Studies in Detoxication

44. THE METABOLISM OF BENZENE. THE MUCONIC ACID EXCRETED BY RABBITS RECEIVING BENZENE. DETERMINATION OF THE ISOMERIC MUCONIC ACIDS

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Muconic acid (hexa-2:4-diene-1:6-dioic acid) was first discovered to be a metabolite of benzene by Jaffé (1909), who isolated it from the urine of dogs and rabbits receiving benzene. There are three geometrical isomers of muconic acid, namely, *cis-cis*, *cis-trans* and *trans-trans*, and in every instance in which the isolation of muconic acid from the urine of men and animals dosed with benzene has been reported, the isomer isolated has been the *trans-trans* form (Jaffé, 1909; Fuchs & Soós, 1916-17; Mori, 1918; Neumaerker, 1923; Thierfelder & Klenk, 1924; Drummond & Finar, 1938; Bernhard & Gressly, 1941). If muconic acid is formed *in vivo* by the opening of the benzene ring, then it would be expected on stereochemical grounds that the isomer produced would be the *cis-cis*-isomer; the oxidation of phenol or of catechol with peracetic acid does, in fact, give rise to *cis-cis*-muconic acid (Böeseken & Engelberts, 1931; Böeseken & Kerkhoven, 1932; Elvidge *et al.* 1950*a*).

The isolation of the *trans-trans*-acid from benzene urine, however, raised doubt as to whether benzene was the source of the acid, until Bernhard & Gressly (1941) showed that benzene containing deuterium gave rise *in vivo* to muconic acid containing deuterium. The *trans-trans*-acid could, however, arise from the *cis-cis*-acid by isomerization during the isolation procedures, but Drummond & Finar (1938) showed that this was probably not the case and they suggested that the isomerization probably took place *in vivo*.

That *cis-cis*-muconic acid is probably the first muconic acid to be derived biologically from benzene is suggested by the observation of Hayaishi & Hashimoto (1950) that an enzyme called pyrocatecase separated from a strain of *Pseudomonas* sp. oxidizes catechol to what they suggest is *cis-cis*-muconic acid, which was isolated in high yield in the crystalline state. Catechol is known to be a metabolite of benzene in the rabbit (Porteous & Williams, 1949*a, b*).

Recently, however, Elvidge, Linstead, Sims & Orkin (1950*b*) identified, for the first time, the third isomer of muconic acid, namely, the *cis-trans*-isomer, and they showed that recrystallization of the *cis-cis*-acid from water (but not ethanol) was sufficient to

convert it into the *cis-trans*-acid. This observation thus casts doubt on much of the earlier work which involved the use of the so-called *cis-cis*-acid.

The objects of the present work were to find methods of estimating and distinguishing between the three isomers of muconic acid, and to find out whether or not the urinary muconic acid was a mixture of isomers, since earlier workers could have lost the more soluble *cis*-isomers during purification. We shall show, however, that all the muconic acid of benzene urine from rabbits is almost certain to be the *trans-trans*-isomer.

EXPERIMENTAL

Preparation of materials

Melting points are uncorrected. *cis-cis*-Muconic acid was readily prepared by the oxidation of phenol by concentrated peracetic acid. (Elvidge *et al.* (1950*a*) used dilute peracetic acid, but in our hands better results were obtained with the concentrated acid.) The peracetic acid was prepared by cautiously mixing in the cold 10 ml. of 70% (w/w) H_2O_2 with 19 ml. of acetic anhydride, the molecular ratio of H_2O_2 to Ac_2O being 2:1.5, and keeping in cold water for 18 hr. Phenol (3 g.) was then added with shaking until it dissolved, and the mixture was kept in the dark. After 24 hr. the crystalline deposit of the *cis-cis*-acid was collected on a sintered-glass filter. Further quantities were obtained and filtered off each day by keeping the solution for 3 more days. The yield was 1.1 g. or 25%. The combined precipitates were washed with a little ethanol and twice recrystallized from ethanol. It formed colourless rhombs and had m.p. 194° as determined by the method of Elvidge *et al.* (1950*b*); the benzhydryl ester (Elvidge *et al.* 1950*b*) had m.p. 156°.

cis-trans-Muconic acid was prepared by twice recrystallizing the *cis-cis*-acid from hot water according to Elvidge *et al.* (1950*b*). It formed colourless prismatic needles from water, m.p. 190°, and its benzhydryl ester had m.p. 142°. A mixture of the *cis-cis*- and *cis-trans*-acids melted at 179-182°.

trans-trans-Muconic acid formed colourless minute prisms, m.p. 300° (decomp.) and was prepared according to Ingold (1921). The benzhydryl ester had m.p. 190° (cf. Elvidge *et al.* 1950*b*).

The lactones related to muconic acid were prepared by the methods of Elvidge *et al.* (1950*b*). The dilactone, butanolido-butanolide (3:7-diketo-2:6-dioxabicyclo[3:3:0]octane) formed pale brown prisms, m.p. 134° (Elvidge *et al.* (1950*b*) give m.p. 131°, depressed by admixture with the mono-

lactone to 90–95°. The monolactone, γ -carboxymethyl- Δ^2 -butenolide, formed very pale brown rhombs, m.p. 110° depressed on admixture with the dilactone to 90–95° (Elvidge *et al.* (1950a) give m.p. 110.5–111.5°).

The colour reaction between muconic acid and phenol

When *cis-cis*- and *cis-trans*-muconic acids were heated at 130° for 5 min. with various phenols, together with a drop of conc. H_2SO_4 , coloured products were obtained which were soluble in water and organic solvents and fluoresced in ultraviolet light. The *cis-trans*-acid gave more intense colours than the *cis-cis*-acid, whereas the *trans-trans*-acid under the same conditions gave no coloured product at all. These observations are summarized in Table 1.

