CLXXXVI.—*The Mechanism of the Degradation of Fatty Acids by Mould Fungi. Part II.*

By PHILIP DALTON COPPOCK, VIRA SUBRAMANIAM, and THOMAS KENNEDY WALKER.

THE importance of data obtained from experiments with mild oxidising agents at normal temperatures *in vitro*, as a basis upon which to conduct physiological studies of fatty acid catabolism, has been stressed by Dakin (*J. Biol. Chem.*, 1908, **4**, 227), who developed the use of hydrogen peroxide for such purposes. Two of the present authors recently indicated (this vol., p. 803) the possibilities of the use of mould fungi in investigations of this nature, and the inquiry has now been extended to a study of the degradations of *n*-butyric, *n*-valeric and *iso*valeric acids by *Aspergillus niger*.

Although numerous investigations have been carried out from the standpoint of Knoop's hypothesis of β-oxidation, the precise mechanism of such oxidation of lower normal saturated fatty acids in the body is not yet known with certainty, previous experimental work indicating that the first product may be either a β-keto-acid, or a β-hydroxy-acid or an αβ-unsaturated acid. According to Dakin (*J. Biol. Chem.*, 1923, **56**, 43), all of these are equally easily oxidised by the liver and, moreover, are readily interconvertible, hence the uncertainty attaching to the question. We find that

when calcium *n*-butyrate is submitted under appropriate conditions to the attack of the mould, acetone is produced, and, according to our experimental evidence, the sequence of changes by which it arises is :

$$\text{butyric acid} \longrightarrow \beta\text{-hydroxybutyric acid} \longrightarrow \text{acetoacetic acid} \longrightarrow \text{acetone.}$$

Experiments are now being undertaken to ascertain the behaviour of the mould towards β-hydroxybutyric acid. Crotonic acid was not detected in the calcium butyrate culture media, neither could the mould be induced to grow upon various media containing salts of crotonic acid as sole sources of carbon.

Dakin's experiments on the oxidation of *n*-butyric acid with hydrogen peroxide (*J. Biol. Chem.*, 1908, **4**, 77) having shown that attack by this reagent commences initially in two directions, namely, at the β-carbon atom and, to some extent, at the α-carbon atom, search was made in the culture media for possible products of the α-oxidation of the acid, but without success.

On a medium containing calcium *n*-valerate as sole carbon supply, *Aspergillus niger* made satisfactory growth and ultimately a mixture of β-hydroxy-*n*-valeric acid and methyl ethyl ketone was obtained in good yield, but further work will be necessary to determine definitely which of these is the first product.

The study of the action of the mould on *iso*valeric acid was of special interest in view of the fact that the work of Embden, of Baer and Blum, and of Friedmann (compare Dakin, " Oxidations and Reductions in the Animal Body," 1922) indicates that in the body this acid suffers both β-oxidation and loss of one methyl group with ultimate production of acetoacetic acid and hence acetone, whereas R. Meyer (*Annalen*, 1883, **219**, 240) found that on oxidation *in vitro* with alkaline permanganate acetone was produced by oxidation at the tertiary hydrogen atom and subsequent splitting off of the *iso*propyl group. The source of the acetone is therefore different in the two cases. Our experiments on the fermentation of *iso*valeric acid have shown that it is degraded in accordance with the results of the physiological studies just mentioned, for the following facts were observed and, in our view, are capable of no other plausible explanation. On the sixth day after inoculation, a culture on calcium *iso*valerate gave rise to a small quantity of a substance which gave the iodoform reaction, and another sample of the liquid gave acetone on oxidation. On the tenth day, a sample on treatment with benzenediazonium chloride and sodium acetate developed a red colour and deposited a minute amount of a reddish-brown compound, an indication of the presence

of traces of a substance possessing a reactive methylene or methine group. This was followed on the sixteenth day by the occurrence of acetone in the fermenting medium. Precisely similar behaviour towards the diazonium salt was exhibited by the cultures of *A. niger* on calcium *n*-butyrate media just prior to the appearance therein of acetone, the reactive intermediate, presumably acetoacetic acid in both cases, having apparently but a transitory existence.

