XXXVII. THE MODE OF OXIDATION OF FATTY ACIDS WITH BRANCHED CHAINS. II. THE FATE IN THE BODY OF HYDRATROPIC, TROPIC, ATROLACTIC AND ATROPIC ACIDS TOGETHER WITH PHENYLACETALDEHYDE.

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In a previous communication, Raper [1914] advanced an hypothesis to explain the mode of oxidation in the body of fatty acids substituted at the α -carbon atom by a methyl group. The experimental evidence available at that time indicated that these acids underwent oxidation in the same way as the straight chain fatty acids from which they were derived. It was suggested that, this behaviour might be accounted for if oxidation of the α -methyl group took place first, giving rise to a derivative of the semi-aldehyde of malonic acid, which being very unstable would lose carbon dioxide to produce a normal fatty aldehyde. This aldehyde, in turn, would give rise to its corresponding fatty acid by further oxidation. The series of reactions would thus be as follows:

In this way an α -methylated acid would give rise to a fatty acid with a straight chain structure but containing one carbon atom less. In support of this hypothesis it was shown that isobutyric acid and α -methylbutyric acid, on oxidation with hydrogen peroxide, yielded propaldehyde and normal butaldehyde respectively.

Owing to the relative scarcity of data concerning the fate of branched chain fatty acids in the animal organism it was decided to investigate the fate of phenyl derivatives of the α -methylated fatty acids. The present communication is concerned with the simplest member of this series, namely, hydratropic acid (α -phenylpropionic acid), and some of its derivatives. Experiments with other members of the α -substituted series are in progress and will be communicated in due course. The results have been disappointing so far as the isolation of intermediate products of oxidation is concerned but the fate of the various acids investigated, (I) to (IV), has enabled conclusions to

be drawn which indicate the probable manner in which hydratropic acid is oxidised.

Hydratropic acid (I) administered to dogs produced slight toxic effects in doses of 0.25 g. per kilo and was oxidised to the extent of about two-thirds. The remaining third was excreted in the urine, partly combined with glycine, and was dextrorotatory. A resolution of hydratropic acid, not yet complete, has shown that this was not the pure dextro form but a mixture of the dextro and laevo modifications in which the dextro component was in excess. No intermediate products of oxidation were detected. Inactive tropic acid (II) was found to be very resistant to oxidation, over 90 % of the acid given being recovered from the urine. The acid excreted, as expected, was optically inactive. Inactive atrolactic acid (III) was also very resistant to oxidation and over 80 % of this acid was excreted unchanged. It also was optically inactive. Atropic acid (IV), on the other hand, was found to be the most toxic of the four acids investigated, but in doses of 0.13 g. per kilo, which were tolerated, it was completely oxidised. No intermediate products were detected in the urine but a small but definite trace of succinic acid was excreted on two occasions and was not found on careful examination of the normal urine of the same dog. It is difficult however to connect this directly with the oxidation of atropic acid.

It is of great general interest to find that tropic acid is much more resistant to oxidation in the body than hydratropic acid, a result which the greater *in vitro* oxidisability of the hydroxylated acids (when acted on by the usual oxidising agents) would hardly lead one to expect. Dakin [1909] has observed the same phenomenon with β -phenylpropionic acid and β -phenyl- β -hydroxypropionic acid. The former is much more easily oxidised than the latter. These results show that the introduction of a hydroxyl group in the β -position in phenyl derivatives of propionic acid does not facilitate their oxidation in the body but rather hinders it. The fact that tropic and atrolactic acids are much less easily oxidised than hydratropic acid excludes them as possible intermediate products in its oxidation. It is also very improbable that hydratropic acid undergoes α -oxidation since either atrolactic acid, phenylglyoxylic acid, mandelic acid or benzoic acid would be expected as intermediate products. A careful search failed to reveal any of these, so that it may be assumed that hydratropic acid is oxidised in the β -position. Further, since the methyl group containing the β -carbon atom is unsubstituted both in atrolactic and hydratropic acids, it would be expected, that the former would be oxidised to a greater extent than was found to be the case. This makes it probable that some other factor besides the non-substitution of the

