

FURTHER STUDIES OF THE MODE OF OXIDATION OF PHENYL DERIVATIVES OF FATTY ACIDS IN THE ANIMAL ORGANISM.

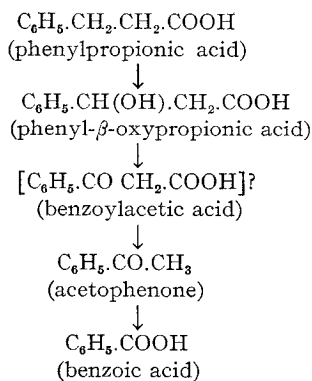
(PHENYLBUTYRIC ACID, PHENYL- β -OXYBUTYRIC ACID, PHENYLACE-
TONE, PHENYLISOCROTONIC ACID, PHENYL- β,γ -DIOXY-
BUTYRIC ACID.)

BY H. D. DAKIN.

(From the Laboratory of Dr. C. A. Herter, New York.)

(Received for publication, August 12, 1908.)

In previous papers¹ it was shown that phenylpropionic acid, at least in part, underwent oxidation in the animal organism in accordance with the following scheme:



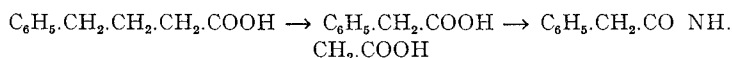
Benzoylactic acid was not detected but its formation was inferred from the production of acetophenone, into which it readily passes through loss of carbon dioxide. At the same time it was thought probable that some benzoic acid was formed without passing through the stage of acetophenone.

The close analogy between the apparent mode of catabolism of phenylpropionic acid and that of butyric acid made it desirable

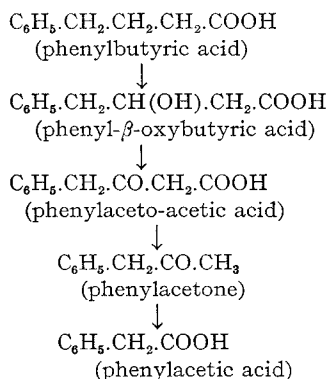
¹ This *Journal*, iv, p. 419, 1908; *Beitr. z. chem. Physiol. u. Pathol.*, xi, p. 404.

to extend the investigation to other aromatic derivatives of the fatty acids in the hope of obtaining further insight into the processes of tissue oxidation of the fatty acids of physiological importance. The present communication deals with the fate in the body of a number of derivatives of phenylbutyric acid.

The fate of phenylbutyric acid in the organism has already been investigated by F. Knoop¹ who administered it by mouth to a dog and observed the subsequent excretion of phenaceturic acid in the urine. This result was interpreted in the light of Knoop's well-known hypothesis of β -oxidation and indeed was one of the most important facts upon which his theory was based. The conversion of phenylbutyric acid into phenaceturic acid involves the intermediate formation of phenylacetic acid which is then paired with glycocoll:



It is clearly desirable to ascertain the intermediate steps in the conversion of phenylbutyric acid into phenylacetic acid. So far as I am aware no picture of the mechanism of the reaction has hitherto been put forward. In order to elucidate this mechanism it was natural to inquire first of all whether the catabolism of phenylbutyric acid did not follow upon the same line as that of phenylpropionic acid. Judging by analogy one might expect the change to be as follows:



¹ *Beitr. z. chem. Physiol. u. Pathol.*, vi, p. 155, 1904.

The occurrence of the first step in this series of changes, namely, the formation of phenyl- β -oxybutyric acid, is highly probable, as is shown by the following facts:

(1) A small quantity of a lævorotatory substance giving the reactions of phenyl- β -oxybutyric acid was isolated from the urine of dogs that had received subcutaneous injections of sodium phenylbutyrate in fairly large doses. The β -oxy-acid could not, however, be isolated in a state of purity.

(2) Phenyl- β -oxybutyric acid injected in the form of its sodium salt was excreted in the form of phenaceturic acid, i. e., the same end-product as phenylbutyric acid itself yields.

