Some Aspects of the Metabolism of Urethane and *N*-Hydroxyurethane in Rodents

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1. Urethane and *N*-hydroxyurethane are interconvertible in C- and C57 mice. 2. In newborn C57/DBA hybrid mice, prior treatment with 3-methylcholanthrene or urethane stimulated the *N*-hydroxylation of urethane; SKF 525A inhibited the *N*-hydroxylation at 24 hr. but stimulated it at 48 hr. after administration. 3. Liver homogenates of CBA and C3H mice, and of Chester Beatty and hooded rats, but not whole-body homogenates of 1-day-old C57/DBA mice or lung homogenate of 3-week-old Chester Beatty rats, metabolized urethane into *N*-hydroxyurethane in small but definite amounts. 4. Nitrite was detected in the bodies of newborn C57/DBA hybrid mice treated with lethal doses of urethane or *N*-hydroxyurethane; nitrite formation from *N*-hydroxyurethane was stimulated by pretreatment of the animals with 3-methylcholanthrene. 5. The rate of catabolism of *N*-hydroxyurethane by C57/DBA mice was faster in 8-day-old than in 1-day-old animals of the same sex, and faster in females than in males of the same age. 6. Liver slices of several species of rats and mice catabolized *N*-hydroxyurethane at rates that varied with the age and sex of animals of the same species; liver homogenates or microsomes were less effective than slices from the same liver. 7. The enzyme activity was destroyed by boiling or freezing the liver; it was inhibited by increasing substrate concentration and by urethane, *n*-butyl carbamate, cyanide, *p*-benzoquinone or 2,4-dinitrophenol, but not by *p*-chloromercuribenzoate or menadione. 8. The catabolism of *N*-hydroxyurethane by liver slices from adult H-strain rats was not oxygen-dependent. 9. Lung homogenates of 4-week-old female Chester Beatty rats catabolized *N*-hydroxyurethane at 40% of the rate of liver slices from the same source. 10. *O*-Acetyl- and *O*-ethoxycarbonyl-*N*-hydroxyurethane were rapidly deacylated by liver homogenates from adult hooded rats and adult C57 mice, and by human erythrocytes. 11. *N*-Hydroxyurethane reacted rapidly with pyridoxal phosphate at pH 7.4 and 37°. 12. The rate of decomposition of *N*-hydroxyurethane in 0.1 *N*-sodium hydroxide was increased by Ni²⁺, Cu²⁺, Mn²⁺ and [Fe(CN)₆]³⁻ and decreased by Cr²⁺, Zn²⁺, Co²⁺, Mg²⁺ and Fe²⁺. 13. Attempts to synthesize sulphonates of *N*-hydroxyurethane gave ethyl hydrogen sulphate, probably via rearrangement of the unstable *O*-sulphonate.

On injection into rats and mice, urethane is quickly distributed throughout the body, and over 90% of the dose is catabolized within 24 hr. (Boyland & Rhoden, 1949; Bryan, Skipper & White, 1949; Skipper *et al.* 1951*a*); about 5% of the dose appears in the urine unchanged (Bryan *et al.* 1949; Boyland & Nery, 1965; Mirvish, 1964, 1966), and as *N*-hydroxyurethane, acetyl-*N*-hydroxyurethane and *S*-ethyl- and *S*-ethoxycarbonyl-*N*-acetylcysteine (Boyland & Nery, 1965). Some residual radioactivity from labelled urethane is present in blood and tissues 48 hr. after injection; tumour-bearing mice retain up to 46% of the

activity after 24 hr. (Bryan *et al.* 1949; Skipper *et al.* 1951*a*).

A small proportion of the administered urethane thus appears to be responsible for a variety of effects produced in plants, animals and micro-organisms, including tumorigenic, mutagenic, anti-tumour, anti-viral and anti-bacterial effects (for review see Haddow, 1963). The evidence suggesting that these effects may be due to a metabolite falls into three groups, namely: (1) urethane is a complete carcinogen only for remote sites including the lung, liver, spleen, thymus, kidney and ear (Haddow, 1963; Liebelt, Yoshida & Gray, 1961; Trainin,

Preerutti & Law, 1964), and an incomplete skin carcinogen (Salaman & Roe, 1953; Tannenbaum & Silverstone, 1958); (2) it is not a direct carcinogen for transplanted foetal mouse lung (Rogers, 1955); and (3) it is inactive or much less active than *N*-hydroxyurethane in many systems *in vitro* in which metabolic activation is unlikely (see the Discussion section).

MATERIALS AND METHODS

Extinctions. These were determined on a Unicam SP.500 spectrophotometer.

Chromatography. Urethane, *N*-hydroxyurethane and *O*-acetyl- or *O*-ethoxycarbonyl-*N*-hydroxyurethane were detected by thin-layer chromatography as described by Boyland & Nery (1965).

Chemicals. These were of commercial origin, except for *N*-hydroxyurethane and its derivatives, which were prepared as described by Boyland & Nery (1965).

Animals and dosing. (a) *Experiments in vivo.* For the collection of urine, mice were housed in metabolism cages lined with two sheets of Whatman 3MM filter paper held in position with glass rods. Litters, which were grouped according to sex and age and subdivided into control and test animals, were kept with their mothers for experiments lasting over 1 hr. after dosing. Animals less than 10 days old were injected subcutaneously; others were injected intraperitoneally. Accurate dosing of young animals was achieved by using a 10 μ l. Hamilton syringe for injection of aqueous solutions and a 0.5 ml. tuberculin syringe graduated in 0.01 ml. and fitted with a no. 18 needle for solutions in arachis oil. The needle was inserted in the pelvic region and slid subcutaneously into the back of the neck; after delivery of the required dose with gentle massaging of the skin to aid dispersal of the droplet, the needle was slowly withdrawn and the skin gently pinched at the point of insertion to avoid loss through seepage of the injected material. Urethane and *N*-hydroxyurethane were used as 10% (w/v) solutions and sodium nitrite as a 0.7% aqueous solution. 3-Methylcholanthrene and SKF 525A (2-diethylaminoethyl 2,2-diphenylvalerate hydrochloride) were used as 0.5% solutions in arachis oil.

