EXPERIMENTS ON THE CATABOLISM OF CAPROIC ACID AND ITS DERIVATIVES.

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The present paper contains the results of experiments aiming at obtaining evidence of the modes of catabolism followed by normal fatty acids when undergoing so called \( \beta \)-oxidation in the animal body. Knoop's theory of \( \beta \)-oxidation simply postulates the removal of successive pairs of carbon atoms without indicating the mechanism of the process. The common excretion of \( \beta \)-hydroxybutyric acid with acetoacetic acid in cases of faulty fatty acid catabolism, especially after administration of butyric acid, at first led to the natural assumption that the saturated fatty acid was successively oxidized to the \( \beta \)-hydroxy- and \( \beta \)-ketonic acid.

\[
\text{CH}_3 \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{COOH} \rightarrow \text{CH}_3 \cdot \text{CHOH} \cdot \text{CH}_2 \cdot \text{COOH} \rightarrow \text{CH}_3 \cdot \text{CO} \cdot \text{CH}_2 \cdot \text{COOH}
\]

Later, when the oxidation \textit{in vitro} of butyric acid to acetoacetic acid was observed it was also thought at first that \( \beta \)-hydroxybutyric acid was an intermediate product. Further investigation failed to confirm this view and in addition it was found that \( \beta \)-hydroxybutyric acid formed \textit{in vivo} was at any rate partly derived by the reduction of acetoacetic acid. Thus the formation of \( \beta \)-hydroxy-acids as the first stage in the oxidation of saturated fatty acids instead of appearing highly probable became rather problematical. The initial formation of \( \beta \)-ketonic acids appeared more probable.

Subsequently, the unsaturated acids acquired importance in connection with the oxidation of saturated fatty acids. In the first place it was found that unsaturated acids, such as cinnamic acid, could be formed \textit{in vivo} from phenylpropionic acid and related compounds and later on the direct oxidation of succinic to
Catabolism of Caproic Acid

Fumaric acid by Battelli and Stern's "succinoxidon"—a tissue enzyme—was well established. Furthermore, the mutual interconversion of the unsaturated and \( \beta \)-hydroxy-acids was observed in a number of cases both within and without the body. The direct formation of unsaturated acids from saturated ones was intelligible in the light of Wieland's dehydrogenation theory of oxidation.

The literature dealing with the reactions just outlined is extensive and it is not proposed to review it in the present communication. Most of it can be found in the writer's monograph (1) on the subject and also in an article in Physiological Reviews (2).

From a consideration of all the facts it would appear that it is possible to advance more or less evidence in favor of regarding either the unsaturated acids, or the \( \beta \)-ketonic acids or, rather less probably, the \( \beta \)-hydroxy-acids, as the initial products of the oxidation of saturated fatty acids.¹ These changes may be represented in the following scheme which also indicates the possible further transformations observed \textit{in vivo} in the case of numerous examples.

\[
\begin{align*}
R \cdot CH = CH \cdot COOH & \rightleftharpoons R \cdot CHOH \cdot CH_2 \cdot COOH \rightleftharpoons R \cdot CO \cdot CH_2 \cdot COOH \\
R' \cdot CH_2 \cdot CH_2 \cdot COOH & \rightarrow R \cdot CO \cdot CH_2 \cdot COOH \rightleftharpoons R \cdot CHOH \cdot CH_2 \cdot COOH \rightleftharpoons R \cdot CH = CH \cdot COOH
\end{align*}
\]

It was thought possible that some evidence might be obtained as to the relative probability of one or other of these three types of change being concerned in the initial oxidation of saturated fatty acids by the following line of experiments: Caproic acid was known from Embden's results to give acetoacetic acid and acetone,

¹ Recently, Armstrong (Armstrong, H. E., J. Soc. Chem. Ind. 1922, xli, 265) without any apparent regard to existing knowledge has pictured saturated fatty acids as being first oxidized to per acids of the type \( R \cdot CH_2 \cdot CO \cdot O \cdot OH \) which then undergo rearrangement with formation of \( \beta \)-hydroxy- and \( \beta \)-ketonic acids. So far as reactions \textit{in vivo} are concerned there is apparently no evidence in favor of this idea and much against it. Since Armstrong produces no evidence of any kind to support his views, the reader is left somewhat in doubt whether to take his pronouncements seriously or to regard them as a pleasant \textit{jeu d'esprit}. 