Processes for the production of acetone by the action of certain bacteria on starch or on sugars have been known for some years, and recently Challenger and two of the present authors (J., 1927, 200) were able to show that it can also arise by the action of *A. niger* on citric acid. Its formation from butyric acid by mycological agency has not been demonstrated previously, however, neither has the production of methyl ethyl ketone by any fermentative organism been described.

EXPERIMENTAL.

The *A. niger* strain and the medium (solution M) containing the necessary inorganic salts were identical with those employed in the work described in Part I (this vol., p. 803). Incubation of the inoculated media was carried out at 32° unless otherwise stated.

Fermentation of Calcium n-*Butyrate. Detection of* (a) β-*Hydroxybutyric Acid*, (b) *Acetoacetic Acid, and* (c) *Acetone.*—A solution of 25 g. of pure *n*-butyric acid (as calcium salt) in 2500 c.c. of solution M was sterilised at 100° for 15 minutes on three successive days. After inoculation with spores of the mould the solution was incubated and portions were withdrawn daily for submission to the following tests:

(i) 10 C.c. for the iodoform test, which, if positive, would indicate the presence of (*a*), (*b*) or (*c*).

(ii) 20 C.c. were treated with benzenediazonium chloride and sodium acetate to detect the appearance of (*b*).

(iii) 100 C.c. were distilled and two successive fractions were collected. The first fraction (30 c.c.) was heated with 500 c.c. of Denigès' mercuric sulphate solution under reflux on a steam-bath (compare J., 1927, 206) to detect acetone and/or acetoacetic acid. The second fraction (10 c.c.) was tested similarly in order to ensure, in the event of acetone being found in the first fraction, that the residue in the distillation flask (60 c.c.) should be quite free from acetone. A mixture of this residue (60 c.c.) with 20 c.c. of 50% sulphuric acid was then gently boiled and 30 c.c. of 2% potassium dichromate solution were added slowly while distillation proceeded. The distillate (30 c.c.) was heated with Denigès' solution (500 c.c.) as before. This served to detect the presence of any β-hydroxybutyric acid. By the adoption of this plan the sequence in which

the products of oxidation made their appearance in the fermenting culture was readily determined.

On the sixth day after inoculation, test (i) was positive and test (iii) indicated the presence of β-hydroxybutyric acid. On the eighth day, test (ii) gave a red solution containing particles of solid matter, but in an insufficient amount to justify an attempt to convert into a formazyl derivative any acetoacetic acid which might be present. A confirmation of the latter reaction was afforded by the fact that another portion of the culture, removed simultaneously and treated with neutral ferric chloride solution, developed a faint purple colour (a reaction of acetoacetic acid). This was followed on the eleventh day by a slight evolution of carbon dioxide and a positive test for acetone in the culture liquid. The whole of the latter was thereupon distilled, the distillate boiled with Denigès' solution, and the resultant copious precipitate washed with water, drained on a tile, and distilled, in portions, with sodium iodide solution. When treated in the appropriate manner with benzaldehyde, caustic soda, and ethyl alcohol, the united distillates readily gave dibenzylideneacetone, m. p. 113° alone or mixed with authentic material (Found : C, 87·0; H, 6·0. Calc. : C, 87·2; H, 6·0%).

The quantity of β-hydroxybutyric acid present at any one time in the fermenting medium, prior to the appearance of acetone, was very small as judged from the quantities of precipitate obtained when the oxidised culture samples were boiled with Denigès' reagent. In this case also the mercury compound was distilled with sodium iodide solution and the resulting acetone was characterised as dibenzylideneacetone, m. p. and mixed m. p. 113°.

In subsequent fermentation experiments with calcium *n*-butyrate it was found that the yields of acetone varied greatly (5% to 60%). Further experiments are in progress with other salts of butyric acid in order to discover the optimum conditions for production of the ketone.