(b) *Experiments in vitro.* Young animals were killed by decapitation and older ones by cervical dislocation. Body tissues were removed and used immediately, unless otherwise stated.

Incubations. Unless otherwise stated, incubations were performed by shaking (100 cycles/min.) in air at 37° in a metabolic shaker (H. Mickle, Gomshall, Surrey). Washed microsome suspensions were prepared as described by Booth & Boyland (1964). Fresh liver or young mice were homogenized in 4 vol. of 0.05 M-phosphate buffer (pH 7.4) in an Ato-Mix blender (Measuring and Scientific Equipment Ltd., London, S.W. 1) for 2 min., and the homogenate was centrifuged in an Angle 50 centrifuge (Measuring and Scientific Equipment Ltd.) at 1480 g_{av} for 20 min. Incubation mixtures containing microsomes were of the following composition: microsomes from 500 mg. of liver; NaH_2PO_4 - Na_2HPO_4 buffer, pH 7.4 (500 μ moles of phosphate); nicotinamide (400 μ moles); $NADP^+$ (1 μ mole); glucose 6-phosphate (20 μ moles); and NADH (2 μ moles), in a total vol. of 5 ml. When liver or whole body supernatant fractions

were used, the mixtures contained NADH (2 μ moles), phosphate buffer (50 μ moles of phosphate), pH 7.4, and supernatant (5 ml.) in a total vol. of 10 ml. Fresh livers were sliced with a sharp blade between frosted microscope slides (Cohen, 1957), and slices of 0.1 g. wet wt. and substrate were shaken in 1 ml. of Krebs-Ringer phosphate buffer, pH 7.4 (Cohen, 1957). In manometric experiments, the main compartment of Warburg flasks contained 250 mg. of slices and 2 ml. of Krebs-Ringer phosphate buffer; substrate, in 1 ml. of buffer, was tipped from the side arm at the beginning of each experiment. All reactions were stopped by adding 0.75 ml. of 36% (w/v) trichloroacetic acid, mixing and cooling in ice-water.

Determination of urethane and *N*-hydroxyurethane. (a) *Determination in vivo.* The filter paper on which mouse urine was collected was eluted with ethanol and the glass rods were washed with ethanol. The ethanolic washing and eluate were combined, dried by Na_2SO_4 and evaporated *in vacuo*, and the residue was dissolved in 6 ml. of ethanol; 2 ml. was used for the estimation of urethane, as described by Boyland & Nery (1965). The remainder was evaporated to dryness *in vacuo*, and the *N*-hydroxyurethane content was determined by the methods described by Boyland & Nery (1964) and Nery (1966). For determination in the bodies of young mice, the animals were killed and homogenized in ice-cold 10% (w/v) trichloroacetic acid (0.5 ml./g. body wt.), the homogenate was centrifuged and the supernatant neutralized with solid $NaHCO_3$. Urethane was estimated on the residue obtained after extraction of samples with three equal volumes of ether, followed by drying by Na_2SO_4 and evaporation *in vacuo* of the combined ethereal extracts. The *N*-hydroxyurethane content of the neutral aqueous supernatant was determined, in triplicate, by the pentacyanoammine ferroate method (Boyland & Nery, 1964; procedure ii) and by the iodine-oxidation method (Nery, 1966; procedure 2); $10^{-3}\epsilon$ values for the two methods were 4.2 and 35.0 respectively, based on the final concentration of *N*-hydroxyurethane. The mean values of each triplicate estimation on the same supernatant agreed within 5%; each *N*-hydroxyurethane concentration shown in Tables 1 and 2 is the mean of these values. Others were determined by the iodine-oxidation method only.

(b) *Determination in vitro.* In incubation mixtures containing liver microsomes, liver homogenates, liver slices or whole-body homogenates, *N*-hydroxyurethane, hydroxylamine or nitrite was determined on 0.5 ml. portions of the supernatant solutions obtained after centrifugation of a mixture of 2 ml. of incubation mixture and 0.5 ml. of 36% (w/v) trichloroacetic acid, as described. This method of protein precipitation gave quantitative (96–102%) recovery of *N*-hydroxyurethane; protein precipitations by $ZnSO_4$ - $NaOH$ gave a recovery of 53% from an incubation mixture that contained buffer, mouse liver homogenate (from 100 mg. of liver) and 0.1 μ mole of *N*-hydroxyurethane/ml.

Determination of nitrite in the bodies of young mice. The animals were homogenized in 20 ml. of ethanol, the homogenate was centrifuged, the residue was shaken with ethanol and centrifuged, and the two supernatant solutions were combined and evaporated to dryness *in vacuo*. The residue was shaken with 0.1 M-phosphate buffer (pH 8.0; 1 ml. of buffer/g. body wt.) and silica gel G (E. Merck A.-G., Darmstadt, West Germany), which removed lipids that interfered with the subsequent determinations, and filtered. Portions (0.2 ml.) were treated with 3.8 ml. and 1 ml. of the

sulphanilamide and *N*-1-naphthylethylenediamine hydrochloride reagents respectively. The *N*-hydroxyurethane content was determined by dilution of 0.2 ml. portions with 0.3 ml. of water and continuing as described by Nery, (1966) (procedure 1). Nitrite or *N*-hydroxyurethane added to whole-body homogenates or injected into newborn mice immediately before they were killed and homogenized, was quantitatively recovered; 10⁻³ε values were 35.0 and 50.0 for *N*-hydroxyurethane and nitrite respectively. When charcoal was substituted for silica gel in the above procedure, only 35% of nitrite and 21% of *N*-hydroxyurethane were recovered from whole-body homogenates, although they were recovered quantitatively from urine after charcoal treatment.

