

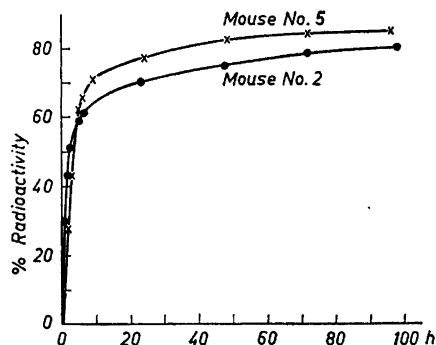
## On the Metabolism of Sorbic Acid in the Mouse

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[1-<sup>14</sup>C]Sorbic acid has been prepared, and its metabolism in mouse has been studied. The main metabolites are carbon dioxide and water. Small amounts of muconic acid and sorbic acid were found in the urine.

Sorbic acid,  $\text{CH}_3\text{CH}=\text{CHCH}=\text{CHCOOH}$ , is an antimicrobial agent, which is used in increasing quantities as a food preservative. This use has led to a marked interest in the metabolism of the compound. Deuel *et al.*<sup>1</sup> in 1953 published an investigation on the fate of sorbic acid in rat, which showed that the intermediary metabolism was similar for sorbic, caproic, and butyric acids. From these results the authors concluded that the end products of the oxidation of sorbic acid in rat are carbon dioxide and water only. In 1962, when the present investigation on the metabolites of [1-<sup>14</sup>C] sorbic acid in mice was almost completed, Fingerhut, Schmidt and Lang<sup>2</sup> published an extensive examination of the metabolism of [1-<sup>14</sup>C]sorbic acid in rats. They found that during 4–10 h 85 % of the radioactivity were exhaled as <sup>14</sup>CO<sub>2</sub> and 1.4 % excreted in the urine as  $\text{NH}_2^{14}\text{CONH}_2$ , 0.2 % as <sup>14</sup>CO<sub>2</sub>. No active sorbic acid or muconic acid were found in the urine. The present inves-



*Fig. 1.* Radioactive carbon dioxide expired after the administration of [1-<sup>14</sup>C] sorbic acid to mouse.

Table 1.

Mouse No.	Sorbic acid given		Radioactivity in % of the given activity found during 4 days in							Time for half the activity given to be expired, hours	Muconic acid activity in % of administered activity	Sorbic acid in the urine in % of given sorbic acid
	mg/kg body weight	counts/min per mouse	the carbon dioxide expired	urine					feces			
				1st day	2nd day	3rd day	4th day	total				
1	40	640 000	82	2.3	0.2	0.1	0.04	2.6	0.9	2	0.2	
2	40	640 000	81	2.6	1.0	0.6	0.1	4.3	0.9	2	0.3	0.7
3	40	640 000	77	2.7	0.4	0.25	0.1	3.5	1.0	5		
4	40	640 000	78	2.7	0.9	0.3	0.2	4.1	0.6	2.5		
5	3000	850 000	85	4.5	0.4	0.1	0.1	5.1		3.5	0.6	0.7
6	3000	850 000	80	4.8	0.5	0.05	0.05	5.4		8	0.5	

tigation differs from that of Fingerhut *et al.* in experiment animals, experimental conditions and also in results.

Mice were given water solutions of the sodium salt of [1-<sup>14</sup>C]sorbic acid by gastric intubation. Contrary to the animals of Fingerhut *et al.* they got water and food *ad libitum*. The expired, radioactive carbon dioxide was collected in sodium hydroxide solution during 4 days. It was precipitated as barium carbonate, the activity of which was measured in "infinitely" thick layers. The amounts of radioactive carbon dioxide expired are shown in Fig. 1 and Table 1. There was still a slow expiration of radioactive carbon dioxide at the time the measurements were interrupted.

