CIX.—*The Mechanism of the Degradation of Fatty Acids by Mould Fungi. Part I.*

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IN investigations designed to throw light on the stages by which fatty acid catabolism proceeds in the animal body, endeavours have often been made to confirm the results of physiological studies by the use of chemical reagents *in vitro*. Certain reactions which proceed in the mammalian body are also of normal occurrence in the metabolic processes of fermentative organisms. This suggested the possibility that the latter might be capable of effecting the enzymic degradation of fatty acids by processes identical with or closely similar to those by which it is believed breakdown is occasioned in living animal tissue, and the present communication deals with a study of the fermentation of propionic acid by the mould *Aspergillus niger*. The case of this acid is of particular interest, since physiological experiments hitherto have not indicated definitely the initial stage of its degradation, and Dakin (*J. Biol. Chem.*, 1926, **67**, 341) has pointed out that its direct conversion into lactic acid, if such could be demonstrated, would furnish a unique example of α-oxidation *in vivo* of a normal fatty acid. The experiments of Ringer (*J. Biol. Chem.*, 1912, **12**, 511) suggest indirectly that oxidation to lactic acid does occur, but Blum and Worringer (*Bull. Soc. Chim. biol.*, 1920, **2**, 88) incline to the view that pyruvic acid is the first product, subsequently undergoing asymmetric reduction to *l*-lactic acid, a conclusion which Knoop has questioned (*Z. physiol. Chem.*, 1923, **130**, 388). Raper (*Biochem. J.*, 1914, **8**, 320) has suggested that, by analogy with the manner in which saturated α-methyl fatty acids react with hydrogen peroxide, propionic acid may be attacked at its β-carbon atom, which forms part of a methyl group. If such should be the case, the reaction might be expected to yield, ultimately, malonic semi-aldehyde together, probably, with malonic acid. Dakin (*J. Biol. Chem.*, 1908, **4**, 227) obtained acetaldehyde by the action of hydrogen peroxide on propionic acid; this may have arisen from decarboxylation of the unstable malonic semi-aldehyde, though its formation from lactic acid is equally probable. In a recent paper (*ibid.*, 1926, **67**, 341) the same author has put forward an hypothesis by which propionic acid might undergo in the body dehydrogenation of the αβ-type, yielding acrylic acid which, in turn, might yield hydracrylic or conceivably lactic acid. This view receives support from the fact that succinic acid is oxidised to fumaric acid by muscle tissue and that acrylic acid, like propionic acid, yields glucose in the phloridzinised dog. Dakin was not able,

however, to obtain experimental evidence in support of this hypothesis.

In an investigation undertaken with a different object, Raistrick and Clark (*Biochem. J.*, 1919, **13**, 329) experienced difficulty in inducing *A. niger* to grow in media containing ammonium or sodium propionate as the sole source of carbon. Only in one instance, on sodium propionate, did development occur and a small yield of oxalic acid result. On salts of lactic acid good growth unaccompanied by oxalic acid formation took place, and ammonium pyruvate gave good growth and small yields of oxalic acid. On this and other evidence the authors questioned whether the oxalic acid had not arisen by purely chemical changes during sterilisation of the media rather than by the biological agency of the mould.

The present authors have found that *A. niger* may be cultivated readily on dilute aqueous solutions of calcium propionate together with the requisite inorganic salts. When incubated at 32°, such cultures gave positive thiophen tests for lactic acid (Fletcher and Gowland Hopkins, *J. Physiol.*, 1907, **35**, 247) on or about the ninth day. Thereafter, the thiophen colour reaction became fainter or disappeared, and about the twelfth or thirteenth day pyruvic acid was detected by the coloration given with benzenediazonium chloride in presence of sodium acetate (Bamberger and Müller, *Ber.*, 1894, **27**, 147). That the latter coloration was due actually to pyruvic acid and not to malonic acid or its semi-aldehyde, which also react with the diazonium acetate, was proved in several experiments in none of which was it possible to isolate a formazyl derivative of malonic acid or of its semi-aldehyde by addition of the diazonium salt to the cultures; whereas, in each instance, the simultaneous addition of aminoguanidine nitrate to other samples of the same cultures yielded its condensation product with pyruvic acid (Wedekind and Bronstein, *Annalen*, 1899, **307**, 298).