methyl group is important in facilitating the oxidation of hydratropic acid. We believe this factor to be the ability to form an unsaturated linkage between the α - and β -carbon atoms by the loss of two atoms of hydrogen. This is not possible in atrolactic acid but it is in hydratropic acid and would result in the formation of atropic acid. Atropic acid is not resistant to oxidation and although it may be urged that it is more toxic than hydratropic acid and therefore is not a likely intermediate product in the oxidation of the latter, it has to be borne in mind that the toxicity of a substance when produced slowly by metabolic processes in the tissues may be much less marked than when a comparatively large dose is introduced quickly by subcutaneous injection. Hydratropic acid, also, is distinctly more toxic than atrolactic and tropic acids. In the first case, doses of 0.25 g. per kilo give toxic effects whereas 1 g. per kilo of either of the other two acids gives no toxic symptoms. For these reasons therefore we are led to believe that atropic acid is the most likely primary oxidation product of hydratropic acid. It follows from this that the change from hydratropic acid to atropic acid is brought about by the direct oxidative removal of two hydrogen atoms and not by the loss of water from either tropic or atrolactic acids, since these are apparently not capable of conversion to atropic acid in the body. If they were they would be oxidised to a greater extent than was found to be the case. It is also possible that atropic acid may be produced from hydratropic acid by a process of dehydrogenation as pictured by Wieland [1912].

As to the further stages in the oxidation of hydratropic and atropic acids we can only speculate. From analogy with cinnamic acid β -oxidation might be expected with the formation of formylphenylacetic acid (V):

This would be unstable and lose carbon dioxide to give phenylacetaldehyde, or by further oxidation it might give phenylmalonic acid. The fate of the latter substance in the body is not known, but Dakin [1909] has described experiments in which phenylacetaldehyde in dilute alcoholic solution was given subcutaneously to dogs. About 84 % was completely oxidised and the remaining 16 % converted into phenylacetic acid which was excreted in the urine as phenaceturic acid. We have repeated and confirmed Dakin's experiment and found that by oral administration also a considerable part of the phenylacetaldehyde administered undergoes complete oxidation. Since neither hydratropic nor atropic acid gave rise to the excretion of phenylacetic acid it is not certain that phenylacetaldehyde represents an intermediate stage in their oxidation, although it would be expected to be such if the original hypothesis put forward previously [Raper, 1914] were correct. It is however not improbable that phenylacetaldehyde produced *in vivo* may undergo complete oxidation in spite of the fact that when introduced orally or subcutaneously it is partially oxidised to phenylacetic acid.

In a recent communication, Hanke and Koessler [1922] have called attention to the phenomena of the oxidation of fatty acids in the body in relation to their electronic structure. The arguments put forward in the case of butyric acid, for which the following electronic structure is developed, will serve as an example:

In this formula the α - and γ -carbon atoms are represented as quadruply negative and the carboxylic carbon atom as quadruply positive, whereas the β -carbon atom is doubly positive. Evidence is brought forward by Hanke and Koessler which indicates that quadruply positive or negative carbon, and especially the former, is resistant to oxidation. This being so, it would be expected that butyric acid when oxidised would be attacked most readily at the β -carbon atom which is only doubly positive. It is therefore suggested that in this way the predominance of β -oxidation of fatty acids in the body may be explained. On our part, we feel that there is, as yet, insufficient knowledge, either qualitative or quantitative, and especially the latter, of the mechanism of oxidation processes on which an explanation in relation to their electronic structure can be based. The idea that β -oxidation is predominant is supported essentially by qualitative evidence for there is little of a quantitative nature. There are no animal experiments on record which enable one to postulate that butyric acid is oxidised only or chiefly in the β -position. Further, Cahen and Hurtley [1917] have shown that when butyric acid is oxidised with hydrogen peroxide, a process which simulates to some extent oxidation in the body, the main product is succinic acid and not acetone, so that oxidation in this case is most marked at the γ -carbon atom. There is also evidence which indicates that in certain cases, β -oxidation, when it occurs, may be preceded by "desaturation" between the α - and β -carbon atoms. The oxidation of hydratropic acid to produce atropic acid, as suggested above, is a case in point, but others are: the production of cinnamic acid from phenylpropionic acid [Dakin, 1909] and the formation of furfuryl-acrylic acid from furfurylpropionic acid [Sasaki, 1910; Friedmann, 1911]. Again, the electronic formulae of β -phenyl- β -hydroxypropionic acid and β -phenyl-propionic acid, developed by Hanke and Koessler, show that in both compounds the β -carbon atom is doubly positive and the α - and carboxylic-carbon atoms quadruply negative and quadruply positive respectively. If the ease of oxidation in the body of a particular carbon atom of a substance is sufficiently explained by the electronic formula then there would be no reason to expect that these two substances should not undergo oxidation equally readily. As a matter of fact the non-hydroxylated acid is much more readily oxidised. The experiments with hydratropic and tropic acids, described in the present communication, show equally marked differences and do not seem to be capable of adequate explanation on an electronic basis

