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The metabolism of 5,5′-methylenedisalicylic acid in various species

CLARKE DAVISON* AND R. T. WILLIAMS

Commercial methylenedisalicylic acid has been shown to be grossly impure. Pure 5,5′-methylenedisalicylic acid (4,4′-dihydroxydiphenylmethane-3,3′-dicarboxylic acid) has been prepared and labelled with ^{14}C. The fate of the pure compound in the rat, mouse, hamster, rhesus monkey, rabbit, guinea-pig and chicken has been investigated. The compound is excreted entirely unchanged in the urine and faeces in all the above species and no metabolites have been found. The biliary excretion of the injected compound is high (50–60%) in the rat and dog and low (5%) in the guinea-pig and rabbit. In the monkey, rabbit and guinea-pig, the compound is excreted almost exclusively in the urine. In the rat about 50% of the dose is excreted in the faeces. In the mouse and hamster, the main route of excretion is the urine, about 10% appearing in the faeces.

THE compound 5,5′-methylenedisalicylic acid (4,4′-dihydroxydiphenylmethane-3,3′-dicarboxylic acid) has been used in the synthesis of triarylmethane dyes and corrosion protectives. It forms complexes with drugs such as phenacetin, theophylline and prednisolone (Higuchi & Pisano, 1964) and salts with organic bases. A salt of considerable interest is bacitracin methylenedisalicylate (Siminoff, Price & Bywater, 1953) which is widely used as an animal feed supplement designed to enhance growth and prevent disease in poultry, swine and mink. Whilst this salt and the acid itself have received toxicological testing (Radomski, Hagan & others, 1954), essentially no studies have been made of the metabolism of the acid. Simon (1962) using a non-specific colorimetric assay reported that 30% of an oral dose of piperidine methylenedisalicylate was excreted in the urine in three days by rabbits.

We find commercial samples of methylenedisalicylic acid and samples synthesized by standard methods to be grossly impure. Purified samples of [^{14}C]methylenedisalicylic acid are not metabolized but excreted unchanged in the urine and faeces of a number of animal species. The species differences in biliary excretion of the acid are similar to those reported by Abou-El-Makarem, Millburn & others (1967a,b) for other compounds.

Experimental

4,4′-Dihydroxydiphenylmethane-3,3′-dicarboxylic acid. Samples of this acid (methylenedisalicylic acid) of m.p. varying from 225–240° (decomp.) were obtained commercially (S. B. Penick, New York; Hopkin & Williams Ltd., Essex) and by preparation from salicylic acid and formaldehyde or paraformaldehyde (e.g. Clemmensen & Heitman, 1911; Kahl, 1898). All these samples were grossly impure and on paper chromatography at least 8 spots, one of which was salicylic acid, were seen by their blue

From the Department of Biochemistry, St. Mary's Hospital Medical School, Paddington, London, W.2, England.

* Present address: Department of Pharmacology, The George Washington University Medical School, Washington, D.C.

fluorescence in ultraviolet light or by the colour given with 1% aqueous ferric chloride. Recrystallization of this material from various solvents or column chromatography on a variety of packings failed to give a product of the desired purity.

Pure samples of methylenedisalicylic acid were eventually obtained by chromatographing 100 mg samples of the crude acid as a band on Whatman No. 3MM paper or on a thin-layer (1/16 in) of Silica Gel G (Merck) without binder, using isopropanol–5N ammonia solution (10:3 v/v) as solvent. The band of Rf 0·6 was extracted from the paper or silica with N ammonia solution. The extract was acidified with 2N hydrochloric acid and the methylenedisalicylic acid extracted with ether. It was purified by adding water to a hot acetone solution from which it formed colourless needles (yield 45–70% of crude material). The 4,4′-dihydroxydiphenylmethane-3,3′-dicarboxylic acid had m.p. 255–257° (decomp.) [Smith, Sager & Siewers (1949) give m.p. 247° (decomp.).] [Found: C, 62·6; H, 4·2%; *M* 266 (Rast), 305 (isothermal dist.); equiv.; 147 (titration). Calc. for $C_{15}H_{12}O_6$; C, 62·5; H, 4·2%; *M* 288; equiv.; 144.] The apparent pK_a by titration was about 3·5. On acetylation, the diacetyl derivative, m.p. 146–149°, was obtained (Clemmensen & Heitman (1911) give m.p. 142°).

