

## XV. MUCONIC ACID AS A METABOLIC PRODUCT OF BENZENE

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JAFFÉ [1909] isolated muconic acid from the urine of rabbits and dogs to which benzene had been administered. The amount was approximately 0.3% of the benzene given, but Jaffé considered that a larger amount was formed in the tissues, possibly as much as 25–30%, by the opening of the benzene ring, because he found that injected muconic acid was metabolized, only about 1% being recovered in the urine. Fuchs & Soos [1916] found muconic acid in the urine of patients who had been treated for leucaemia with benzene. The yield was also small (0.11%).

Higher yields, corresponding to about 3.7% of the benzene administered, have been recorded by Thierfelder & Klenk [1924], but not every investigation has confirmed Jaffé's work. Neumaerker [1923] failed, in spite of using an improved method of extraction, to separate any muconic acid from the urine of a rabbit which had been injected subcutaneously with 3 g. of benzene. The question which needed further study was that of the origin of the muconic acid, because, apart from some uncertainty whether it is always produced, there was the important consideration, based on the work of Böeseken and his colleagues [1929; 1932], that one would expect the *cis-cis* form of the acid to be formed, whereas the evidence indicated that the acid isolated from urine was the *trans-trans* form. Böeseken & Slooff [1929] have remarked, "If it should really be true that on addition of benzene to the food, *trans-trans* acid was separated by the test animals, this is more likely the consequence of a reduction and dehydration, e.g. of galactose, as the grouping of the atoms of this sugar and the closely allied mucic acid renders the formation of *trans-trans* muconic acid possible." The suspicion that the muconic acid in the urine might arise not directly from the benzene but as a result of disturbances of metabolism caused by it seemed to be strengthened by the discrepancies regarding the fate of the acid after it is injected into animals. Jaffé [1909] recovered less than 1%; Neumaerker [1923] as much as 53%; Mori [1918] the very high yield of 73%. The last-mentioned investigator was inclined to doubt that the acid was formed after giving benzene.

### EXPERIMENTAL

Preliminary experiments indicated that the methods already described for isolating muconic acid from urine were unsatisfactory. Recovery of added acid was always poor.

The basis of a better method was found in the fact that the acid, although very slightly soluble in dry alcohol or acetone is very readily soluble in both solvents in the presence of moisture and acid. It is also very soluble in a wet acetone-ether mixture.

*trans-trans* Muconic acid was prepared by the bromination of adipic acid chloride [Stephen & Weizmann, 1913]. The dibromoacid chloride was converted

into the ethyl ester [Ingold, 1921] and by debromination and hydrolysis into the muconic acid, decomp. p. 301°.

The *cis-cis* acid was prepared by the oxidation of phenol by peracetic acid [Böeseken & Engelberts, 1932]. It decomposed at 186°.

#### Recovery trials

(a) *trans-trans Muconic acid*. 0.2 g. of the *trans-trans* acid as the Na salt was added to 50 ml. of rabbit urine, acidified to Congo red with HCl and the volume made up to 75 ml. Equal volumes of acetone and ether were added and the mixture well shaken. On standing the layers separated, the upper nearly colourless, the lower red. The former was separated and the solvents removed. The residual aqueous fluid contained a small amount of brown suspended solid which went into solution on adding an excess of ammonia. Boiling with norite decolored the solution and on acidification a white precipitate was formed immediately. The crude product melted with decomposition at 299°. The yield was 84%.

The method was found suitable for the recovery of smaller amounts, and could detect as little as 10 mg./100 ml.

Exp.	Wt. of muconic acid added to 50 ml. of normal urine g.	Wt. of muconic acid recovered g.	Recovery %
a	0.4	0.3549	88.7
b	0.2	0.1741	87
c	0.1	0.0840	84
d	0.05	0.0378	75.6
e	0.025	0.0186	74.4
f	0.010	0.0057	57
g	0.005	Recovered a trace of impure substance decomp. 233°	

The crude preparations of the acid were often purified by sublimation at 0.001 mm. Quite small quantities could be obtained in a pure condition by this procedure.

(b) *cis-cis Muconic acid*. The first experiments on the recovery of added *cis-cis* acid indicated that our method of extraction tended to convert it into the *trans-trans* form. Later it was found that provided that the precaution was taken of evaporating the alcohol-ether fractions at low temperature the *cis-cis* acid could be recovered from normal urine.

0.3 g. *cis-cis* muconic acid, as the Na salt, was added to 400 ml. of "normal" rabbit urine and the solution kept for 24 hr. The urine was made acid to Congo red with HCl and an equal volume of acetone (400 ml.) added and the mixture shaken vigorously. Then 400 ml. of ether were added and the mixture again shaken vigorously. After a short time two layers separated. The lower layer was run off and again extracted with a mixture of 100 ml. of acetone and 100 ml. of ether. The lower layer obtained again was extracted with another mixture of 100 ml. of acetone and 100 ml. of ether. It was run off and again extracted as before. All four acetone-ether extracts were combined and made alkaline with ammonia, whereupon an aqueous phase separated at the bottom. The volume of this lower layer was 120 ml. It was acidified with HCl and kept overnight. No precipitate formed. It was therefore extracted four times with acetone-ether mixtures as before, using smaller volumes of solvent. The four extracts were combined and made alkaline with ammonia, whereupon an

aqueous phase separated. This was run off, and the acetone and ether dissolved in it were removed by evaporation under reduced pressure in the cold. It was then acidified with HCl and kept overnight. A precipitate formed which was collected, washed with water and dried *in vacuo* over P<sub>2</sub>O<sub>5</sub>. The yellow powder was dissolved in dilute ammonia and reprecipitated.