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or at least a ketone with similar reactions, on perfusion through a surviving liver. The writer’s observations have confirmed this and show that the ketone obtained was acetone and not propylmethyl ketone as might possibly be the case. The formation of \( \beta \)-hydroxybutyric acid was also established. The next step was to perfuse through surviving livers under as nearly similar conditions, the unsaturated, \( \beta \)-hydroxy-, and \( \beta \)-ketonic acids derivable from caproic acid. An experiment was also made with the doubly unsaturated sorbic acid. The relation of these acids to each other is shown by the following formulas.

\[
\begin{align*}
\text{Caproic acid} & \quad \cdots \cdots \cdots \quad \text{CH}_3 \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{COOH} \\
\alpha \beta \text{-Hexenic acid} & \quad \cdots \cdots \cdots \quad \text{CH}_3 \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CH} = \quad \text{CH} \cdot \text{COOH} \\
\beta \text{-Hydroxyacaproic acid} & \quad \cdots \cdots \cdots \quad \text{CH}_3 \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CHOH} \cdot \text{CH}_2 \cdot \text{COOH} \\
\text{Butyrylacetic acid} & \quad \cdots \cdots \cdots \quad \text{CH}_3 \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CO} \cdot \text{CH}_2 \cdot \text{COOH} \\
\text{Sorbic acid} & \quad \cdots \cdots \cdots \quad \text{CH}_3 \cdot \text{CH} = \text{CH} \cdot \text{CH} = \quad \text{CH} \cdot \text{COOH}
\end{align*}
\]

For the correct interpretation of the results it was necessary to devise methods for the estimation of acetone in the presence of propylmethyl ketone and, as will be seen in the experimental portion of the paper, this was adequately accomplished. With the exception of sorbic acid for which at present there is no good reason for believing it to be a metabolite of caproic acid, it was found that each and all of the remaining acids under similar conditions of perfusion gave rise to large amounts of acetoacetic acid, acetone, and \( \beta \)-hydroxybutyric acid, but that the quantitative differences between the various acids as precursors of “acetone bodies” were insufficient to indicate which, if any, was preferentially produced from caproic acid. The total amount of acetone bodies obtained from the four acids was not widely different. It is therefore not possible to obtain any satisfactory answer from the present experiments as to whether an unsaturated, \( \beta \)-hydroxy-, or \( \beta \)-ketonic acid, is first formed by the oxidation of caproic acid. It would appear more probable that all the acids are in readily shifting equilibrium with each other and are easily interconvertible.

**EXPERIMENTAL.**

**Methods.**

The perfusions were made with dog’s blood diluted with not more than one-third volume of saline solution. The livers of
medium sized animals of 10 to 14 kilos weight, which were not fed during the day preceding the operation, were employed using the customary technique. The volume of perfusion fluid varied from 1,200 to 1,600 cc. and the concentration of acid added in the form of neutral ammonium or sodium salt was 2 gm. per liter. The perfusion was continued for 60 minutes only. The blood was then collected, measured, and precipitated with 2 volumes of acid mercuric chloride solution in the usual fashion. An aliquot part of the filtrate was then distilled. The distillate was used for the estimation of acetone and other ketones, while the residue was used for the estimation of $\beta$-hydroxybutyric acid.

The estimation of acetone presented some difficulties for it was clear that propylmethyl ketone might accompany the acetone in all experiments and would certainly be present in the butyryl-acetic acid perfusions. The first distillate referred to above was redistilled with 2 per cent hydrogen peroxide (15 cc.) and sodium hydroxide (15 cc. of 30 per cent solution). The volume of the second distillate was adjusted to 100 cc. In an aliquot part of this the total ketones were determined by titration with iodine solution in the customary fashion. A separate determination of acetone, excluding propylmethyl ketone, was based on the following observations.

