XXI. THE MODE OF OXIDATION OF FATTY ACIDS WITH BRANCHED CHAINS III. THE FATE IN THE BODY OF α -METHYLCINNAMIC ACID, β -PHENYL-*ISO*-BUTYRIC ACID, AND γ -PHENYL-*ISO*-VALERIC ACID.

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THE work described in the present paper, is a continuation of that previously described [Kay and Raper, 1922].

The enquiry has been extended to phenyl derivatives of fatty acids with a methyl group attached to the α -carbon atom and with a straight chain of three or four carbon atoms.

Although the investigation was undertaken with the hope of finding out the method by which this α -methyl group was oxidised, it has in this particular been unsuccessful. No intermediate products of oxidation which might have thrown light on this process have been discovered, but in other directions, the results are not without interest.

The series of acids investigated up to the present behave on oxidation, in so far as the production of benzoic and phenylacetic acids is concerned, exactly like the similar series described in Knoop's classical experiments on β -oxidation, the only exception being hydratropic acid, which was expected to be, like phenylacetic acid, relatively resistant to attack in the body. Actually as described previously [Kay and Raper, 1922] it was oxidised to a considerable extent, but it did not yield either benzoic or phenylacetic acid as oxidation product.

The results are collected in Table I and with them, for reference, the results of the similar experiments by Knoop with the corresponding straight chain series.

Table I.

α -Methyl series		Straight chain series	
Acid administered	End product	Acid administered	End product
$C_6H_5 \cdot CH(CH_3) \cdot COOH$	Completely oxidised	$C_6H_5 \cdot CH_2 \cdot COOH$	Not oxidised
$C_6H_5 \cdot CH_2 \cdot CH(CH_3) \cdot COOH$	Benzoic acid	$C_6H_5 \cdot CH_2 \cdot CH_2 \cdot COOH$	Benzoic acid
$C_6H_5 \cdot CH : C(CH_3) \cdot COOH$	"	$C_6H_5 \cdot CH : CH \cdot COOH$	"
$C_6H_5 \cdot CH_2 \cdot CH_2 \cdot CH(CH_3) \cdot COOH$	Phenylacetic acid	$C_6H_5 \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot COOH$	Phenylacetic acid

The results on the whole, therefore, bear out the generalisation that acids with branched chains are oxidised in the same way as the parent acids with normal chains. In further support of this statement, another similarity between β -phenylpropionic acid and β -phenyl- α -methylpropionic acid was discovered in the present investigation. Dakin [1909] on administering the former acid to cats, was able to isolate cinnamic acid from the urine, as one of the products of oxidation. In similar conditions, as described in the experimental section, it has been possible to isolate α -methylcinnamic acid as one of the products of the oxidation of β -phenyl- α -methylpropionic acid. This, therefore, provides yet another example of the production of unsaturated from saturated acids in the body.

The results with phenylpropionic acid and phenylmethylpropionic acid just mentioned have another significance which is of interest in connection with the mechanism of oxidation of these acids. It is still unsettled whether direct attack of fatty acids at the β -carbon atom with the formation of β -keto-acids is preceded by the formation of an α - β -unsaturated acid or a β -hydroxy acid. Recent work by Dakin [1923] suggests that the three types of acid— β -keto, β -hydroxy and α - β unsaturated—are in equilibrium in the tissues and that neither the α - β -unsaturated acid nor the β -hydroxy acid can be considered as obligatory stages preceding the formation, by further oxidation, of the β -keto-acid.

The use of the quantitative method, in so far as the experiments will allow, appears to be the only fruitful one at present with which this kind of problem can be attacked, and in the present work, as far as possible, it has been followed.

Although not strictly accurate, because of the type of experiment which has to be used, the results obtained suggest that experiments of this kind are capable of quantitative interpretation where large differences are concerned. In two experiments for instance, in which α -methylcinnamic acid was fed to the same dog, yields of 30 % and 33 % of the expected benzoic acid were obtained. In another dog the yield was 27 %. These results show the order of accuracy that may be obtained. When β -phenyl- α -methylpropionic acid was administered to dogs, the yield of benzoic acid was 77 % of the expected amount.