Table 1. Colours obtained by condensing *cis-cis*- and *cis-trans*-muconic acids with phenols at 130° for 5 min.

Condensing phenol	Colour of product	
	In acid solution	In alkaline solution
Phenol	Crimson*	Blue-green*
Resorcinol	Red-brown*	Red†
Catechol	Brownish violet	Olive green*
Quinol	Brown	Yellow brown‡
1-Naphthol	Brown*	Brown*
2-Naphthol	Brown*	Yellow†

* Light blue fluorescence in ultraviolet light.

† Green fluorescence in visible light.

‡ Blue fluorescence in visible light.

Identification of the muconic acids by the above colour reaction

The phenol most suitable for our purposes was phenol itself. The *cis-cis*- and *cis-trans*-acids give an intense crimson product in 5 min., whereas with the *trans-trans*-acid only a faint pink colour is obtained after several hours' heating. If 1 mg. of *cis-trans*-muconic acid is heated with 500 mg. phenol and 0.1 ml. conc. H_2SO_4 for 10 min. at 130° and the product dissolved in 50 ml. ethanol, this solution gives an extinction ($E_{1\text{ cm.}}$) of 0.7 on the Spekker absorptiometer using Ilford Spectrum green filter no. 604 and heat filter H 503. *trans-trans*-Muconic acid under the same conditions gives $E_{1\text{ cm.}} = 0.01$. With *cis-cis*-muconic acid the maximum colour is attained in 45 min. when $E_{1\text{ cm.}} = 0.76$, whereas with *trans-trans*-muconic acid in 45 min. $E_{1\text{ cm.}}$ is only 0.05.

If the temperature of the reaction is raised above 140°, *trans-trans*-muconic acid gives the same crimson colour as its isomers, maximum colour being attained in 20 min. at 160°. At 100°, the *cis-cis*- and *cis-trans*-acids give crimson colours after several hours' heating, but the *trans-trans*-acid gives no colour at all at this temperature. Thus by carrying out the test at different temperatures the *trans-trans*-acid can readily be distinguished from its isomers.

The two lactones, γ -carboxymethyl- Δ^2 -butenolide and butanolido-butenolide, give the same colour at the same rate as *cis-trans*-muconic acid, and it is possible that one of these lactones is the precursor of the colour, although Elvidge *et al.* (1950a) were unable to convert the *trans-trans*-acid into these lactones. Elvidge *et al.*, however, point out that when the *trans-trans*-acid is heated at 160–165° with 95% H_2SO_4 , none can be recovered.

Quantitative estimation of muconic acid

The above colour reaction can be used for the quantitative estimation of muconic acid and the conditions for its use were investigated as follows.

Quantity of phenol and sulphuric acid. Experiments showed that the optimum amounts of phenol and conc. H_2SO_4 for development of the colour were 500 mg. and 0.1 ml., respectively, to about 1 mg. of muconic acid.

Effect of time of heating. In order to estimate the *cis-cis*- and *cis-trans*- in the presence of the *trans-trans*-acid it became necessary to carry out colour development at 100° for the former two acids and at 160° for the latter. The time of development of maximum colour at these temperatures

Fig. 1. The effect of time of heating on the production of the red colour obtained by condensing muconic acid (0.5 mg.) with phenol (0.5 g.) and concentrated H_2SO_4 (0.1 ml.) and then dissolving in 50 ml. absolute ethanol. x—x, *cis-trans*-Muconic acid condensed at 100°; ●—●, *cis-cis*-muconic acid condensed at 100°; ○—○, *trans-trans*-muconic acid condensed at 160°. E measured in the Spekker absorptiometer.

had to be determined. Muconic acid (0.5 mg.) was condensed with 0.5 g. phenol and 0.1 ml. conc. H_2SO_4 in corked tubes, at $100 \pm 1^\circ$ for the *cis-cis*- and *cis-trans*-isomers and at $160 \pm 2^\circ$ for the *trans-trans*-acid. Heating was continued for varying lengths of time, and then the coloured melts were dissolved in ethanol to a volume of 50 ml. The colour was measured in 1 cm. cells in the Spekker absorptiometer using colour filters as above. Maximum colour development was attained at 100° in 4 hr. with *cis-trans*- and the two lactones, and in 6 hr. with *cis-cis*-muconic acid. The *trans-trans*-acid gave no colour in 6 hr. at 100°, but gave the same maximum colour in 20 min. at 160° (see Fig. 1) as the *cis*-acids did in 6 hr. at 100°.

Effect of temperature. The above experiments showed that *cis-cis*- and *cis-trans*-muconic acids could be estimated by

heating with phenol and H_2SO_4 at 100° for 6 hr., whereas the *trans-trans*-acid could be estimated in the same way except that the heating had to be done at 160° for 20 min. In order to estimate the *cis*-acids in the presence of the *trans-trans*-acid, it was necessary to find out whether the *cis*-acids gave the same amount of colour at 160° as at 100° . It was found, however, that if the *cis*-acids were condensed at 160° , more colour was produced than at 100° . This was not seen with the *trans-trans*-acid, for temperatures above 160° merely accelerated the destruction of the colour produced by this acid. The effect of temperature on the development of colour with the three acids is shown in Fig. 2. In these experiments colour density was measured at $520 \text{ m}\mu$. (see below) in a Unicam spectrophotometer, the instrument finally adopted for all measurements of this colour. This extra colour

Fig. 2. The effect of temperature on the intensity of the red colour produced by heating the muconic acids with phenol and sulphuric acid. $\times$ — $\times$, 0.1 mg. *trans-trans*-acid condensed for varying times at different temperatures and the product dissolved in 50 ml. ethanol; $\bullet$ — $\bullet$, 0.1 mg. *cis-cis*-acid treated similarly; $\times$ — $\times$, 0.1 mg. *cis-trans*-acid treated similarly. E measured in the Unicam spectrophotometer.

production at 160° with the *cis*-acids, although constant, showed that the *cis*-acids could not be readily estimated in the presence of the *trans-trans*-acid, although if the *trans-trans*-acid occurred alone it could be estimated. In benzene urine only one isomer, the *trans-trans*-, occurred, so that it could be readily estimated by this colour reaction. The elimination of this difficulty is described later (see below).

