

METABOLIC STUDIES WITH DI(AZIRIDIN-1-YL) SULPHOXIDE (DIETHYLENEIMINOSULPHOXIDE)

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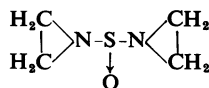
A. W. CRAIG, H. JACKSON AND R. M. V. JAMES

From the Christie Hospital and Holt Radium Institute, Manchester 20

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The work described was undertaken after the observation that the diuresis induced by several derivatives of ethyleneimine could be correlated with their content of ethyleneimine. The preparation of ^{35}S -labelled di(aziridin-1-yl) sulphoxide (diethyleneiminosulphoxide) is described together with its metabolism in rat, mouse, rabbit and dog. The drug was completely metabolized in all species and, with the exception of the dog, most of the activity was excreted in the urine within 3 days. The main radioactive metabolite in all species was sulphate. *In vitro* studies demonstrated that the compound was slowly broken down to sulphite and free ethyleneimine; this hydrolysis was greatly accelerated in the presence of phosphate.

Ethyleneimine and several derivatives structurally resembling urea cause polyuria in nonhydrated rats (Jackson & James, 1963) and this effect is apparently related to the ethyleneimine content of the compounds. The metabolism of one of these compounds, di(aziridin-1-yl) sulphoxide (diethyleneiminosulphoxide), labelled with radioactive sulphur, has therefore been examined.



METHODS

Preparation of ^{35}S -labelled di(aziridin-1-yl) sulphoxide

Thionyl chloride (0.25 g labelled with 16 mC of ^{35}S) in dry ether (12 ml.) was added slowly with stirring to a mixture of triethylamine (0.43 g) and ethyleneimine (0.3 g) in 12 ml. of dry ether, cooled with solid carbon dioxide. The precipitated triethylamine hydrochloride was then removed by filtration, the ether removed *in vacuo* and the di(aziridin-1-yl) sulphoxide distilled (boiling point $60^\circ\text{C}/0.1\text{ mm Hg}$). Chromatography in a butanol:dioxan:2*N*-ammonia (4:1:5) mixture and water-saturated butanol gave single radioactive spots with R_f values of 0.62 and 0.69 respectively.

Assay of radioactivity in biological material

The radioactivity present in urine was measured as barium ^{35}S -sulphate. Aliquots of urine (0.5 to 4.0 ml.) were heated at 75°C for 20 min with 1 ml. of 0.4 *N*-sulphuric acid in 7.5 ml. screw-capped containers. Barium chloride solution (2 ml., 10% w/v) was then added and the mixture left overnight at 75°C . The precipitate of barium sulphate was then collected and washed successively with water and methanol, using a filtering device developed by Dale & Gilbert (1956). The uniform layer of barium sulphate on a 1 in. diameter disc of

filter-paper (Whatman No. 42) was dried, weighed and counted using an end-windowed Geiger counter. To determine the degree of self-absorption, a curve relating weight of barium sulphate against count-rate was plotted for a known amount of radioactive sulphate precipitated with different amounts of barium sulphate. In each assay the weight of sulphate was noted and the necessary corrections were made. Radioactivities present in blood and plasma were measured by solid counting of 0.1 ml. aliquots dried on aluminium planchettes, the self-absorption factor being determined using known amounts of radioactivity incorporated into aliquots of blood and plasma. The distribution of radioactivity on chromatograms was determined using a multicounter system (Gilbert & Keene, 1958) and confirmed by contact autoradiography.

Metabolic studies

Urine was collected from rats, mice, a rabbit and two dogs. Aliquots were taken for estimating total radioactivity and for chromatography.

Rats. Six male Wistar rats (220 ± 5 g) were injected with an aqueous solution of the labelled drug (2.5 mg/kg, intraperitoneally).

Mice. Two groups of twelve male mice (32 ± 4 g) were given intraperitoneal injections of the labelled drug dissolved in water at dose levels of 2.5 and 5.0 mg/kg.

Rabbit. A female rabbit (2.2 kg) was injected with the labelled drug dissolved in water (2.5 mg/kg, intraperitoneally).

