CCXVI.—*The Formation of* l-*Malic Acid from Fumaric Acid by* Aspergillus niger.

By Frederick Challenger and Louis Klein.

The observation of the present authors with Walker and Subramaniam (*Nature*, 1928, 244) that the growth of *A. niger* on muconic acid gave rise to citric acid suggested that hydroxylation of the two double linkages might first occur. Consequently a study of the action of the mould on simpler unsaturated acids appeared desirable in order to determine its behaviour towards a conjugated ethylene linkage.

The fermentation of potassium fumarate by *A. niger* gave rise to *l*-malic acid, which was characterised by several independent methods. Evidence has been afforded by Walker (unpublished observation) that this reaction is reversible.

The conversion of fumaric acid into *l*-malic acid has been observed in other biochemical processes. Dakin (*J. Biol. Chem.*, 1922, **52**, 183) found that fumaric acid in presence of muscle pulp yielded *l*-malic acid, and Clutterbuck (*Biochem. J.*, 1927, **21**, 512) obtained a similar result with succinic and fumaric acids. The change succinic acid⟶fumaric and malic acids has been effected anaërobically by muscle in presence of methylene-blue (Fischer, *Ber.*, 1927, **60**, 2257), and Butkewitsch and Fedorov (*Biochem. Z.*, 1929, **207**, 302) have shown that the growth of *Mucor stolonifer* on acetic acid gives succinic and fumaric acids. Dakin (*J. Biol. Chem.*, 1924, **61**, 139) observed the formation of *l*-malic acid during alcoholic fermentation, the amount being increased in presence of sodium fumarate.

Fumaric acid is easily converted into *dl*-malic acid by heating with dilute sodium hydroxide solution, or with water at 150° (Lloyd, *Annalen*, 1878, **192**, 80; Weiss and Downs, *J. Amer. Chem. Soc.*, 1922, **44**, 1118).

The production of *l*-malic acid in this research might be explained on the assumption that the *dl*-acid is first formed and the *d*-isomeride is then selectively assimilated by the mould. This is, however, not the case, since Walker and Stent (private communication) have shown that when *A. niger* grows on *dl*-malic acid the liquid culture gradually becomes dextrorotatory. The mould therefore preferentially assimilates the *l*-acid. The formation of *l*-malic acid from fumaric acid must consequently occur by asymmetric addition of water to the double linking. The biological reactions already mentioned are in agreement with this conclusion.

Takahashi and Sakaguchi (*Bull. Agric. Chem. Soc. Japan*, 1927, **3**, No. 4—6, p. 59) have shown that *l*-malic acid is produced from fumaric acid by moulds of the *Rhizopus* species. These moulds produce fumaric acid from sugar, a reaction which has not yet been effected by *A. niger*.

EXPERIMENTAL.

The *A. niger* strain, and the medium containing the inorganic salts, designated " medium M," were the same as those employed by Challenger, Subramaniam, and Walker (J., 1927, 200). The mould was kept in a young virile condition by repeated sub-culturing in tubes of solid medium containing 5% glucose, 1½% agar-agar, and the salts of medium M. The usual precautions were taken to ensure aseptic conditions in working, and the results checked by control experiments on the uninoculated media. The temperature of incubation was 31—32°.

Fermentation of Potassium Fumarate.—Experiment 1. Preliminary experiments having shown that the strain of *A. niger* did not grow well on a 1% solution of potassium fumarate in medium M, we trained the mould to grow on the fumarate by using at first solutions containing 4% of glucose and relatively small amounts (0·5%) of fumarate, and then transferring the spores to solutions poorer in glucose (1%) and richer in fumarate (0·75%) until finally good growth took place on sugar-free fumarate solutions. One litre of 1% potassium fumarate in medium M was inoculated from young test-tube cultures of *A. niger* on the same medium. There was a fairly good growth in 5 days. On the 13th day a positive Denigès's test for malic acid (*Compt. rend.*, 1900, **130**, 34) was obtained, the test being modified as follows : 100 c.c. of the filtered culture were concentrated, treated with a slight excess of hydrochloric acid, and filtered from fumaric acid. Sodium acetate was then added, followed by an equal volume of Denigès's acetic acid solution of mercuric acetate. The clear solution was heated to boiling, and a 2% solution of potassium permanganate added drop by drop; the presence of malic acid was then indicated by the formation of a white precipitate (frequently slightly brown). Denigès assumed that the precipitate thus obtained from malic acid consisted of a basic mercury salt of oxaloacetic acid, and the authors find that, on distillation of the precipitate obtained from authentic malic acid with aqueous potassium iodide, acetaldehyde is evolved and can be characterised as the 2 : 4-dinitrophenylhydrazone. Neglecting mercury salts, the change is doubtless to be represented thus :