On heating dilute acetone solutions with 7 per cent mercuric sulfate, dissolved in 20 per cent sulfuric acid, the whole of the acetone is precipitated in the form of a white granular precipitate (Denigès, 3). This reaction has been utilized by Oppenheimer (4) for the gravimetric estimation of acetone in urine, working in closed vessels. The writer has found that while propylmethyl ketone readily combines with mercuric sulfate so that it is not recoverable on distillation, the compound formed is much more soluble in dilute sulfuric acid, especially when hot, than the corresponding acetone compound. The following procedure for the determination of acetone in the presence of propylmethyl ketone was found adequate for the purpose in view. A portion of the distillate, obtained as previously described (25 or 50 cc.), is acidified with sulfuric acid (1:1) so that the solution contains 10 per cent sulfuric acid by volume. Mercuric sulfate solution (25 to 50 cc.) is then added and the mixture, contained in a conical flask provided with a reflux tube drawn to a fine point at the end,
is heated for 30 minutes in a rapidly boiling water bath. The precipitate containing the acetone is filtered off while the solution is still hot and washed with water and alcohol. It is then dried in a steam bath. The weight of precipitate multiplied by 0.055 gives the acetone with very fair accuracy. Under the above conditions propylmethyl ketone gives almost no precipitate though some settles out on standing in the cold. A solution of acetone containing 47.5 mg. in 25 cc. according to iodometric analysis gave 47.3 by gravimetric analysis. A similar solution of propylmethyl ketone (100 mg.) gave 0.0075 gm. precipitate, corresponding to 0.4 mg. of acetone. Mixtures of both ketones gave results for acetone of more than sufficient accuracy for the purpose in view.

The formation of $\beta$-hydroxybutyric acid as the result of the perfusion of acids, such as caproic acid, through a surviving liver apparently has not been previously investigated though the reduction of acetoacetic acid, which is known to be formed, to $\beta$-hydroxybutyric acid is well established as occurring in the liver. In order to gain some idea of the extent of its production use was made of Shaffer’s (5) method of estimation, suitably modified for present purposes. The residue left after the distillation of ketones from the blood filtrate was acidified with concentrated sulfuric acid (25 cc.) and then potassium bichromate solution (6 per cent) was added, a few drops at a time as fast as reduced during a rapid distillation. The distillate was then redistilled with hydrogen peroxide and sodium hydroxide as previously described. The second distillate (100 cc.), containing essentially acetone and propylmethyl ketone, was then analyzed (a) iodometrically for total ketones, and (b) gravimetrically for acetone as above described. When the results of the two analyses approximated each other it was inferred that no significant amount of the higher ketone was present.

Results of Perfusion Experiments.—In Table I are recorded the results of the various perfusions. In the first column under I the results of the estimations of total volatile ketones in terms of acetone are recorded with the true acetone values as determined gravimetrically. With the exception of the experiments with butyrylacetic acid, in which the unchanged acid gives propylmethyl ketone on distillation, it would appear that no significant
amount of any other ketone than acetone was present. The columns under II contain the results of oxidizing with chromic acid the residue from the first distillation. The total ketone estimations here are probably of very little value, since unaltered $\alpha\beta$-hexenic acid and $\beta$-hydroxycaproic undoubtedly furnish some propylmethyl ketone on oxidation. The actual acetone determinations made gravimetrically are probably a fair index of the $\beta$-hydroxybutyric acid formation and it is interesting to note that the results in many cases are not very much lower than those for the acetone derived from acetoacetic acid.

A few notes on the preparation of the substances used and the results obtained with them are appended.

_Caproic Acid._—The acid used was a redistilled specimen of the synthetic acid. The results on perfusion indicate a pronounced formation of acetoacetic acid and $\beta$-hydroxybutyric acid. The close concordance between the "total ketone" and acetone figures shows that very little, if any, $\beta$-hydroxycaproic acid or butyryl-"acid were in the blood at the close of perfusion, for both of these acids would yield propylmethyl ketone and so increase the figures for total ketone.
αβ-Hexenic Acid.—Caproic acid was brominated with bromine and phosphorus in the usual way and then converted into α-bromo-caproic ester by treatment with alcohol. The ester was boiled with diethyl aniline according to the directions of Blaise and Luttringer (6) in order to remove hydrobromic acid. The resulting ester was hydrolyzed and the free acid carefully fractionated. The product used was entirely free from bromine compounds.