There was little hope of isolating the substance corresponding to the second hypothetical step in the reaction, namely, phenyl-aceto-acetic acid, for this body has not yet been synthesized and would doubtless be unstable. It is improbable, however, that it was present in the urines of the dogs which had received injections of sodium phenylbutyrate, because in this case phenylacetone would have been found in the distillates, for a β -ketonic acid of the type of phenylaceto-acetic acid doubtless would lose carbon dioxide on boiling, with formation of the corresponding ketone.

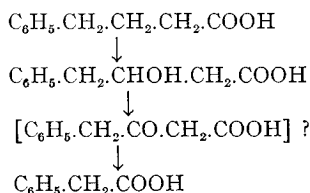
The third possible stage in the catabolism of phenylbutyric acid involving the formation of phenylacetone, corresponding to the intermediate production of acetophenone from phenyl-propionic acid, was definitely excluded on the basis of the following experimental results:

(1) No trace of phenylacetone could be detected in the urines of animals which had received injections of considerable quantities of phenylbutyric acid.

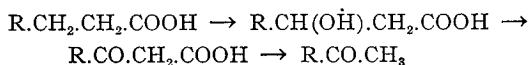
(2) Phenylacetone cannot be an intermediate stage in the catabolism of phenylbutyric acid for when administered to dogs it results in the excretion of *hippuric acid*. Phenylbutyric acid under similar conditions gives *phenaceturic acid*.

To sum up: Evidence has been obtained that phenylbutyric acid when oxidized in the body passes through the stage of phenyl- β -oxybutyric acid. No evidence could be obtained of the formation of phenylaceto-acetic acid and it could not be detected in the urine. The possibility of its formation as an intermediate product is not, however, excluded. Phenylacetone is certainly *not* a product of the catabolism of phenylbutyric acid.

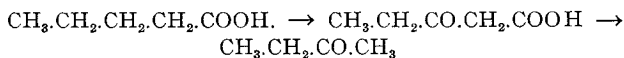
It is clear, therefore, that the modes of catabolism of phenylpropionic acid and of phenylbutyric acid, though similar as regards the primary formation of a β -oxy-acid, differ in that ketone formation takes place in the case of phenylpropionic acid but not in that of phenylbutyric acid. The catabolism of phenylbutyric acid is therefore to be represented as follows:



The mechanism of the catabolism of phenylbutyric acid appears to be of interest from several points of view. In the first place it furnishes an additional example of the primary oxidation of the hydrogen attached to the β -carbon atom of a phenyl-fatty acid with formation of a laevorotatory β -oxy-acid. In the second place it indicates the possibility, which has been insisted on in previous papers, of a β -oxy or β -ketonic acid undergoing oxidation without intermediate ketone formation.¹ *Indeed it would appear as if ketone formation were restricted to the simplest members of the fatty acids and phenyl-fatty acids, theoretically capable of ketone formation, namely, butyric acid and phenylpropionic acid:*

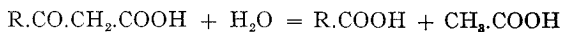


This hypothesis furnishes an explanation of why under conditions such as diabetes in which large quantities of ketones are excreted only acetone has been detected. If the above scheme represented a perfectly general type of reaction, it would be hard to explain why in diabetes the catabolism of acids such as caproic and valeric do not give rise to the excretion of propylmethyl ketone or ethylmethyl ketone respectively:



¹ Unpublished experiments upon the mode of catabolism of phenylvaleric and phenyl- β -oxyvaleric acid indicate that the substances resemble phenylbutyric acid in this respect.

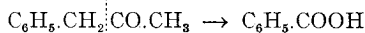
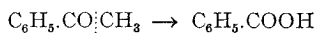
It is at present impossible to decide what rôle, if any, the β -ketonic acids, other than aceto-acetic acid play in intermediary metabolism. Apart from aceto-acetic acid and benzoylacetic acid few if any of these acids of a type which might be formed in metabolism, have been prepared. It may be that they are extremely unstable or even incapable of more than momentary existence. It is well, however, to bear in mind that not only are β -ketonic acids capable of undergoing hydrolysis according to the well-known scheme



a reaction which fits in well with the progressive degradation of long-chain fatty acids by the loss of two carbon atoms at a time, but also that they are extremely easily oxidizable substances.¹