Dogs. A bitch (7.5 kg) was given the labelled drug dissolved in saline (2.5 mg/kg, intraperitoneally). A second bitch (13 kg) was injected intravenously at the same dose level and 5 ml. samples of heparinized blood were taken 0.5, 6 and 48 hr after the injection. Urine samples were collected from each animal.

Assay of ethyleneimine in urine and buffered solution

Food was withheld overnight from groups of ten male rats before intraperitoneal injection of the drugs. Ethyleneimine excretion was measured at intervals of 2 hr after injection of ethyleneimine (2.9 mg/kg) and of di(aziridin-1-yl) sulphoxide (4.4 mg/kg, equivalent to 2.9 mg/kg of ethyleneimine). The method of assay involved chloroform extraction of the dye formed between ethyleneimine and sodium 3,4-dihydro-3,4-dioxonaphthalene-1-sulphonate (sodium 1,2-naphthaquinone-4-sulphonate) (Rosenblatt, Hlinka & Epstein, 1955). Aliquots of urine were added to 7.5 ml. of 0.067 M-phosphate buffer (pH 7.4) and 1 ml. of sodium 3,4-dihydro-3,4-dioxonaphthalene-4-sulphonate solution (1% w/v). The mixture was shaken for 5 min with chloroform (10 ml.), and the chloroform extract dried with sodium sulphate and filtered. After determining the absorption at $420\text{ m}\mu$, the ethyleneimine content was calculated by reference to a standard curve.

In vitro experiments

The hydrolysis rate of di(aziridin-1-yl) sulphoxide (2 mg/ml.) at 37°C was followed at pH 7.0 in 0.067 M-phosphate buffer, 0.1 M-tris(hydroxymethyl)aminomethane buffer and 0.1 M-barbitone buffer. Di(aziridin-1-yl) sulphoxide (2 mg/ml.) was also incubated in water, barbitone buffer and Ringer solution for 4 hr before addition of an equal volume of 0.13 M-phosphate buffer. Ethyleneimine formation after addition of the latter was then compared with ethyleneimine production when incubating di(aziridin-1-yl) sulphoxide (1 mg/ml.) in 0.067 M-phosphate buffer.

RESULTS

Metabolism of di(aziridin-1-yl) sulphoxide

With the exception of the dog, all species excreted 80% or more of the administered radioactivity within 24 hr and nearly 100% by 48 hr. Details of the relative rates of excretion by the various species are shown in Table 1. A more

TABLE 1
THE RATE OF URINARY EXCRETION OF RADIOACTIVE SULPHUR AFTER ADMINISTRATION OF ^{35}S -LABELLED DI(AZIRIDIN-1-YL) SULPHOXIDE
i.p.=intraperitoneal; i.v.=intravenous

Species	No. of animals	Dose (mg/kg)	Route of administration	Cumulative urinary excretion (% of dose) after				
				2 hr	4 hr	8 hr	24 hr	2 days
Mouse	12	2.5	i.p.	6.6	31	69	86	99
Mouse	12	5.0	i.p.	3.5	19	63	81	97
Rat	6	2.5	i.p.	28	56	79	97	102
Rabbit	1	2.5	i.p.	9.3	24	24	88	97
Dog	1	2.5	i.p.	—	—	—	30	38
Dog	1	2.5	i.v.	—	—	1.3	1.3	52

detailed analysis, given in Tables 2 and 3, shows that the principal labelled metabolite in all species was sulphate. Only one other labelled substance, S_1 , was excreted in appreciable amounts (R_f 0.25 in butanol; dioxan: ammonia, 0.1 in butanol: water). Details for each species are given below.

Mouse. After 2.5 mg/kg of di(aziridin-1-yl) sulphoxide, 78% of the radioactivity in the urine was recovered as sulphate and 16% as S_1 . Small amounts of four other metabolites were present. At the higher dose level (5 mg/kg) there was a similar metabolic picture, 69% of the urine activity being as sulphate and 12% as S_1 (Table 2).