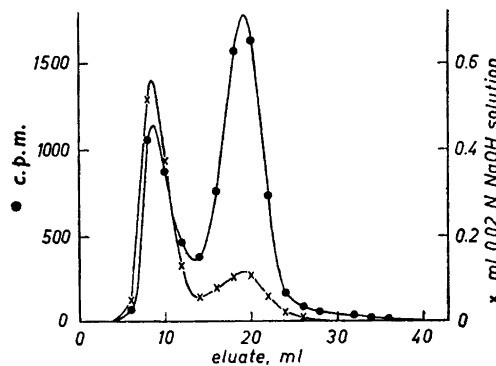


Fig. 2. Partition chromatography of the urinary metabolites of [1-<sup>14</sup>C]sorbic acid.

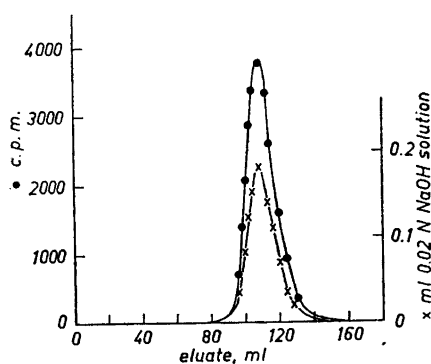


Fig. 3. Partition chromatography of [1-<sup>14</sup>C]sorbic acid.

Table 2.

Recrystallization	Specific activity, c.p.m./mg
1	146
2	124
3	120
4	133
5	134
6	129

For each animal the total radioactivity of the neutralized urine was measured daily on thin layers of evaporated aliquots. About 4 % of the radioactivity administered to the mice were found in the urine during 4 days. Part of the metabolites (30–35 % of the radioactivity in the urine sample) could be extracted with ether from the acidified urine of the first 24 h and separated by partition chromatography. The activity in the ether solution was distributed amongst at least two different metabolites (Fig. 2). The compound or compounds giving rise to the first activity peak are probably conjugates, but they have not been identified. The main ether extractable metabolite (second peak, 0.2–0.6 % of the total activity given to the mouse) has been identified as *trans-trans*-muconic acid,  $\text{HOOCCH}=\text{CHCH}=\text{CHCOOH}$ , both by chromatography with addition of unlabelled muconic acid, when the activity peak and the alkali titration peak were in the same fractions (Fig. 4), and by repeated crystallization from water and ethanol with inactive muconic acid, when the specific activity soon remained constant (Table 2).

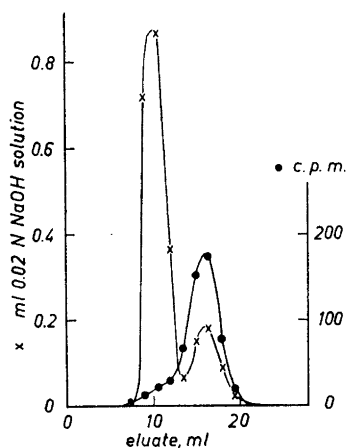


Fig. 4. Partition chromatography of the substance from the second peak of Fig. 2 together with a small amount of unlabelled *trans-trans*-muconic acid and a great amount of unlabelled malonic acid.

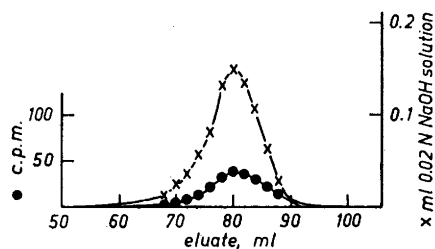


Fig. 5. Partition chromatography of part of an extract of a first day urine to which unlabelled sorbic acid has been added.

A small amount of unchanged sorbic acid was also found in the ether extract of the urine from the first 24 h. In the chromatogram (Fig. 2) it has been lost on account of its volatility and oxidability. The acid could be retained by inactive sorbic acid added to the urine before the ether extraction. When the evaporated ether extract was then chromatographed, the titration peak of sorbic acid was found in the same fractions as a radioactivity peak (Fig. 5). Quantitative microanalysis of sorbic acid on a steam distillate of the urine from mice given sorbic acid gave the amounts of sorbic acid present (about 0.7 % in the urine from the first 24 h).

