XXXVI. THE MECHANISM OF THE FORMATION OF ORGANIC ACIDS BY MOULD FUNGI.

I. THE ACTION OF *ASPERGILLUS NIGER* ON SODIUM HEXOSEDIPHOSPHATE IN THE PRESENCE OF TOLUENE.

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EXAMINATION of the various hypotheses which have been formulated in explanation of the mycological conversion of hexoses into citric acid shows that the chemical changes involved in this transformation have been regarded from at least four different points of view.

Kostytschew and Tschesnokow [1927] advanced the opinion that citric acid is a by-product of protein synthesis from sugar. Raistrick and Clark [1919] considered it possible that a molecule of glucose might by loss of two molecules of water and oxidation at the terminal carbon atoms yield β : δ -diketoadipic acid which, on hydrolysis, should split readily into acetic and oxaloacetic acids. In the view of these authors an aldol condensation between the two latter acids might then be presumed to yield citric acid.

Now the formation of citric acid by mould fungi is usually accompanied or followed by the formation of oxalic acid, and the suggestions of Raistrick and Clark provided a reasonable explanation of this fact, since they supposed oxalic acid to be derived from acetic acid by oxidation and from oxaloacetic acid by hydrolytic fission to acetic acid followed by oxidation. It has been suggested that citric acid might arise *via* compounds of 3 carbon atoms formed by preliminary fission of the sugar molecule [*cf.* Euler, 1909; Amelung, 1927; Chrzęszcz and Tiukow, 1930]. Other views have involved suggestions that the hexose chain may be converted into a molecule of citric acid by intramolecular change preceded or followed by processes of dehydration and/or oxidation [*cf.* Knoop and Windaus, 1905; Buchner and Wüstenfeld, 1909; Butkewitsch, 1924; Franzen and Schmitt, 1925; Challenger, Subramaniam and Walker, 1927].

The last named authors, employing a single strain of *A. niger*, obtained small yields of saccharic acid from cultures on glucose and on calcium gluconate, and also detected citric acid in cultures of this mould on potassium saccharate media. On this evidence and in view of the observation of Franzen and Schmitt [1925] that β : γ -diketoadipic acid on treatment with alkali metal

hydroxides undergoes a "benzilic acid transformation" to citric acid, the suggestion was made that the conversion of glucose to citric acid by *A. niger* might proceed by the stages:

gluconic acid $\rightarrow$ saccharic acid $\rightarrow$ β : γ -diketoadipic acid $\rightarrow$ citric acid.

The subsequent work of one of us with other strains of *A. niger* has not, however, supported this view, nor has it been found possible to detect the presence of β : γ -diketoadipic acid in cultures of the original strain of *A. niger* on glucose and on saccharic acid.

In view of these facts and, in particular, in view of the recent failure of Bernhauer, Siebenäuger and Tschinkel [1931] to obtain evidence of citric acid formation in cultures of numerous strains of *A. niger* on saccharic acid or potassium saccharate media, it must be concluded that the conversion of saccharic acid into citric acid in the single case mentioned above is to be regarded as a strain characteristic, and is not of general significance in the scheme of reactions leading to the formation of citric acid.

On the grounds that those species of *Citromyces* and of *Aspergillus* which are typical producers of citric acid give rise simultaneously to little or no ethyl alcohol, whilst, on the other hand, those *Mucor* species, e.g. *M. racemosus*, which form appreciable amounts of ethyl alcohol from sugar are incapable of also giving as end-products more than traces of acids, Butkewitsch [1923] concluded that the processes leading to citric acid formation cannot be considered to be closely related to those which give rise to ethyl alcohol.

Bernhauer and Schön [1928] were also inclined to the same opinion since they were unable to "trap" more than traces of acetaldehyde in numerous experiments in which strains of *A. niger*, known to be capable of forming citric acid, were cultivated on glucose, fructose and glycerol [cf. Neuberg and Cohen, 1921].

Recently, however, Chrzęszcz and Tiukow [1930] established the facts that the action of *Penicillium citrogenum* on sodium acetate affords fumaric, *l*-malic and citric acids, and that cultures of the same mould on potassium acetate give rise to the formation of succinic, *l*-malic, citric and oxalic acids.

More recently still, Bernhauer and Siebenäuger [1931] have been able to reproduce these results by the employment of a number of strains of *A. niger*. The latter authors have also obtained citric acid by the action of *A. niger* on ethyl alcohol in the presence of sodium bicarbonate or of calcium carbonate, and Chrzęszcz and Tiukow (private communication) have observed the formation of citric acid from the same source, thus confirming a much earlier observation of Mazé and Perrier [1904] which had hitherto lacked substantiation.