4,4′-Diaminodiphenylmethane (Koch-Light & Co. Ltd., Colnbrook, Bucks) was converted into 4,4′-dihydroxydiphenylmethane, m.p. 158–160° according to Haase & Moyat (1894). This phenol showed on Whatman No. 1 paper, Rf values of 0·65 with 2% aqueous sodium carbonate, 0·80 with 20% aqueous acetic acid and 0·71 with benzene–acetic acid–water (100:32:0·5 by vol.) as solvents. On fusing a little pure methylenedisalicylic acid with potassium hydroxide according to Clemmensen & Heitman (1911), a small amount of material was obtained which was chromatographically identical in the above three solvents with 4,4′-dihydroxydiphenylmethane. 5,5′-Methylenedisalicylic acid was further characterized by conversion to aurin tricarboxylic acid with salicylic acid and nitrous acid according to Smith & others (1949). The aurin tricarboxylic acid behaved chromatographically on paper identically with an authentic sample (British Drug Houses Ltd., Poole, Dorset).

TABLE 1. PARTITION OF RADIOACTIVITY IN CRUDE [^{14}C]METHYLENEDISALICYLIC ACID BY THIN-LAYER CHROMATOGRAPHY. See text for details of solvent and supporting medium. The values given are the mean for four plates.

Rf	% of ^{14}C	Specific activity μc/mg
Above 0·6	0·1	—
0·6*	42·9	0·57
0·45†	34·0	0·75
0·1–0·2 (2 bands)	17·7	0·71
0·0–0·1 (3 bands)	5·6	0·64

* 5,5′-methylenedisalicylic acid.
† Trimeric form, see text.

The major contaminant (Rf 0·45; see Table 1) occurred to the extent of up to 35% of the crude methylenedisalicylic acid. This was isolated as above and crystallized from acetone–water mixtures as needles which

softened at 264° and decomposed at 272°. This was probably a trimeric form derived from three salicylic acid molecules. [Found: C, 62·2; H, 4·3%; *M* 509 (isothermal dist.). Calc. for $C_{23}H_{18}O_9$; C, 63·0; H, 4·1%; *M* 438.] Several other impurities at lower Rf values amounted to 25%, whereas free salicylic acid was only 3% of the crude material.

4,4'-Dihydroxydiphenyl[^{14}C]methane-3,3'-dicarboxylic acid. Salicylic acid (1·05 g; 0·076 mole) and [^{14}C]paraformaldehyde (108·9 mg; 0·036 mole; 500 μc; Radiochemical Centre, Amersham) were dissolved in glacial acetic acid (1·4 ml) by warming at 95° for 5 min in a filtration tube (20 ml) fitted with a separatory funnel. The side arm of the filtration tube was connected to successive dinitrophenylhydrazine and silver nitrate solution traps to collect any [^{14}C]formaldehyde. A mixture of conc. sulphuric acid and glacial acetic acid (0·4 ml, 1 : 5 by vol.) was introduced and the mixture left in a boiling water bath for 3 hr. Air was sucked through the traps and hot distilled water (100 ml) then added to the reaction tube. After cooling, the crude [^{14}C]methylenedisalicylic acid was filtered, washed with water three times and dried (yield 0·76 g, 73%). The crude [^{14}C]acid in quantities of 78–80 mg was chromatographed on thin-layer silica gel plates and the pure [^{14}C]acid (Rf 0·6) separated as before. The average recoveries from four plates of methylenedisalicylic acid and other products are shown in Table 1.

ANIMALS

The animals used were Wistar albino rats, rhesus monkeys, European hamsters, albino mice (I.C.I. strain), New Zealand White rabbits, Abyssinian guinea-pigs, mongrel dogs and Light Sussex hens. These animals had free access to water and suitable food.

The methylenedisalicylic acid was administered as the sodium salt in water. Biliary cannulation of the animals was performed by Dr. M. M. Abou-El-Makarem (St. Mary's Hospital Medical School).