RESULTS

Interconversion of urethane and N-hydroxyurethane in mice. Groups of 12–20 animals were housed in metabolism cages and given two daily injections of urethane or *N*-hydroxyurethane. Control animals were injected with the same volume of water. The urine from each group was collected as described (see the Materials and Methods section) for the periods indicated (see Table 1) and the urethane and *N*-hydroxyurethane contents were determined (see the Materials and Methods section). *N*-Hydroxyurethane was determined on each sample by the sodium pentacyanoammine ferroate

(Boyland & Nery, 1964) and iodine-oxidation (Nery, 1966) methods. The results shown are means of the two values thus obtained; duplicate figures (where shown) were obtained similarly from repeat experiments. Samples of each urine or body extract were also examined by thin-layer chromatography as described by Boyland & Nery (1965); the results confirmed the presence of urethane and *N*-hydroxyurethane in those extracts that contained at least 0.1% of the administered dose (see Table 1).

Effect of pretreatment of young mice with various agents on the N-hydroxylation of urethane. Five groups, each of four male C57/DBA hybrid mice, were injected subcutaneously with urethane, 3-methylcholanthrene or SKF 525A, as outlined in Table 2. Five similar groups, which served as controls, were injected with the corresponding solvents only. Forty 1-day-old female C57/DBA hybrid mice were similarly treated. The results (Table 2) show (a) a slight stimulation of *N*-hydroxylation of urethane by a dose of urethane or 3-methylcholanthrene given 24 hr. earlier or (b) a slight stimulation by SKF 525A given 48 hr. earlier, but complete inhibition if it was given 24 hr. earlier.

In experiments *in vitro*, urethane was incubated with liver homogenates from 1-day-old or adult

Table 1. *Interconversion in vivo of urethane and N-hydroxyurethane by mice*

Animals received urethane (1g./kg. body wt.) or *N*-hydroxyurethane (0.4g./kg.) at the age indicated and a second similar dose 24 hr. later. Other details are given in the text. Where two values are given, they are from duplicate experiments. —, Not determined.

Strain	Sex	Age (days) at first dose	Duration of experiment (hr.)	% of dose found			
				As urethane		As <i>N</i> -hydroxyurethane	
				In body	In urine	In body	In urine
Compound given: urethane							
C-	MF	1	0	68	—	0	—
	M	1	46	—	—	—	0.5
	F	1	46	—	—	0.02	0.1
	M	4	40	4.6, 2.0	1.2	0, 0	0.3, 0.4
	F	4	40	9.3, 6.6	2.6, 2.9	0, 0	0.3, 0.4
	M	42	40	—	0.1, 0.2	—	0.01, 0.03
	F	42	40	—	0.2	—	0.5
	C57	M	3	40	—	—	—
F		3	40	—	—	—	0.16
M		42	40	—	0.1	—	0.13
F		42	40	—	0.1	—	0.13
Compound given: <i>N</i> -hydroxyurethane							
C-	MF	2	0	—	—	95	—
	M	4	40	3.1	0.1	30	2.9
	F	4	40	5.1	0.2	32	1.5
	M	42	40	—	0.1	—	3.2
	F	42	40	—	0.05	—	2.0

Table 2. *N*-Hydroxylation in vivo of urethane by young C57/DBA hybrid mice: effect of pretreatment with various agents

Animals received urethane (1g./kg. body wt.), 3-methylcholanthrene or SKF525A (25mg./kg. body wt.). They were killed 4hr. after receiving the final dose indicated, and the *N*-hydroxyurethane content of the bodies was determined. Other details are given in the text.

Age (days) ...	Substance given			Sex	% of dose detected as <i>N</i> -hydroxyurethane
	1	2	3		
Urethane		—	—	M	0, 0
				F	0, 0.002
Urethane		Urethane	—	M	0.01, 0.02
				F	0.025, 0.03
3-Methylcholanthrene		Urethane	—	M	0.05, 0.04
				F	0.05, 0.06
SKF525A		Urethane	—	M	0, 0
				F	0, 0
SKF525A		—	Urethane	M	0.04, 0.03
				F	0.04, 0.04

CBA, C57/DBA hybrid and C3H mice, and from adult Chester Beatty or hooded rats, and *N*-hydroxyurethane was estimated after 0.5, 1 and 1.5 hr. Duplicate results (see Table 3) were obtained from repeat experiments in which tissue preparations from fresh animals were used. No *N*-hydroxyurethane was found when (i) lung homogenates or liver microsomes from 21-day-old male or female Chester Beatty rats were used, (ii) liver or whole-body homogenates or liver slices from newborn C57/DBA hybrid mice were used or (iii) incubations were conducted in an atmosphere of nitrogen.

Formation of nitrite in the bodies of young mice dosed with N-hydroxyurethane. Newborn C57/DBA hybrid mice were injected subcutaneously with urethane, *N*-hydroxyurethane or 3-methylcholanthrene during the first three days of life, as outlined in Table 4. Control animals were dosed with vehicle only. The animals were killed 3 hr. after receiving the final or only dose, and the nitrite and *N*-hydroxyurethane contents of their bodies were estimated as described. The blood of animals in whose bodies nitrite was found (see Table 4) often appeared dark, and the animals were moribund. Animals killed after recovery from the effects of the drugs (about 18–24 hr. after receiving the final dose) contained no nitrite. When 1-day-old C57/DBA mice were given subcutaneous injections of 70 mg. of sodium nitrite/kg. body wt., the concentrations of nitrite in the body after 30, 80 and 220 min. were 37, 28 and 13 mg./kg. body wt. in males, and 35, 21 and 8 mg./kg. body wt. in females.

