The Distribution of Urethane in Animal Tissues, as Determined by a Microdiffusion Method, and the Effect of Urethane Treatment on Enzymes

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The narcotic action of urethane has been known for a long time (Schmiedeberg, 1885), and the leucopenic action was described by Hawkins & Murphy (1925) almost 20 years before its first clinical use in treatment of leukaemia (Paterson, ApThomas, Haddow & Watkinson, 1946). Urethane has a relatively slow toxic action on cell nuclei causing pycnosis. It is a radiomimetic poison like tryptamine and the nitrogen mustards (Dustin, 1947), and causes chromosome breaks (Boyland & Koller, 1949) when given in larger doses. This radiomimetic action may be associated with the induction by urethane of lung tumours (Nettleship & Henshaw, 1943).

The nature of the biochemical lesions underlying the narcotic and radiomimetic effects is still unknown, although Fisher & Henry (1944) suggest that the respiration associated with activity rather than that concerned with the basal metabolism is primarily inhibited. Meyerhof & Wilson (1948) have shown that phenylurethane inhibits hexokinase of mammalian cells.

An improved method of estimating urethane in tissues is described in this paper, and this has been used to study the distribution and excretion of urethane in animals. Urethane is hydrolyzed by caustic alkalis to yield ethanol and this reaction is the basis of the method of estimation described by Archer, Chapman, Rhoden & Warren (1948), and the present method.

EXPERIMENTAL

Preliminary trials showed that urethane was hydrolyzed at room temperature and at 37° by KOH, and the rate of this reaction in Conway units with different concentrations of alkali was followed by the method described by Winnick (1942) for estimation of the ethanol formed.

The course of the reaction, shown in Table 1, is such that the reaction is only complete in 16 hr. if the final concentration of KOH is 2N. If higher concentrations of alkali are used with whole blood, precipitation occurs and the results are irregular. Urethane is not decomposed by K_2CO_3 under these conditions and for this reason K_2CO_3 was added to the blank unit in carrying out determinations.

Method of estimation. A standard volume of 1 ml. 0.04N- $K_2Cr_2O_7$ in 10N- H_2SO_4 is placed in the central compartment of a Conway unit. The sample, which should be less than 0.7 mg. urethane, contained in 1 ml. fluid, and 1 ml. 4N-KOH, are placed in the outer ring of the unit. A blank is set up with a 1 ml. sample of urethane and 1 ml. 2M- K_2CO_3 . The alkalis used must not contain volatile reducing substances and are kept in glass-stoppered bottles. The units are sealed and incubated at 37° for 16 hr. or more. At the end of the incubation KI is added to the acid $K_2Cr_2O_7$, and the liberated I_2 titrated with 0.02N- $Na_2S_2O_3$. The difference in titre between the two units is proportional to the amount of urethane present; 1 ml. 0.02N- $Na_2S_2O_3$ is equivalent to 0.445 mg. urethane.

Table 1. *Hydrolysis of urethane at 37°*

(Yield of ethanol as % of theory at different times)

Alkali used ...	KOH								K_2CO_3	
Final conc. in outer ring ...	5N	2N	N				0.5N	M		
Urethane present (mg.) ...	0.5	0.25	0.5	0.25	0.5	0.25	0.5	0.25	0.5	0.5
Time (hr.)	1	40	—	—	—	—	—	—	—	—
2	70	19	13	8	8	8	0	0	0	—
4	101	83	58	67	56	12	23	4	0	—
8	101	85	76	74	67	12	33	0	0	—
16	—	100	98	84	92	80	75	0	0	—
32	—	98	95	98	100	92	85	0	0	—

Alternatively, the titration can be carried out with a Conway burette using 0.1N- $Na_2S_2O_3$. With this modification 1 ml. of 0.025N- $K_2Cr_2O_7$ in 10N- H_2SO_4 is placed in the central compartment and the urethane sample (e.g. 0.1 ml. blood) is incubated with 2 ml. of M- K_2CO_3 as the blank, and a further 0.1 ml. blood treated with 2 ml. 2N-KOH in the outer ring of the second unit. Thus by using a sensitive microburette the estimation can be carried out on 0.2 ml. blood. The amount of urethane and volatile reducing matter in the sample must not be more than the equivalent of 0.5 mg. urethane.

Duplicate estimations agreed within 5% and the method could be applied directly to blood, urine or tissue extracts deproteinized with tungstic acid or trichloroacetic acid. The distillation method could not be used with urine and some extracts on account of the frothing which occurred. Determinations on normal human blood and on blood of normal rats and rabbits gave no indication of the presence of any substance reacting as urethane in this reaction.