Stability of the colour. The colour in ethanol when exposed to air was stable for 15 min. after which time it slowly deteriorated probably due to absorption of moisture. E values at 0, 0.25, 0.5, 1, 2, 3 and 4 hr. were 0.78, 0.79, 0.75, 0.68, 0.55, 0.49 and 0.44 respectively. All readings were therefore carried out within 15 min. of development. Water (1–2%) reduced the intensity of the colour by a half.

Absorption spectrum of the colour and possible interference by other dicarboxylic acids. It seems possible from the mode of production of the colour, that a xanthone derivative is formed by the condensation of one of the lactones of muconic acid with two molecules of phenol. If this is true, then the possibility that other dicarboxylic acids can form such coloured complexes must be borne in mind especially when the method is applied to urine.

The absorption spectrum of the coloured complex in ethanol in the visible region showed a single maximum at $520 \text{ m}\mu$. with $E_{1 \text{ cm.}} = 0.56$ for a solution prepared from 0.2 mg. muconic acid (*cis-cis*- and *cis-trans*- by condensation at 100° and *trans-trans*- at 160°) and made up to 50 ml. with ethanol, measurements now being made with a Unicam spectrophotometer (see Fig. 3 curve A). All three isomers condensed as mentioned above gave the same $E_{1 \text{ cm.}}$

Fig. 3. Absorption spectra of the colours obtained by condensing at 160° for 20 min., certain organic acids with phenol and concentrated H_2SO_4 and dissolving the product in 50 ml. ethanol. Each curve corresponds to 1 mg. of the acid. A, *trans-trans*-muconic acid; B, oxalic acid; C, adipic acid; D, pimelic acid; E, succinic acid; F, suberic acid.

Common acids of the urine were first tested, namely benzoic, hippuric, phenylacetic, salicylic and *p*-hydroxybenzoic acids. When condensed with phenol and sulphuric acid at 160° , these acids give pale pink melts which dissolved with ethanol to give nearly colourless solutions. These acids did not interfere.

The dicarboxylic acids, oxalic, succinic, adipic, pimelic, suberic and azelaic acids were tested and all were found to give coloured melts at 160° , the spectra of which are recorded in Fig. 3. At $520 \text{ m}\mu$., the principal interference would come from adipic acid whose coloured complex (formed at 160°) at this wavelength has 33% of the absorption of the phenol condensation product of muconic acid. In the same way, pimelic and oxalic acids would interfere to the extent of 7 and 5% respectively. In the estimation of *cis-cis*- and *cis-trans*-muconic acids, that is with a condensation temperature of 100° , the interference would be much less being 1% for oxalic, 0.9% for adipic and 0.4% for pimelic acid.

The amounts of these dicarboxylic acids in rabbit urine are likely to be very small, although values are known only

for human urine. Hanson (1943) has reported traces of succinic, adipic and suberic acids in normal human urine, and gives the content of $\text{COOH}(\text{CH}_2)_n\text{COOH}$ as 15 mg./l. The succinic acid content of normal human urine has been reported by Weitzel (1947) as being 1.9–8.8 mg./l. in twelve subjects. According to Mori (1918) rabbits excrete about 4–9 mg./day of oxalic acid. The amounts of these dicarboxylic acids excreted, however, depend upon diet (Hanson, 1943; Weitzel, 1947).

Recovery of muconic acid from aqueous solutions. Solutions of *cis-cis*- or *trans-trans*-muconic acids (0.5 or 0.2 mg.) in 0.1N- Na_2CO_3 (1 ml.) were treated with 2N-HCl (0.05 ml.), and the solutions were evaporated at 105°. Phenol and H_2SO_4 were then added as above, and the tubes containing the *cis-cis*-acid heated for 6 hr. at 100° and those containing the *trans-trans*-acid at 160° for 20 min. The colour was measured as before. Recoveries were satisfactory (95–102%). The standard curves for the three acids are shown in Fig. 4.

Fig. 4. Standard curve for the estimation of muconic acid, prepared by condensing muconic acid with phenol (0.5 g.) and conc. H_2SO_4 (0.1 ml.) at 100° for 6 hr. with the *cis-cis*- and *cis-trans*-acids and at 160° for 20 min. with the *trans-trans*-acid and then dissolving in 50 ml. absolute ethanol. $E_{1\text{ cm.}}$ at 520 mμ. measured in the Unicam spectrophotometer. x, *cis-cis*; ●, *cis-trans*; ○, *trans-trans*.

Estimation of muconic acid in urine. The above method was now applied to rabbit urine. If this urine were dried at 105°, subsequent treatment with phenol and H_2SO_4 yielded dark-brown solutions. Much of this darkening could be avoided if the urines were dried at room temperature *in vacuo* in a desiccator over P_2O_5 . This also had the advantages of avoiding heat (which might cause isomerization), treatment with organic solvents and non-quantitative extraction procedures.