The authors feel, therefore, that this result indicates clearly that the formation of α -methylcinnamic acid (although it was isolated as an intermediate product) is not an obligatory stage in the oxidation of β -phenyl- α -methylpropionic acid to benzoic acid, for if it were, the yield of benzoic acid, which these two acids give on oxidation in the body, should be approximately the same.

The low yield of benzoic acid from the unsaturated acid suggests that this does not undergo chiefly β -oxidation, but is oxidised with rupture of the aromatic nucleus.

Whether it is a function of the double bond in α -methylcinnamic acid

and other similar substances, to make the aromatic nucleus more susceptible to oxidation, must be left to further experiment.

One other point of interest in connection with α -methylcinnamic acid may be mentioned. As ordinarily prepared, two forms of the acid are obtained which were previously thought to be *cis* and *trans* isomers. According to Stoermer and Voht [1915] these two products are simply isomorphous forms. In solution, they would be expected therefore to show the same chemical behaviour and this was found to be so on submitting them to oxidation in the body. An almost identical yield of benzoic acid was obtained from each. Whether *cis* and *trans* isomers behave alike in the body, has not been determined but it would appear likely that some difference in their ease of oxidation might be expected. The biological evidence tends therefore to support the purely chemical evidence on the nature of these two products.

EXPERIMENTAL PART.

Methylcinnamic acid.

The acid used in these experiments, was prepared partly by Erdmann's method [1885] and partly by Edeleano's method [1887]. Both isomorphous forms of the acid were obtained, the higher melting form (M.P. 82°) was separated by recrystallisation from aqueous alcohol, and was obtained pure after two crystallisations of the crude product.

The mother liquor, containing a mixture of the two forms, was precipitated by evaporating to remove the alcohol and the acid thus obtained crystallised from light petroleum. It was obtained pure, in long prisms, after two recrystallisations and melted at 72°.

For convenience, the two forms are designated *A* (M.P. 82°) and *B* (M.P. 72°).

Form A. The acid was administered to a dog (11 kilos.), as the sodium salt by hypodermic injection, 3 g. of the acid being given in this way on each of two successive days. The urine was collected as usual, from the time of giving the first dose until 48 hours after the last dose had been given.

Distillation in steam of the urine collected during the experiment, did not yield any detectable ketones. The urine was concentrated and after acidifying strongly with syrupy phosphoric acid, was shaken out twice with ether and then extracted for 48 hours with the same solvent in a continuous extraction apparatus. The ether extract, obtained as a residue when the ether was removed by distillation, was extracted several times with warm light petroleum. On removal of the light petroleum, a small amount of acid remained as a crystalline residue. It was converted into the sodium salt and this dissolved in a small volume of water was precipitated with barium chloride. The precipitated barium salts were filtered off and washed with a few cc. of cold water. The acids both from the precipitate and the filtrate plus washings, were recovered by treatment with hydrochloric acid and extraction with ether.

The acid from the barium precipitate was crystallised from light petroleum and was obtained in needles, m.p. 72° ; it absorbed bromine and reduced permanganate in the cold and was obviously unchanged α -methylcinnamic acid, but in the *B* form: 0.1 g. was obtained. The acid obtained from the filtrate and washings from the barium precipitation was obtained as a crystalline residue from ether. It was recrystallised from light petroleum, and obtained in glistening plates, m.p. 120 – 121° . It sublimed on heating and gave a strong smell of ethyl benzoate on warming with ethyl alcohol and strong sulphuric acid, and was therefore benzoic acid: 0.1 g. was obtained.

