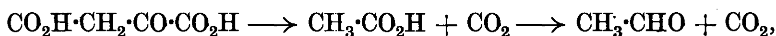
CCCXXX.—*The Mechanism of the Degradation of Fatty Acids by Mould Fungi. Part IV.*

By Vira Subramaniam, Howard Braithwaite Stent, and Thomas Kennedy Walker.

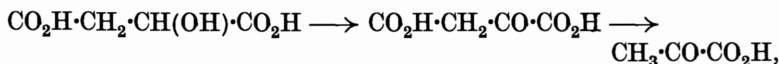
In continuation of the investigations detailed in Part III (this vol., p. 1987) further study has been made of the manner in which succinic acid is degraded by the enzymes of *Aspergillus niger*; and it has been found that the formation of malic acid in cultures on calcium succinate media is followed, after an interval of 24—72 hours, by the simultaneous appearance therein of malonic and pyruvic acids, both of which have been characterised as derivatives. The intimate chemical and biological relationships subsisting between malic, fumaric, and oxaloacetic acids have been emphasised by a number of investigations (compare Mayer, *Biochem. Z.*, 1913, **50**, 283; Neuberg and Gorr, *ibid.*, 1924, **154**, 495; Raistrick and Clark, *Biochem. J.*, 1919, **13**, 329; Blanchetière, *Ann. Inst. Past.*, 1920, **34**, 392; Aubel, *Compt. rend.*, 1921, **173**, 179; Quastel, *Biochem. J.*, 1924, **18**, 365). Moreover, Neuberg and Karczag (*Biochem. Z.*, 1911, **36**, 68) found that under the influence of the enzyme carboxylase oxaloacetic acid undergoes the degradation



$$CO_2H{\cdot}CH_2{\cdot}CO{\cdot}CO_2H \longrightarrow CH_3{\cdot}CO_2H + CO_2 \longrightarrow CH_3{\cdot}CHO + CO_2,$$

and Walker and Coppock (J., 1928, 805) have shown that *A. niger* secretes carboxylase. Hence it is inferred that oxaloacetic acid is the immediate precursor of the pyruvic acid detected in the present case. The existence of the former in the culture media must be regarded as transitory, since means were not found to characterise it, an experience in accordance with that of Quastel (*loc. cit.*), who failed to detect this acid during the conversion of fumaric acid into pyruvic acid by *Bacillus pyocyaneus*, although, in this case also, its intermediate formation cannot be doubted.

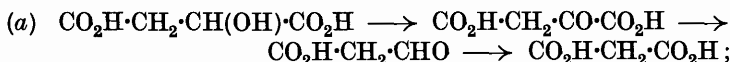
Quastel and Whetham (*ibid.*, p. 519) have suggested that pyruvic acid can be formed from malic acid by bacterial agency through the intermediate production of fumaric acid, followed by that of oxaloacetic acid. They consider that it is unlikely to arise by the more direct transformations



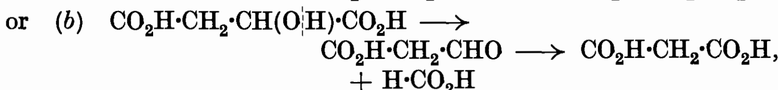
$$CO_2H\cdot CH_2\cdot CH(OH)\cdot CO_2H \longrightarrow CO_2H\cdot CH_2\cdot CO\cdot CO_2H \longrightarrow CH_3\cdot CO\cdot CO_2H,$$

since they found malic acid to be incapable of donating hydrogen to methylene-blue in the presence of non-proliferating *B. coli*. More recently, however, Hahn and Haarmann (*Z. Biol.*, 1929, **88**, 587) have shown that dehydrogenation of malic acid occurs in the presence of washed muscle tissue and methylene-blue, which proves that the inability of malic acid to donate hydrogen is not general. They isolated the intermediate compound, oxaloacetic acid, in the form of its semicarbazone. On this evidence, together with the facts that the pyruvic acid formed in our cultures occurred in relatively substantial yields following malic acid, whilst fumaric acid was not present in sufficient amounts to be detected, we hold the opinion that the pyruvic acid obtained by us arose *via* direct dehydrogenation of malic acid to oxaloacetic acid.