RESULTS

Excretion of urethane. Groups each of six rats kept in metabolism cages were given intraperitoneal injections of 500 mg. urethane/kg. body weight, as a 5% (w/v) solution. The urine was collected and the urethane content determined. No urethane was found in the urine of control animals, which were injected with 10 ml. water/kg. body weight. Results of a typical experiment are shown in Table 2. The injected animals excreted more urine than the controls so that the drug had a diuretic action, but only about 4% of the injected urethane was excreted in urine.

Table 2. *Excretion of urethane by rats*

(Six rats injected with 500 mg. urethane/kg. body wt. in 5% (w/v) solution. Six control rats injected with 10 ml. water/kg. body wt.)

Time after injection (hr.)	Vol. of urine collected		Total urethane excreted (mg./kg. body wt.)	Urethane excreted (percentage of dose)
	Controls (ml.)	Treated (ml.)		
1	Nil	2.5	1.0	0.3
2	0.5	8.0	6.9	1.4
3	0.5	1.8	8.1	1.6
6	2.0	3.0	10.1	2.0
24	4.0	25.0	20.4	4.1
Total urine	7.0	40.3	—	—

Distribution of urethane in tissues. Rats were injected subcutaneously with 1 g. urethane/kg. body weight, and killed at different times after injection. A specimen of blood was taken and organs were removed and extracted with 2 vol. of 10% trichloroacetic acid. Urethane was estimated in the blood and in the organ extracts.

Deproteinized extracts of rat liver, but not of other tissues, prepared with trichloroacetic acid, metaphosphoric acid or tungstic acid, produced volatile reducing substances on standing. The nature of this material was not determined. Determinations carried out on fresh liver extracts appeared to be quite satisfactory.

The results shown in Table 3 indicate that in normal rats the urethane of a dose of 1 g./kg. disappears from the body within 24 hr. As very little urethane is excreted in urine and none could be detected in faeces most of the drug must be metabolized. The metabolism is very much slower in tumour-bearing rats, in which some urethane remains even after 48 hr.

Observation of the behaviour of rats indicated that the threshold concentration of urethane for narcosis was between 60 and 80 mg./100 ml. blood. Rats with blood concentrations of 60 mg./100 ml. or below were not anaesthetized, while those with concentrations of 80 mg./100 ml. or over were anaesthetized. The same dose of urethane produced much

more prolonged narcosis in tumour-bearing than in normal rats. A dose of 1 g./kg. body weight induced anaesthesia for 8–12 hr. in normal rats, but for 24 hr. in tumour-bearing rats.

Table 3. *Urethane content of rat tissues after injection of 1 g./kg. body weight*

Time after injection (hr.)	A. Normal rats			
	Urethane present (mg./100 g.)			
	Blood	Brain	Liver	Spleen
1	140	138	—	—
2	100	85	100	110
5	90	—	80	90
18	57	75	—	40
24	0	0	2	2
40	0	0	0	0
80	0	0	0	0

Time after injection (hr.)	B. Rats with Walker carcinoma	
	Urethane present (mg./100 ml.)	
	Blood	Tumour
1.5	100	100
2	75	90
5	93	80
18	110	65
20	120	110
24	77	55
40	32	15
49	10	25
55	7	0

The injected urethane was rapidly distributed in the organs of rats or rabbits with no indication of localization of the drug in any one organ or tissue.

Urethane in blood of patients. The method described was used for estimation of urethane in blood from patients under treatment with urethane. The blood was collected in tubes containing fluoride. The highest concentration found in human blood was 59 mg./100 ml. in a leukaemia patient receiving 7 g. urethane daily. This patient was sleepy, but not anaesthetized, which suggests that the concentration of urethane inducing narcosis is higher in man than in rats. Another leukaemia patient who was given 6 g. urethane daily had blood levels of 23, 19 and 40 mg./100 ml. on samples taken at weekly intervals. Patients dosed with 3 g. urethane daily had blood concentrations of 10, 12, 12, 14, 16, 18 and 19 mg./100 ml.

Blood cholinesterase in patients treated with urethane. As many potent inhibitors of cholinesterase are urethane derivatives, the continuous treatment with urethane might cause reduction in blood cholinesterase. The esterase activity of the plasma with acetylcholine, benzoylcholine and acetyl- β -methylcholine as measured by liberation of carbon dioxide from sodium bicarbonate in the Warburg apparatus (following the methods of Hawkins & Gunter, 1946) are shown in Table 4. The

Table 4. *Esterase activity of human blood constituents under urethane treatment*

(Subjects received 3 g./day by mouth.)

