CCLIX.—*The Mechanism of the Degradation of Fatty Acids by Mould Fungi. Part III.*

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In Part II of this series (J., 1928, 1422) it was shown that the cultivation of *Aspergillus niger* on calcium *n*-butyrate results in β-oxidation of the latter and production of acetone. We have now examined cultures of the mould on calcium *n*-butyrate for the presence of non-volatile products, and have obtained small yields of succinic acid. The latter may arise either (i) by oxidation of the butyric acid at the γ-carbon atom, or (ii) by oxidation at the β-carbon atom with ultimate formation of acetic acid, followed by dehydrogenation of the latter according to the Thunberg–Wieland hypothesis. The experiments of Raper (*Biochem. J.*, 1914, **8**, 320), Cahen and Hurtley (*ibid.*, 1917, **11**, 164), and Clutterbuck and Raper (*ibid.*, 1925, **19**, 385, 911; 1926, **20**, 59) on the oxidation of fatty acids by hydrogen peroxide support mechanism (i), but (ii) receives support from the work of Moritz and Wolffenstein (*Ber.*, 1899, **32**, 2534), of Kühnau (*Biochem. Z.*, 1928, **200**, 29), and of Wishart (*Biochem. J.*, 1923, **17**, 103). The last found that acetic acid yields hydrogen to methylene-blue in the presence of liver tissue. Moreover, Takahashi and Asai (*Bull. Agric. Chem. Soc. Japan*, 1928, 113) and Butkewitsch and Fedoroff (*Biochem. Z.*, 1929, **207**, 302) have shown that *Rhizopus* species produce succinic acid when placed in media containing salts of acetic acid as sole source of carbon. It may be pointed out that it is not essential to assume the presence of a true dehydrogenating enzyme in *A. niger* in order to account for the dehydrogenation of acetic acid to succinic acid, since Knoop and Gehrke (*Z. physiol. Chem.*, 1925, **146**, 63) obtained succinic acid in small yield by treatment of acetone with hydrogen peroxide at 37°. We consider that the only plausible explanation of the latter conversion is that shown by the scheme

$$3CH_3\cdot CO\cdot CH_3 \longrightarrow CH_3\cdot CO\cdot CO_2H + 2CH_3\cdot CO_2H \longrightarrow CH_3\cdot CH(OH)\cdot CO_2H + CO_2H\cdot CH_2\cdot CH_2\cdot CO_2H$$

and the result indicates that under certain conditions hydrogen peroxide can function as a dehydrase system. Since we have shown that the mould produces acetone from calcium butyrate by what is evidently a peroxide oxidation, the necessary conditions are present for the formation of succinic acid according to Knoop and Gehrke's process; and such a method of formation seems as probable as that postulated under (i) above.

The mould grew rapidly when introduced into a solution of succinic acid half neutralised with calcium carbonate, but no products of degradation could be detected. When, however, the normal calcium salt was employed it was possible to detect the presence of malic acid in the media, usually on the 12th to the 15th day after inoculation. At these times such cultures were proved to possess slight lævorotatory properties. In subsequent experiments the malic acid was isolated, and in every case it was found to consist of a mixture of the *dl*- and the *l*-form. It was established by a control experiment that *l*-malic acid is not racemised by the treatment adopted for the extraction of the malic acid from the culture solutions; hence the *dl*-malic acid produced in our media had not arisen by purely chemical treatment of preformed *l*-malic acid. Neither have we found any evidence that under enzymic influence racemisation of either the *d*- or the *l*-acid occurs. Consequently, it is to be inferred that the *dl*-malic acid owed its formation to the direct hydroxylation of succinic acid, a process which presumably could be effected by an aerobic oxydase system.

It remained to account for the formation of the *l*-malic acid in the calcium succinate cultures, and this could be brought about either (*a*) from *dl*-malic acid owing to more rapid utilisation by the mould of the *d*-form than of the *l*-form, or (*b*) by asymmetric addition of water to fumaric acid produced by dehydrogenation of succinic acid. The observation of McKenzie and Harden (J., 1903, **83**, 424) that a strain of *A. niger* used by them decomposed *d*-malic acid more rapidly than the *l*-isomeride when grown on the inactive acid appeared to support view (*a*); it was found, however, that the strain employed in our work behaved in the reverse manner, for the media developed dextrorotatory properties when the mould was cultivated on the racemic acid. In order to obtain further data on this point, a different strain of *A. niger*, kindly supplied by Dr. C. Neuberg of Berlin-Dahlem, was then employed, and this exhibited a somewhat stronger preference for the *l*-isomeride than did the original strain. Such a marked difference in the behaviour of mould fungi species is not unusual, however, since McKenzie and Harden (*loc. cit.*) noted the preferential utilisation of the *l*-isomeride when *A. griseus* was cultivated on *dl*-malic acid.