If the normal course of metabolism of a straight-chain fatty acid other than butyric acid and phenylpropionic acid, proceeds through the β -oxy-acids and possibly the β -ketonic acids, but does not involve ketone formation, is it not probable that the catabolism of butyric and phenylpropionic acid in part does not involve the intermediate formation of acetone and acetophenone respectively? There is a certain amount of physiological evidence in support of this view, and so far as I know, there is none opposed to it. The results of oxidation experiments *in vitro* furnish complete chemical analogy for these reactions.²

The excretion of hippuric acid following the administration of phenylacetone is of interest especially when it is considered that its lower homologue, acetophenone, also yields hippuric acid. These changes are similar to the action of ordinary oxidizing agents (including hydrogen peroxide) which oxidize both ketones to benzoic acid. It will probably be found that most aromatic methyl ketones primarily undergo oxidation in the body, so as to yield acids with two less carbon atoms, except in the case of acetophenone, in which the carbonyl group is directly attached to the nucleus:

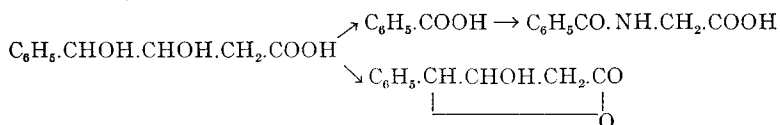


¹ A communication upon this subject will be made shortly.

² This *Journal*, iv, p. 77.

The fate of phenylisocrotonic acid was determined by injecting solutions of the sodium salt (=2.0 grams of acid) subcutaneously into cats. The fate of this substance had already been determined by Knoop¹ who fed it by mouth to a dog and found that it was excreted in the urine in the form of phenaceturic acid. The explanation of this rather remarkable result might conceivably depend upon reduction in the intestinal canal. That this was not the case was proved by obtaining the same end-product when the acid was subcutaneously injected.

The fate in the organism of another derivative of phenylbutyric acid, phenyl- β,γ -dioxibutyric acid was also determined. This acid is certainly not an intermediary product of the catabolism of phenylbutyric acid since on oxidation in the animal body hippuric acid together with a little phenyl- β -oxybutyrolactone produced by the removal of the elements of water from the original substance, were isolated from the urine.



Whether the lactone was excreted as such or formed from unchanged acid during the analysis is not clear. Lactone formation is very easily brought about by merely boiling with water or dilute acid and the lactones of aromatic acids as a class appear to be rather resistant to animal oxidation..

The formation of hippuric acid from phenyldioxybutyric acid is of special interest since it is an example of tissue oxidation taking place at the γ -carbon atom. So far as I am aware it is the only example of its kind so far recorded. Judging by analogy with some other tissue oxidations,² one might anticipate the formation of mandelic acid according to the following scheme:



No definite indication of the presence of this acid could be obtained, however.

¹ *Loc. cit.*, p. 159.

² E.g., phenyl- β -oxypropionic acid and phenyl- β -oxybutyric acid.

EXPERIMENTAL PART.

Preparation of phenylbutyric acid. Phenylbutyric acid has been synthesized by several methods, the most direct of these being the reduction of phenylisocrotonic acid with sodium amalgam as described by Fittig and Jayne.¹ The reduction, however, is not very easily carried out as was found by Jayne and by Knoop and also in experiments of my own. Kipping and Hill,² on the other hand, apparently had no difficulty in effecting the reduction at the ordinary temperature. On the whole, it was found more advantageous to employ the following method, mainly, based on the investigations of Fittig and his pupils. Phenylparaconic acid, prepared by means of Perkin's reaction from benzaldehyde, sodium succinate and acetic anhydride,³ is distilled *in vacuo*. The distillate, consisting mainly of phenylisocrotonic acid with a little phenylbutyrolactone, is boiled with twenty-five parts of hydrochloric acid (1 part concentrated acid, 3 parts water by volume) under a reflux condenser, for six hours. The result of this procedure is to convert about 65 per cent of the phenylisocrotonic acid into phenylbutyrolactone.⁴ The acid is separated from the lactone by adding sodium carbonate till faintly alkaline to the ethereal extract containing the two substances.⁵ The acid remaining in the aqueous layer is again treated with hydrochloric acid. In this way an 80 per cent yield of phenylbutyrolactone is readily obtained. The phenylbutyrolactone is reduced to phenylbutyric acid according to Shield's method⁶ by boiling it with ten parts of hydriodic acid (b. p. 127°) and 1.5 parts of red phosphorus for ten hours. The phenylbutyric acid is obtained by ether extraction after previous dilution with water and after removing the ether by evaporation readily crystallizes. The method gives an excellent yield. Twenty-five grams of phenylparaconic acid gave on the average

¹ *Ann. d. Chem.*, ccxvi, p. 108.