Catabolism of N-hydroxyurethane. (a) *Catabolism in vitro.* Whole-body homogenates of 1-day-old CBA or C3H mice, or liver slices or homogenates of

mice or rats of both sexes and of the various strains and ages shown in Table 5, were incubated in 4 ml. portions with 20 μ moles of *N*-hydroxyurethane for 0.5, 1.0 and 1.5 hr. Parallel control mixtures containing all ingredients (see the Materials and Methods section) except *N*-hydroxyurethane, or tissue boiled for 10 min. and cooled before *N*-hydroxyurethane was added, were also incubated. Each reaction was stopped by cooling the mixture in ice-water and adding 1 ml. of aq. 36% (w/v) trichloroacetic acid; the *N*-hydroxyurethane content of the supernatant obtained after centrifugation of the mixture was determined as described in the Materials and Methods section. The results are shown in Table 5.

(b) *Catabolism in vivo.* Litters of 1-day-old and 8-day-old C57/DBA mice were divided into sexes, and each group of 20 animals was dosed with *N*-hydroxyurethane (5 μ moles/g. body wt.). Control animals were dosed with equal volumes of water. Four animals from each group were killed at each of the times indicated (see Fig. 1) and the *N*-hydroxyurethane content of the body of each animal was determined separately (see the Materials and Methods section). The results (see Fig. 1) show that the 8-day-old animals catabolized *N*-hydroxyurethane about 1.2 times as fast as the 1-day-old animals of the same sex, and females about 1.5 times as fast as males of the same age.

Importance of cellular organization in the catabolism of N-hydroxyurethane by rat liver. Livers from 6-week-old female hooded rats were sliced, homogenized or fractionated into microsomal and cell-sap fractions. Part of the homogenate was boiled for 10 min. and cooled. Slices and homogenates were

Table 3. *N*-Hydroxylation of urethane by rodent tissue homogenates

Incubation mixtures contained 10 μ moles of urethane and supernatant from 100 mg. wet wt. of liver/ml., unless otherwise indicated. Other details are given in the text. Where two values are given, they are from duplicate experiments. —, Not determined.

			<i>N</i> -Hydroxyurethane found (μ mole/g. of tissue)		
Age (days)	Strain	Sex	At 0.5 hr.	At 1.0 hr.	At 1.5 hr.
Mouse					
1*	CBA	M	0, 0	0, 0.01	0
		F	0.01, 0.01	0.05, 0.08	0.03, 0.06
21		M	—	0.03, 0.02	0.01, 0.02
		F	—	0, 0.02	0
70-84		M	0, 0	0, 0.01	0
		F	0, 0.02	0.04, 0.07	0.03, 0.03
70-84	C3H	M	0, 0	0.01, 0.01	—
		F	0.01, 0.01	0.04, 0.07	—
1*	C57/DBA	M	0	0	—
		F	0	0	—
Rat					
19	CB	M	0, 0	0, 0.02	0, 0.04
		F	0.01, 0.01	0, 0.02	0.01, 0.02
21†		M	—	0	—
		F	—	0	—
112-117		M	—	0	0
		F	—	0	0.004
28	Hooded	M	—	0.02, 0.07	—
		F	—	0.04, 0.07	—
42		M	—	0	0
		F	—	0	0.03

* Whole-body homogenate. † Lung homogenate.

also prepared from the livers previously frozen for 48 hr. and thawed. Each preparation was incubated with *N*-hydroxyurethane at an initial concentration of 0.5 μ mole/ml., and the *N*-hydroxyurethane contents were determined at 15 min. intervals; corresponding mixtures without substrate served as controls (see Fig. 2). Liver slices were more effective than homogenates or suspensions of microsomes from the same source. The enzyme activity was completely destroyed by boiling or freezing the tissue preparations before incubation.

Inhibition of the catabolism of N-hydroxyurethane by rat liver slices by substrate and other compounds. Liver slices from adult hooded or H-strain rats were incubated with *N*-hydroxyurethane and the various inhibitors at the concentrations shown in Table 6. After 45 min., the *N*-hydroxyurethane contents were determined. The results (see Table 6) show marked inhibition by increasing substrate concentration, and also show that inhibition by *n*-butyl carbamate is strain-specific. *p*-Chloromercuribenzoate, parathion or menadione (each at 1 μ mole/g. wet wt. of liver), when added to mixtures containing 1 g. of liver slices from 6-week-old

female hooded rats and 5 μ moles of *N*-hydroxyurethane in a final volume of 10 ml., showed no inhibition after 45 min.

Effect of oxygen on the catabolism of N-hydroxyurethane by rat liver slices. Mixtures containing 250 mg. of liver slices from 7-week-old male H-strain rats and 0.6, 1.0, 1.5 or 3.0 μ moles of *N*-hydroxyurethane in 3 ml. of Krebs-Ringer phosphate buffer, pH 7.4, were incubated in a nitrogen or oxygen atmosphere at 37° in Warburg constant-volume respirometers, and the *N*-hydroxyurethane contents were determined after 45 min. The amounts of substrate catabolized were 1.8, 1.7, 1.1 and 0.3 μ moles/g. wet wt. of liver in nitrogen and 1.8, 1.8, 1.2 and 0.3 μ moles/g. wet wt. of liver in oxygen. These results show that both the catabolism of *N*-hydroxyurethane and its inhibition by increasing substrate concentration were not oxygen-dependent. On incubation of 1 μ mole of hydroxyurea and 250 mg. of liver slices for 45 min., 1.5 μ moles (in nitrogen) and 1.6 μ moles (in oxygen) of hydroxyurea disappeared/g. wet wt. of liver.

Enzymic deacylation of O-acetyl- and O-ethoxycarbonyl-N-hydroxyurethane. Homogenates of livers

Table 4. *Metabolism of urethane and N-hydroxyurethane by newborn C57/DBA mice: formation of nitrite*

Animals received 3-methylcholanthrene (25mg./kg. body wt.), urethane or *N*-hydroxyurethane during the first 3 days of life, as shown. Nitrite and *N*-hydroxyurethane contents of the whole bodies were determined 3 hr. after the final dose. Other details are given in the text. —, Not determined.