Normal urines from rabbits were collected in flasks containing 0.5 ml. 1% HgCl_2 . Each 24 hr. urine (slightly less than 100 ml.) was diluted with water to 100 ml. Solutions of

cis-cis- and *trans-trans*-muconic acids (5 mg./ml. in N- NaHCO_3) were added to 25 ml. of these urines which were then diluted with water so that the final concentration of muconic acid in each solution was 0.1 mg./ml. In this way, solutions of muconic acid corresponding to outputs of muconic acid of 20 and 100 mg./day were made up. 1 ml. portions of these diluted urine-muconic acid solutions in test tubes ($3 \times \frac{5}{8}$ in.) were then dried *in vacuo* over P_2O_5 at room temperature. Phenol (0.5 g.) and conc. H_2SO_4 (0.1 ml.) were then added to each dry residue. The tubes containing the *cis-cis*-acid were then heated at 100° for 6 hr. and those containing the *trans-trans*-acid at 160° for 20 min. The product from each tube was then dissolved in 50 ml. ethanol and filtered and the $E_{1\text{ cm.}}$ at 520 mμ. then measured in the spectrophotometer. The recoveries were *cis-cis*- (at 20 mg./day), 78–85% and at 100 mg./day 95–98%; *trans-trans*- (at 20 mg./day), 89–95% and at 100 mg./day, 95–99%.

Recovery of muconic acid from benzene urine. Muconic acid (20 mg. in the least amount of N- NaHCO_3) was added to the 24 hr. filtered urine of a rabbit which had received a dose of 0.5 g./kg. of benzene, so that the concentration of added muconic acid was 0.2 mg./ml. Then 1 ml. samples of this urine were dried *in vacuo* and condensed with phenol as above. Blank estimations were carried out simultaneously on the benzene urine. The recoveries of added muconic acid were *cis-cis*-, 102–108; *cis-trans*-, 103–105; *trans-trans*-, 90–97%.

Muconic acid in the urine of rabbits receiving benzene

Benzene was administered orally to rabbits and the urine collected daily, made up to 100 ml. where necessary, and centrifuged. For each urine, eight tubes were set up containing 1 ml. of urine which was evaporated *in vacuo* in a desiccator over P_2O_5 . When dry, 500 mg. phenol and 0.1 ml. conc. H_2SO_4 were added to each tube. Three tubes were heated at 100° for 6 hr., three at 160° for 20 min. and two tubes (blanks) kept at room temperature for 6 hr. Each melt was then dissolved in ethanol, made up to 50 ml., filtered from any precipitated inorganic salts and the optical density measured at 520 mμ. in the Unicam spectrophotometer. Normal urines collected before and after the feeding of benzene were treated in the same way. The colour (a general absorption) given by normal urine after heating at 100° for 6 hr. or at 160° for 20 min. remained very constant and gave a reading of $E_{1\text{ cm.}}$ approx. 0.1. In none of the experiments with benzene was there any increase in absorption at 520 mμ. above that of the normal urines after condensation at 100° for 6 hr. From this it was concluded that the urine of rabbits fed benzene does not contain *cis-cis*- and *cis-trans*-muconic acids in amounts greater than 0.05% of the dose of benzene. The figure 0.05% is the limit imposed by the method. The amounts of *trans-trans*-muconic acid found are given in Table 2, which shows that at a dose level of 0.5 g./kg. 0.5% of the benzene appears as *trans-trans*-muconic acid. Fig. 5 shows an individual result.

Metabolism of muconic acid by micro-organisms

Since Evans & Smith (1951) have shown that *cis-cis*-muconic acid, but not the *cis-trans*- and *trans-trans*-isomers, is metabolized by certain micro-organisms, we had to consider the possibility that this acid might be formed and excreted by rabbits receiving benzene and then be rapidly

Table 2. *trans-trans-Muconic acid excretion by rabbits receiving benzene orally*

(*cis-cis*- and *cis-trans*-Muconic acids were absent from all these urines since no colour was obtained on prolonged heating at 100° with phenol and sulphuric acid. Dose level 0.5 g./kg. body wt. except for the two experiments at the foot of the table.)

Rabbit no.	Dose of benzene (g.)	Period of excretion (days)	<i>trans-trans</i> -Muconic acid excreted (mg.)	Benzene excreted as <i>trans-trans</i> -muconic acid (% of dose)
51	1.58	2	17.6	0.6
53	1.41	1	11.3	0.45
67	1.41	2	12.3	0.5
69	1.23	1	22.3	1.0
70	1.76	3	17.2	0.55
75	1.32*	2	4.9	0.15
82	1.32*	2	9.3	0.4
87	1.32*	1	14.7	0.65
69	2.46	3	17.1	0.4
70	3.52	3	22.0	0.35

* Urine collected in the presence of HgCl₂.

destroyed by micro-organisms infecting the urine. We found that the recovery of added *cis-cis*-muconic acid from very stale rabbit urine was negligible. In two experiments we recovered no *cis-cis*-muconic acid after 48 hr. when it was

Fig. 5. The excretion of *trans-trans*-muconic acid by rabbit no. 70 which had received orally 1.76 g. of benzene on day 3 and 3.52 g. of benzene on day 8. The muconic acid excretion is expressed in terms of the $E_{1 \text{ cm.}}$ at 520 mμ. of the phenol-muconic acid colour in ethanol (see text).

added to stale urine in concentrations of 20 and 100 mg./100 ml., whereas the *trans-trans*-acid could be recovered almost quantitatively.