Two views are also permissible as to the precise mechanism by which the malonic acid is formed in the cultures. The stages may be :



$$(a)\quad CO_2H\cdot CH_2\cdot CH(OH)\cdot CO_2H \longrightarrow CO_2H\cdot CH_2\cdot CO\cdot CO_2H \longrightarrow CO_2H\cdot CH_2\cdot CHO \longrightarrow CO_2H\cdot CH_2\cdot CO_2H;$$



$$\text{or}\quad (b)\quad CO_2H\cdot CH_2\cdot CH(O|H)\cdot CO_2H \longrightarrow CO_2H\cdot CH_2\cdot CHO + H\cdot CO_2H \longrightarrow CO_2H\cdot CH_2\cdot CO_2H,$$

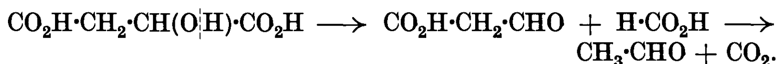
fission of malic acid being the first step.

Scheme (*b*) has already been put forward by Aubel (*loc. cit.*) to account for the occurrence of malonic and formic acids in cultures of *B. pyocyaneus* on asparagine, in addition to the formation therein of malic acid. There is still less experimental evidence for or against scheme (*a*), since information as to whether oxaloacetic acid can undergo biological decarboxylation in the α-position as readily as in the β-position is lacking. Wieland (*Annalen*, 1924, **436**, 229) considers that oxaloacetic acid is oxidised to malonic acid by hydrogen peroxide.

Pyruvic acid is now recognised to be an essential reactant in numerous fermentation phenomena, oxaloacetic acid being but one of its known precursors, and its probable function as a particularly suitable source of nutrition has been discussed by Quastel (*Biochem J.*, 1925, **19**, 641).

On the contrary, the formation of malonic acid by micro-organisms has been recorded in a few cases only (Blanchetière, *loc. cit.*; von Lippmann, *Ber.*, 1920, **53**, 2070; Challenger, Subramaniam, and Walker, J., 1927, 3044) and, so far as we are aware, in no previous instance has the mycological production of either this acid or pyruvic acid, from succinic acid, been directly demonstrated.

Although much work has already been carried out on the mechanism of the oxidation of succinic acid *in vitro*, no substances intermediate between it and acetaldehyde, which is the first product to be recognised, have hitherto been detected. In view of the objects of the present research it was necessary to reinvestigate this problem. Fenton (J., 1900, **77**, 77) found succinic acid to be unchanged after contact for 30 minutes with a 6% solution of hydrogen peroxide and a ferrous salt in a freezing mixture, whereas under similar conditions malic acid yielded oxaloacetic acid. Further oxidation of the last at 0° afforded first dihydroxymaleic acid and secondly mesoxalic acid semialdehyde. From malic acid and the reagent at biologically normal temperatures, Fenton obtained carbon dioxide and pyruvic acid. Neuberg (*Biochem. Z.*, 1914, **67**, 59) obtained acetaldehyde by treatment of succinic acid with 1·2 mols. of hydrogen peroxide and a ferrous salt at 40°, and found that under similar conditions the other four-carbon dibasic acids of this group gave the same product, though in smaller yields. His data fail to show which of these acids is the immediate precursor of the aldehyde arising from succinic acid. Wieland (*Annalen*, 1924, **436**, 229) oxidised the same acids with a large excess (80 mols. : 1 mol.) of hydrogen peroxide at 100°. Malic acid gave more acetaldehyde than did fumaric acid, and oxaloacetic acid gave none, whence he concluded that the aldehyde is derived according to the scheme



$$CO_2H\cdot CH_2\cdot CH(O|H)\cdot CO_2H \longrightarrow CO_2H\cdot CH_2\cdot CHO + H\cdot CO_2H \longrightarrow CH_3\cdot CHO + CO_2.$$

In our opinion the yield of acetaldehyde is scarcely a sound criterion on which to base a view of the mechanism of the oxidation, since such a comparison does not take into account the relative reactivities of the acids to hydrogen peroxide.