These results rendered necessary a reconsideration of the question as to whether compounds of 3 carbon atoms, derived from sugars, are precursors of citric acid, and Chrzęszcz and Tiukow expressed the view that the transformation of glucose into citric acid occurs through reactions which may be divided into two phases, in the first of which the stages are presumed to be: hexose $\rightarrow$ a hexosephosphoric ester $\rightarrow$ methylglyoxal $\rightarrow$ pyruvic acid $\rightarrow$ acetaldehyde $\rightarrow$ alcohol, and in the second of which it is supposed that acetalde-

hyde and ethyl alcohol give acetic acid $\rightarrow$ succinic acid $\rightarrow$ fumaric acid $\rightarrow$ *l*-malic acid. This last acid is then presumed to condense with acetic acid (by dehydrogenation) yielding citric acid. This assumption as to the course of the reactions in the first phase is supported by the fact that Molliard [1922] and Raistrick and his collaborators [1931] have identified glycerol among the metabolic products from glucose of two different species of *Aspergillus*, whilst the view as to the course of the reactions in the second phase receives support (1) from the experiments of Butkewitsch and Fedoroff [1930], which demonstrated the formation of succinic and fumaric acids when *Mucor stolonifer* was allowed to act upon calcium acetate or upon ethyl alcohol in the presence of calcium carbonate, (2) from the observation of Challenger and Klein [1929] that the action of a strain of *A. niger* on fumaric acid afforded a high yield of *l*-malic acid, and (3) from the work of Stent, Subramaniam and Walker [1929] which established the fact that *A. niger* converts succinic acid *via* fumaric acid into *l*-malic acid.

Whilst there is thus strong experimental evidence in favour of Chrzęszcz and Tiukow's explanation of the formation of citric acid from acetic acid, the production of methylglyoxal by mould fungi has not hitherto been established. It might be claimed that the formation of acetaldehyde and of ethyl alcohol, which has been observed during the development of various moulds on sugar media, constitutes indirect evidence of a prior formation of methylglyoxal but, on the other hand, it might also be argued that acetaldehyde and ethyl alcohol could be derived from oxaloacetic acid produced from glucose according to the mechanism suggested by Raistrick and Clark.

The experiments of Quastel [1924] with bacteria, of Stent, Subramaniam and Walker [1929] with *A. niger*, and of Hahn, Haarmann and Fischbach [1929] with muscle provide ample evidence of the extreme readiness with which oxaloacetic acid, in the presence of certain biological agents, undergoes decarboxylation to pyruvic acid. Further, Nagayama [1921] and Walker and Coppock [1928] have shown that the development of various moulds, including *A. niger*, on calcium pyruvate, affords acetaldehyde and ethyl alcohol.

Accordingly, the present authors have commenced a further study of the reactions which give rise to citric acid, and in this communication it is shown that the action of *A. niger* on sodium hexosediphosphate in the presence of toluene leads to the formation of methylglyoxal in quantity sufficient to permit of its isolation as the 2:4-dinitrophenylosazone.

EXPERIMENTAL.

Neuberg and Kobel [1930] have shown that in order to demonstrate the formation of methylglyoxal by the desmolysis of sugar under the influence of yeast, it is not essential to carry out preliminary liquefaction of pressed yeast by plasmolytic agents. The desired inhibition or weakening of the co-enzyme can be effected equally well in the presence of magnesium hexosediphosphate

which, in aqueous solution, is shaken with fresh "top-fermentation" yeast together with toluene or other suitable plasmolytic agent. On now allowing the mixture to stand at 37° methylglyoxal and pyruvic acid are produced and accumulate in the medium.

Our experiments with *A. niger* were designed, so far as possible, on similar lines. In preliminary work use was made of boiling-tubes each containing 15 cc. of a 5 % solution of glucose together with inorganic salts (NH_4NO_3 0.2 %; KH_2PO_4 0.1 %; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.05 %).

After sterilisation these were given heavy sowings of spores and were incubated for various lengths of time; after which the media were removed by sterile pipettes and sterilised tap-water at 30° was added in the same manner, the tubes being then returned to the incubator for 3 or 4 hours.

At the end of this time the water was removed and a second treatment with sterile water was given, followed by a further period of incubation for 3 hours.

The second wash-water was now withdrawn as before, and solutions of sodium hexosediphosphate containing 1 % of toluene were added. The tubes were again placed in the incubator at 30° and, after various intervals of time, samples were withdrawn and tested for the presence of methylglyoxal by means of a solution of 2:4-dinitrophenylhydrazine hydrochloride in 2*N* hydrochloric acid (1200 cc. contained 4 g. of the base).

Six strains of *A. niger*, all of which are known to be capable of citric acid formation, were employed in the preliminary experiments, and are designated N2, B8, B16, No. 3528.7, W16 and W17. Of these, N2 was supplied by Prof. C. Neuberg, B8 and B16 were received together with other strains from Dr K. Bernhauer of Prague and No. 3528.7 was sent to us by Dr C. Thom of the United States Department of Agriculture. We have pleasure in expressing our indebtedness to these gentlemen for their assistance. Strains W16 and W17 were isolated by one of us from plates of suitable agar medium which had been exposed to the open air.