On culturing this urine on McConkey agar two predominant organisms were isolated. One was a late-lactose-fermenting paracolon bacillus and the other a faecal-type streptococcus (enterococcus). These organisms grew very scantily in 48 hr. on synthetic media of salts and vitamins which contained (NH₄)₂SO₄ and urea as sources of nitrogen and *cis-cis*-muconic acid as the source of carbon. None of the muconic acid was metabolized. These organisms however grew well on fresh, autoclaved rabbit urine containing *cis*-

cis-muconic acid, and in 96 hr. the faecal-type streptococcus metabolized 100% and the paracolon bacillus, 96% of the added *cis-cis*-muconic acid (0.5 mg./ml.). On subculturing 4 or 5 times on glucose nutrient agar media the organisms lost their ability to grow well in rabbit urine containing *cis-cis*-muconic acid.

It was concluded from these observations that in our experiments these organisms did not occur in sufficient numbers and did not metabolize *cis-cis*-muconic acid sufficiently rapidly to affect our results. In any case heavy growth of these organisms only occurred when rabbit urine had been allowed to stand for several days at room temperature. However, in order to eliminate this possibility, the urine from rabbits receiving benzene was collected in flasks containing 0.5 ml. of 1% HgCl₂. In this concentration HgCl₂ had no effect on the colour development. The results of these experiments (see Table 2) were essentially the same as those in which no HgCl₂ was used, and it was concluded that the absence of *cis-cis*-muconic acid was not due to its destruction by micro-organisms.

Isolation of muconic acid from benzene urine

A total of 7 g. of benzene (0.5 g./kg.) was fed by stomach tube with water to four rabbits. The 48 hr. urine (450 ml.) was made 0.2N with respect to HCl by adding conc. HCl. It was then saturated with (NH₄)₂SO₄ and continuously extracted for 6 hr. with peroxide-free ether. (In separate experiments it was shown that 150 mg. of *cis-cis*-muconic acid added to 100 ml. normal rabbit urine could be recovered almost quantitatively by ether extraction for 6 hr. at pH 2, as a muconic acid m.p. 185–186°, probably a mixture of *cis-cis* and *cis-trans*. No *trans-trans*-muconic acid was formed in this procedure.) The ethereal extract was decanted from a small quantity of wet tarry material and allowed to concentrate at room temperature. After cooling at 0° for several hours, the nearly colourless crystals (69 mg. or 0.6% of the dose) which had separated were filtered and washed with a little ether. Evaporation of the mother liquors yielded another 51 mg. of semi-crystalline material.

The colourless crystals were impure, for they began to melt at 180–185° and finally decomposed at 295–300°. On colorimetric assay, as described earlier, they contained no *cis-cis* or *cis-trans*-muconic acid and 20% of *trans-trans*-

muconic. This crude material was also tested microbiologically by Dr W. C. Evans of Aberystwyth (Evans & Smith, 1951) who reports that it contained no *cis-cis*-muconic acid.

Recrystallization of this material from ethanol eventually yielded pure *trans-trans*-muconic acid, m.p. 300° (decomp.); the yield of pure material in several experiments was 0.1–0.2% of the dose of benzene. The acid was further identified by the preparation of benzhydryl *trans-trans*-muconate, m.p. and mixed m.p. 190° (the *cis-cis*-ester melts at 156° and the *cis-trans*- at 142°). These isolation experiments strongly suggest that benzene urine contains only *trans-trans*-muconic acid.

Experiments on the injection of the three isomers of muconic acid

The estimation of cis-cis- (and/or cis-trans-) muconic acid in the presence of trans-trans-muconic acid. The colour reaction developed above for the estimation of the muconic acids could not be applied as such when more than one isomer occurred in the test solution, because of the different amounts of colour produced by the *cis*-acids at 100 and 160°. A solution of this difficulty, however, was found in the observation of Grundmann & Trischmann (1936) (cf. Elvidge *et al.* 1950*b*) that the *cis-cis*- and *cis-trans*-acids may be quantitatively converted to the *trans-trans*-acid by irradiation with ultraviolet light in the presence of small amounts of I_2 . The conditions of this transformation when the acids occurred in urine were therefore investigated. When these conditions were known the *cis*-acids could then be estimated in the presence of the *trans-trans*-acid by first estimating the *cis*-acids in a mixture by the colour reaction with phenol at 100°, then irradiating with ultraviolet light to convert the *cis*-acids into the *trans-trans* and finally estimating the total muconic acid as the *trans-trans*-isomer by the colour reaction with phenol carried out at 160°.

In pure solution it was found that 100% conversion of the *cis*-acids (5 ml. of 1 mg./ml. solution) to the *trans-trans*-acid occurred on irradiation from above, for 1–2 hr., of the solution containing 0.5 ml. of 0.1% ethanolic I_2 in small beakers placed 10 cm. below the ultraviolet lamp. In all experiments a Gallenkamp ultraviolet lamp was used, stated by the makers to contain a '125 watt mercury discharge unit enclosed by an ultraviolet glass filter from which there is practically no visible light, 95% of the radiation being at 3650 Å.' The solutions were thus in effect irradiated by light of wavelength 365 mμ, which is some distance from the absorption maximum of muconic acid which is at 260 mμ. However, irradiation of the solutions with a carbon arc lamp produced no better results than the Gallenkamp lamp.

It was also found that the isomerization could be carried out just as efficiently in 3 × ½ in. test tubes kept upright and irradiated from above, as in open beakers. Furthermore, the isomerization took place equally well in dilute urine, dilute H_2SO_4 or $NaHCO_3$ and in the presence of $HgCl_2$ which was used as an antiseptic for the urines. The amount of I_2 used as a catalyst, however, was important. In the first place it was found that solutions of I_2 in KI interfered with the subsequent development of the red colour with phenol, but ethanolic solutions of I_2 were satisfactory because the I_2 could be removed by evaporation at the end of the irradiation period.