In two of the experiments on the action of *A. niger* on *dl*-malic acid the formation of fumaric acid was noted. This was isolated and characterised. Challenger and Klein (this vol., p. 1644) have shown recently that the strain of *A. niger* used by us affords excellent yields exclusively of *l*-malic acid when cultivated on a fumarate medium. Hence *A. niger* secretes the enzyme fumarase. The mycological conversion of fumaric acid to *l*-malic acid has been observed previously by Takahashi and Sakaguchi (*Bull. Agric. Chem. Soc. Japan*, 1927, **3**, 59) in experiments with a *Rhizopus* species, and they also noted that the same mould effected the reverse change. The same equilibrium is set up between fumaric and *l*-malic acids under other biological conditions in which fumarase is present (compare Dakin, *J. Biol. Chem.*,1920, **50**, 1; 1922, **52**, 183; 1924, **59**, 11; Quastel and co-workers, *Biochem. J.*, 1924, **18**, 365, 519; 1925, **19**, 304; Clutterbuck, *ibid.*, 1927, **21**, 512; 1928, **22**, 1193; Alwall, *Scand. Arch. Physiol.*, 1928, **54**, 11).

To sum up, it would appear probable that, whilst the development of dextrorotatory properties in cultures of *A. niger* on solutions of *dl*-malic acid was due partly to the conversion of a portion of the *l*-isomeride to fumaric acid, it was occasioned also by the preferential utilisation of this isomeride for protoplasmic synthesis. Hence, it follows that the *l*-malic acid, which was produced when the mould was grown on calcium succinate media, must have arisen through preliminary dehydrogenation of the substrate to fumaric acid (scheme *b*).

The production of inactive malic acid from succinic acid in one stage by *A. niger*, as now described, appears to be the first recorded instance in which this change has been experimentally demonstrated by biological means, and it is of interest in view of the comparatively few cases known in which a racemic compound may be isolated from the products of a living organism.

EXPERIMENTAL.

The strain of *A. niger* employed was that used in the work recorded in Parts I and II of this series, and the aqueous solution of inorganic salts (solution M) used in the media, unless otherwise stated, was of the same composition as that used previously. In all experiments the mould was cultivated at 28°.

Enzymic Conversion of n-*Butyric Acid to Succinic Acid.*—A sterilised solution of 25 g. of pure *n*-butyric acid, as calcium salt, in 2500 c.c. of solution M was inoculated from test-tube cultures on the same medium. After 18 days the contents of the flask were filtered from the scanty mycelium, concentrated to small bulk, acidified with phosphoric acid, and thoroughly distilled in steam. The distillate

contained only butyric acid. The residual liquid was treated with ether in a continuous-extraction apparatus, and evaporation of the ethereal extract yielded crystals (0·1 g.). These were drained on a tile, washed with a few drops of ether, and dried in a vacuum; m. p. 184·5°, raised to 185° in admixture with authentic succinic acid (m. p. 185°). In a similar experiment in which the mould was cultivated on a medium containing 70 g. of calcium *n*-butyrate, the solution after 21 days was worked up as before and yielded 0·32 g. of crude solid material, presumably succinic acid. This was converted to the normal sodium salt, and thence to the di-*p*-nitrobenzyl ester (Lyman and Reid, *J. Amer. Chem. Soc.*, 1917, **39**, 708); the resultant ester, when recrystallised four times from aqueous alcohol, had m. p. 90°; mixed m. p. with authentic di-*p*-nitrobenzyl succinate (m. p. 88·6°), 89°.

In a control experiment, 20 g. of the same sample of calcium *n*-butyrate as that employed for the cultures were dissolved in solution M, the liquid was concentrated, treated with phosphoric acid, and submitted to distillation in steam for several hours. The residual liquid contained no trace of succinic acid.