The activity of the feces was measured daily for each animal. Thin layers of evaporated ether extracts, obtained after alkaline digestion and acidification of the feces, were used for the measurements. The fecal, ether extractable radioactivity being only about 1 % of the activity given to the mice, no efforts have been made to identify the active products.

Thus in 4 days 81 %  $\pm$  10 % of the sorbic acid given was oxidized to carbon dioxide and water. About 4 % of the activity left the mice with the urine, part of it as muconic acid (in the urine from the first 24 h about 0.4 %) another part as sorbic acid (in the urine from the first 24 h about 0.7 %).

#### EXPERIMENTAL

*Preparation of [1-<sup>14</sup>C]sorbic acid.* A sorbic acid labelled with <sup>14</sup>C in the 1 position was prepared by refluxing 0.102 g of [1-<sup>14</sup>C]malonic acid, 0.080 ml of crotonic aldehyde, and 0.105 ml of pyridine for 2  $\frac{1}{2}$  h (cf. Miller and Nord <sup>3</sup>). The solvent was evaporated under reduced pressure, and the residue was purified by chromatography on equal parts of kieselguhr (Hyflo supercel) and silica gel with benzene as solvent. In order to diminish the loss of radioactive acid in the column, 0.025 g of pure, inactive sorbic acid was placed on the column together with the reaction product.

The [1-<sup>14</sup>C]sorbic acid obtained was further purified by repeated crystallization from 50 % ethanol and by chromatography on hydrophobic kieselguhr (Hyflo supercel treated with gaseous dichlorodimethylsilane, washed with 10 % ethanolic acetic acid and ethanol and dried at 110°–120°). The stationary phase was butanol and the moving phase water.

A portion of weakly labelled [1-<sup>14</sup>C]sorbic acid has been bought. The absence of radioactive impurities is seen from Fig. 3, which shows the radioactivity and titration peaks, when the product was chromatographed as described above.

*Stock solutions and administration of [1-<sup>14</sup>C]sorbic acid to mouse.* Stock solutions of sodium [1-<sup>14</sup>C]sorbate (0.136 % and 15.5 %) in water were prepared from the two labelled sorbic acid samples. For radioactivity determinations the solutions were diluted with ethanol, aliquots were plated on copper planchets and measured with a gas flow counter (background 45 c.p.m.) in "infinitely" thin layers. The activities of the solutions were found to be 1 280 000 c.p.m./ml and 1 700 000 c.p.m./ml.

The animals used in this investigation were white, adult, female mice, weighing about 22 g. 0.5 ml of a stock solution (640 000 c.p.m. or 850 000 c.p.m.) was given by gastric intubation to each mouse (unanesthetized). Before and during the experiments the usual food and water were fed *ad libitum*.

*<sup>14</sup>C recovery in carbon dioxide expired after administration of [1-<sup>14</sup>C]sorbic acid.* Each mouse given a <sup>14</sup>C-sorbate stock solution was immediately placed in a metabolism cage for mice according to Roth *et al.* <sup>4</sup> Through the cage a stream of CO<sub>2</sub>-free air was passed at a constant rate of about 225 ml/min. The air with the radioactive carbon dioxide expired by the mouse was passed through two effective absorption vessels containing 3 N sodium hydroxide solution (50 ml in each) for collection during 1 h or 50 % potassium hydroxide solution (50 ml in each) for collection during 24 h. The carbonate formed was precipitated from the boiling solution with 2 N barium chloride solution in the presence of ammonium chloride. The activity of the barium carbonate was measured with

an automatic flow counter in "infinitely" thick layers. The results are seen in Fig. 1 and Table 1.