In these experiments, lactic acid was detected in every case prior to the appearance of pyruvic acid. The presence of lactic acid was confirmed by its conversion by sulphuric acid into acetaldehyde and formic acid, the latter being analysed as the lead salt. These results made it probable that the pyruvic acid had arisen by oxidation of the lactic acid, a biological transformation which has already been demonstrated in the cases of other fermentative organisms (compare Mazé, *Compt. rend. Soc. Biol.*, 1918, **81**, 1150; Quastel, *Biochem. J.*, 1925, **19**, 304; Kayser, *Bull. Soc. Chim. biol.*, 1924, **6**, 345). The mould was therefore cultivated on a calcium lactate medium and was found to yield pyruvic acid, which was detected by benzenediazonium acetate on the tenth day and isolated subsequently in the form of its compound with aminoguanidine (Wede-

kind and Bronstein, *loc. cit.*). In another experiment pyruvic acid was produced on the seventh day and was characterised as the *p*-nitrophenylhydrazone.

Nagayama (*Biochem. Z.*, 1921, **116**, 303) has shown that a number of moulds, including *A. niger mutante*, produce acetaldehyde when grown on calcium pyruvate as sole source of carbon. The fact that cultures of *A. niger* on calcium propionate developed a distinct odour of acetaldehyde led us to attempt to isolate the latter from the various culture solutions by carrying out fermentations in the presence of sodium sulphite and of calcium sulphite according to the method of Neuberg and Reinfurth (*Biochem. Z.*, 1918, **89**, 389). The results were entirely negative and in the case of calcium lactate cultures strong reduction occurred accompanied by darkening of the liquid and evolution of hydrogen sulphide. Nevertheless, in pyruvate and lactate cultures the transitory existence of acetaldehyde was indicated by the fact that after 28 days these were found to contain traces of ethyl alcohol, produced presumably either by direct reduction or by dismutation (biologically induced Cannizzaro reaction) of the aldehyde. The alcohol was oxidised to acetaldehyde and characterised as aldomedon (Neuberg and Reinfurth, *Biochem. Z.*, 1920, **106**, 281). By the action of certain yeasts on calcium lactate Kayser (*loc. cit.*) obtained ethyl alcohol, in addition to pyruvic acid. Blank experiments in which sterile solutions of calcium pyruvate and the inorganic salts of the medium were mixed and maintained for several weeks at 32° gave no traces of acetaldehyde or ethyl alcohol. Hence it seems probable that *A. niger* brings about the conversion of pyruvic acid into acetaldehyde by the agency of the enzyme carboxylase. If the ethyl alcohol produced in these fermentations arises by dismutation of acetaldehyde, then acetic acid must be formed simultaneously; and Neuberg (*Biochem. Z.*, 1914, **67**, 90) obtained this acid as the main product of the putrefactive fermentation of pyruvic acid. Its presence in our fermenting media has not yet been proved, but indirect evidence of its occurrence was furnished by the following facts: (*a*) Separate cultures of *A. niger* on the calcium salts of propionic, lactic, and pyruvic acids in the later stages of fermentation gave positive naphtharesorcinol tests for glyoxylic acid in all cases; (*b*) a calcium lactate culture yielded a small amount of calcium oxalate; and (*c*) Challenger, Subramaniam, and the present authors (J., 1927, 200, 3044) had already shown that the fermentation of calcium acetate by *A. niger* follows the course: acetic acid → glycollic acid → glyoxylic acid → oxalic acid. Hence it is to be inferred that the oxalic acid isolated in the present instance had been produced in this manner. Incidentally, in view of the above, the mycological genesis of the oxalic acid

obtained by Raistrick and Clark (*loc. cit.*) must be regarded as verified.

