08, 0.1, 0.1 (0.08)	—, —, —, 0.01, 0.01, 0.03, 0.03, 0.02 (0.02)
Ethyl	Rabbit	1.0	0.4, 1.2, 1.6 (1.1)	0.5, 0.6, 0.7 (0.6)	0.08, 0.08, 0.2 (0.12)	0.01, 0.02, 0.02 (0.02)
Ethyl	Man	2 g./adult male/day	0.04, 0.04, 0.8, 1.0 (0.5)	—	0.09, 0.1, 0.2, 0.2 (0.15)	—
n-Propyl	Rat	1.0	1.9, 2.6, 2.6 (2.4)	1.6, 1.9, 2.4 (2.0)	0.001, 0.005, 0.01 (0.005)	0, 0.002, 0.002 (0.001)
n-Butyl	Rat	0.3	0.8	0.8	0.09	0.06
n-Butyl	Rat	0.4	1.0	1.1	0.08	0.08
(B) N-Hydroxycarbamates (R.O.CO.NH.OH)						
R = Methyl	Rat	0.4	2.5, 2.9, 6.8 (4.1)	4.8, 5.6, 6.6 (5.7)	27, 30, 30 (29)	1.5, 5.1, 5.2 (3.9)
Ethyl	Rat	0.02	0.3, 0.5, 0.8 (0.5)	—	0.2, 0.5, 0.5 (0.4)	—
Ethyl	Rat	0.05	0.4, 0.9, 1.3 (0.9)	0.1, 0.2, 0.4 (0.2)	2.2, 3.0, 4.0 (3.1)	0.8, 1.0, 1.0 (0.9)
Ethyl	Rat	0.10	0.4, 1.0, 2.0 (1.1)	0.2, 0.4, 0.6 (0.4)	3.2, 3.9, 4.3 (3.8)	0.8, 1.2, 1.2 (1.1)
Ethyl	Rat	0.20	0.6, 2.1, 2.8 (1.8)	0.2, 0.5, 0.6 (0.4)	5.2, 7.0, 9.8 (7.3)	0.9, 1.0, 1.2 (1.0)
Ethyl	Rat	0.40	1.1, 2.3, 2.5 (2.0)	0.4, 0.5, 0.6 (0.5)	30, 34.0, 35 (33)	2.0, 2.2, 2.6 (2.3)
Ethyl	Rabbit	0.20	0.1, 0.2, 0.3 (0.2)	0, 0.06, 0.08 (0.05)	15, 18, 20 (18)	2.0, 2.1, 2.6 (2.2)
n-Propyl	Rat	0.20	2.4	1.9	5.5	1.4
n-Butyl	Rat	0.20	0.3, 0.6, 0.6 (0.5)	0.5, 0.6, 0.6 (0.6)	6.1, 8.8, 9.1 (8.0)	3.8, 3.9, 4.2 (4.0)

neutralized with N-sodium hydroxide, evaporated *in vacuo* at 40°, the residue shaken with ethanol (20 ml.), the mixture filtered and the residue washed with ethanol (10 ml.); the combined filtrate and washings were evaporated *in vacuo*, the residue was dissolved in water (8 ml.) and the N-hydroxycarbamate content determined as previously described. For N-hydroxycarbamate urines, portions (3.5 ml.) of the decolorized samples were treated with 8% (w/v) sodium amminoprusside and the N-hydroxycarbamate content was determined as previously described. Carbamates were determined on the remainder of the urine samples, as previously described. The results obtained are given in Table 3.

Gastric juice of the rat and hamster after dosing with urethane or N-hydroxyurethane

Adult female rats (approx. body wt. 250 g.) and male hamsters (approx. body wt. 85 g.) established with gastric fistulae were given intraperitoneal doses of urethane (0.8 g./kg.) or N-hydroxyurethane

(0.32 g./kg.). The gastric juice was withdrawn after varying time-intervals, neutralized with aq. 5% (w/v) sodium carbonate, evaporated *in vacuo*, the residue shaken with chloroform (10 ml.), filtered and washed with chloroform (10 ml.); the combined chloroform solutions were dried (sodium sulphate), evaporated *in vacuo*, the residue was dissolved in water (3.5 ml.) and the N-hydroxyurethane content determined with the 0.8% (w/v) sodium amminoprusside reagent. The results obtained are shown in Table 4.