DETERMINATION OF ^{14}C

A Packard Tri-Carb scintillation counter (Model 3204) and a Packard radiochromatogram scanner (Model 7200) were used. For the assay of urine, bile and other clear solutions, the scintillator used was that of Bray (1960) (POPOP/PPO/dioxan). For plasma and homogenates of tissues and faeces in dioxan-methanol (1 : 1 by vol.), a thixotropic gel (Cab-O-Sil, Packard Instrument Co.) was added to the above scintillator solution.

CHROMATOGRAPHY

Urine and various extracts were chromatographed on Whatman No. 3MM paper or thin-layer silica gel as referred to earlier. The solvent systems used were isopropanol–5N ammonia solution (10 : 3 by vol.) or phenol–water (4 : 1 by vol.). For detecting possible metabolites on chromatograms, 1% aqueous ferric chloride and Gibbs reagent (dichloroquinonechloroimide) was used for phenols, naphthresorcinol for glucuronides, and *p*-dimethylaminobenzaldehyde in acetic anhydride for glycine conjugates. Since no metabolite of methylenedisalicylic acid

is formed *in vivo* further details are omitted. Methylenedisalicylic acid itself is readily detected on chromatograms by its blue fluorescence in ultraviolet light and Rf value.

DETERMINATION OF METHYLENEDISALICYLIC ACID BY ISOTOPE DILUTION

To a sample of radioactive urine (enough to contain 0·05–0·2 μc) was added 0·8 g of pure methylenedisalicylic acid. The mixture was made alkaline with sodium hydroxide and filtered. The methylenedisalicylic acid was precipitated by acidification and this process was repeated eight times on each sample. Dissolution in acetone and precipitation with water was also used as an alternative method of purification. Constant specific activity was usually attained after four precipitations by either method.

DETERMINATION OF THE PLASMA HALF-LIFE OF METHYLENEDISALICYLIC ACID

The [^{14}C]acid (10 mg/kg) was injected intravenously into rats, dogs, guinea-pigs or rabbits. Plasma samples were prepared every 5–10 min for the first hr and subsequently at longer intervals. The ^{14}C of each sample was determined by scintillation counting. No radioactive compound other than methylenedisalicylic acid was found in the plasma.

TABLE 2. THE EXCRETION OF [^{14}C]METHYLENEDISALICYLIC ACID IN RATS. The dose of methylenedisalicylic acid was 10 mg/kg and of ^{14}C 5·8 μc/kg. The compound was administered as the sodium salt in 0·21 ml of water. The excreta of the animals in each group were pooled for analysis.

	Males	Females	Males
Number of rats used	9	3	6
Weight, mean, g	280	235	295
Route of administration	oral	oral	i.p.
	% of dose of ^{14}C excreted* in		
Urine, day 1	25·3	29·0	55·4
2	7·3	10·3	2·4
3	1·6	0·0	0·1
Total	34·2†	39·3	57·9
Faeces, day 1 and 2	50·2	48·6	30·3
Total excreted	84·4	87·9	88·2

* In a separate experiment it was shown that in female rats receiving the above dose of [^{14}C]methylenedisalicylic acid, the amount of radioactive CO_2 in the expired air collected for 6 hr after dosing was <0·03% of the dose.

† By isotope dilution 98% of this material was shown to be 4,4′-dihydroxydiphenylmethane-3,3′-dicarboxylic acid.

Results and discussion

Preliminary studies in rats injected with non-radioactive methylenedisalicylic acid (10–200 mg/kg) indicated that the drug was not metabolized and only the original substance appeared in the urine and faeces. Chromatograms of the urine before and after acid hydrolysis showed one and the same spot characterized as methylenedisalicylic acid. No glycine or glucuronic acid conjugates were detected. This was confirmed, using the [^{14}C]acid, by isotope dilution and by use of the radiochromatogram scanner.