$$\mathrm{COOH{\cdot}CH(OH){\cdot}CH_2{\cdot}COOH} \longrightarrow \boxed{\mathrm{COO}}\mathrm{H{\cdot}CO{\cdot}CH_2{\cdot}}\boxed{\mathrm{COO}}\mathrm{H} \longrightarrow \mathrm{CH_3{\cdot}CHO} + 2\mathrm{CO_2}.$$

[Compare also the action of potassium iodide on the similar mercury derivative obtained from citric acid (J., 1927, 200).]

Experiment 2. 1500 C.c. of 1% potassium fumarate in medium M were inoculated from young test-tube cultures of *A. niger* which had been inoculated with a strain from Experiment 1. There was a very good growth in 4 days. On the 12th day a strong positive Denigès's test for malic acid was obtained, so 150 c.c. of the filtered culture were treated with Denigès's reagent and potassium permanganate as described above, and the precipitate was decomposed by distillation with aqueous potassium iodide. The acetaldehyde evolved was identified by conversion into the 2 : 4-dinitrophenylhydrazone (compare Brady and Elsmie, *Analyst*, 1926, **51**, 77) and this after two crystallisations from alcohol had m. p. and mixed m. p. 164°. Another portion of the culture (500 c.c.) was concentrated and precipitated with lead acetate. The lead salt in aqueous suspension was decomposed with hydrogen sulphide, and the filtrate concentrated; a little fumaric acid then separated. The clear liquid on evaporation to a syrup and keeping in a vacuum desiccator gave 1·5 g. of *l*-malic acid (yield, about 40%). One half was converted into the *p*-nitrobenzyl ester, which, after recrystallisation from alcohol and then from alcohol–acetone, melted at 125° alone and in admixture with di-*p*-nitrobenzyl *l*-malate prepared from authentic *l*-malic acid (Lyman and Reid, *J. Amer. Chem.*

Soc., 1917, **39**, 708) (Found by microanalysis: C, 53·4; H, 3·7; N, 6·8. Calc.: C, 53·5; H, 4·0; N, 6·9%). The corresponding dl-*malate*, m. p. 109°, which has not been described, was prepared (Found: C, 53·1; H, 4·5; N, 7·0%).

The rest of the *l*-malic acid, after recrystallisation from dry ether–petroleum, had m. p. 95—100° alone and in admixture with recrystallised Schuchardt's *l*-malic acid (Found by titration: equiv., 67, 66. Calc.: equiv., 67). The *l*-malic acid obtained from a third portion of the culture (400 c.c.) was recrystallised and converted into the cinchonine salt (compare Dakin, *J. Biol. Chem.*, 1924, **59**, 7; **61**, 139), which after recrystallisation from hot water had m. p. and mixed m. p. 197°.

A control flask of 1% potassium fumarate in medium M was kept in the incubator for the same period and at the same time as the flask which had been inoculated, but after concentration and removal of the fumaric acid in the usual way, Denigès's test failed to show the presence of malic acid.

Experiment 3. 1500 C.c. of 1% potassium fumarate in medium M were used and the *l*-malic acid (1·7 g.) was isolated from 1400 c.c. of the culture as already described. After recrystallisation from dry ether–petroleum it had m. p. and mixed m. p. 95—100° (Found: equiv., 68, 68. Calc.: equiv., 67). It also gave a cinchonine salt, m. p. and mixed m. p. 197°. Another portion was examined polarimetrically after its rotation had been enhanced by addition of uranyl acetate (Dakin, *J. Biol. Chem.*, 1924, **59**, 11): 0·1843 g. was neutralised with sodium hydroxide, and one drop of glacial acetic acid added, followed by 0·9215 g. of powdered uranyl acetate. The mixture was then diluted to 50 c.c. After 1 hour the specific rotation was $[\alpha]_D^{21^\circ} = -480^\circ$. Dakin (*loc. cit.*) gives $[\alpha]_D^{18^\circ} = -482^\circ$ for *l*-malic acid similarly treated.

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