TABLE 2
THE PROPORTION OF RADIOACTIVE METABOLITES PRESENT IN MOUSE URINE AT DIFFERENT TIMES AFTER INJECTION OF DI(AZIRIDIN-1-YL) SULPHOXIDE

Time (hr)	Radioactivity excreted (% of dose)	Urine content (% of dose) of	
		Sulphate	S ₁
<i>After 2.5 mg/kg intraperitoneally</i>			
0-2	6.6	2.8	2.4
2-4	24.5	11.1	6.8
4-8	38.2	30.5	6.8
8-24	16.7	16.7	—
24-48	12.8	12.8	—
48-72	4.0	4.0	—
0-72	102.8	77.9	16
<i>After 5.0 mg/kg intraperitoneally</i>			
0-2	3.5	1.5	0.8
2-4	15.5	8.5	4.6
4-8	44.4	28.3	5.0
8-24	17.1	14.4	1.1
24-48	16.2	16.2	—
0-48	96.7	68.9	11.5

Rat. All the radioactivity was recovered in the urine during the 48 hr after injection; 84% was sulphate (Table 3) and there were traces of several other labelled metabolites. Rats given di(aziridin-1-yl) sulphoxide (4.4 mg/kg) excreted 28% of the available ethyleneimine content in 6 hr compared with the excretion of 30% of an equivalent dose of ethyleneimine (2.9 mg/kg) in the same test period.

Rabbit. In the first 48 hr after injection, 82% of the radioactive sulphur was excreted as sulphate and 6% as S_1 (Table 3).

TABLE 3
THE PROPORTION OF RADIOACTIVE METABOLITES PRESENT IN RAT, RABBIT
AND DOG URINE AT DIFFERENT TIMES AFTER INJECTION OF DI(AZIRIDIN-1-YL)
SULPHOXIDE

Animals of each species received 2.5 mg/kg of the drug

Time (hr)	Radioactivity excreted (% of dose)	Urine content (% of dose) of	
		Sulphate	S ₁
<i>Rat, intraperitoneal injection</i>			
0-2	27.9	22.8	1.5
2-4	28.4	24.6	0.5
4-8	22.8	17.2	1.0
8-24	18.2	15.2	—
24-48	4.2	4.2	—
0-48	101.5	84.0	3.0
<i>Rabbit, intraperitoneal injection</i>			
0-2	9.3	3.8	2.2
2-4	14.2	8.3	2.5
4-24	64.7	60.9	1.0
24-48	9.0	9.0	—
0-48	97.2	82.0	5.7
<i>Dog, intravenous injection</i>			
0-8	1.3	0.4	0.5
8-48	50.5	37.4	4.0
0-48	51.8	37.8	4.5
<i>Dog, intraperitoneal injection</i>			
0-24	29.8	15.5	10.8
24-48	8.6	—	—
0-48	38.4	15.5	10.8

Dog. After intravenous and intraperitoneal injections, 52% and 38% respectively of the radioactivity were excreted in 48 hr. After intraperitoneal administration, chromatographic analysis was only possible with urine collected during the first 24 hr, 16% of the injected material being excreted as sulphate and 11% as S₁. After intravenous injection, several metabolites, including S₁ (4.5%), were excreted in the urine together with 38% as sulphate. Blood from the animal, after intravenous injection, contained 13% of the total activity after 30 min, with a fall to 8% in 6 hr and 2% by 48 hr.

In vitro studies

Identification of chromatographic material. A major *in vitro* product of di(aziridin-1-yl) sulphoxide hydrolysis was shown to be sulphite, because addition of barium chloride to an incubated solution gave an acid-soluble nitrogen-free precipitate. Analysis of the urine of animals given di(aziridin-1-yl) sulphoxide showed the principal *in vivo* metabolite to be sulphate.