Sex	Substance given			Total or daily dose of urethane or <i>N</i> -hydroxyurethane (g./kg.)	Found in body (μ moles/g.)	
	Age (days) ...	1	2	3	<i>N</i> -Hydroxyurethane	Nitrite
F	Urethane	—	—	0.5	0	0
F	Urethane	—	—	1.0	0	0
F	Urethane	—	—	1.4	0.006	0.02
F	Urethane	Urethane	—	1.0	0.002	0
F	Urethane	Urethane	Urethane	1.0	0.05	0.04
F	3-Methylcholanthrene	Urethane	—	1.0	0.005	0.02
F	3-Methylcholanthrene	Urethane	—	0.5	0.001	0
M	3-Methylcholanthrene	Urethane	Urethane	1.0	0.05	0.01
F	<i>N</i> -Hydroxyurethane	—	—	0.5	3.8	0
F	<i>N</i> -Hydroxyurethane	—	—	1.0	—	0.02
F	3-Methylcholanthrene	<i>N</i> -Hydroxyurethane	—	0.5	3.3	0.05
M	3-Methylcholanthrene	<i>N</i> -Hydroxyurethane	—	0.5	3.5	0.06

Table 5. *Catabolism of N-hydroxyurethane in vitro*

Each 1ml. of incubation mixture contained homogenate or slices from 100mg. of tissue, as indicated, and 0.5 μ mole of *N*-hydroxyurethane. Other details are given in the text. h, Liver homogenate; w, whole-body homogenate; s, liver slices; lu, lung homogenate. —, Not determined.

<i>N</i> -hydroxyurethane catabolized (μ moles/g. of tissue)											
Strain	Sex	Age (days)	After 0.5 hr.			After 1.0 hr.			After 1.5 hr.		
			Tissue ...	...	...	h	w	s	h	w	s
Mouse CBA	M or F	1	0	0	0	0	0	0	0	0	0
	M	21	0.75	—	2.0	0.8	—	3.2	1.0	—	3.4
	F	21	0.8	—	2.1	0.9	—	3.5	—	—	—
	F	21	0.8	—	2.1	0.9	—	3.5	—	—	—
	M	28	0.9	—	1.8	1.0	—	3.0	—	—	—
	F	28	1.4	—	2.9	1.6	—	3.8	—	—	—
C3H	M or F	1	0	0	0.1	0	0	0.2	0	0	0.25
	M	42	—	—	—	0.5	—	2.8	—	—	—
	F	1	—	—	—	0.2	—	2.1	—	—	—
C57/DBA	M or F	1	—	—	—	0	0	0	—	—	—
			After 0.5 hr.			After 1.0 hr.			After 1.5 hr.		
			Tissue ...	...	...	h	s	lu	h	s	
Rat CB	F	28	—	—	—	—	—	0.6	—	—	—
	M	28	0	—	—	0	0.4	—	0	—	0.9
	F	28	0	—	—	0	1.5	—	—	—	—
Hooded	F	28	0	—	1.4	0.1	2.8	—	0.15	—	4.0
	M	42	0	—	1.2	0	2.9	—	0.1	—	3.9
	F	42	0.1	—	1.8	0.15	2.5	—	0.2	—	3.5

Fig. 1. Influence of age and sex on the catabolism of *N*-hydroxyurethane by C57/DBA mice. Animals were dosed subcutaneously with $5\mu\text{moles}$ of *N*-hydroxyurethane/g. body wt. on the first or eighth day of life. Four animals from each group were killed at the times indicated and the *N*-hydroxyurethane content of each animal was determined. Experimental points are $\pm$ s.d. Δ , 1-day-old females; $\blacktriangle$, 1-day-old males; $\square$, 8-day-old males; $\bullet$, 8-day-old females.

Fig. 2. Catabolism of *N*-hydroxyurethane by various liver preparations. Incubation mixtures contained $5\mu\text{moles}$ of *N*-hydroxyurethane and the appropriate preparation from 1g. of liver from a 6-week-old female hooded rat, in a total volume of 10ml. Catabolism by: $\circ$, boiled homogenate or boiled or frozen slices; Δ , microsomes; $\square$, homogenate; $\bullet$, slices. The points shown are means of results of three to five experiments with $\pm$ s.d. 0.03–0.15.

from hooded rats or from C57 mice (both aged 6 weeks) were incubated with *O*-acetyl-*N*-hydroxyurethane ($5\mu\text{moles}$ of substrate/g. wet wt. of liver).

Control mixtures contained boiled homogenates and substrate. Portions (25ml.) of each mixture, after incubation for 10min., were extracted with two equal volumes of ether, the extracts were combined, dried by sodium sulphate and evaporated *in vacuo*, and the residues were examined by chromatography (see the Materials and Methods section); control mixtures contained only unchanged substrate; others contained *N*-hydroxyurethane but no substrate. Portions (2ml.) of each incubation mixture were treated with 0.5ml. of 35% (w/v) trichloroacetic acid at the times indicated (see Fig. 3) and the *N*-hydroxyurethane content was determined by the iodine-oxidation method (see the Materials and Methods section). *O*-Ethoxycarbonyl-*N*-hydroxyurethane (not shown) gave similar results. The pH-activity relationship showed a plateau of maximum activity between pH 7.2 and pH 7.9; at pH 7.4, liver from a 6-week-old male hooded rat showed a maximum activity of deacetylating $105\mu\text{moles}$ of *O*-acetyl-*N*-hydroxyurethane/g. wet wt. of liver, after 10min. No inhibition occurred when the substrate concentration was varied from 5 to $500\mu\text{moles/g.}$ wet wt. of liver. A mixture containing homogenate from 0.5g. of liver from a 9-week-old male hooded rat and $5\mu\text{moles}$ of *O*-acetyl-*N*-hydroxyurethane in 0.05M-Krebs-Ringer phosphate, pH 7.4, in a final volume of 10ml., after 10min. at 37° , contained $3.5\mu\text{moles}$ of *N*-hydroxyurethane, $0.4\mu\text{mole}$ of nitrite and no hydroxylamine. The experiment was repeated with $5\mu\text{moles}$ of *N*-hydroxyurethane or $5\mu\text{moles}$ of *O*-ethoxycarbonyl-*N*-hydroxyurethane as substrate. That containing *N*-hydroxyurethane contained unchanged substrate ($4.9\mu\text{moles}$) and no nitrite or hydroxylamine; the other contained $3.3\mu\text{moles}$ of *N*-hydroxyurethane, $0.3\mu\text{mole}$ of nitrite and no hydroxylamine.