alone. It would seem that until we have a more definite knowledge of the mechanism of the oxidation process, its explanation, based solely on views of polarity in the substance oxidised, or on its electronic structure, cannot be entirely successful. These views, developed largely by a study of the phenomena of addition and substitution in organic compounds, have given a satisfactory general explanation of previously inexplicable phenomena; but with the meagre quantitative data at our disposal and our lack of knowledge as to how oxidation of very simple substances takes place either inside or outside the body, the polarity alone of the atoms in the substance oxidised is insufficient as a basis of explanation. As an instance of the probable complexity of an apparently simple process such as the oxidation of carbon monoxide to carbon dioxide when the former is burnt in moist air, the work of von Wartenberg and Sieg [1920] may be referred to. It is suggested that in this oxidation four intermediate reactions are concerned. Firstly the production of formic acid by the addition of water, secondly its dehydrogenation to produce carbon dioxide and hydrogen, thirdly, oxidation of the hydrogen to produce hydrogen peroxide and lastly decomposition of the peroxide with the formation of water and oxygen. If the oxidation of carbon monoxide is as complicated as this scheme represents, then it is at least probable that the processes of oxidation of fatty acids are not less complex.

EXPERIMENTAL.

Methods. Unless stated otherwise, the acids used in this investigation were administered hypodermically as sodium salts in aqueous solution. The urine was collected from the time of the first dose until 36 hours after the last dose was given. Urines were preserved on ice. Volatile products were sought for by distilling all, or part of the urine in steam and examining the distillate. The urine was then concentrated under reduced pressure to about one-fifth of its original volume and acidified strongly with syrupy phosphoric acid. Subsequently it was extracted with ether in a continuous extractor for 30 to 48 hours. The ether extract was then examined for unchanged acid or products of oxidation.

Hydratropic acid. The acid was obtained in two ways. Firstly, by the reduction of atropic acid with sodium amalgam and secondly, by the oxidation of hydratropic aldehyde with silver oxide in the presence of a slight excess of sodium hydroxide. The hydratropic aldehyde was prepared by the method of Tiffeneau [1907]. The former method was found to be the more expeditious. The acid was purified by distillation under reduced pressure and had B.P. 148–9° (14 mm.). In doses of 0.25 g. per kilo a slight lassitude was developed but no other ill effects were noted. Dogs, only, were used in this experiment and larger doses were not tried. No volatile products of oxidation were detected when the urine was distilled with steam. The ether extract from the acidified urine was obtained as a light brown syrup which refused to crystallise. Extraction with warm light petroleum removed some unchanged hydratropic

acid and traces of fatty acids. The latter were removed as insoluble barium salts and the hydratropic acid recovered was weighed. In this way, in two experiments, 3 and 22 % of the acid originally given was recovered. Its equivalent was 151 (calculated 150), and it was dextrorotatory. In absolute alcohol (concentration 3 %), it had $[\alpha]_D^{20} + 21.2^\circ$ to $+ 26.9^\circ$. The ether extract, after removal of the hydratropic acid as just outlined, was treated in many ways to induce it to crystallise but all were fruitless. It was dextrorotatory, reduced Fehling's solution on boiling and gave the orcinol reaction, indicating the presence of a glycuronic acid derivative. The original urine also gave these reactions. In addition, the ether extract contained some nitrogenous substance. Having failed to obtain any crystalline compound from this extract, and suspecting the presence of a glycine derivative because of the marked nitrogen content, it was hydrolysed by boiling with concentrated hydrochloric acid for an hour. After hydrolysis, the solution was diluted and extracted with light petroleum and then with ether. The latter yielded only a trace of oily substance which was neglected, but the former was found to contain dextrorotatory hydratropic acid (equivalent 150.8). In 3 % solution in absolute alcohol it gave $[\alpha]_D^{20} + 27.2^\circ$. The hydrolysate after ether extraction was evaporated to small bulk under reduced pressure and esterified with absolute alcohol saturated with hydrochloric acid. It was seeded with a crystal of glycine ester hydrochloride and placed on ice for two days, whereby a mass of crystals of glycine ester hydrochloride was obtained. The identity of the glycine was confirmed by benzoylation, when hippuric acid, m.p. $186-7^\circ$, was obtained. Careful search failed to reveal any other definite products of hydrolysis, phenylacetic, tropic and atropic acids being specially sought for. The chief substance present in the ether extract of the urine thus appeared to be hydratropylglycine. A sample of this substance was made by the Schotten-Baumann method from hydratropyl chloride and glycine and was found to be more difficult to crystallise than the commoner acyl derivatives of glycine such as phenaceturic and hippuric acids, and its contamination with some oily impurity, possibly a glycuronate of hydratropic acid, with about the same solubilities, explains its non-crystallisation from the ether extract. In a later experiment it was found possible to obtain crystalline hydratropylglycine from this extract with considerable loss, by the following method. The acids extracted from the urine by ether, after removal of unchanged hydratropic acid, were converted in turn into sodium salts and ferric salts. The acids obtained from the latter by decomposition with dilute sulphuric acid and extraction with ether were mixed with half their equivalent of sodium hydroxide and the free acids present in the solution shaken out with ether. The remaining sodium salts were now decomposed with dilute sulphuric acid and the liberated acid extracted with ether. This extract crystallised readily on inoculation with hydratropyl glycine and after two recrystallisations from water had m.p. 103° , which agreed with that of the synthetic product made from the dextrorotatory hydratropic acid recovered