The purified material melted at 183° and was identified as the *cis-cis* acid. The recovery was 56%. Satisfied, therefore, that both acids could be separated from rabbit urine we proceeded to make experiments on the effect of the administration of benzene. The adult rabbits were given the hydrocarbon dissolved in olive oil by means of a stomach tube. When 0.25 or 0.5 ml. of benzene was given over a period of 8–10 days the urine did not yield any muconic acid. After giving 2 ml. dissolved in 5 ml. of olive oil for 6 days a few mg. of impure *trans-trans* acid were isolated. When the dose was raised to 3 ml. of benzene daily the results were satisfactory. From the urine representing 5 days' dosing at this level 146 mg. of acid were isolated. It was the *trans-trans* form and no trace of the *cis-cis* isomeride was found. The yield in several experiments was about 0.4%; a figure agreeing reasonably well with Jaffé's original observations.

It was now necessary to satisfy ourselves of the fate of the two isomerides when injected into the body.

*Injection experiments*

(a) *trans-trans Acid*. Rabbits were injected subcutaneously with 0.89 of the acid given in 20 ml. of an aqueous solution of the Na salt.

The urines were collected during 72 hr. and examined by the method just described. By examination of each day's urine it was found that practically the whole of the muconic acid excreted was present in the samples taken over the first 24 hr. Only a few mg. were isolated from the urine of the second day and none from that of the third.

Rabbit	Muconic acid injected g.	Volume of urine ml.	Reaction of urine to litmus	Muconic acid in urine g.	Recovery %
A	0.8000	104	Neutral	0.4807	60
B	0.8000	122	Alkaline	0.5101	63.8
C	0.8000	87	„	0.4240	53
A	0.8000	115	„	0.4520	56.5
B	0.8000	153	„	0.4015	50.2
C	0.8000	107	„	0.4183	52.3
Average					56

The acid was in all cases unchanged *trans-trans*. These yields are much better than Jaffé obtained and more in agreement with those recorded by Mori.

(b) *cis-cis Acid*. Two rabbits were given 0.8 g. of the *cis-cis* acid as the Na salt. It was injected subcutaneously in 20 ml. of water. The acid recovered from the urine was also *cis-cis* and the yields were respectively 47.5 and 45.3%. No *trans-trans* acid was isolated and it is important to remember that had it been present its smaller solubility would probably have led to its detection before the *cis-cis* acid separated out.

*Origin of trans-trans acid in "benzene" urine*

We failed to isolate any *cis-cis* acid from the urine of the animals which had received benzene. As the experiments on the recovery of injected *cis-cis* acid gave nearly a 50% yield and provided no evidence of conversion into the *trans-trans* we were inclined to think that had benzene given rise to the former acid it would have been found in the urine. One thing had, however, aroused our suspicion. The urine voided by rabbits given rather large doses of benzene is rich in phenolic substances and darkens.

In one experiment on the recovery of added *cis-cis* acid from urine it was found that conversion into the *trans-trans* form occurred rather readily when "benzene" urine was used. No evidence of conversion was obtained when the process was applied to normal rabbit urine.

0.4 g. of *cis-cis* muconic acid, as the Na salt, was added to

- (1) 400 ml. of "normal" rabbit urine.
- (2) 400 ml. of "benzene" rabbit urine.

Both were kept for 24 hr. Each was made acid to Congo red with HCl and extracted four times with acetone-ether mixtures, as previously described. The acetone-ether extracts were combined and made alkaline with ammonia, whereupon an aqueous phase separated. The lower aqueous phase was run off and evaporated on the water bath to small bulk, the liquid being kept alkaline with ammonia during the course of the evaporation. The solution was cooled, strongly acidified with HCl and kept overnight. A precipitate formed. This was collected, washed, and dried *in vacuo* over  $P_2O_5$ .

- (1) Decomposition point was  $181^\circ$  (*cis-cis*).
- (2) Decomposition point was  $293^\circ$  (*trans-trans*).

By modifying the process so as to eliminate the heating of the extracts, it was found possible to recover added *cis-cis* acid in the presence of *trans-trans* acid in "benzene" urine.

(a) *Urine without addition of cis-cis muconic acid.* An impure substance was isolated (about 19 mg.) the decomposition point of which was  $291^\circ$ . This, therefore, was *trans-trans* muconic acid.

(b) *Urine to which cis-cis muconic acid had been added.* When the second aqueous solution was obtained, the acetone and ether dissolved in it were removed under reduced pressure in the cold. It was then acidified with HCl, and after about an hour a small precipitate formed. This was collected and dried *in vacuo* over  $P_2O_5$ . The weight of this precipitate was 28 mg., and its decomposition point  $293^\circ$ . This was *trans-trans* muconic acid.