The fate of the compound in rats is shown in Table 2. There is no sex difference in its excretion, for in both male and female rats 30–40% of an oral dose is excreted in the urine in 3 days and about 50% in the faeces in 2 days. These figures may suggest that the acid is not completely absorbed, but as shown in Table 4 there is a considerable biliary excretion of the acid in the rat after intravenous injection. When given intraperitoneally, nearly 60% is excreted in the urine and 30% in the faeces. Thus, both incomplete absorption and biliary excretion could account for the faecal excretion after oral administration. The faecal excretion after intraperitoneal injection is largely the result of biliary excretion.

TABLE 3. ELIMINATION OF ^{14}C IN VARIOUS SPECIES RECEIVING [^{14}C]METHYLENEDISALICYLIC ACID. The dose of [^{14}C]methylenedisalicylic acid was 10 mg/kg. except in mice which received 20 mg/kg. The monkeys and chickens were females, whilst the guinea-pigs, hamsters, mice and rabbits were males.

Animal (No.)	Monkey (2)*	Rabbit (3)†	Guinea-pig (3)†	Hamster (3)‡	Mouse (8)‡	Chicken (3)‡§
Dose of ^{14}C, μc/kg	0·58	0·58	0·58	5·8	11·6	1·16
Body wt, mean, kg	3	3·8	0·72	0·1	0·025	2·9
Route of administration	s.c.	i.v.	i.p.	i.p.	i.p.	i.p.
			% of the dose of ^{14}C excreted in			
Urine, day 1 ..	89, 92	43 (0–93)	82 (78–89)	69	87	68
2 ..	1, 1	34 (2–92)	0·7 (0·4–1)	6	7	15
3 ..	—	19 (1–50)	—	—	—	—
Total	90, 93	96 (79–90)	83 (79–90)	75	94	83
Faeces, day 1 and 2	0·4, 0·7	0	0	15	8	—
Expired air day 1 ..	—	—	—	< 0·1	< 0·1	—
Total excreted	90·4, 93·7	96	83	90	102	83
			% of ^{14}C excreted in urine			
% of urinary ^{14}C present as methylenedisalicylic acid‖	97	97	102	98	97	90

* For monkeys individual values are given.
† Mean values are given with ranges in parentheses.
‡ Excreta were pooled.
§ The chickens were fasted for one day to reduce faeces; values are for total excreta.
‖ Determined by isotope dilution.

The fate of the compound after injection in six other species is shown in Table 3. In the monkey, rabbit and guinea-pig, methylenedisalicylic acid is almost entirely excreted in the urine, whereas in the hamster and mouse, although the urine is the major channel of excretion, some 15 and 8%, respectively, is excreted in the faeces. With the hen, the urine and faeces are not readily separated, and although the birds were starved for a day the urine collected did contain a small amount of faeces. The figure of 82% excretion therefore does not clearly distinguish between urinary and faecal excretion. By isotope dilution it was shown that the material excreted in the urine of the monkey, hamster, mouse, rabbit and guinea-pig was unchanged methylenedisalicylic acid, the value found 97–102% (Table 3) being within the experimental error of the method. The value for hens was 90% but there was no evidence of any other material than methylenedisalicylic acid in the excreta. The expired air of the rats (Table 2), mice and hamsters (Table 3) was also examined for

radioactivity. None was found and it was concluded that methylenedisalicylic acid is not degraded to carbon dioxide.

Table 4 summarizes the findings on the biliary excretion of methylenedisalicylic acid in four species. Excretion is high in the rat (54% of the dose) and dog (60–70%), and low in the rabbit and guinea-pig (4–5%); at the dose level used (10 mg/kg) biliary excretion is practically complete in all these species in 6 hr. These observations on species differences

TABLE 4. BILIARY EXCRETION OF [^{14}C]METHYLENEDISALICYLIC ACID IN VARIOUS SPECIES. The acid (10 mg/kg) as the sodium salt in water was injected intravenously into biliary cannulated animals. The rats and rabbits were females, the guinea-pigs males and one dog was male and the other female. Bile and urine were collected for 6 hr after dosing.