from the urine, and glycine. The pure inactive glycine derivative has m.p. 105°. In one experiment carried out as quantitatively as possible for the estimation of the hydratropic acid excreted, either free or combined, three doses, each of 1.5 g., of the acid were given on three successive days. It was found that 0.156 g. (= 3.5 %) of the acid administered was present in the free state and 1.225 g. (= 27 %) in combination with glycine, or possibly partly with glycuronic acid. In order to determine whether any derivative was present in the urine as a glycuronate the concentrated urine after the usual ether extraction was freed from phosphoric acid by baryta and the reducing substance precipitated by basic lead acetate. This precipitate was decomposed with hydrogen sulphide and to the resulting solution, after removal of hydrogen sulphide in a current of air, dilute sulphuric acid was added to make the concentration about 5 %. It was boiled for eight hours and the resulting solution, after clarification with charcoal, extracted continuously with ether for eight hours. The ether extract contained only a trace of resinous substance. The reducing substance was probably, therefore, free glycuronic acid which had originally been combined with hydratropic acid and which had been split off during the ether extraction of the concentrated and strongly acidified urine. It is well known that many glycuronates are easily hydrolysed under such conditions.

Tropic acid. Inactive tropic acid was used in these experiments and its fate in both cats and dogs was determined. Doses of 1 g. per kilo caused no noticeable ill effects. The urine on steam distillation yielded no volatile oxidation products. The ether extract of the concentrated urine, on removal of the ether, gave a brown mass which crystallised at once on cooling and after one recrystallisation gave pure tropic acid, m.p. 118–119°. The acid was optically inactive. In two preliminary experiments, one on a cat and the other on a dog, the urine was extracted with ether for only 12 hours. In the first, 2.8 g. of the acid were given and 2.25 g. (80 %) of the pure acid were recovered from the urine. In the second, 5.9 g. of the acid yielded 4.4 g. (74 %) in the urine. A third experiment was carried out and the extraction with ether continued for 30 hours, when all the acid had been extracted. Of 2.94 g. of the acid administered, 2.77 g. were recovered, i.e. 94 %. Examination of the mother liquors after the separation of the tropic acid failed to reveal the presence of any oxidation products.

Atrolactic acid. The inactive acid was prepared from acetophenone by Spiegel's method using the modification described by McKenzie and Clough [1912]. Both dogs and cats were used for the experiments and doses of 0.9 g. per kilo were well tolerated, no ill effects being noted. No volatile oxidation products were detected in the steam distillate. The residue from the ether extract crystallised on cooling and was extracted with hot benzene to remove unchanged atrolactic acid. Most of the residue was soluble in benzene but a small amount of brown tarry substance remained after the extraction. This was dissolved in water and re-extracted with ether. The ether residue was

then repeatedly extracted with moist light petroleum whereby a further quantity of atrolactic acid was obtained. A small amount of brown oily substance remained which was insoluble in cold water, benzene or light petroleum and would not crystallise. The only definite substance obtained from the urine, therefore, was atrolactic acid. It was optically inactive and melted at $92-3^{\circ}$ after one recrystallisation from water. In one experiment on a dog, 5.8 g. of the acid were administered and 4.9 g. (84 %) recovered from the urine. In another case two cats were used and of 4.5 g. of the acid administered, 3.42 g. (76 %) were recovered from the urine.