The effect of I_2 was investigated as follows. *cis-trans*-Muconic acid was dissolved in tenfold diluted normal rabbit urine to a concentration of 0.1 mg./ml. To 1 ml. of this

solution there was added from 0.05 to 0.2 ml. of 1% ethanolic I_2 solution. These solutions were irradiated with ultraviolet light for 2 hr. in 3 × ½ in. test tubes, held upright 10 cm. below the lamp as mentioned above. The solutions were then dried *in vacuo* at room temperature over P_2O_5 and the residue condensed with 0.5 g. phenol and 0.1 ml. conc. H_2SO_4 at 160° for 20 min. as described for the estimation of *trans-trans*-muconic acid. The red colour was measured as before. Similar solutions without the irradiation were dried *in vacuo* and condensed with phenol at 100° as in the estimation of *cis-cis*-muconic acid. The results are shown graphically in Fig. 6, which shows that in 1 ml. of diluted rabbit

Fig. 6. The effect of the amount of iodine present on the isomerization by ultraviolet light of *cis-cis*- to *trans-trans*-muconic acid (0.1 mg./ml.).

urine 1 mg. of I_2 (0.1 ml. of 1% ethanolic I_2) gives 100% conversion of 0.1 mg. of the *cis*-acids to the *trans-trans*-acid on irradiation for 2 hr. This somewhat large amount of I_2 is necessary to allow for the consumption of I_2 by normal rabbit urine (approx. 4.5 ml. of 1% ethanolic I_2 /100 ml.) and for that lost by evaporation during the irradiation.

Mixtures of all three acids were now estimated in tenfold diluted rabbit urine. The procedure which was finally used for benzene urine was to take 1 ml. of urine appropriately diluted with water so as to contain 0.1 mg. of muconic acid and the procedure for the estimation of *cis-cis*-muconic acid was then followed. To another 1 ml. of the urine made up in the same way there was added 0.1 ml. of 1% ethanolic I_2 , then irradiated for 2 hr. with ultraviolet light and the procedures for the estimation of *cis-cis*- and *trans-trans*-muconic acids followed. With still another 1 ml. the *trans-trans*-estimation was carried out without irradiation. A blank estimation was also carried out on 1 ml. urine containing 0.1 ml. 1% I_2 but without muconic acid, condensations being carried out at 100 and 160°. With benzene urine the blank estimations were carried out on urine collected prior to dosing. Recovery experiments are given in Table 3.

Injection of trans-trans-muconic acid. The acid was dissolved in an equivalent amount of saturated aqueous $NaHCO_3$. Such a solution contained approx. 100 mg./ml. This solution was injected intraperitoneally into rabbits, each rabbit receiving 100 mg. muconic acid/kg. The urines were collected daily in flasks containing 0.5 ml. of 1%

Table 3. *Recovery of isomeric muconic acids from diluted rabbit urine*

(*E* values are for the red colour obtained by condensation of muconic acid with phenol and H_2SO_4 and then dissolving in ethanol (see text). Corrected values (for 160° condensations) are obtained by subtracting the urine blank values.)

Material	Ultraviolet irradiation treatment	<i>E</i> at 520 m μ . Condensation temperature			Recovery (%)
		100° for 6 hr.	160° for 20 min.		
			Found	Corrected	
In aqueous solution (1 ml.):					
0.1 mg. <i>cis-cis</i>	None	0.275	0.53	—	—
0.1 mg. <i>cis-trans</i>	None	0.28	0.54	—	—
0.1 mg. <i>trans-trans</i>	None	0.02	0.27	0.27	—
In urine (1 ml.) + I ₂ (0.1 ml.):					
None (urine blank)	None	0.155	0.185	—	—
0.1 mg. <i>cis-cis</i>	2 hr.	0.185	0.445	0.26	95
0.1 mg. <i>cis-trans</i>	2 hr.	0.160	0.43	0.245	88
0.05 mg. each of <i>cis-cis</i> and <i>trans-trans</i>	2 hr.	0.135	0.485	0.300	109
0.05 mg. each of <i>cis-trans</i> and <i>trans-trans</i>	2 hr.	0.140	0.435	0.250	91

Table 4. *Excretion of muconic acid by rabbits after the intraperitoneal injection of the three isomeric muconic acids*

Muconic acid injected	Rabbit no.	Weight (kg.)	Dose (mg.)	Muconic acid recovered		
				As <i>cis-cis</i> or <i>cis-trans</i> (mg.)	As <i>trans-trans</i> (mg.)	Percentage of dose
<i>trans-trans</i>	75	2.8	250	0	137	56
	82	2.8	250	0	119	48
	87	2.2	175	0	86	49
	67	2.45	240	0	132	55
<i>cis-cis</i>	75	2.8	250	154	0	62
	82	2.8	250	179	0	72
	86	3.1	280	189	0	66
	51	2.4	240	145	0	61
<i>cis-trans</i>	75	2.8	250	150	0	60
	82	2.8	250	118	0	47
	86	3.1	280	159	0	58

Table 5. *Estimation of cis-cis-muconic acid in the urine of rabbits injected with the cis-cis-acid, and demonstrating the absence of the trans-trans-acid*

(Each rabbit was injected with 100 mg./kg. of *cis-cis*-muconic acid. *E* values are for the red colour obtained by condensation of muconic acid with phenol and H_2SO_4 and then dissolving in ethanol.)