After many attempts we have established what appears to be the first stage of the degradation of succinic acid by hydrogen peroxide, namely, oxidation to malic acid. Malonic acid also has been isolated from the reaction products. The method employed was a modification of Fenton's process and involved the use of a relatively large concentration of ferrous salt at 30—37°. The malonic acid was separated as its insoluble compound with mercuric acetate (Denigès, *Ann. Chim.*, 1907, **12**, 402) and the malic acid was then

characterised by oxidation in the presence of mercuric acetate, whereby an insoluble basic mercury salt of oxaloacetic acid was obtained. This procedure gave better results than the action of hydrogen peroxide on an ammoniacal solution of ammonium succinate, although application of the latter method of oxidation to malic acid afforded a relatively high yield of malonic acid. During the course of these experiments tartaric acid was sought amongst the products, but was not detected. It is unlikely, therefore, that fumaric acid is the first product of the action of hydrogen peroxide on succinic acid, since, were it formed, it might suffer conversion into tartaric acid in a manner similar to the conversion of crotonic acid into αβ-dihydroxybutyric acid by hydrogen peroxide as experimentally demonstrated by Wieland (*loc. cit.*).

The results recorded here and in previous papers of this series supply ample proof that the degradations undergone by the lower fatty acids, when attacked by hydrogen peroxide in the one case and by *A. niger* in the other, proceed by almost identical stages, that is to say, the mould operates principally by a series of peroxidase reactions (compare Harrison and Thurlow, *Biochem. J.*, 1926, **20**, 227).

On the other hand, the recent comprehensive work of Kühnau (*Biochem. Z.*, 1928, **200**, 29), of Hahn and Haarmann (*loc. cit.*), and of Hahn, Fischbach, and Haarmann (*ibid.*, p. 516) illustrates very clearly the close parallels which exist between a number of degradative processes undergone by fatty acids in the animal body and processes which we have found to occur when the same acids are subjected to the attack of the mould; hence this secretes certain enzymes (succino-dehydrogenase, fumarase, and β-carboxylase) common to animal tissues.

It can be concluded, therefore, that *A. niger* may be regarded as a reagent capable in certain instances of affording helpful information in studies of catabolism, and it was with the object of testing its applicability in this direction that the present investigations were undertaken. In work of this type the use of the mould has advantage in some cases over that of hydrogen peroxide, since the former operates under milder conditions and, generally, at a slower rate. For these reasons intermediate stages can sometimes be established in reactions induced by *A. niger* when this is not possible with hydrogen peroxide as the reagent. In illustration of this final point may be cited the production of relatively large quantities of lactic and β-hydroxyvaleric acids in cultures of *A. niger* on the corresponding saturated fatty acids (compare Parts I and II).

EXPERIMENTAL.

Enzymic Conversion of Succinic Acid into a Mixture of Malonic and Pyruvic Acids.—2000 C.c. of a sterile saturated solution of calcium succinate in medium M (as used in the experiments described in Part III, *loc. cit.*) were inoculated with a vigorous sporulating culture of *A. niger* (of the strain used previously, Parts I—III) and incubated, samples being withdrawn for examination daily after the mycelium had developed. Malic acid was detected after 13 days. On the 17th day a sample gave a strong red coloration on treatment with benzenediazonium chloride and sodium acetate, and a separate sample gave a yellow faint precipitate with a solution of 2 : 4-dinitrophenylhydrazine hydrochloride. The whole of the solution was filtered, strongly cooled, and treated with a solution containing the diazonium salt from 17 g. of aniline, followed by excess of sodium acetate. Separation of a red product commenced immediately. This was removed at intervals (to avoid decomposition) and washed with cold water. A small quantity of the dried substance added to concentrated sulphuric acid gave a deep bluish-green coloration, and a trace of the substance added to concentrated hydrochloric acid produced a colour similar to that of a dilute aqueous solution of potassium permanganate; this shade soon changed to ruby-red. These reactions are characteristic of hydrogen formazyl. The product, after crystallising several times from aqueous methyl alcohol and then from benzene–light petroleum, melted at 117—118°, alone or in admixture with authentic hydrogen formazyl prepared from malonic acid and benzenediazonium acetate (Found: C, 69·2; H, 5·4. Calc.: C, 69·6; H, 5·35%). The yield (6·1 g.) corresponds to the presence of 2·8 g. of malonic acid in the culture solution.