The bulk of the ether extract obtained from the concentrated urine was insoluble in light petroleum. It was dissolved in hot water, subjected to steam distillation to remove volatile fatty acids, clarified with charcoal and concentrated to about 50 cc. and left to crystallise. 1.93 g. of slightly impure hippuric acid separated and this was obtained pure, m.p. 186 – 187° , after one crystallisation from hot water (yield 1.67 g.).

The mother liquor contained some substance which reduced permanganate at once in the cold and on warming with the same reagent gave a strong odour of benzaldehyde. The presence of some unchanged α -methylcinnamic acid as the glycine derivative was suspected, and the whole was therefore hydrolysed by adding enough sulphuric acid to make the concentration 20 % and boiling for two hours under a reflux condenser. The solution was extracted several times with light petroleum and the petroleum removed from the combined extracts by distillation. A semi-crystalline residue of acids remained. These were converted into sodium salts, the solution extracted with ether to remove any neutral substances present, and then precipitated with barium chloride, the precipitate being washed with small amounts of water. The barium precipitate and the filtrate plus washings, were treated separately to recover the acids they contained. In each case, hydrochloric acid was added and the solution shaken out several times with ether.

From the barium precipitate 0.12 g. of a crystalline acid was obtained which had all the properties of α -methylcinnamic acid. It crystallised from light petroleum in long needles, m.p. 71 – 72° , reduced permanganate at once in the cold and decolorised bromine water. On warming with permanganate, it gave a smell of benzaldehyde. The filtrate and washings from the barium precipitate yielded 0.18 g. of benzoic acid, m.p. 121° , which was crystallised from aqueous alcohol.

In this experiment, therefore, of 6 g. of α -methylcinnamic acid administered, 0.22 g. reappeared in the urine, partly free and partly combined (probably with glycine). The rest was oxidised and yielded 1.42 g. of benzoic acid which was excreted almost entirely as hippuric acid. This represents a yield of 30 % of the theoretical (4.35 g.) assuming that the whole of the acid given and not recovered in the urine had undergone β -oxidation to yield benzoic acid.

A second experiment on a dog (8 kilos.) in which 2 g. of the acid were

given on each of two successive days, yielded 1.03 g. of hippuric acid, 0.8 g. of benzoic acid, and 0.1 g. of unchanged acid. This was carried out before the method of isolating the unchanged acid present in combined form in the ether extract was worked out. Possibly a little more unchanged acid was present than the 0.1 g. which was isolated in the free state. The yield of benzoic acid in this experiment was 27 % of the theoretical.

Form B. The procedure used in this experiment was identical with that described in the first experiment with *Form A*. The same dog was used and the dosage was 6 g. of acid as before. The results were almost the same as those given by the acid in its higher melting isomorphous form. A small amount of the acid (0.15 g.) was excreted unchanged in the urine, some of this being free and some probably combined with glycine. The benzoic acid excreted, either in the free state or as hippuric acid, amounted to 1.45 g. This represents a yield of 33 % of the theoretical amount of benzoic acid expected if all the acid had undergone β -oxidation.

The good quantitative agreement between the results of the three experiments with α -methylcinnamic acid, when the methods of extraction and purification of the oxidation products are taken into account, suggests, that the greater part (upwards of 70 %) of the α -methylcinnamic acid, does not undergo β -oxidation to yield benzoic acid, but is oxidised in some other way, which involves rupture of the aromatic nucleus.

Phenyl-iso-butyric acid (β -Phenyl- α -methylpropionic acid).

The acid was prepared in two ways, (a) by the malonic ester method from benzyl chloride and methylmalonic ester, (b) by reduction with sodium amalgam of α -methylcinnamic acid. It was purified by distillation under reduced pressure, B.P. 155–156° (11 mm.).

Experiments were carried out with cats and dogs. The general method was the same with both. The acid was given hypodermically as the sodium salt and the urine collected from the first administration until 48 hours after the last dose had been given. The urine was distilled in steam and the distillate examined for ketones by the use of *p*-nitrophenylhydrazine. In no case were any detected although it was expected that acetophenone or propiophenone might be obtained. The urine was now concentrated, and after strongly acidifying with phosphoric acid, extracted with ether. The oxidation products were sought for in the ether extract.