Rabbit no.	Ultraviolet irradiation treatment of urine	<i>E</i> at 520 m μ . condensation temperature	
		100° for 6 hr.	160° for 15 min.
75	None	0.415	—
	2 hr.	—	0.45
82	None	0.48	—
	2 hr.	—	0.48
86	None	0.51	—
	2 hr.	—	0.50

HgCl_2 . Each urine was then diluted to 1000 ml. with water, and 1 ml. of this diluted solution used for the estimation of muconic acid. Only traces of colour which were within the error of the method were obtained when the condensation

with phenol was carried out for 6 hr. at 100°, thus showing the absence of *cis-cis*- and *cis-trans*-muconic acid. The main colour production occurred on condensing with phenol at 160° for 20 min. In four experiments an average of 52% of the injected *trans-trans*-muconic acid was recovered in the urine as such within 24 hr. of dosing, there being no conversion into the other isomers (see Table 4). On continuous ether extraction of the urine after acidification, only the *trans-trans*-isomer was isolated, m.p. and mixed m.p. 300°; 80% of the original urine yielded 204 mg. of the acid which is equivalent to 36% of that injected.

Injection of cis-cis-muconic acid. In a similar manner, 100 mg./kg. of *cis-cis*-muconic acid was injected into rabbits and the urine collected and diluted as before. On developing the colour with phenol at 100° for 6 hr. an average of 66% of the injected acid was recovered unchanged (see Table 4). On developing the colour at 160° for 20 min. an increase in the amount of colour was observed, but after irradiation of the urine with ultraviolet light and then estimating *trans-trans*-muconic acid, the colour produced was the same as for the procedure for the *cis-cis*-acid (see Table 5). This indicated that urine contained no *trans-trans*-acid.

On continuous extraction of the urine with ether, the muconic acid isolated had m.p. 189° after two recrystalliza-

tions from ethanol. It was thus not *trans-trans*, but one or a mixture of the *cis*-acids.

Injection of *cis-trans*-muconic acid. In a similar way, *cis-trans*-muconic acid was injected and 55% was found again in the urine (Table 4). Ether extraction of the urine yielded the *cis-trans*-acid, m.p. 189–190°, after two recrystallizations from water (yield approx. 50% of dose).

DISCUSSION

The discovery by Elvidge *et al.* (1950*b*) that mere recrystallization of *cis-cis*-muconic acid (II and IIa) from water yields the *cis-trans*-isomer (III) makes much of the earlier biochemical work, in which the so-called *cis-cis*-muconic acid was used, difficult to interpret. If samples of *cis-cis*-muconic acid which had been crystallized from water were used, it appears very probable that earlier workers were handling either mixtures of the *cis-cis*- and *cis-trans*-isomers or the *cis-trans*-acid alone. This point is an important one since Evans & Smith (1951) have shown that the *cis-cis*-acid behaves biologically in a different manner from the *cis-trans*-acid.

(Elvidge *et al.* (1950*b*) suggest that the *cis-cis*- and *cis-trans*-acids probably occur in extended *s-trans*-configuration (IIa and III). II is the *s-cis*-configuration of *cis-cis*-muconic acid showing its relationship to benzene, I.)

The present work confirms the findings of earlier workers that only *trans-trans*-muconic acid (IV) is found in the urine of rabbits receiving benzene (I). Furthermore, evidence has been presented to show that this isomer could not have arisen by isomerization in the urine of the *cis-cis*-isomer after excretion. It is also shown that if any *cis-cis*-muconic acid were excreted it could not have been destroyed, before estimations could be carried out, by micro-organisms infecting the urine. *trans-trans*-Muconic acid, therefore, appears to be a genuine metabolite of benzene and not an artifact. By our method of estimation the amount of benzene, at a dose level of 0.5 g./kg., excreted as this acid, is about 0.5% (range 0.15–1.0%) of the dose. It is thus a minor metabolite of benzene.

Recently Ravdin & Crandall (1951) have obtained a water-soluble enzyme system from rat liver which oxidatively cleaves homogentisic acid to 4-fumarylacetoacetate,

The configuration of the carbon-carbon double bond in this compound is *trans*, whereas the original configuration of this bond in the benzene ring of homogentisic acid is *cis*. Thus we have here another problem similar to that of the muconic acids.

Drummond & Finar (1938) concluded from their experiments that *cis-cis*-muconic acid was not isomerized to the *trans-trans*-acid in the animal organism. On injection of '*cis-cis*-muconic acid' they recovered by isolation in two experiments 47 and 45% of the acid unchanged. With injected *trans-trans*-muconic acid they recovered by isolation an average of 56% of doses of 800 mg., and similar results were obtained by Mori (1918) who recovered 55–60%. In most of these experiments the yields were based on crude materials and are therefore probably high. Our experiments on the injection of the three isomers in rabbits confirm the views of Drummond & Finar. Whichever isomer is injected, the same isomer is excreted (when allowance is made for the easy isomerization in aqueous solution of the *cis-cis*- into the *cis-trans*-acid). We found that at a

dose level of 100 mg./kg., 50–65% of the acids were excreted unchanged in about 24 hr. The metabolites of muconic acid are not known, but it seems likely that the acid undergoes β -oxidation in the usual way, for Hensel & Reisser (1913) observed that in the perfused liver, muconic acid (presumably the *trans-trans*-acid as this was the only isomer known in 1913) gives rise to acetone. According to Raizon & Yamamoto (1940) perfusion of dog liver with the *trans-trans*-acid leads to the production of acetone bodies, but the *cis-cis*-acid does not. Mori (1918), however, reports that the *trans-trans*-acid does not increase the output of oxalic acid in rabbits.