In another experiment separate samples taken on the 16th day from a culture on 2500 c.c. of calcium succinate medium gave a strong coloration with benzenediazonium acetate and an immediate yellow precipitate with a solution of 2 : 4-dinitrophenylhydrazine hydrochloride, respectively. The contents of the flask were filtered and divided into portions: (*a*) 500 c.c. were treated with 1000 c.c. of 2 : 4-dinitrophenylhydrazine hydrochloride solution containing 4 g. of this reagent and gave 2·5 g. of precipitate; (*b*) 500 c.c. were treated with a solution of 2 g. of *p*-nitrophenylhydrazine in a mixture of 5 c.c. of alcohol and 5 c.c. of glacial acetic acid and yielded 1·4 g. of precipitate; (*c*) 400 c.c. were treated with 4 g. of phenylhydrazine dissolved in a little acetic acid and yielded 3·1 g. of derivative; (*d*) 400 c.c. were treated with a solution of 4 g. of aminoguanidine nitrate in a little water and deposited 1·9 g. of precipitate; (*e*) the residual culture solution (600 c.c.) was treated with a solution of

benzenediazonium acetate prepared from 7 g. of aniline and yielded 3·95 g. of hydrogen formazyl. Derivative (*a*) was recrystallised several times from alcohol; it then melted at 221°, alone or mixed with a genuine specimen of the 2 : 4-dinitrophenylhydrazone of pyruvic acid, m. p. 221—222°. Derivative (*b*) after recrystallisation from alcohol had m. p. 229—230° (decomp.), alone or in admixture with the *p*-nitrophenylhydrazone of pyruvic acid, m. p. 228° (decomp.). Derivative (*c*) on purification melted at 191° (decomp.), alone or mixed with the phenylhydrazone of pyruvic acid (Found by micro-analysis: C, 60·7; H, 5·7. Calc.: C, 60·7; H, 5·6%). Derivative (*d*) was recrystallised several times from large quantities of hot water. It did not melt at 350° and was identical in physical properties with the aminoguanidine nitrate derivative of pyruvic acid.

Oxidation of Succinic Acid by Hydrogen Peroxide in the Presence of Ferrous Sulphate. Detection of Malic Acid and of Malonic Acid.— (A) Ferrous sulphate heptahydrate (32 g.) and concentrated sulphuric acid (4 c.c.) were brought into solution in 200 c.c. of water at 30°. (B) Succinic acid (20 g.; 1 mol.) and 6% hydrogen peroxide (64 c.c.; 1 atom of oxygen) were dissolved in 800 c.c. of water at 30°. (A) was added to (B) with vigorous agitation. The temperature rose immediately to 37°, the solution assumed a port-wine colour, and after 20 minutes no hydrogen peroxide remained. The mixture was heated almost to boiling; an odour of acetaldehyde was then observed and sufficient caustic soda was added to precipitate the iron as hydroxide. The cooled, filtered, pale yellow solution, which was free from iron, was acidified with acetic acid, treated with one-tenth of its volume of a 5% solution of mercuric acetate in dilute acetic acid, and boiled. The white precipitate (*X*) formed was removed after 5 hours' standing and the filtrate, containing mercuric acetate and, presumably, malic acid, was oxidised, 50 c.c. at a time, by a 2% aqueous solution of potassium permanganate which was added drop by drop, at 100°; a greyish mercury compound, presumably that of oxaloacetic acid, separated. These precipitates were combined, filtered off when cold, and washed free from acetic acid. The total quantity was mixed with a 20% aqueous solution of sodium iodide and cautiously distilled, the distillate being collected in a cooled receiver. One half (*a*) of the distillate was mixed with a solution of dimethyldihydroresorcinol (1 g.) in 25 c.c. of ethyl alcohol free from acetaldehyde, and the other half (*b*) was treated with 150 c.c. of a solution of 2 : 4-dinitrophenylhydrazine hydrochloride. After 12 hours, solution (*a*) was poured into 200 c.c. of cold water; a white crystalline precipitate (0·3 g.) was then deposited. This was redissolved in alcohol and poured

again into 200 c.c. of water; after drying, the substance then had m. p. 140—141°, alone or in admixture with an authentic specimen of aldomedon, m. p. 140—141° (Found by micro-analysis: C, 70·6; H, 8·3. Calc.: C, 70·6; H, 8·5%). Solution (*b*) rapidly deposited orange-coloured crystals (0·2 g.), which were washed and recrystallised twice from ethyl alcohol; they then had m. p. 162°, alone or mixed with an authentic specimen of acetaldehyde 2 : 4-dinitrophenylhydrazone, m. p. 161—162°. The degradation of the mercury compound of oxaloacetic acid to acetaldehyde in the above manner has been demonstrated in previous work (compare Challenger and Klein, this vol., p. 1644; see also Part III, *loc. cit.*).