² *Trans. Chem. Soc.*, lxxv, p. 147.

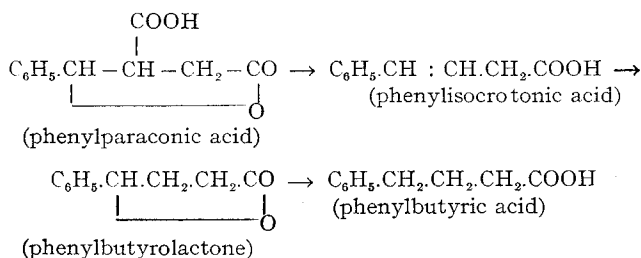
³ *Ann. d. Chem.*, ccxvi, p. 100.

⁴ Fittig and Hadorff: *Ibid.*, cccxxxiv, p. 117

⁵ This method of converting phenylisocrotonic acid into phenylbutyrolactone was found far superior to that of Erdmann who employed 33 per cent sulphuric acid. *Ibid.*, ccxxviii, p. 178.

⁶ *Ibid.*, cclxxxviii, p. 207

12.5 grams of crude phenylbutyric acid, melting at about 46° to 49°. After a single recrystallization it melts at 49° to 50°. The synthesis may be represented as follows:



Fate of phenylbutyric acid in the organism. Phenylbutyric acid in amounts varying from 5 to 6 grams was converted into the sodium salt and injected in aqueous solution subcutaneously into a small dog weighing about 6 kilos. The site of injection was usually the loose tissue at the back of the animal's neck and no ill effects followed the injection. The urine passed during the next three days was collected and analyzed. It was first distilled in order to test for the presence of phenylacetone (and indirectly phenylaceto-acetic acid). In no case could any indications of this substance be obtained. The distillates were examined with the aid of paranitrophenylhydrazine,¹ with the sodium nitroprusside reaction and with the iodoform test. On one occasion a minimal iodoform reaction was obtained but it was not due to an aromatic ketone nor was the amount more than a negligible trace. A portion of the urine was tested with ferric chloride but no color reaction was obtained, such as a β -ketonic acid would be expected to yield. Phenylacetone and phenylaceto-acetic acid were therefore absent.

The urine after distillation was concentrated, acidified with phosphoric acid and extracted with ether in a continuous extractor for thirty-six hours. The ether residue was distilled in steam and the aqueous solution decolorized with charcoal, con-

¹ Phenylacetone forms a beautifully crystalline paranitrophenylhydrazine melting after crystallization from alcohol or pyridin at 145° to 145.5°. It usually crystallizes in rosettes of platelets which are only moderately soluble, even in hot alcohol. The substance serves well for the identification of phenylacetone.

centrated and allowed to crystallize. A little over 2 grams of well crystallized phenaceturic acid were obtained in this way. The substance crystallized in well formed platelets and melted after a single recrystallization at 142° to 143° . The mother liquors from the phenaceturic acid were examined in the polarimeter and found to be decidedly laevorotatory (0.35° to 0.51°). The optically active substance was found to be insoluble in benzene but readily soluble in chloroform, in this respect agreeing with the known properties of phenyl- β -oxybutyric acid. This substance, however, could not be isolated in a state of purity. Its presence was made almost certain, however, by the following reactions: (1) Part of the solution was neutralized with ammonia and distilled with hydrogen peroxide in the same way as was employed for the detection of phenyloxypropionic acid.¹ On successively redistilling the distillate with ammoniacal silver solution to remove aldehydes and then with phosphoric acid, a liquid was obtained with a strong aromatic smell, similar to phenylacetone and the solution gave a strong iodoform reaction and gave with sodium nitroprusside deep red coloration in both alkaline and acid solution, identical with those obtained by using pure phenylacetone. With paranitrophenylhydrazine in acetic acid solution a yellow precipitate was obtained. The amount was too small for complete purification. (2) On treating another portion of the solution with a little sodium carbonate and then adding a little strong, cold potassium permanganate solution, a strong odor of an aromatic aldehyde similar to phenylacetaldehyde was at once obtained.