DISCUSSION

The process of N-hydroxylation appears to be an essential step in carcinogenesis by some aromatic amino and acetamido derivatives (Cramer, Miller & Miller, 1960; Nelson & Troll, 1961; Uehleke, 1963; Boyland, Manson & Nery, 1963). Acyl derivatives of N-hydroxyamino acids have also been isolated from microbial fermentations. These include N-formyl-N-hydroxyglycine (hadacidin; Kaczka,

Table 4. *Secretion of N-hydroxyurethane in the stomachs of rats and hamsters*

Compound given	Species	No. of animals	Dose (g./kg.)	Time after dosing (hr.)	Vol. of gastric juice collected (ml.)	Concn. of <i>N</i> -hydroxyurethane (μg./ml.)
Urethane	Rat	4	0.8	4	20	4.8
				20	20	1.1
				28	18	0
Urethane	Hamster	3	0.8	4	7.5	18
				20	7.0	6.3
				28	7.0	3.0
				44	6.2	0
<i>N</i> -Hydroxyurethane	Rat	2	0.32	4	4.2	68
				20	4.2	6

Gitterman, Dulaney & Folkers, 1962), *N*-hydroxyisoleucine, which occurs in aspergillilic acid (Dutcher, 1958), *N*-hydroxylysine in mycobactin (Snow, 1954) and *N*-hydroxytyrosine in mycelianamide (Birch, 1957). Hydroxamic acids are produced by the chemical oxidation of amides, aldehyde-ammonias and primary amines by hydrogen peroxide or persulphuric acid (Bamberger, 1900, 1902); the addition of hydroxylamine to fumaric acid to yield *N*-hydroxyaspartic acid is catalysed by aspartase (Emery, 1963). *N*-Formyl-*N*-hydroxyglycine (had-acidin), *N*-hydroxyurea (Stearns, Losee & Bernstein, 1963) and hydroxylamine (Cudkiewicz, 1955) have anti-tumour properties. Hydroxyurethane is carcinogenic (Berenblum *et al.* 1959) and causes chromosome breaks in the root tips of *Vicia faba* (Boyland, Nery, Peggie & Williams, 1963).

When urethane is administered to the rat, rabbit and man, it is oxidized by a process of *N*-hydroxylation to yield *N*-hydroxyurethane and its *N*- and *O*-acetyl derivatives (see Table 2). The latter compound is probably formed from the chemical rearrangement of the *N*-isomer. The fact that only small amounts of these metabolites are excreted in the urine may not be indicative of the extent to which *N*-hydroxylation actually occurs in the animal since, in the rat, small doses of administered *N*-hydroxyurethane are metabolized (see Table 3). Thus, of a dose of 20 mg./kg., less than 0.5% is excreted in the urine in 24 hr., whereas over 30% of a dose of 400 mg./kg. is excreted unchanged during the same period.

The presence of *S*-ethyl derivatives of cysteine in the urines of animals dosed with urethane and *N*-hydroxyurethane indicates that, like many other classes of radiomimetic carcinogens, these substances are biological alkylating agents and, as such, their carcinogenic effects may be partly explained. However, this process apparently occurs only to a minor extent since *S*-ethylcysteine derivatives are excreted in small amounts only. A process which appears to occur to a larger extent is that of *S*-

carbethoxylation; but whether *N*-acetyl-*S*-carbethoxycysteine is in itself a carcinogen is not known. *N*-Hydroxyurethane, but not urethane, reacts *in vitro* with cysteine and *N*-acetylcysteine in aqueous sodium hydrogen carbonate solutions to yield small amounts of the *S*-ethyl and *S*-carbethoxy derivatives. A solution of *N*-acetyl-*S*-carbethoxycysteine in 2 *N*-sodium hydroxide at 23° slowly yields *S*-ethylcysteine, a reaction analogous to that described by Otto & Rossing (1886) for the alkaline degradation of the *S*-ethyl and *S*-phenyl esters of ethyl thiocarbonate, i.e.

where R is ethyl, phenyl or *N*-acetylcysteinyl. Thus at least two metabolites of urethane, i.e. *N*-hydroxyurethane and *N*-acetyl-*S*-carbethoxycysteine, may produce *S*-ethylcysteinyl derivatives *in vivo*. However, Berenblum *et al.* (1959) have shown that the *N*-carbethoxy derivatives of several amino acids are not lung carcinogens and do not have an initiating action on skin carcinogenesis, but diethyl carbonate is an initiator of skin carcinogenesis.

In view of these findings it is probable that *N*-hydroxyurethane plays an important role in urethane carcinogenesis, but the mechanism of its action is still obscure. In addition to its alkylating and carbethoxylating properties, *N*-hydroxyurethane may give rise to hydroxylamine, nitroxyl or hyponitrous acid. In this connexion, reference may be made to the fact that the ability, in descending order of reactivity, of carbamates, *N*-hydroxyurea and urea to give cyanate in alkaline media is related to the relative stabilities of the alkoxy, hydroxy-amido and amido anions (A⁻) produced in the reaction:

N-Hydroxycarbamates do not give this reaction but may produce hydroxyamido anions via the S_N2 -type transition state (II):