EXPERIMENTAL.

The *A. niger* strain, and the medium containing the inorganic salts, designated "Solution M," were the same as those employed by Challenger, Subramaniam, and one of us (*loc. cit.*). The usual precautions were observed to ensure aseptic conditions in working and results were checked by blank experiments carried out under identical conditions.

Fermentation of Calcium Propionate. Detection of Lactic and Pyruvic Acids.—Three separate experiments were carried out in which, in each case, calcium propionate (25 g.) in solution M (2500 c.c.) was inoculated by young test-tube cultures of *A. niger* on the same medium, and the mixture incubated at 32°. In these three cases lactic acid was detected qualitatively after 9, 10, and 9 days, and pyruvic acid after 14, 14, and 13 days, respectively. In one of these experiments the thiophen colour test was markedly distinct on the eleventh day, whereupon 1000 c.c. of the medium were removed, filtered, and evaporated to dryness and the residue was distilled with 60 c.c. of 60% sulphuric acid. A strong smell of acetaldehyde, which gave way to that of formic acid, was observed. The distillate was neutralised with caustic soda and evaporated to dryness, and the residue extracted several times with methyl alcohol, which removed sodium propionate and left 0·76 g. of insoluble material, presumably sodium formate. The latter, in 5 c.c. of water, gave with 1·6 g. of lead nitrate in 8 c.c. of water, a white precipitate (1·4 g.), which was washed with cold water, crystallised twice from hot water, and dried over calcium chloride (Found : Pb, 69·6. Calc. : Pb, 69·7%).

In another of the experiments in which a positive test for pyruvic acid had been obtained faintly on the thirteenth and very definitely on the fourteenth day, 2000 c.c. of the medium were immediately filtered and treated with 10 g. of aminoguanidine nitrate and the mixture was strongly cooled. After 48 hours the precipitate (3·4 g.) was removed, washed with cold water, and recrystallised three times from large bulks of hot water (100°). The m. p. of the condensation product of aminoguanidine nitrate with pyruvic acid is given in the literature (*loc. cit.*) as 206°, but our product did not melt at 350°, doubtless owing to polymerisation during the treatment with hot water (Found for the anhydrous substance : C, 22·7 ; H, 4·5. Calc. : C, 23·2 ; H, 4·35%). In calcium propionate cultures fermented as described above, the pyruvic acid formed was found to diminish after 2 or 3 days and a little later the solutions gave positive naphtharesorcinol tests indicative of the presence of

glyoxylic acid. The latter could not be characterised owing to the smallness of the amounts present and to interference from traces of pyruvic acid which still persisted in the media.

Fermentation of Lactic Acid. Detection of Pyruvic Acid.—A solution of Kahlbaum's calcium lactate (50 g.) in 2500 c.c. of solution M was sterilised, inoculated, and incubated at 32°. After 10 days a sample (25 c.c.) of the medium gave a faint red coloration when treated at 0° with a few c.c. of a benzenediazonium chloride solution, followed by sodium acetate. This coloration was much deeper when the test was performed after 12 days, but no solid condensation product separated. The culture medium was then filtered and treated with 15 g. of aminoguanidine acetate and 5 c.c. of glacial acetic acid. After 48 hours the precipitate was washed with cold water. The mother-liquor on strong cooling yielded another smaller crop and the total precipitate (1·71 g.) was recrystallised successively from large bulks of hot water (100°) and boiling methyl alcohol. A sample did not melt at 350° (Found : C, 33·3; H, 5·6. Calc. : C, 33·3; H, 5·6%). A quantity of the tripolymeride, $[CO_2H{\cdot}CMe{:}N{\cdot}NH{\cdot}C(NH_2){:}NH]_3$ (*loc. cit.*), made for purposes of comparison was identical in physical properties with the derivative under investigation.