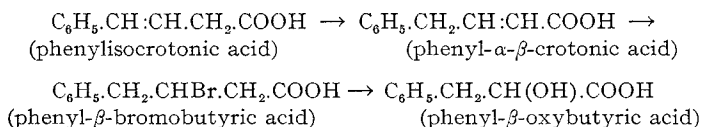
These reactions, combined with the observed laevorotation of the solution make it extremely probable that phenyl- β -oxybutyric acid was present. However, until the substance can be isolated in the pure state, complete proof must be considered lacking.

Preparation of phenyl- β -oxybutyric acid. Great difficulty was experienced in obtaining this acid. A small quantity was eventually obtained by a modification of Fittig and Luib's methods.² Phenylisocrotonic acid (40 grams) was boiled with 10 mol. parts of 10 per cent caustic soda solution. The solution was then

¹ This *Journal*, iv, p. 430.

² *Ann. d. Chem.*, cclxxxiii, p. 302.

acidified and extracted with ether. The ethereal residue dissolved almost completely in carbon bisulphide so that the quantity of oxy-acid formed must have been very small. The residual acids consisting of a mixture of phenylisocrotonic acid and phenyl- α,β -crotonic acid were then crystallized from water which removed the bulk of the former acid. The more soluble acid remaining in solution was then extracted with ether. On evaporation of the ether the residue, without further purification, was allowed to stand for three days with 4 parts of glacial acetic acid saturated with hydrobromic acid gas. On pouring the solution into water an oil separated which was boiled for three hours with 15 parts of water to which a little sodium acetate had been added. After cooling, the aqueous portion was filtered off and extracted with ether. The ethereal residue readily crystallized and was purified by washing with carbon bisulphide. The yield of pure acid was only about 3 per cent.¹ The changes may be represented as follows:



Fate of phenyl- β -oxybutyric acid in the organism. One gram of the acid was converted into the sodium salt and injected subcutaneously into a cat weighing about 2.5 kilos. The urine during the next three days was carefully collected and analyzed exactly as in the case of phenylbutyric acid. No phenylacetone or phenylaceto-acetic acid could be detected. Four-tenths of a gram of phenaceturic acid was obtained in the form of crystals which melted at 142° after a single recrystallization. A very small amount of unchanged substance appeared to be present, for the mother liquor from the phenaceturic acid was feebly lævorotatory ($-\text{o.10}^\circ$) and faint indications were also obtained of its presence by oxidation with hydrogen peroxide and with potassium permanganate.

¹ The acid obtained crystallized in platelets and melted at 98° to 100° . I have been unable to find any record of the melting point of this acid which was obtained by Fittig and Luib.

Fate of phenylacetone in the organism. Phenylacetone (Kahlbaum) was injected subcutaneously into dogs in dilute alcoholic solution in doses varying from 3 to 4 grams. The absorption of the ketone appeared to be slow and in one case comparatively little hippuric acid was obtained (0.3 gram). In all the other cases an abundant yield of hippuric acid was obtained—in one case as much as 3.0 grams being recovered. A small amount of unchanged ketone was excreted in the urine. The hippuric acid after recrystallization, melted sharply at 187° .

Fate of phenylisocrotonic acid in the organism. Phenylisocrotonic acid was prepared by distilling phenylparaconic acid *in vacuo* and crystallizing the distillate from carbon bisulphide. Two grams of the acid were dissolved in alcohol and almost neutralized with caustic soda. The solution was injected subcutaneously into a cat (two kilos). The urine was collected for three days. It contained a trace of acetone but no aromatic ketone. The urine was concentrated to about 100 cc., acidified with phosphoric acid and extracted in a continuous extractor with ethyl acetate. After purifying the ethyl acetate extract by steam distillation and by boiling the aqueous solution with charcoal, 0.65 gram of pure phenaceturic acid, melting at 142° , was obtained. No hippuric acid could be detected.