These results lead to the conclusion that *cis-cis*-muconic acid is not necessarily an intermediate in the metabolism of benzene in the animal body. Evans & Smith (1951), however, have suggested from simultaneous adaptation studies that *cis-cis*-muconic acid is an intermediate in the microbiological oxidation of phenol, catechol and benzoic acid to β -ketoadipic acid. Hayaishi & Hashimoto (1950) have isolated *cis-cis*-muconic acid (or more probably *cis-trans*- in view of the treatment the material received during the isolation) from the oxidation of catechol by an enzyme from *Pseudomonas* sp. Thus *cis-cis*-muconic acid may be an intermediate in the bacterial oxidation of phenol,

although its microbiological production from benzene itself has not yet been studied.

Pathways of metabolism of benzene to *trans-trans*-muconic acid without the intermediate formation of the *cis-cis*-acid can readily be postulated. Thus hydroxyquinol (V) which is a metabolite of benzene (Porteous & Williams, 1949b) could give rise to fumarylacetic acid (VII) in the same way as homogentisic acid gives rise to fumarylacetoacetic acid (Ravdin & Crandall, 1951), although one would expect maleoylacetic acid (VI) as an intermediate. Reduction followed by dehydration of fumarylacetic acid could then give rise to *trans-trans*-muconic acid (IV) thus:

There are obvious difficulties in this postulate, such as the conversion of maleoylacetic to fumarylacetic acid. Another possibility is the formation of $\beta\beta'$ -dihydroxyadipic acid (VIII) which might give the *trans-trans*-acid on dehydration. Dihydroxyadipic acid could arise from hydroxyquinol or 1:2:4:5-tetrahydroxybenzene (IX) (this last phenol has not been found as a metabolite of benzene), thus:

According to Stanier, Sleeper, Tsuchida & Macdonald (1950) hydroxyquinol is probably not an intermediate in the bacterial oxidation of phenol and other compounds to β -keto adipic acid. The latter is a common intermediate in the bacterial oxidation of several aromatic compounds, but we have never detected it in the urine of rabbits receiving benzene.

It is possible that the solution of the stereochemical problems involved in the formation of *trans-trans*-muconic acid from benzene in the intact animal and of fumarylacetoacetic acid from homogentisic acid in tissue extracts may lie in the chemistry of unsaturated hydroxy compounds which undergo ready isomerization.

SUMMARY

1. A colorimetric method for the determination of the three geometrical isomers of muconic acid has been studied. The method is based on the production of a red colour by heating muconic acid with phenol and concentrated sulphuric acid and then dissolving the product in absolute ethanol.

2. The same amount of colour is produced on heating the *cis-cis*- and *cis-trans*-acids at 100° for 6 hr. as on heating the *trans-trans*-acid at 160° for 20 min. By this method the *cis*-acids can be distinguished from the *trans-trans*-acid and estimated

when they occur separately. This method has also been applied to urine.

3. When the *cis*-acids and the *trans-trans*-acid occur together the same colorimetric method can be used for their estimation, if the *cis*-acids are first estimated in the mixture and then converted to the *trans-trans*-acid by ultraviolet light followed by an estimation of the total muconic acid as the *trans-trans*-isomer.

4. By this colorimetric method it has been shown that rabbits receiving an oral dose of benzene excrete about 0.5% of the dose as *trans-trans*-muconic acid, there being none of the *cis*-isomers in the urine. *trans-trans*-Muconic acid was isolated from the urine and characterized as its benzhydryl ester.

5. It has been shown that if *cis-cis*-muconic acid had been excreted after administration of benzene it could not have been destroyed by bacteria.

6. The three isomers of muconic acid have also been injected into rabbits and no evidence was found for their *in vivo* isomerization, 50–65% of unchanged acid being recovered in the urine.

7. It is concluded that *trans-trans*-muconic acid is probably a true metabolite of benzene and is not produced *in vivo* from the *cis-cis*-acid. It is suggested that the *cis-cis*-acid need not be an intermediate in the formation of the *trans-trans*-acid in the animal body.

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The Separation of Esters of Choline by Filter-paper Chromatography

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The technique of paper chromatography (Consden, Gordon & Martin, 1944) has been applied in recent years to the separation of numerous classes of substances. It has now been applied to esters of choline in connexion with work on the identification of choline esters in tissue extracts and on the mechanism of enzymic reactions involving choline esters. Preliminary accounts have been given by Whittaker (1951) and Whittaker & Wijesundera (1951).

METHODS

Chromatographic procedure

Whatman no. 4 filter paper was used throughout. For preliminary screening of solvents, tall inverted beakers or wide-mouthed bottles with screw caps were employed as containers. Solutions of esters in water (10-50 µg. in 10 µl.) were delivered, 3 cm. from the lower edge, on to a rectangle of filter paper of suitable dimensions by means of an 'Agla'

micropipette (Burroughs Wellcome Ltd.). The paper was rolled into a cylinder whose axis was parallel to the intended direction of solvent flow, and the apposed ends of the paper secured by wire staples. The cylinders were then placed upright in a pool of solvent at the bottom of the container and irrigated by capillary ascent, a 10 cm. run taking 2-4 hr. Promising solvents were then tried with longer runs, using both upward and downward irrigation in containers of conventional design.

Reading of chromatograms

Esters were rendered visible on the paper by three methods, one specific for carboxylic esters and certain other compounds containing an acyl linkage (e.g. lactones, acyl phosphates), the other two relatively non-specific.

Carboxylic reagent

The first method employed a reaction first used by Feigl, Anger & Frehden (1934, cf. Feigl, 1939) as the basis of a spot test, and later applied to the quantitative estimation of acyl phosphates (Lipmann, 1946) and acetylcholine (Hestrin, 1949). The reaction involves the conversion of the carboxylic ester into a hydroxamic acid with alkaline hydroxylamine, followed by the formation of a purple

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