Atropic acid. The acid used in these experiments was prepared from tropic acid by boiling with 10 % aqueous baryta for 18 hours. It was obtained quite pure after one crystallisation from alcohol and had m.p. $106-7^{\circ}$. The yield was 67 % of the theoretical. Doses of 0.4 g. per kilo were invariably fatal both in cats and dogs within 30 hours and no oxidation products or unchanged acid were found in the urine. A dose of 0.2 g. per kilo, given to a dog, produced only slight lassitude, but when repeated after 24 hours caused death at the end of 60 hours. Doses of 0.13 g. per kilo were well tolerated. In one instance a dose of 0.18 g. per kilo, given to a dog, caused haematuria but the animal recovered. The urine on steam distillation yielded no detectable oxidation products. The residue of the ether extract was very small in amount. It was dissolved in hot water, clarified with charcoal, and on standing deposited a small amount of a colourless, crystalline substance. In one experiment in which 1.9 g. of the acid were given, 0.04 g. of this crystalline substance was obtained, and in another, 2.8 g. yielded 0.05 g. It was recrystallised from a mixture of nine parts benzene and one part alcohol and had m.p. $178-9^{\circ}$. On heating above its melting point it gave a crystalline sublimate. It was soluble in water and alcohol, slightly in ether and almost insoluble in chloroform and benzene. It gave insoluble ferric and lead salts but a soluble calcium salt. When recrystallised from dilute nitric acid it melted at 183° . The ammonium salt heated with zinc dust gave the pyrrole reaction. These properties agree with those of succinic acid but enough material was not obtained for analytical identification. No other substance was isolated from the urine. A control experiment with a 48 hours sample of urine from the same dog to which atropic acid had been given yielded none of the crystalline acid on ether extraction.

Phenylacetaldehyde. In a mixture of four parts alcohol to six parts water (by volume), 0.93 g. of phenylacetaldehyde was dissolved and given subcutaneously to a cat weighing 2 kilos. The cat appeared to be affected by the alcohol and was drowsy for two days, but recovered. The urine was collected for four days and on steam distillation yielded no detectable oxidation products. The ether extract was dissolved in water, distilled with steam to remove acids, again acidified with phosphoric acid and extracted with ether. The ether extract yielded a small amount of oily substance which crystallised after standing for two days. The mass was rubbed up with a little water and

filtered. In this way 0.26 g. of a crystalline acid was obtained. After recrystallisation from water it gave a very unsharp m.p., the main part melting at 135–140° and the rest at 170–180°. It appeared therefore to be phenaceturic acid mixed with some higher melting substance. By boiling with 20 % sulphuric acid for a short time the phenaceturic acid was hydrolysed and the resulting phenylacetic acid was extracted with benzene. It weighed 0.102 g. and was not quite pure. The higher melting substance was extracted by ether from the acid liquid and proved to be hippuric acid, which is more resistant to hydrolysis with hot dilute acids than phenaceturic acid. In this experiment therefore, not more than 10 % of the phenylacetaldehyde given appeared as phenaceturic acid. A second experiment was carried out on a dog to test the effect of oral administration, the use of alcohol as a solvent for subcutaneous injection being objectionable. 1.9 g. of phenylacetaldehyde in its own volume of alcohol were administered to a dog by the mouth in gelatin capsules. No abnormal symptoms were observed. The urine was collected for the ensuing 48 hours and was treated in the same way as that from the cat. A fairly pure crop of crystals of phenaceturic acid was obtained (m.p. 141–3°). It weighed 0.85 g. The mother liquors on hydrolysis yielded 0.1 g. of impure phenylacetic acid and again a little hippuric acid was isolated. The phenaceturic and phenylacetic acids thus obtained correspond to 0.62 g. of phenylacetaldehyde, which represents 32 % of that administered. Nearly 70 % had therefore been completely oxidised. These experiments thus confirm those of Dakin [1909].

SUMMARY AND CONCLUSIONS.

1. The experiments described in this paper were carried out to determine the mode of oxidation in the body of phenyl derivatives of fatty acids with a branched chain structure.

2. The acids investigated were, hydratropic, tropic, atrolactic and atropic acids. In addition previous observations by Dakin on the fate of phenylacetaldehyde were confirmed.

3. Hydratropic acid is moderately well oxidised and undergoes a partial resolution in the body, the unoxidised acid found in the urine being dextro-rotatory. Atropic acid is also well oxidised and is more toxic than the other three acids. Tropic and atrolactic acids are comparatively resistant to oxidation.

4. From the observations made, it is concluded that hydratropic acid on oxidation is converted directly into atropic acid (though this was not directly proved), and that this then undergoes complete oxidation.

5. Phenylacetaldehyde largely undergoes complete oxidation in the body and may therefore be an intermediate product in the oxidation of hydratropic and tropic acids.

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