Fate of phenyl- β , γ -dioxymyric acid. This acid was prepared by Fittig and Obermüller's method¹ by oxidizing phenylisocrotonic acid in dilute alkaline solution at 0° with dilute potassium permanganate. The product obtained was a mixture of the free acid and lactone and was converted into the sodium salt by boiling with excess of caustic soda, neutralizing with acetic acid and injecting the dilute solution subcutaneously into a cat of about 2.5 kilos weight. One and a quarter grams of the acid in the form of sodium salt gave about 0.45 gram of pure crystalline hippuric acid (m.p. 185° to 187°) and about 0.2 gram of phenyl- β -oxybutyrolactone. The lactone was separated from the hippuric acid as follows: The urine was concentrated, acidified and extracted with ether in the usual way. The ethereal residue was distilled in steam for a very short time only, then decolorized with charcoal and concentrated to about 5 cc. The

¹ Liebig's *Ann. d. Chem.*, cclxviii, p. 44.

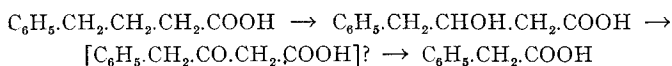
solution was then made just alkaline with sodium carbonate solution and extracted with ether to remove the lactone. The alkaline residue was acidified and again repeatedly extracted with ether. On evaporation, hippuric acid crystals were obtained in abundance. The aqueous solution of the ethereal extract was optically inactive and no positive indication of the presence of mandelic acid could be obtained.

SUMMARY.

The subcutaneous injection of phenylbutyric acid in the form of its sodium salt in aqueous solution results in the excretion of phenaceturic acid as found by Knoop. In addition a small quantity of a laevorotatory acid possessing the properties of phenyl- β -oxybutyric acid was excreted. No phenylacetone was excreted and phenylaceto-acetic acid could not be detected.

Phenyl- β -oxybutyric acid administered under similar conditions results in the excretion of phenaceturic acid. No phenylacetone could be detected. A part of the oxy-acid is apparently excreted unchanged and is laevorotatory.

Administration of phenylacetone results in the excretion of hippuric acid, no phenaceturic acid being formed. Phenylacetone cannot therefore be an intermediate product of the catabolism of phenylbutyric acid. The probable mode of oxidation of phenylbutyric acid in the body may be represented as follows:



The phenylacetic acid is excreted in the form of phenaceturic acid.

A comparison is made between the mode of oxidation of phenylbutyric acid and that of phenylpropionic acid. The first step in the catabolism of both acids apparently consists in the formation of a β -oxy-acid but in the case of phenylbutyric acid there is no formation of the corresponding ketone as a product of further oxidation. The intermediate formation of ketones observed in the catabolism of butyric and phenylpropionic acids is probably confined to these two acids and is not a general reaction.

These results are in harmony with the view that in normal metabolism probably only part of the butyric acid and phenylpropionic acid undergoing oxidation passes through the stages of acetone and acetophenone respectively.

Phenylisocrotonic acid administered subcutaneously to cats in the form of its sodium salt is excreted in the form of phenaceturic acid.

Phenyl- β,γ -dioxymybutyric acid administered to cats in the form of its sodium salt resulted in the excretion of hippuric acid together with a little phenyl- β -oxybutyrolactone. No indications could be obtained of the formation of mandelic acid. Phenyl-dioxymybutyric acid therefore does not undergo β -oxidation but oxidation takes place at the γ -carbon atom. Phenyl-dioxymybutyric acid is not a product of the catabolism of phenylbutyric acid.

Phenylacetone is readily identified by conversion into its par-nitrophenylhydrazone which crystallizes from alcohol or pyridin in sparingly soluble rosettes of platelets melting at 145° to 145.5° .

Note added during proof correction. The investigation of the fate of phenylvaleric and phenyl- β -oxyvaleric acid has shown that while the end product of catabolism in both cases is hippuric acid, *cinnamylglycocol*, $C_6H_5.CH:CH.CO.NH.CH_2.COOH$ m. p., 193° , is an intermediate product of their catabolism. This substance is also produced in the oxidation of phenylpropionic acid in the animal body. These observations throw considerable light upon the mechanism of fatty acid metabolism and will form the subject of a separate communication.