The filtrate after scraping the sides of the vessel with a glass rod began to form a second precipitate the amount of which gradually increased. After a few hours this precipitate was collected, washed with water and dried *in vacuo* over  $P_2O_5$ . The weight of the crude substance obtained was 0.33 g. and its decomposition point was  $176^\circ$ . It was further purified by reprecipitation from its ammonium salt. The decomposition point of the purified product was  $184^\circ$ . It was identified as the *cis-cis* acid. This experiment was repeated four times with the same result.

The evidence seemed to be pointing to a possible action of some substances in "benzene" urine which might under suitable conditions lead to a conversion of the *cis-cis* into the *trans-trans* acid.

To throw light on this question a rabbit was given the usual dose of 3 ml. benzene for two periods of 3 days. It was then injected with 0.22 g. of the

*cis-cis* acid (Na salt), and the benzene administration continued for 3 days. Finally there was another 3-day control period of benzene. The results were:

Period	Vol. urine ml.	Acid isolated	Wt. mg.
1 benzene	225	<i>t-t</i>	58
2 benzene	175	<i>t-t</i>	80
3 benzene and <i>cis-cis</i> acid	510	<i>t-t</i>	107
4 benzene	500	<i>t-t</i>	43

The difference between the yields of *trans-trans* acid in the three control periods makes it difficult to decide whether the increased excretion during period 4 is significant. It probably is, because no *cis-cis* acid was isolated whereas from the results on p. 82 a yield of about 100 mg. might have been expected.

Although the evidence is not conclusive it seems to indicate that the *cis-cis* acid may be converted into the *trans-trans* form during the metabolism of benzene.

Why this happens under these conditions but does not in the normal rabbit when the *cis-cis* acid is given cannot yet be explained.

*Other substances present in "benzene" urine*

The urine of the rabbits to which benzene had been given was examined for other products. Free benzene was detected by using the apparatus devised by Cannan & Sulzer [1924] for the micro-estimation of alcohol in body fluids.

The aspirated air passed through a nitrating mixture composed of equal parts of  $H_2SO_4$  and fuming  $HNO_3$ . This fluid was examined after several hours' aeration by applying the colour reaction described by Schrenk *et al.* [1935] and Pearce *et al.* [1936].

The reaction was negative when amounts smaller than 3 ml. of benzene were administered, but with that quantity or with larger doses free benzene was detected in the urine. The amount of phenolic substance rose considerably during the periods when benzene was being given. The results of a typical experiment which show the rise in ethereal sulphates are recorded below.

Day	Rabbit	mg. per kg. body weight				
		Inorganic S		Ethereal S		
		I	II	I	II	
1	93	121	4.9	4.5	5 ml. olive oil daily by stomach tube	
2	82.5	121	5.2	1.5		
3	71.5	110.5	3.7	4.1		
4	60.5	84	2.3	6.8		
5	—	—	—	—		
6	28.6	7.5	33	32	3 ml. of benzene in 5 ml. of olive oil daily	
7	2.1	43.7	38.6	33.8		
8	31.2	61	34.2	26.5		
9	8.7	32	34.6	29.2		
10	27.8	42.4	35.2	30.7		

The increased excretion of ethereal sulphates was accompanied, as was expected, by a rise in the excretion of glycuronates but this was not actually estimated. Several attempts were made to isolate phenylmercapturic acid from the "benzene" urine but without success. Neither the method used by Bourne & Young [1934] for the isolation of  $\alpha$ -naphthylmercapturic acid, nor the preparation of a mercury compound yielded material which could be adequately

purified. Strong evidence that these fractions contained phenylmercapturic acid was obtained by treating them with acids when thiophenol was produced. The production of phenols by the oxidation of benzene made it desirable to ascertain whether muconic acid can be found in the urine after administering either phenol itself or catechol.

The largest amount of phenol which it was found practicable to give a fully-grown rabbit by stomach tube was 0.25 g. in 50 ml. of water. This dose for 4 days produced a dark urine rich in phenolic sulphates and glycuronates but no muconic acid was isolated.

In all probability the dose of phenol was too small for the amount of muconic acid formed, if any, to be recoverable by our methods. The permissible dosages of catechol were even smaller, so it is not surprising that these also failed to yield muconic acid. Negative results were also recorded in an experiment in which 3 g. of tyrosine dissolved in 80 ml. of warm milk were given to a rabbit for 6 days. This confirms Kotake (cited by Mori [1918]).

#### DISCUSSION

Our results are not wholly conclusive but they tend to support the view that the *trans-trans* muconic acid found in the urine of animals to which benzene has been administered does arise from the hydrocarbon molecule. It is a curious finding for which our work has not provided a satisfactory explanation, that the *cis-cis* acid, which, as Böeseken has pointed out, would be the one directly derived from the opening of the benzene ring, is rather readily converted into the *trans-trans* isomeride in the presence of other products formed when benzene is metabolized. In striking contrast one finds the *cis-cis* acid not converted in the normal organism or in contact with the substances present in normal urine.

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