Enzymic Conversion of Succinic Acid to dl-*Malic and to* l-*Malic Acid.*—To a nearly saturated solution of calcium succinate in 1800 c.c. of cold tap-water was added a concentrated solution of the requisite amounts of the necessary inorganic salts, and the whole was diluted to 2000 c.c. with tap-water and sterilised at 100° for a few minutes on each of three successive days. This medium contained approximately 0·7% of calcium succinate. The solution was inoculated by the addition of 100 c.c. of a vigorous sporulated culture of *A. niger* on the same medium. Growth was rapid and after 4 days the thick mycelium showed spore formation, whereupon portions of the solution were withdrawn daily and tested for the presence of malic acid by Denigès's mercuric acetate reagent (*Compt. rend.*, 1900, **130**, 34). On the 13th day, 10 c.c. of culture liquid were mixed with 2 c.c. of this reagent, heated to boiling, filtered hot from a slight cloudy precipitate, and the filtrate was oxidised at the boiling point by the cautious addition of a 2% aqueous solution of potassium permanganate. The greyish-white precipitate formed was washed by decantation, filtered off, and treated with hydrochloric acid; the odour of acetaldehyde was observed and the resultant liquid gave a positive reaction for pyruvic acid when treated with an alcoholic solution of guaiacol and strong sulphuric acid, a very delicate test (Quastel, *Biochem. J.*, 1924, **18**, 371). Confirmatory tests showed that the mercury compound obtained by oxidation of authentic malic acid in presence of mercuric acetate exhibited similar behaviour. The main bulk of the culture solution

was then filtered and oxidised in presence of Denigès's reagent, and the mercury compound (6 g.) so obtained was washed, drained, and cautiously distilled with a solution of sodium iodide (7 g.) in water (50 c.c.). The distillate (10 c.c.) was collected below the surface of a solution of 2 : 4-dinitrophenylhydrazine hydrochloride (Brady and Elsmie, *Analyst*, 1926, **51**, 77). An orange-coloured precipitate (0·2 g.) was obtained immediately, and after being washed with water was recrystallised several times from hot ethyl alcohol; its m. p. was then 162° alone or in admixture with a pure specimen of acetaldehyde-2 : 4-dinitrophenylhydrazone. A quantity of the mercury compound obtained by oxidation of authentic malic acid in presence of mercuric acetate also yielded acetaldehyde when heated with aqueous sodium iodide.

In a subsequent experiment the presence of malic acid was detected in the culture after 12 days and was isolated as follows: 4000 C.c. of medium were filtered and concentrated on the water-bath under reduced pressure until solid matter commenced to separate; a little hot water was then added and the clear saturated solution was treated with lead acetate (118 g., hydrated) in water (600 c.c.), after which the mixture was made just alkaline with ammonia, and alcohol was added until it constituted one-third of the total volume. After standing over-night, the lead salt which had separated was removed, triturated with cold water, and washed by decantation. It was then suspended in water, the lead precipitated by hydrogen sulphide, and the filtrate boiled under reduced pressure and concentrated to 25 c.c. On standing over-night practically all the succinic acid present crystallised and was removed. In order to free the malic acid present in the syrupy mother-liquor from the last traces of succinic acid, the liquid was treated with ether in a continuous-extraction apparatus, whereby the two acids were removed from the water layer at different rates. Colourless, slightly deliquescent crystals (0·8 g.) were thus obtained, and, after drying, these melted at 116°, and at 120° in admixture with authentic *dl*-malic acid (m. p. 129—130°). The crystals were divided into two portions, (A) and (B). Portion (A) was analysed without further purification (Found, by micro-method: C, 35·7; H, 4·4. Calc.: C, 35·8; H, 4·5%); a further small quantity (0·111 g.) of portion (A) was found to contain 45% of *l*-malic acid by examination in the polarimeter in the presence of uranium acetate.

Portion (B) was recrystallised three times from ether–light petroleum; a heavy loss of material occurred, and the residue melted at 129° alone or mixed with authentic *dl*-malic acid (m. p. 129—130°) (Found: equiv., 67·0, 66·4. Calc.: 67·0. Found, by micro-analysis: C, 35·6; H, 4·5. Calc.: C, 35·8; H, 4·5%). A small

quantity (0·2 g.) of this purified material (m. p. 129—130°) was converted to the di-*p*-nitrobenzyl ester by boiling the sodium salt with an aqueous-alcoholic solution of *p*-nitrobenzyl bromide; the ester after purification had m. p. 107° and did not depress the m. p. (108—109°) of an authentic specimen of di-*p*-nitrobenzyl *dl*-malate.