Animal (No.)	Rat (4)	Dog (2)	Guinea-pig (2)	Rabbit (3)
Body wt, mean, kg	0·265	8 (m), 7·2 (f)	0·88	3·8
Bile vol. ml /hr	1·2	5	4	12·0
Plasma half life, hr	2·0	1	3	3·0
	% of ^{14}C* administered excreted in			
Bile 0–0·5 hr	17 (8–26)	39, 32	1·2, 2·6	1·2 (0·9–1·8)
0·5–1·0	14 (12–16)	14, 13	1·5, 0·5	1·6 (1·3–1·7)
1–2	11 (9–13)	9, 10	1·2, 0·6	1·2 (1·1–1·4)
2–3	6 (4– 8)	4, 3	0·4, 0·6	0·6 (0·6–0·5)
3–4	3 (1– 5)	2, 2	0·0, 0·2	0·3 (0·2–0·5)
4–5	2 (0·4–3)	1, 1	—	—
5–6	1 (0·2–2)	—	—	—
Total	54 (46–62)	69, 61	4·3, 4·5	4·9 (3·8–5·3)
Urine 0–6 hr	15, 23†	1, 11	59, 73	44 (37–52)

* The values for the rat and rabbit are given as mean values with ranges in parentheses; individual values are given for the dog and guinea-pig.
† Individual values for two of the rats.

agree with others found in this laboratory (Abou-El-Makarem & others, 1966, 1967a,b). According to Millburn, Smith & Williams (1967), for extensive biliary excretion of a compound to take place in the rat the compound should have a molecular weight of not less than 325 $\pm$ 50, and a strongly polar anionic group, or it must be converted into such a compound by metabolism. 5,5′-Methylenedisalicylic acid has a molecular weight of 288 and a pK_a of about 3·5 and appears to fit these criteria without undergoing conjugation.

No metabolite of methylenedisalicylic acid was found in the bile or in the urine. Since it is closely related to salicylic acid, there was the possibility that, like salicylic acid, it might have formed a glycine or glucuronic acid conjugate or an oxidation product by further hydroxylation (see Williams, 1959). However, none of these were found in any of the species examined.

METABOLISM OF CRUDE METHYLENEDISALICYLIC ACID

Methylenedisalicylic acid as used commercially, contains large amounts of impurities probably from the variety of ways in which salicylic acid and formaldehyde can be condensed. The major impurity (Rf 0·45; see Table 1) is probably a product containing three salicylic acid residues and two methylene groups and is referred to below as "trimeric product." In the experiments from which Table 1 was constructed, it can be calculated that the specific activity of a product containing three salicylic acid

residues and two methylene groups should be about 0·75 which agrees with the value found (Table 1). In some experiments using crude [^{14}C]methylenedisalicylic acid, only 26% of the ^{14}C of an injected dose of this material (10 mg/kg) was excreted in the urine in 6 hr by the rat, whilst in a parallel experiment with purified [^{14}C]methylenedisalicylic acid, 46% of the ^{14}C was excreted in the same time. Furthermore, the urinary material from the crude compound contained appreciably less of the contaminants than the injected compound. Since the "trimeric product" has a molecular weight (calc. 438) higher than methylenedisalicylic acid it might be expected to have a higher biliary excretion and a greater faecal excretion than the latter. A solution of the ^{14}C-labelled "trimeric product" (10 mg/kg) as the sodium salt in water, was injected intraperitoneally into three biliary cannulated female rats and the bile and urine collected for 3 hr. In this time an average of 74% of the injected ^{14}C appeared in the bile and 1% in the urine. From Table 4 it can be seen that the biliary excretion of methylenedisalicylic acid is about 47% in 3 hr. In another experiment the ^{14}C-labelled "trimeric product" (10 mg/kg) was injected intraperitoneally into three normal male rats with anal cups and the urine and faeces collected for 48 hr. In this time an average of 92% of the injected ^{14}C appeared in the faeces and 4% in the urine and these figures should be compared with those for purified methylenedisalicylic acid (Table 2) which are 50% in the faeces and 34% in urine. It appears, therefore, that the contaminants may have a markedly different pattern of excretion from that of 5,5′-methylenedisalicylic acid. However, no further work was done on these contaminants due to the difficulty of separating and characterizing them in sufficient quantities.

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