 CO_2 ($\mu\text{L}/\text{hr.}$) evolved by 1.0 ml. plasma or 1.0 ml. washed whole erythrocytes in presence of 0.025 M- NaHCO_3 at 37°

					Plasma			Erythrocytes	
Substrate					0.06 M- Acetylcholine bromide	0.06 M- Benzoylcholine chloride	0.03 M- Acetyl- β - methylcholine chloride	0.06 M- Acetylcholine bromide	0.03 M- Acetyl- β - methylcholine chloride
Mr E.:									
1 day before treatment					4750	1765	312	1170	1295
2 days after beginning of treatment					4550	2100	240	810	1074
5	"	"	"	"	4685	1020	585	1780	1270
11	"	"	"	"	4230	—	280	800	1015
13	"	"	"	"	4010	—	300	1000	1067
20	"	"	"	"	3350	1280	140	1400	1100
Mrs A.:									
4 days before treatment					1805	600	135	2700	1960
2 hr.	"	"	"	"	1840	480	175	1180	—
7 days after beginning of treatment					1030	572	100	1370	1430
9	"	"	"	"	1371	554	114	—	1600

results indicate that urethane treatment causes a decrease in the ability of the plasma to hydrolyze acetylcholine. In one case (Mr E.) this seems to be due to a fall in pseudocholinesterase as indicated by the decreased hydrolysis of benzoylcholine. In the second series (Mrs A.) the plasma showed a fall in true cholinesterase, as the samples taken during treatment hydrolyzed acetyl- β -methylcholine more slowly.

Effect of urethane on tissue metabolism. Urethane given to tumour-bearing rats or mice in doses sufficient to inhibit mitosis had no inhibitory effect on respiration or glycolysis of tissue slices as measured in the Warburg apparatus (Table 5). Treatment with urethane caused a slight increase in respiration of tumour tissue. The doses of urethane given would be

sufficient to induce cancer of the lung, and did inhibit mitosis of the jejunal mucosa and Walker rat carcinoma as shown by examination of histological preparations of tissue from animals used for the manometric measurements. The glycolysis and respiration of mouse lung and jejunum and of Walker carcinoma tissue from rats were not affected by 0.011M-urethane added *in vitro*. This concentration is equivalent to that which produces cytological effects in these tissues *in vivo*.

DISCUSSION

The results show that urethane disappears from the body of normal rats for the most part without being excreted. It is presumably metabolized. Experiments carried out with urethane containing ^{14}C in the carbamate radical by Skipper, Bryan, White & Hutchison (1948) have shown that very little of the carbamate is excreted in urine, and that about 90 % of the radioactivity passes into the respiratory gases in 24 hr.

The work with radioactive urethane also supports the findings in the present paper, that there is no accumulation of urethane in any particular organ and that the breakdown is slower in tumour-bearing animals than in normal animals (Skipper, personal communication). The reduced metabolism of urethane in tumour-bearing rats is not due to retention in the tumour, but is probably due to deficiency of enzymes concerned in the breakdown of the urethane molecule. This would be comparable with the reduced content of other enzymes such as catalase (Greenstein, 1943; Weil-Malherbe & Schade, 1948), D-amino-acid oxidase (Westphal, 1943), or arginase (Greenstein, Jenrette, Mider & White, 1941) in the livers of animals with tumours.

Table 5. *Effect of urethane on tissue metabolism in vitro*

(Measurements during 1 hr. made in the appropriate Krebs-Ringer solution with Warburg manometers at 38° . Q_{O_2} in O_2 and Q_{CO_2} values in $\mu\text{L}/\text{mg. dry wt.}/\text{hr.}$)

Tissue	Tissue from normal animals without addition		Tissue from animals treated with urethane		Tissue from normal animals with 0.011 M-urethane	
	$Q_{\text{CO}_2}^{\text{N}}$	Q_{O_2}	$Q_{\text{CO}_2}^{\text{N}}$	Q_{O_2}	$Q_{\text{CO}_2}^{\text{N}}$	Q_{O_2}
Mouse lung	4.2	10.2	3.9*	7.5*	4.2	11.0
Mouse jejunum (whole wall)	12.6	19.2	16.7*	—	11.2	18.3
Rat Walker carcinoma	29.0	9.2	28.9†	9.9†	28.7	9.9
Rat-jejunal mucosa	5.35	—	4.95†	—	—	—
Rat-kidney medulla	25.4	—	25.9†	—	—	—
Rat-brain homogenate	7.0	—	6.1†	—	—	—

* Mice fed on diet containing 2 % urethane for 3 days before measurements.