The white precipitate (*X*) of a mercury compound, which had been removed previously by filtration from the mother-liquor containing the malic acid, was dried, suspended in ether, and the mercury removed by hydrogen sulphide. The ethereal solution was extracted with a slight excess of a saturated solution of sodium carbonate. The alkaline extracts were neutralised with acetic acid and evaporated and the residue was dried in a vacuum. It was finely powdered, intimately mixed with 25 drops of freshly distilled cinnamaldehyde and 3 c.c. of glacial acetic acid, and the whole heated in a sealed tube at 100° for 10 hours. The mixture was dissolved in a little water and treated with excess of sodium carbonate, and unchanged cinnamaldehyde extracted by ether. The alkaline solution was filtered and acidified with hydrochloric acid; a very small quantity of a lemon-yellow precipitate was then obtained. After drying, this had m. p. 204—206° (decomp.). Cinnamylidenemalonic acid melts at 206—208°. There was insufficient material for recrystallisation. The substance became white superficially on exposure to light, a property of cinnamylidenemalonic acid.

After further experiments had been made to determine the optimum conditions for the formation of malonic acid by oxidation of succinic acid, the following relative proportions of reagents were employed: succinic acid : hydrogen peroxide : ferrous ion = 1 : 3 : 0·14. Appropriate quantities of these in the necessary volumes of water were mixed and the subsequent formation of malonic acid was detected by benzenediazonium acetate. After removal of iron as ferric hydroxide the reaction mixture was treated as in the previous case. The sodium carbonate extract of the product, presumably containing sodium malonate, was evaporated and 0·8 g. of the dry residue was powdered finely and treated with cinnamaldehyde as before. The condensation product (0·12 g.), when purified from alcohol, had m. p. 208° (decomp.), alone or mixed with authentic cinnamylidenemalonic acid of m. p. 208° (decomp.) (0·1120 g. required 20·4 c.c. of *N*/20-NaOH. Calc., 20·30 c.c.).

Oxidation of Malic Acid by Hydrogen Peroxide in the Presence of Ammonia. Isolation of Malonic Acid.—The oxidation mixture (ammoniacal hydrogen peroxide) employed in the following experiment is similar to that used for the oxidation of butyric acid by Weitzmann (*J. Biol. Chem.*, 1921, **49**, 123). *dl*-Malic acid (5 g.) was dissolved in a 20-volume (6%) solution of hydrogen peroxide (100 c.c.), and the liquid was made just alkaline with concentrated aqueous ammonia and diluted to 150 c.c. with water. The whole was kept at 20° and samples were tested periodically at —2° by means of a solution of benzenediazonium acetate. After 6 hours, malonic acid was detected by the red coloration thus produced. The quantity increased and later samples yielded precipitates of hydrogen formazyl. After 30 hours, the formation of the latter reached a maximum, whereupon the bulk of the reaction mixture was shaken with animal charcoal (5 g.) to remove the undecomposed hydrogen peroxide still present. The solution was filtered, boiled, and treated with excess of calcium chloride solution. After concentration to 100 c.c. and subsequent standing for 3 days, the white crystalline precipitate (5·25 g.) was collected, washed with water, and dried in a vacuum [Found: Ca, 21·7. Calc. for $CH_2(CO{\cdot}O)_2Ca,2H_2O$: Ca, 22·3%]. A quantity of this calcium salt (1·5 g.) was heated in a sealed tube with glacial acetic acid (5 c.c.) and freshly distilled cinnamaldehyde (30 drops) at 100° for 10 hours. The product was purified as detailed in a previous case and yielded 0·32 g., which melted at 208° (decomp.), alone or mixed with authentic cinnamylidenemalonic acid of m. p. 208° (decomp.) (0·1510 g. required 27·4 c.c. of *N*/20-NaOH. Calc., 27·7 c.c.).

The original *Aspergillus niger* strain employed in the work which has formed the subject of the present series of papers was kindly presented to us by Dr. C. Neuberg of Berlin-Dahlem. It has also been used in investigations on the mechanism of citric acid formation (J., 1927, 200, 3044). Cultures have been lodged at the National Collection of Type Cultures, Lister Institute, and a complete report on the mould will be published shortly in a paper by Dr. F. Challenger and one of us.

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