Hydrolysis of O-acetyl-N-hydroxyurethane by washed human erythrocytes. Blood (group B positive, 5ml., from the Royal Marsden Hospital, used a few days after expiry; haematocrit, 40%) from a human subject was diluted with 10ml. of 0.9% sodium chloride and centrifuged, the erythrocytes (2ml., packed) were separated and washed twice more with 10ml. portions of the 0.9% sodium chloride and suspended in 10ml. of Krebs-Ringer phosphate (pH 7.4). The suspension was incubated with an equal volume of (a) the buffer or (b) a 1mM solution of *O*-acetyl-*N*-hydroxyurethane in the buffer. At the times indicated (see Fig. 3), 2ml. portions of (a) or (b) were centrifuged and the supernatant from (b) was assayed for *N*-hydroxyurethane, nitrite and hydroxylamine. The results (see Fig. 3) showed that the substrate was rapidly hydrolysed to form *N*-hydroxyurethane. Nitrite and hydroxylamine were not formed.

Reaction between N-hydroxyurethane and pyridoxal

Table 6. *Inhibition of the catabolism of N-hydroxyurethane by rat liver slices*

Each 1 ml. of incubation mixture contained 100mg. of sliced liver, 0.5 μ mole (unless otherwise indicated) of *N*-hydroxyurethane and inhibitor as indicated. Where higher concentrations of substrate were used, the total concentration is given in column 2. Livers (a) from 6-week-old female hooded rats; (b) from 6-week-old female H-strain rats; (c) from 8-week-old male H-strain rats; (d) from 8-week-old male hooded rats. *p*-Chloromercurobenzoate, parathion or menadione, all used at concentrations of 1 μ mole/g. of liver in incubation mixtures containing *N*-hydroxyurethane and (a), gave no inhibition. —, Not determined.

Inhibitor	Concn. of inhibitor (μ moles/g. of liver)	N-Hydroxyurethane catabolized (μ moles/g. of liver) after 45 min.				Inhibition* (%)			
		(a)	(b)	(c)	(d)	(a)	(b)	(c)	(d)
None	None	1.5	2.5	2.5	2.3	0	0	0	0
Substrate	10	—	—	—	1.7	—	—	—	26
	20	—	—	—	1.0	—	—	—	56
	50	—	—	—	0	—	—	—	100
Urethane	10	0.9	—	—	—	40	—	—	—
<i>n</i> -Butyl carbamate	10	0.36	1.6	2.3	—	76	36	8	—
Sodium cyanide	1	0.6	—	1.4	—	60	—	44	—
	2	—	—	1.2	—	—	—	52	—
	10	—	—	0.4	—	—	—	84	—
<i>p</i> -Benzoquinone†	5	—	2.25	2.4	—	—	10	4	—
2,4-Dinitrophenol†	5	—	2.1	2.4	—	—	16	4	—

* Amount of *N*-hydroxyurethane catabolized after 45 min. at an initial *N*-hydroxyurethane concentration of 0.5 μ mole/ml. of incubation mixture is taken as 0% inhibition.

† Inhibition after 20 min. incubation.

phosphate, and the effect of metal ions. Solutions containing 1 μ mole of *N*-hydroxyurethane and 0.25–8 μ moles of pyridoxal phosphate/ml. in 0.2M-phosphate buffer (pH 7.4) were incubated at 37° for 10 min. The *N*-hydroxyurethane content of each mixture was determined. About 80% of the *N*-hydroxyurethane reacted when the two components were present in equimolar concentrations and about 85% when there was a 2–8-fold excess of pyridoxal phosphate. Similar results were obtained when the reaction proceeded in 0.2M-acetate buffer (pH 4.5), or 0.2M-phosphate buffer (pH 6, 7, or 8), or when pyridoxal phosphate was replaced with pyridoxal. The nature of the reaction product was not investigated.

Mixtures containing 1 μ mole of *N*-hydroxyurethane/ml. in 0.2M-phosphate buffer (pH 6, 7, or 8), or in 0.1N-NaOH were incubated with or without 2 molar equivalents of pyridoxal 5'-phosphate or 1 molar equivalent each of the following salts: cupric sulphate, magnesium sulphate, manganese sulphate, sodium vanadate, ferrous sulphate, ferric chloride, cobalt chloride, nickel sulphate, zinc sulphate, chromous chloride and potassium ferricyanide. The mixtures were shaken in air at 37° for 2 hr. The *N*-hydroxyurethane content of each mixture was determined by treating 0.2 ml. portions with 1.8 ml. of 0.5M-phosphate buffer (pH 8.0) and then continuing as

described by Nery (1966) (procedure 1), but with only 0.9 ml. of the *N*-1-naphthylethylenediamine reagent; the nitrite content was determined by treating similar portions with 2.4 ml. each of the sulphanilamide and *N*-1-naphthylethylenediamine reagents. The $10^{-3}\epsilon$ values were 35.8 and 50.0 for *N*-hydroxyurethane and nitrite respectively. At pH 6, 7 and 8, the metal ions did not catalyse the breakdown of the *N*-hydroxyurethane–pyridoxal phosphate reaction product after 2 hr., with the exception of VO_4^{3-} , which also caused some breakdown of *N*-hydroxyurethane with the production of nitrite (see Table 7). In 0.1N-NaOH, *N*-hydroxyurethane was decomposed to the extent of 20 moles/100 moles after 2 hr.; this decomposition was decreased to 2–4 moles/100 moles on addition of Cr^{2+} , Cu^{2+} , Co^{2+} , Mg^{2+} or Fe^{2+} and increased to 27, 92, 100 and 100 moles/100 moles in the presence of Ni^{2+} , Cu^{2+} , Mn^{2+} , or $\text{Fe}(\text{CN})_6^{3-}$ respectively. The Mn^{2+} -catalysed decomposition produced 32 moles of nitrite/100 moles.