† Rats given doses of 1 g. urethane/kg. body wt., on 3 successive days before measurements.

The absence of inhibitory effect of urethane (given in doses which reduce mitosis) on tissue metabolism indicates that urethane possibly inhibits some specific cell process or prevents the utilization of the energy of glycolysis or respiration from synthetic processes. Studies on the effect of methyl-di(2-chloroethyl)-amine (HN2) on the metabolism of the Walker carcinoma showed a decrease in glycolysis some days after treatment (Boyland, Clegg, Koller, Rhoden & Warwick, 1948). Urethane resembles nitrogen mustard in its cytological effects, and a similar effect on metabolism had been expected. The inhibition of glycolysis observed with nitrogen mustard is probably due to effects on hexokinase, and this may not be the fundamental cause of the nuclear damage, but rather an indication that phosphokinases are affected. Urethane may inhibit such phosphokinases (as yet unidentified) which are concerned with nucleoprotein metabolism, but the biochemical mechanism of its action is as yet unknown.

SUMMARY

1. As urethane is hydrolyzed by cold caustic alkali to give quantitative yields of ethanol, it can be

estimated by incubation with potassium hydroxide in Conway units and determination of the ethanol absorbed in acid potassium dichromate. This method can be applied directly to blood (using 0.2 ml.), urine or tissue extracts.

2. Following injection, urethane becomes evenly distributed in all the tissues of the body. Only a small amount is excreted in urine but the greater part is metabolized. The metabolism is slower in tumour-bearing rats than in normal rats.

3. Of two patients treated with urethane the blood of one showed a decrease in true cholinesterase while the blood pseudocholinesterase of the other patient decreased.

4. Treatment of rats or mice with urethane in doses sufficient to cause inhibition of mitosis or induce cancer of the lung did not reduce the respiration or glycolysis of lung, jejunum or Walker carcinoma tissue.

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REFERENCES

- Archer, H. E., Chapman, L., Rhoden, E. & Warren, F. L. (1948). *Biochem. J.* **42**, 58.
 Boyland, E., Clegg, J. W., Koller, P. C., Rhoden, E. & Warwick, O. H. (1948). *Brit. J. Cancer*, **2**, 17.
 Boyland, E. & Koller, P. C. (1949). (To be published.)
 Dustin, P. (1947). *Nature, Lond.*, **159**, 794.
 Fisher, K. C. & Henry, R. J. (1944). *J. gen. Physiol.* **27**, 469.
 Greenstein, J. P. (1943). *J. Nat. Cancer Inst.* **3**, 397.
 Greenstein, J. P., Jenrette, W. V., Mider, G. B. & White, J. (1941). *J. Nat. Cancer Inst.* **1**, 687.
 Hawkins, J. A. & Murphy, J. B. (1925). *J. exp. Med.* **42**, 609.
 Hawkins, R. D. & Gunter, J. M. (1946). *Biochem. J.* **40**, 192.
 Meyerhof, O. & Wilson, J. R. (1948). *Arch. Biochem.* **17**, 153.
 Nettleship, A. & Henshaw, P. S. (1943). *J. Nat. Cancer Inst.* **4**, 309.
 Paterson, E., ApThomas, I., Haddow, A. & Watkinson, J. M. (1946). *Lancet*, **i**, 677.
 Schmiedeberg, O. (1885). *Arch. exp. Path. Pharmacol.* **20**, 203.
 Skipper, H. E., Bryan, C. E., White, C. & Hutchison, O. S. (1948). *J. biol. Chem.* **173**, 371.
 Weil-Malherbe, H. & Schade, R. (1948). *Biochem. J.* **43**, 118.
 Westphal, U. (1943). *Hoppe-Seyl. Z.* **276**, 213.
 Winnick, I. (1942). *Industr. Engng Chem. (Anal. Ed.)*, **14**, 523.

An Immune Globulin Fraction from Bovine Precolostrum

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This paper describes the preparation and partial characterization of an immune globulin fraction from bovine precolostrum. This material was studied as part of a joint programme on diseases of young calves because of its possible relation to colostrum, and the role of the latter in the transference of passive immunity to the suckling animal.

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Bovine precolostrum is a viscous honey-like substance obtained from the udders of pregnant heifers at about half term. It was first described by Woodman & Hammond (1923) and Asdell (1925), who showed that it was deficient in the normal constituents of milk, but very rich in globulin. They suggested that it might prove rich in antibodies, a point recently demonstrated by Blakemore (1947), and that its accumulation in the udder well before