The above data were confirmed by the results of three more fermentations of succinic acid in each of which 2000 c.c. of culture medium were submitted to the action of the mould for 15 days. Malic acid was isolated in every case, and by m. p. determination followed by analysis of the three specimens, which all melted at different temperatures, it was proved to consist in each case of a mixture of the *dl*- and the *l*-form. From an m. p. curve based upon the m. p.'s of known mixtures of authentic specimens of the *dl*- and *l*-acids, the approximate compositions of the mixtures obtained by fermentation were ascertained. The data of these three experiments were :

Expt.	Yield (g.) from 15 g. succinic acid.	M. p.	Found, by micro-method. C, %.	Found, by micro-method. H, %.	Approx. comp. (from m. p. curve). *dl*-, %.	Approx. comp. (from m. p. curve). *l*-, %.
1	1·25	116—117°	35·60	4·31	50	50
2	0·9	117—118	35·70	4·38	55	45
3	1·1	107—108	35·68	4·42	30	70

It is probable that the ratio of *dl*- to *l*-acid as formed in the culture solution is greater than is shown by these figures, since the method of isolation tends to result in the conservation of a larger percentage of the *l*- than of the *dl*-acid.

Development of Optical Activity in Solutions of dl-*Malic Acid fermented by* A. niger. *Isolation of Fumaric Acid.*—A 1% solution of calcium *dl*-malate in medium M was inoculated with spores of the mould. A sample of the solution tested after 7 days showed no optical activity although the mould had grown well. After 14 days, 25 c.c. of the culture were treated with 1·5 c.c. of glacial acetic acid and 10 c.c. of an 8% solution of uranium acetate and filtered. The filtrate, after being kept in the dark during 2 hours and then examined in a 2-dm. tube, was found to be slightly dextrorotatory (+ 0·5°, Ventzke scale). Subsequent small-scale experiments on similar lines, but with a different strain of *A. niger*, confirmed this behaviour, the following results being obtained :

Medium.	Polarimeter reading (Ventzke scale). 7 days.	14 days.
Calcium *dl*-malate, 1%.....	+0·9°	+1·3°
Sodium ,, 1%.....	+1·3	+1·2
Sodium ,, 4%.....	+0·9	+2·9

In a further experiment, in which 1500 c.c. of a 4% solution of sodium *dl*-malate were submitted to the action of the mould, the

medium soon became dextrorotatory, readings (Ventzke scale) after increasing periods being :

Days after inoculation	0	8	11	14	18
Polarimeter reading	0	1·5°	2·3°	3·7°	4·1°
Days after inoculation	20	21	25	36	43
Polarimeter reading	3·5°	3·0°	3·0°	3·7°	5·2°

When it was noted on the 20th day that the reading had decreased, a sample (A) (320 c.c.) of the medium was withdrawn. The fermentation was stopped on the 43rd day and the medium remaining (B) was worked up separately.

Solution (B) yielded with lime-water a white precipitate, which was boiled with dilute sulphuric acid and filtered; the filtrate decolorised both dilute potassium permanganate solution and bromine water in the cold. The bulk of (B) was evaporated to 100 c.c., acidified with syrupy phosphoric acid (20 c.c.), and thoroughly extracted with ether for 2 hours. The ethereal solution was washed with water, and on evaporation yielded white crystals; these were recrystallised from hot water (50 c.c.) (yield, 0·73 g.), and were then found to melt in a sealed tube at 287°, alone or mixed with authentic fumaric acid (0·440 g. required 30·60 c.c. of $N/4$-NaOH. Calc., 30·35 c.c.). A quantity of the acid was converted to the di-*p*-nitrobenzyl ester, m. p. 151°, alone or mixed with the same ester of fumaric acid. In a similar manner a small quantity of fumaric acid was isolated from sample (A).

The solution (B) from which the fumaric acid had been removed was treated with a concentrated solution of barium hydroxide until no further precipitate separated. The barium phosphate was filtered off, the solution evaporated to 120 c.c., and then treated with lead acetate (90 g., hydrated) in water (200 c.c.). Two drops of concentrated ammonia solution were added, followed by ethyl alcohol (250 c.c.), and the precipitated lead salt was collected after two days. From this salt malic acid was isolated in the manner described previously (p. 1991). The quantity recovered was 5·7 g., or 44% of that present originally in a similar volume of solution, and it melted at 115°. Polarimetric examination showed that it contained 12% of the *d*-acid.

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