*Isotope recovery in the urine. Identification of the main isotopic compounds extractable with ether from urine collected during the first 24 h after the administration of [1-<sup>14</sup>C] sorbic acid.* For each mouse the urine was collected daily during 4 days after the administration of the labelled sorbic acid. The urine samples were diluted to 5 ml each. From each sample 0.2 ml was neutralized and evaporated on a copper planchet, and the activity was measured. The results are seen in Table 1.

The urines obtained during the first 24 h after the administration of the [1-<sup>14</sup>C] sorbic acid were acidified (pH < 2) and extracted 3 times with equal volumes of ether in order to find out if other active products than carbonate and urea, already pointed out by Fingerhut *et al.* in rats, were present in the urine. The combined ether extracts were dried with anhydrous sodium sulphate and evaporated at reduced pressure just to dryness. Aliquots of the residue were chromatographed on hydrophobic kieselguhr (3.5 g) with butanol (3.0 ml) as stationary phase and water as moving phase. Fractions of 1.5 or 2 ml were collected, titrated (Aglameter syringe) with 0.02 N sodium hydroxide solution, using thymol blue as indicator, and then evaporated to dryness at 85°–105°. The residues in the tubes were dissolved in 50 % ethanol, evaporated on copper planchets, and the radioactivities were counted. The active peaks obtained are seen in Fig. 2.

Part of the second peak of the chromatogram of Fig. 2 was rechromatographed together with a small amount of unlabelled *trans-trans*-muconic acid and a great amount of unlabelled malonic acid on hydrophobic kieselguhr (4.5 g) with chloroform–2-ethylhexanol–methanol (4 ml) as stationary phase and water–methanol as moving phase. Water (180 ml), methanol (170 ml), chloroform (25 ml), and 2-ethylhexanol (25 ml) were equilibrated to form the phases. Fractions were collected and treated as in the preceding chromatogram. The radioactivity peak and the acidity peak, which emanated from the muconic acid, were found in the same fractions (Fig. 4).

The definite proof that the second peak really contained radioactive *trans-trans*-muconic acid was obtained by repeated crystallization of the fraction concerned with inactive *trans-trans*-muconic acid alternately from water and ethanol and determination of the specific activity of the crystals. Table 2 shows that the specific activity soon became constant, which demonstrates the identity of the labelled and unlabelled products.

No sorbic acid was found in the chromatograms of the ether extracts of the urine of the first day (no radioactive peak at 90–130 ml of eluate; *cf.* Fig. 3), but sorbic acid being rather volatile and also easily oxidized might be present in spite of that. A special search for sorbic acid has therefore been made.

To a 1st day urine were added 10.4 mg of unlabelled sorbic acid, and the urine was extracted 3 times with ether. The ether extract was evaporated just to dryness under reduced pressure, and part of the residue was chromatographed on hydrophobic kieselguhr (3 g) with butanol (2 ml) as stationary phase and water as moving phase. The fractions were titrated and the radioactivity of their dry substance was measured. As seen from Fig. 5 the titration peak of the unlabelled sorbic acid is in the same fractions as a radioactivity peak.

A quantitative analysis of the sorbic acid percentage in the urine was also performed according to Schmidt.<sup>5</sup> 0.7 % of the sorbic acid administered to the mice were found in the urine from the first 24 h.

*Isotope recovery in feces after administration of [1-<sup>14</sup>C]sorbic acid.* The feces from each mouse were collected daily during 4 days after the administration of the labelled sorbic acid. They were homogenized and hydrolysed with boiling 5 N ethanolic potassium hydroxide solution for 30 h. The alcohol was evaporated, the residues were acidified with 6 N hydrochloric acid and extracted three times with ether. The ether solutions were evaporated to dryness and the residues were dissolved in 50 % ethanol. The activities were measured on evaporated aliquots. In the feces from the first two days there was found about 1 % of the radioactivity given (mean of 5 mice). In the feces from the third and fourth days the activities were negligible.

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