Fermentation of Calcium n-*Valerate.—Isolation of methyl ethyl ketone.* 2000 C.c. of solution M to which 20 g. of *n*-valeric acid (as calcium salt) had been added were sterilised as in the case of calcium butyrate, inoculated, and incubated at 32°. Growth was slow and products of enzymic action could not be detected in the medium during the first 21 days; vegetative growth then appeared to cease. After remaining at 20° for a further 14 days, a test portion having given an immediate precipitate on treatment with a solution of *p*-nitrophenylhydrazine acetate, the culture liquid was submitted to three careful fractional distillations through a 12-pear Young column. The final distillate was turbid, and clarified on the

separation of an oil; this was identified as methyl ethyl ketone (yield, *ca.* 50%) by conversion into the semicarbazone, m. p. 143°, and into the *p*-nitrophenylhydrazone (Found: C, 57·7; H, 6·4. Calc.: C, 58·0; H, 6·3%), m. p. 128°, each of which did not depress the m. p. of authentic material.

Detection of β-hydroxyvaleric acid. The residue in the distillation flask, from which the methyl ethyl ketone had been removed as described above, was oxidised by means of the usual mixture of sulphuric acid and sodium dichromate, and submitted to distillation; a further considerable quantity of methyl ethyl ketone (identified in the forms of the *p*-nitrophenylhydrazone and the semicarbazone) was then obtained. A control experiment in which several grams of pure *n*-valeric acid were distilled in the presence of a considerable quantity of a similar oxidising mixture gave no trace of methyl ethyl ketone.

Fermentation of Calcium iso*Valerate. Detection of Acetone and of its Precursors.*—The medium used consisted of solution M (2500 c.c.) to which had been added 25 g. of calcium *iso*valerate (Kahlbaum), and the whole was sterilised and inoculated as before. During incubation daily tests were performed as in the case of the calcium butyrate cultures. After 6 days, the distillate from an oxidised test portion, on being heated under reflux on the steam-bath with excess of Denigès' reagent, gave a white precipitate (A). After 10 days, a red coloration and a slight precipitate were produced on addition of benzenediazonium chloride and sodium acetate, and this behaviour continued until the sixteenth day; acetone, in small quantity, was then first detected in the culture. In this particular experiment the yields of all three products were very small, but in a subsequent fermentation 25 g. of calcium *iso*valerate, after 14 days' undisturbed incubation, gave 3·5 g. of acetone, calculated on the weight of Denigès' mercury compound (B) obtained from it. Both lots (A and B) of mercury compound were decomposed separately by treatment with sodium iodide solution, and the liberated acetone was characterised in each case as dibenzylideneacetone (m. p. 113°) and as the *p*-nitrophenylhydrazone (m. p. 148·5°). In no case did the mixed-m. p. determination show depression. The dibenzylidene compound derived from (A) was analysed (Found: C, 86·9; H, 6·1. Calc.: C, 87·2; H, 6·0%). Blank experiments showed that pure *iso*valeric acid gives no acetone on being heated with a sodium dichromate and sulphuric acid mixture of the concentration used in this work.

(Note added, May 16th, 1928).—Since this paper was prepared for publication W. N. Stokoe (*Biochem. J.*, 1928, **22**, 80) has made known the results of an investigation of the action of moulds,

principally of the *Penicillium* genus, on coco-nut oil. Our attention has also been directed to the observations of H. G. Derx (*Proc. K. Akad. Wetensch. Amsterdam*, 1925, **28**, 96) on the oxidative cleavage of fats by fungi and, further, we have noted the very recent work of W. O. Tausson (*Biochem. Z.*, 1928, **193**, 85) on the oxidation of waxes by micro-organisms. By the employment of experimental conditions somewhat different from those adopted by us, in that we fermented salts of the fatty acids in the entire absence of any other sources of carbon, Stokoe and Derx obtained methyl ketones from several fatty acids which are higher in the series than valeric acid and are normally present in the glycerides of coco-nut oil. Derx did not observe ketone formation, however, when butyric and valeric acids were submitted to the action of cultures of a *Penicillium* species in a glucose medium. In general principle, the experimental results recorded in these papers are in substantial agreement with those obtained by us, inasmuch as they all demonstrate clearly the origin of the methyl ketones found in certain plant products (compare Dakin, *J. Biol. Chem.*, 1908, **4**, 221). In the interpretation of the precise course of the oxidation in the case of a straight-chain acid we differ from Stokoe and Derx, who presume that a β-keto-acid is the first product, whereas our evidence points to the initial formation of a β-hydroxy-acid and its subsequent oxidation to the corresponding β-keto-acid.

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MUNICIPAL COLLEGE OF TECHNOLOGY,
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