Attempts to prepare sulphonates of N-hydroxyurethane. A mixture of *N*-hydroxyurethane (3g.) and pyridine-sulphur trioxide (10g.) in chloroform (100ml.) was stirred at 25° for 16 hr. The solvent was evaporated *in vacuo*, the residue was extracted with methanol (100ml.) and the extracts were treated with dry ammonia at 0° and filtered; the filtrate was evaporated *in vacuo* to 50 ml. and treated

with dry ether (100 ml.). Ethyl ammonium sulphate (3.6 g., 90%) separated as glistening plates, m.p. and mixed m.p. 99° (Found: C, 17.1; H, 6.2; N, 9.7; S, 22.5. Calc. for $C_2H_9NO_4S$: C, 16.8; H, 6.3; N, 9.8;

S, 22.4%). When similar mixtures were stirred for 4 or 16 hr. at 0°, or when methylene chloride was used as a solvent, varying amounts of ethyl ammonium sulphate and starting materials were recovered.

DISCUSSION

Urethane is inactive, or much less active than *N*-hydroxyurethane, in those systems *in vitro* in which further metabolism of the drug is unlikely, e.g. in the inactivation of transforming DNA (Freese, 1965), in causing aberrations in plant (Boyland, Nery & Pegg, 1965) and animal (Borenfreund, Krim & Bendich, 1964) chromosomes, as an anti-viral agent (de Sousa, Boyland & Nery, 1965) and as a specific inhibitor of DNA synthesis (Young & Hodas, 1964). Urethane is metabolically activated *in vivo* and becomes a teratogen (Takaori, Tanabe & Shimamoto, 1966) of lower activity than *N*-hydroxyurethane (Murphy & Chaube, 1964), a mutagen, a chromosome-damaging agent and capable of changing mitotic activity (for reviews see Cornman, 1954; Wakonig-Vaartaja, 1964), an inhibitor of nucleic acid synthesis (Skipper *et al.* 1951b) and an anti-viral agent (Link, Blaskovic & Raus, 1961). The mutagenic effects observed for urethane in many organisms including *Antirrhinum*, *Drosophila* and *Neurospora* indicate (a) that there is a need for the metabolic conversion of urethane by a 'cytoplasmic factor' into the active agent and (b) that such effects may be induced by guanosine and cytidine-adenosine and thymidine transitions (see Freese, 1965).

Urethane is metabolized into products that are quickly eliminated from the system (Boyland & Rhoden, 1949; Skipper *et al.* 1951a). A small fraction of the dose is *N*-hydroxylated (see Table 3; also Boyland & Nery, 1965); the *N*-hydroxy derivative is largely reduced to urethane (Boyland & Nery, 1965; Mirvish, 1964, 1966), ethylates and ethoxycarbonylates tissue thiol groups (Boyland &

Fig. 3. Enzymic deacylation of *O*-acetyl-*N*-hydroxyurethane. Incubation mixtures contained 5 μ moles of *O*-acetyl-*N*-hydroxyurethane, and supernatant from 1 g. of liver or erythrocytes from 2.5 ml. of human blood, in a total volume of 10 ml. Liver supernatant from an adult hooded rat ($\circ$, male; $\triangle$, female) and from an adult C57 mouse ($\bullet$, male; $\square$, female); boiled liver supernatant from above sources ($\blacktriangle$); and human erythrocytes ($\blacksquare$). The points ($\pm$ S.D.) are means of three separate experiments.

Table 7. Reaction between *N*-hydroxyurethane, pyridoxal phosphate and sodium vanadate

Reaction mixtures contained 1 μ mole of *N*-hydroxyurethane and, where indicated, 2 μ moles of pyridoxal phosphate and 1 μ mole of sodium vanadate/ml. of phosphate buffer; the *N*-hydroxyurethane and nitrite contents of the mixtures were determined after 2 hr. at 37°. Other details are given in the text.

		Concns. (μ mole/ml.)					
		pH 6.0		pH 7.0		pH 8.0	
VO_4^{3-}	Pyridoxal phosphate	<i>N</i> -Hydroxyurethane	Nitrite	<i>N</i> -Hydroxyurethane	Nitrite	<i>N</i> -Hydroxyurethane	Nitrite
None	None	1.0	0	1.0	0	1.0	0
None	+	0.18	0	0.22	0	0.24	0
+	None	0.95	0.03	0.93	0.06	0.90	0.08
+	+	0.20	0.02	0.23	0.02	0.025	0.02

Nery, 1965) or forms a conjugate with glucuronic acid (Mirvish, 1966). The *N*-hydroxylation of urethane *in vivo* is stimulated by pretreatment of mice with 3-methylcholanthrene (see Table 4), which stimulates microsomal oxidations and which has a synergistic action with urethane in leukaemogenesis (Kawamoto, Ida, Kirschbaum & Taylor, 1958), and by repeated urethane injections (see Table 4). The inhibitor SKF525A showed a biphasic effect, i.e. inhibition of *N*-hydroxylation during the 24–28 hr. period and apparent stimulation during the 48–52 hr. (see Table 2) period following administration. Following administration of a single dose of 1.4 g. of urethane/kg., or of 1 g. of *N*-hydroxyurethane/kg., or of three daily doses of 1 g. of urethane/kg. to newborn C57/DBA mice, small amounts of nitrite were detected in the bodies of the animals 4 hr. after receiving the final dose. This effect was increased when the animals were pretreated with 3-methylcholanthrene during the first day of life (see Table 4). The animals in whose bodies nitrite was found were moribund and their blood appeared dark, owing probably to methaemoglobinemia. The nitrite may have arisen from further metabolic oxidation of *N*-hydroxyurethane or from the catabolism of acetyl-*N*-hydroxyurethane (see below). Analogous metabolic reactions are the conversions of arylamines into nitroso-aryls (Kiese, 1959; Jagow, Kieser & Renner, 1966) and of hydroxylamine into nitrite (Cuisa & Luzzatto, 1908). Chemical oxidation of *N*-hydroxyurethane produced *O*-ethoxycarbonyl-*N*-hydroxyurethane and nitrite (Boyland & Nery, 1966*b*; Nery, 1966).