Two experiments were carried out with dogs, and both gave the same result. The second one, carried out quantitatively, was as follows: 3.86 g. of the acid were administered to a dog of 7.5 kilos. It produced no ill effects. The residue from the ether extract of the urine was extracted with light petroleum and from it 0.3 g. of benzoic acid obtained (m.p. 121.5°). The portion of the ether extract which was insoluble in light petroleum was crystallised from hot water and yielded 2.82 g. of pure hippuric acid. No intermediate products of oxidation were detected. The yield of benzoic acid, free and

combined, in this experiment, was 77 % of the theoretical, assuming that β -oxidation alone had taken place. The later experiments were carried out with cats and a larger dose of acid per kilo. was given in order to induce the appearance of intermediate products of oxidation in the urine. This was successfully accomplished in one instance. When the dosage was greater than 1 g. per kilo. the animals died; but with a slightly smaller dose (0.9 g. per kilo.) they recovered after a period of lassitude lasting as a rule for 24 hours. The results of one successful experiment are described. A cat of 2.5 kilos. received two doses of the acid (2 g. and 1.8 g. respectively) a week intervening between the first dose and the second. The urines were distilled in steam in both instances but no ketones were obtained. The residue from the ether extract of the concentrated and acidified urines was repeatedly extracted with warm light petroleum and yielded a small quantity (0.18 g.) of an oily acid, which gave a barium salt soluble in water. It was probably uncharged phenyl-*iso*-butyric acid, but the amount obtained was too small for identification.

The acids from the ether extract which were insoluble in light petroleum were dissolved in hot water, distilled in steam to remove volatile fatty acids, and clarified with charcoal. On cooling, a considerable crop of hippuric acid was obtained. It was recrystallised from hot water and had m.p. 186° (yield, 0.35 g.). The mother liquor reduced permanganate immediately in the cold, suggesting that an unsaturated acid was present. To separate the acids contained in it, the following procedure was used. The mother liquor was neutralised with sodium hydroxide, evaporated to small bulk and precipitated with barium chloride. The precipitate after washing with a little cold water was decomposed with hydrochloric acid and the solution extracted with ether. A small amount of a syrupy acid was obtained which was insoluble in light petroleum but easily soluble in water. It did not reduce permanganate in the cold, but only on warming, when it gave a strong smell of benzaldehyde. This suggested the presence of either β -hydroxy- β -phenyl-*iso*-butyric acid or β -hydroxy- β -phenylpropionic acid, but the amount of acid obtained was too small for further investigation.

The filtrate from the barium precipitate was concentrated to about 5 cc. and then made strongly acid by adding 10 cc. concentrated hydrochloric acid. The solution was boiled under a reflux condenser for two hours to hydrolyse any glycine compounds, diluted and extracted with light petroleum. The extract on evaporation left a colourless, oily, acid residue, which was converted into the sodium salt and precipitated with barium chloride. The acid recovered from the precipitate was colourless and crystalline, and on recrystallising from light petroleum was obtained in needles m.p. 70–71°. 0.1 g. was obtained. It reduced permanganate immediately in the cold, giving a smell of benzaldehyde. After fusion the m.p. rose to 74–76°. This behaviour is characteristic of the form of α -methylcinnamic acid, which when quite pure, melts at 72°, and on melting is partially transformed into the higher

melting variety. A mixed melting point determination confirmed the identity, the two acids after fusing together gave the m.p. 75–77°. The acid recovered from the filtrate of the barium precipitate was small in amount (0.15 g.); it was a viscous liquid, soluble in light petroleum, and was optically inactive. It contained benzoic acid, but the amount was too small to be separated from the liquid component of the mixture. It was therefore not further investigated.

Phenyl-iso-valeric Acid (α -Methyl- γ -phenylbutyric Acid).