The results on perfusion indicate a strong acetoacetic acid formation unaccompanied by butyrylacetic acid. The acetone derived from β-hydroxybutyric acid is also considerable in amount. The excess of total ketones over acetone on chromic acid oxidation may well be assumed to be due to the formation of some β-hydroxy-caproic acid during perfusion.

β-Hydroxycaproic Acid.—Two preparations of this acid were used. One was made according to Fittig and Baker’s method (7) from β-bromo-caproic acid which in turn was prepared from αβ-hexenic acid. The other preparation was prepared by the following rather more convenient method. Butyrylacetic ester (20 gm.), prepared as described in the next section, dissolved in alcohol (20 cc.), was mixed with water (180 cc.) and the whole cooled in an ice bath. Sodium amalgam (200 gm. of 4 per cent) was added fairly rapidly with good mechanical stirring. Less than a gram of oil was left undissolved and this was removed the following day by extraction with ether. Nine-tenths of the solution were made just acid with sulfuric acid (1:1), then the remaining one-tenth was added and the whole concentrated on a water bath. On making the residue just acid to Congo red with sulfuric acid the oily acid partly separated and was extracted with ether. On evaporation of the ether extracts and drying in vacuo, 10 gm. of acid remained which appeared to be identical in all respects with Fittig and Baker’s product.

The perfusion results showed that the acid gave rise to both acetoacetic and β-hydroxybutyric acid in marked degree. In one case the difference between total ketone and acetone in the blood filtrate distillate indicated a possible butyrylacetic acid production. On oxidation with chromic acid much propylmethyl ketone was naturally formed thus giving a high total ketone figure, but acetone derived from β-hydroxybutyric acid was undoubtedly present in fair amount.
Butyrylacetic Acid.—The ethyl ester of this acid was prepared by the condensation of butyric and acetic esters by means of sodium as outlined by Wahl and Doll (8). The details of the apparatus and mode of operation were the same as those used by Dakin and Dudley (9) for the preparation of ethyl \( \gamma \)-diethoxy-acetoacetate. The proportions used were the following: ethyl butyrate 232 gm., ethyl acetate 176 gm., and sodium 46 gm. The yield of crude ester was 76 gm. of oil, boiling from 80–105° at 15 mm. pressure. The crude product was purified by repeated shaking with sodium bisulfite solution with which it does not combine, and then refractionated. The main, middle fraction, boiling steadily at 87–89° at 8 to 10 mm. pressure, was collected and used for the preparation of the acid. The hydrolysis of the ester was effected in the cold with sodium hydroxide and the product worked up as is customary with acetoacetic acid. The dilute solution of the sodium salt was well boiled at room temperature under greatly diminished pressure in order to remove traces of alcohol or ether. Its concentration was determined by distilling a portion of the solution and estimating the propylmethyl ketone in the distillate with standard iodine solution. The results of the perfusion indicate a considerable production of both acetoacetic acid and \( \beta \)-hydroxybutyric acid. The excess of total ketones obtained by chromic acid oxidation compared with the actual acetone concentration appears to indicate a considerable reduction of the \( \beta \)-ketonic acid to \( \beta \)-hydroxypropionic acid. This change is, of course, analogous to the known reduction of acetoacetic acid to \( \beta \)-hydroxybutyric acid.

Sorbic Acid.—The substance was obtained by the condensation of crotonic aldehyde and malonic acid with pyridine according to Doebner’s (10) method. The perfusion results indicate that the acid, while definitely giving some acetoacetic acid and \( \beta \)-hydroxybutyric acid, is much less effective in this respect than the other acids examined. There would seem no good reason to regard it as a probable normal metabolite of caproic acid.

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