In another experiment in which calcium lactate (10 g.) in solution M (500 c.c.) was fermented as above, the liquid yielded a positive test for pyruvic acid on the seventh day. The whole of the solution was at once filtered and treated with *p*-nitrophenylhydrazine (2 g.), dissolved in a mixture of glacial acetic acid (5 c.c.) and water (20 c.c.). After 24 hours the precipitate (1·0 g.) was recrystallised three times from ethyl alcohol; it then had m. p. 221°, alone or mixed with authentic pyruvic acid *p*-nitrophenylhydrazone (m. p. 221°) (Found, by micro-analysis : C, 49·0; H, 4·2. Calc. : C, 48·4; H, 4·0%).

Fermentation of Lactic Acid. Isolation of Oxalic Acid.—In the previous experiment, the mixture of mycelium and traces of insoluble matter which remained when the calcium lactate medium had been filtered was washed with water and extracted several times with warm dilute hydrochloric acid. The combined extracts were filtered, neutralised exactly with ammonia, acidified with acetic acid, and again filtered. This procedure removed all the inorganic constituents of solution M which are soluble in acetic acid. The small residue on the filter, presumably calcium oxalate, was dissolved in a little dilute hydrochloric acid, and the solution was neutralised with ammonia and acidified with acetic acid; the small amount of white precipitate obtained reduced potassium permanganate in dilute acidified solution (Found : CaO, 44·0. Calc. : CaO, 43·75%).

Fermentation of Pyruvic Acid. Detection of Ethyl Alcohol.—Pure sterile calcium carbonate (1 mol.) in sterile water was treated with freshly fractionated pyruvic acid (2 mols.). When neutralisation was completed the solution was diluted with a sterile aqueous solution of the necessary inorganic salts to give 1000 c.c. of medium containing 10 g. of pyruvic acid (as calcium salt). It was inoculated and incubated at 26° for 28 days; a test portion then yielded a distillate, which gave a strong iodoform reaction but contained no acetaldehyde, since the test with piperidine and sodium nitroprusside (Rimini, *Z. anal. Chem.*, 1904, **43**, 517) and other colour reactions were negative. The whole culture was then very carefully distilled and redistilled. The second distillate (100 c.c.) was treated with sodium dichromate (30 g.) and concentrated sulphuric acid (15 c.c.), and the mixture heated cautiously. The vapours were passed through two spiral-condensers and the distillate (30 c.c.) was collected in a receiver at 0°. A small portion of the distillate, when treated with a few drops of a dilute aqueous solution of piperidine and 0·25 c.c. of a very dilute aqueous solution of sodium nitroprusside, gave the distinct blue coloration characteristic of acetaldehyde (*loc. cit.*). The remainder of the distillate was treated with 1 g. of 5 : 5-dimethyl*cyclo*hexane-1 : 3-dione (dimethyldihydroresorcinol) (Neuberg and Reinfurth, *Biochem. Z.*, 1920, **106**, 286) dissolved in a little ethyl alcohol (free from acetaldehyde), and the resulting clear solution was kept at room temperature. The crystals that had separated after 48 hours were washed with cold water, dried in a vacuum, and dissolved in ethyl alcohol (10 c.c.); the solution was poured into cold water (200 c.c.). After repetition of this procedure and drying, the substance had m. p. 140° and did not depress the m. p. (140°) of an authentic specimen of aldomedon. Yield, 0·31 g. (Found : C, 70·4; H, 8·4. Calc. : C, 70·6; H, 8·5%).

Fermentation of Lactic Acid. Detection of Ethyl Alcohol.—A sterile solution of 50 g. of lactic acid (as calcium salt) in 2500 c.c. of solution M was inoculated and incubated at 26° for 28 days. The whole of the solution was then distilled, and the distillate oxidised as described above. A portion of the distillate from the oxidation mixture gave a positive colour test for acetaldehyde by Rimini's method (*loc. cit.*). Treatment of the rest of the distillate with dimethyldihydroresorcinol gave a very small amount of a crystalline precipitate, insufficient for analysis. It had m. p. 138·5° when alone and 139° when in admixture with an authentic specimen of aldomedon (m. p. 140°).

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