This acid was prepared by the action of phenylethyl chloride on the sodium compound of methylmalonic ester and distillation of the malonic acid derivative. The chief difficulty met with was the preparation of a pure phenylethyl halide. After two unsuccessful attempts by Fittig's method (the chlorination of ethylbenzene, which gives a mixture of chlorides, of which the fraction boiling at 86–87° (21 mm.) contains at least two, probably $C_6H_5 \cdot CH_2 \cdot CH_2Cl$ and $C_6H_5 \cdot CHCl \cdot CH_3$), and an equally unsuccessful attempt by the addition in the cold of hydrobromic acid to freshly distilled styrene (which gives a mixture of the corresponding bromides), Von Braun's method [1911] was employed and a pure ω -chloride was thus obtained. The phenyl-iso-valeric acid obtained by the malonic ester method was purified by distillation under reduced pressure and boiled at 169–172° (15 mm.). It was a colourless liquid and did not solidify on standing for several weeks at room temperature. Equivalent, 176; calculated 178. On analysis it gave C, 73.58 %; H, 7.59 %. Calculated, C, 74.11 %; H, 7.91 %. The acid was administered hypodermically as the sodium salt to a cat (2.5 kilos.) on two occasions, 1.2 and 1.5 g. of the acid being given respectively. No ill effects were noted. The urine was collected for 48 hours following each injection, the two portions united, concentrated on the water-bath, and acidified with phosphoric acid. It was extracted with ether containing 10 % alcohol for 48 hours continuously. The residue from the ether extract was purified by distillation in steam to remove volatile acids, clarified with charcoal and evaporated to small bulk. On standing, 0.47 g. of phenaceturic acid crystallised out. On crystallisation from hot water it gave m.p. 143°. A portion hydrolysed with hydrochloric acid, yielded phenylacetic acid, m.p. 77°.

The mother liquor from the phenaceturic acid yielded a further crop of this acid on evaporation, but it was contaminated with a small amount of oily substance. The mother liquor also reduced permanganate in the cold, indicating the presence of unsaturated acids. To isolate the phenylacetic acid, the whole of this crop of crystals and the mother liquor were boiled with strong hydrochloric acid for two hours and the phenylacetic acid extracted with benzene. It was purified by conversion into the lead salt which was crystallised from warm acetone. The acid liberated from the purified lead salt melted at 77°; 0.54 g. was obtained. A search for oxidation products other than phenylacetic acid was fruitless.

The total amount of phenylacetic acid isolated either free or combined

with glycine was thus 0.87 g. or 42 % of the amount to be expected if β -oxidation alone had taken place. The method of isolation was not strictly quantitative but the results suggest that probably not more than half of the phenyl-*iso*-valeric acid yields phenylacetic acid.

SUMMARY.

(1) β -Phenyl-*iso*-butyric acid on oxidation in the body yielded benzoic acid. The yield was 77 % of the theoretical, assuming that β -oxidation alone had taken place. α -Methylcinnamic acid was isolated as an intermediate product of the oxidation.

(2) α -Methylcinnamic acid also yielded benzoic acid on oxidation but no intermediate products of oxidation were isolated. The yield of benzoic acid was only 33 % of that expected if β -oxidation alone had taken place. This suggests that oxidation involving rupture of the aromatic nucleus takes place to a considerable extent.

(3) No difference was discovered in the ease of oxidation in the body of the two isomorphous forms of α -methylcinnamic acid.

(4) γ -Phenyl-*iso*-valeric acid gave rise to phenylacetic acid on oxidation in the body. The yield of this was not more than 50 % of the amount expected if β -oxidation alone had taken place.

(5) The results in general indicate that fatty acids with an α -methyl group undergo oxidation to yield the same products as the parent acids with a straight chain of carbon atoms. No evidence has been obtained which shows whether the α -methyl group is primarily attacked or is removed along with the two end carbon atoms, as in the scheme of β -oxidation suggested by Knoop.

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