Hydrolysis in water and buffered solution. Ethyleneimine liberation from di(aziridin-1-yl) sulphoxide in the presence of phosphate buffer (pH 7) greatly exceeded that produced in water alone. Ethyleneimine was only slowly liberated from di(aziridin-1-yl) sulphoxide in the presence of barbitone and tris-(hydroxy-

TABLE 4
ETHYLENEIMINE FORMATION ON INCUBATING DI(AZIRIDIN-1-YL) SULPHOXIDE
(2 MG/ML.) WITH WATER AND VARIOUS BUFFERS AT pH 7

Solution	Conversion (%) to ethyleneimine after		
	1 hr	2 hr	4 hr
Water	3	4	9
0.067 M-phosphate buffer	41	71	75
0.1 M-barbitone buffer	—	4	3
0.1 M-tris(hydroxymethyl)-aminomethane buffer	3	5	7

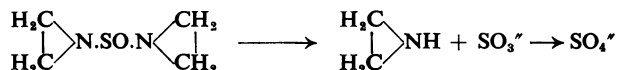
TABLE 5
RESULTS OF EXPERIMENTS IN WHICH DI(AZIRIDIN-1-YL) SULPHOXIDE (2 MG/ML.) WAS INITIALLY INCUBATED IN VARIOUS SOLUTIONS FOR 4 HR BEFORE ADDITION OF 0.133 M-PHOSPHATE BUFFER AT pH 7.0, TO PRODUCE A FINAL CONCENTRATION OF 1 MG/ML.

Solution	Conversion (%) to ethyleneimine after 4 hr	Conversion (%) to ethyleneimine at different times after addition of phosphate buffer	
		1 hr	2 hr
Water	4	25	51
0.1 M-barbitone buffer	2	58	71
Ringer solution	2	50	—
Compare Di(aziridin-1-yl) sulphoxide (1 mg/ml.) in 0.067 M-phosphate buffer (pH 7.0)	—	61	73

methylaminomethane buffers (Table 4). Even after incubation for 4 hr in barbitone buffer, Ringer solution and water, subsequent addition of phosphate buffer (pH 7.0) resulted in the rapid formation of ethyleneimine (Table 5).

DISCUSSION

With the exception of the dog, the species studied rapidly excreted the administered radioactive material (Table 1). In all species the principal labelled metabolite was sulphate, presumably formed by the *in vivo* oxidation of sulphite. Apart from a labelled metabolite, S₁, which occurred to a variable extent (3 to 16%) in all species, only traces of other labelled materials were excreted. The other principal metabolite was ethyleneimine, indicating that the main metabolic pathway was by the fission of the sulphoxide, liberating free ethyleneimine:



Similar amounts of ethyleneimine were found in the urine of rats given di(aziridin-1-yl) sulphoxide or an equivalent dose of ethyleneimine. This strongly supports the view that the effectiveness of di(aziridin-1-yl) sulphoxide and other simple ethyleneimine derivatives in producing polyuria is directly related to the production of free ethyleneimine, which is a powerful diuretic agent (Jackson & James, 1963). The conversion of di(aziridin-1-yl) sulphoxide to ethyleneimine could

explain its lack of tumour-inhibitory activity (Jackson, Fox & Craig, 1959), because ethyleneimine is ineffective in this respect (Hendry, Homer, Rose & Walpole, 1951).

In vitro, di(aziridin-1-yl) sulphoxide is hydrolysed to ethyleneimine and sulphite. This hydrolysis occurs slowly in aqueous solution, but much more rapidly in phosphate buffer at pH 7.0 (Table 4). At the same pH it was found that barbitone and tris(hydroxymethyl)aminomethane buffer failed to accelerate the hydrolysis rate. Addition of phosphate buffer to a solution of di(aziridin-1-yl) sulphoxide incubated for 4 hr in barbitone and tris(hydroxymethyl)aminomethane buffers still resulted in ethyleneimine formation at a rate similar to that in phosphate alone (Table 5). This result indicates that the enhancement of di(aziridin-1-yl) sulphoxide hydrolysis in phosphate buffer is not due to the hydrogen-ion concentration, but is related to the presence of phosphate; also that the compound was not undergoing any other transformation to a significant extent. These studies are now being extended over a range of pH's and in the presence of a number of different anions. This may be significant if release of ethyleneimine *in vivo* occurs selectively at sites where the concentration of phosphate is greatest.

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