O-Ethoxycarbonyl- and *O*-acetyl-*N*-hydroxyurethane were deacylated and oxidized in liver preparations so that some nitrite was formed. Human blood contains esterases that liberate a stoichiometric amount of *N*-hydroxyurethane from the *O*-acetyl derivative after incubation for 10 min., and the product is not metabolized further during at least 1 hr. (see Fig. 3).

The mechanism of urethane carcinogenesis is probably similar to that being elucidated for aromatic amine carcinogenesis (reviewed by Miller & Miller, 1966). Further metabolic activation of *N*-hydroxyurethane by acetylation (Boyland & Nery, 1965) or glucosiduronidation (Mirvish, 1966) may produce derivatives capable of generating the radical (3) (Scheme 1), which may be the proximate carcinogen. Esters of 2-*N*-hydroxyacetamido-fluorene readily react with DNA or proteins to give products in which the arylamino analogue of radical (3) or an isomer is directly bound to guanine or to methionine sulphur (Miller & Miller, 1966); acyl esters of *N*-hydroxyurethane react *in vitro* at neutral pH with cytosine, cysteine, glutathione and methionine more readily than does *N*-hydroxy-

urethane (E. Boyland & R. Nery, unpublished work). Although a sulphuric acid conjugate has not been detected as a metabolite of urethane or *N*-hydroxyurethane, such a metabolite, if formed, may react *in situ* as an alkylating agent; attempts to synthesize it under conditions that favour the sulphonation of arylhydroxylamines (Boyland & Nery, 1962) gave almost quantitative yields of ethyl ammonium sulphate (see the Results section), probably via the unstable *O*-sulphonate, which may be the active species responsible for the ethylation of thiol groups previously reported (Boyland & Nery, 1965).

It has been suggested (Mirvish, 1966; Kaye & Trainin, 1966) that urethane is the proximate carcinogen and that *N*-hydroxyurethane is carcinogenic by virtue of its reduction to urethane. This is supported by the findings that (i) the major metabolic reaction of *N*-hydroxyurethane in rodents is conversion into urethane (Boyland & Nery, 1965; Mirvish, 1964, 1966), this metabolic *N*-dehydroxylation apparently being a general one for *N*-hydroxy compounds (Williams, 1959; Walker & Walker, 1959; Boyland & Nery, 1965; Lotlikar, Miller, Miller & Margreth, 1965; Adamson, Ague, Hess & Davidson, 1965); (ii) *N*-hydroxyurethane has been detected only in small amounts in animals dosed with urethane, so that *N*-hydroxylation is probably only a minor pathway of urethane metabolism; and (iii) *N*-hydroxyurethane has been shown to be approximately equally (Berenblum *et al.* 1959; Miller, Cramer & Miller, 1960; Boiato, Mirvish & Berenblum, 1966) or less (Kaye & Trainin, 1966), but not more, carcinogenic than urethane, although a slightly higher incidence of thymic lymphosarcomas and a shorter latent period for leukaemogenesis have been observed (Boiato *et al.* 1966). The theory suggests that the greater susceptibility of young rodents to urethane carcinogenesis is due to the slower catabolism of urethane; however, *n*-butyl carbamate, which inhibits urethane catabolism (Mirvish & Kaye, 1964), decreases the carcinogenic response (Garcia, 1963); X-rays, which have a synergistic action with urethane in leukaemogenesis, do not affect the rate of urethane catabolism (Cividalli, Mirvish & Berenblum, 1965; Kaye, 1960); and differences in neoplastic response of mice of different strains and sex are not reflected in differences in the rates of urethane catabolism (Cividalli *et al.* 1965). A recent report (Kaye & Trainin, 1966) that in small doses urethane is about five times as carcinogenic as *N*-hydroxyurethane in comparable doses contrasts with the fact that *N*-hydroxyurethane is converted into urethane to the extent of at least 70% both in young and in adult mice (Boiato *et al.* 1966; Mirvish, 1966).

Because of the greater reactivity of *N*-hydroxy-

other hydroxamic acids; reaction (a) is an irreversible and detoxifying process; and (2) is directly responsible for anaesthesia, since urethane is the more efficient anaesthetic (Spriggs & Stockham, 1966). The metabolites (1), (2), (4), (5) and (10) have been described (see above); the radical (6) has been detected by electron-spin-resonance studies during the chemical oxidation of *N*-hydroxyurethane (Gutch & Waters, 1965), and radical (8) as *S*-cysteinyl derivatives during the metabolism of urethane and *N*-hydroxyurethane (Boyland & Nery, 1965). The nitroxyl radical, (9), which is formed in hot alkaline solutions of hydroxamic acids (Boyland & Nery, 1966a), is probably responsible for chromosomal aberrations produced by several hydroxamic acids (Borenfreund *et al.* 1964).

It is suggested that analogous free-radical mechanisms may operate during aromatic amine carcinogenesis [cf. the ready reactivity of *N*-acyloxy derivatives of aromatic amines with cellular macromolecules at neutral pH (Miller & Miller, 1966)], whereas the *N*-hydroxy derivatives require acidic conditions (Kriek, 1965) and probably react through ionic intermediates (Heller, Hughes & Ingold, 1951).

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