The sodium hexosediphosphate employed was highly purified material supplied by I. G. Farbenindustrie A.G. (Elberfeld), and some experiments were also undertaken with a pure specimen of calcium hexosediphosphate which was made available through the courtesy of Dr F. F. Nord.

The data obtained from a large series of small-scale experiments showed that dilute solutions (from 0.25 to 1 %) of sodium hexosediphosphate gave larger percentage yields of what, presumably, was methylglyoxal, than were obtained when higher concentrations of the salt were employed, and that it was advantageous to use young cultures of the mould before sporulation was far advanced. The optimum yields of the 2:4-dinitrophenylosazone were obtained when the reagent was added to the hexosediphosphate solutions 24 hours after they had been placed in contact with the mould.

Precipitates of the osazone were obtained from experiments with each of the six *A. niger* strains. These precipitates melted at about 250° but after they had been washed separately with 2*N* hydrochloric acid, water, 20 % sodium

carbonate solution, and water, in that order, their melting points were raised to about 280°. On treatment with alcoholic potassium hydroxide solution they all gave rise to the intense blue-violet coloration described by Neuberg and Kobel as characteristic of the 2 : 4-dinitrophenylosazone of methylglyoxal.

In further experiments calcium hexosediphosphate was employed. Owing to its slight solubility this salt could not be used in concentrations higher than approximately 0.4 to 0.5 %, but such solutions gave yields approximating to those obtained from the use of 1 % solutions of the sodium salt.

In order to accumulate sufficient precipitate for purification and analysis a series of experiments was set up in flasks. In one of these experiments, the details of which are cited below, three concentrations of sodium hexosediphosphate were adopted, in order to provide quantitative data as to the yields.

Ten 300 cc. Erlenmeyer flasks, each containing 100 cc. of a 5 % solution of glucose together with the necessary salts, were heavily inoculated with spores of strain W16 and incubated at 30° for 2 days. The media were then removed, the mycelia washed as already described, and the flasks were divided into three groups (A), (B) and (C), to which were then added 1 %, 0.5 % and 0.25 % aqueous solutions of sodium hexosediphosphate, respectively, together in each case with 1 % of toluene. The liquid was removed from the mycelia after contact for 24 hours at 30° and the filtered solutions were combined in their respective groups (A), (B) and (C), and each was treated with 500 cc. of 2 : 4-dinitrophenylhydrazine hydrochloride solution.

After standing for 2 hours the resulting precipitates were filtered off, washed as described above, dried and weighed. Each precipitate melted at 280°. The yields of methylglyoxal calculated on the actual weights of sodium hexosediphosphate used in the several cases were as follows.

Group (A). From 1 % hexosediphosphate, equivalent to	5 %.
Group (B). From 0.5 %	13 %.
Group (C). From 0.25 %	16 %.

The sodium carbonate washings in no case gave more than traces of deposit when acidified with hydrochloric acid, hence pyruvic acid 2 : 4-dinitrophenylhydrazone was not present in appreciable amounts.

In this experiment "control" flasks were also set up in which mycelia were allowed to stand in contact with (a) water and (b) a 1 % solution of sodium hexosediphosphate, in both cases without toluene, and (c) water *plus* 1 % of toluene. In no case did subsequent addition of 2 : 4-dinitrophenylhydrazine hydrochloride solution cause either formation of a precipitate or clouding of the liquid. It should also be remarked that precautions were adopted, throughout the course of these experiments, to exclude the possibility of bacterial contamination of the cultures or of the hexosediphosphate solutions. Careful microscopical examination and sub-culturing failed to detect bacterial contamination in any single experiment.

The precipitates from (A), (B) and (C) were combined (0.47 g.) and re-

crystallised twice from nitrobenzene at 150–160°, when, after washing with alcohol and ether and drying at 100°, the melting-point was 298°. (Found, by micro-analysis, N 25.5 %; 26.03 %; calculated, N 25.93 %.)

Starting from glycerol a specimen of the 2:4-dinitrophenylosazone of methylglyoxal was prepared by the method of Neuberg and Hofmann [1930]. After purification this melted at 298° and a mixture of the two specimens also melted at the same temperature.

SUMMARY.

It has been found that by suitable adaptation of the method whereby Neuberg and Kobel demonstrated the formation of methylglyoxal by the action of yeast on sodium hexosediphosphate it is possible to show that the latter substance affords methylglyoxal when submitted to the action of *Aspergillus niger* in the presence of toluene. The product was characterised as the 2:4-dinitrophenylosazone. This behaviour of the mould is in harmony with the view of Chrzęszcz and Tiukow that the early stages of the transformation of glucose into citric acid by mould fungi involve the formation of a hexose-phosphate and its conversion into methylglyoxal.

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