This occurs when B is RS^- (e.g. cysteinyl), HO^- or EtO^- , yielding an *S*-carbethoxy thiol ester, an alkyl hydrogen carbonate and a dialkyl carbonate respectively and, probably, hydroxylamine. Similarly when B is HO^- , *N*- and *O*-alkyl-*N*-hydroxyurethanes yield the corresponding *N*- and *O*-alkylhydroxylamines (Nicolaus, Pagani & Testa, 1962). When B is ammonia, the products are *N*-hydroxyurea and an alcohol, probably formed via transition state (III) (E. Boyland & R. Nery, unpublished work). A similar reaction occurs between urethane and hydroxylamine in alkaline media, the products being *N*-hydroxyurea and ethanol (Deghenghi, 1960). Acid hydrolysis of hydroxamic acids or their *N*- and *O*-alkyl derivatives also yields hydroxylamine or the corresponding alkylhydroxylamines (Yale, 1943).

Administration of *N*-hydroxyurea to human subjects gives rise to hydroxylamine, which specifically cleaves thiol esters (e.g. acetyl-coenzyme A) and acyl phosphates, thereby producing aceto-hydroxamic acid (Fishbein & Carbone, 1963). Hydroxylamine is readily oxidized to nitroxyl (NOH), which rapidly dimerizes to hyponitrous acid (Thorne & Roberts, 1948), or it may disproportionate via the nitroxyl radical, again to form hyponitrous acid (Nast & Foppl, 1950). The latter reaction is also given by organic *N*-substituted hydroxylamines (Sidgwick, 1945). Nitroxyl, which is also an intermediate during photosynthesis of nitrogenous compounds in plants (Baly, Heilbron & Hudson, 1922), may react with aldehydes to give hydroxamic acids. Such reactions between aldehydes and nitroxyl or compounds capable of yielding nitroxyl, e.g. Piloty's acid, are known (Angeli, 1897, 1908). Bendich, Borenfreund, Korngold, Krim & Balis (1963) have shown that hydroxylamine, hyponitrous acid, *N*-hydroxyurea and *N*-hydroxyurethane are capable of producing similar main-chain cleavages in human leucocyte DNA in addition to breakage and rearrangement of metaphase chromosomes in Chinese hamster cells. Hydroxylamine fragments mammalian and bacteriophage DNA (Bendich, Borenfreund, Korngold & Krim, 1963), is a mutagen (Freese, Bautz & Freese, 1961) and is an inhibitor of catalase (Boyland & Gallico, 1952).

These results indicate that urethane may exert

its carcinogenic effects after conversion into *N*-hydroxyurethane by one or more of the following processes: (1) biological alkylation, (2) biological carbethoxylation, probably an intermediate process leading to (1), and (3) reaction of hydroxylamine, nitroxyl or hyponitrous acid.

The enhanced carcinogenicity of urethane, as compared with the other analogues (e.g. methyl, *n*-propyl and *n*-butyl carbamates) may be due to the following: (1) urethane is *N*-hydroxylated apparently to a greater extent in the animal body; (2) urethane is metabolized more rapidly, thus presenting a higher concentration of carcinogen to the tissues during a shorter time; (3) the apparent specificity of the ethyl group, since variation of this in the carbamate (or, possibly, *N*-hydroxycarbamate) series results in less activity. Urethane and *N*-hydroxyurethane have the same activity in inducing cancer and in inhibiting the growth of the Walker carcinoma in rats.

If *N*-hydroxylation is the critical process in carcinogenesis, or chemotherapy by urethane, it is difficult to understand why the *N*-hydroxy analogue should not be more potent; this may be due to the fact that, above a certain critical concentration, *N*-hydroxyurethane is rapidly excreted unchanged in the urine to a large extent (see Table 3). Since the carbamates and their *N*-hydroxy analogues are interconvertible in the animal body, it is possible that a certain optimum carbamate:*N*-hydroxycarbamate concentration ratio is necessary for maximum carcinogenic activity. If the production of hydroxylamine, nitroxyl and hyponitrous acid *in vivo* is critical, then variation of the alkyl group in the *N*-hydroxycarbamate series may not produce significant differences in activity.

Urethane and *N*-hydroxyurethane both produce chromosome damage in the tissues of rats (P. C. Koller, personal communication). *N*-Hydroxyurethane is an essential and active metabolite in the biological action of urethane, as is indicated by the production of chromosome damage in root tips of *Vicia faba* by *N*-hydroxyurethane, whereas urethane is inactive in this system. Rat tissues can convert urethane into the active *N*-hydroxy derivative but it is probable that the plant tissues are unable to carry out this biochemical conversion. *N*-Hydroxyurethane and urethane are similar in action to hydroxyurea, in which case the alkylating and carbethoxylating functions of *N*-hydroxyurethane are probably less important than the hydroxylamine residue of the molecule.

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