The Mechanism of the Formation of Organic Acids by Mould Fungi

4. THE FORMATION OF ACETIC AND PYRUVIC ACIDS IN *ASPERGILLUS NIGER* GROWING IN GLUCOSE MEDIA

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This communication describes the isolation of acetic acid from the mycelial felts of young cultures of citric acid-forming strains of *Aspergillus niger* grown on a medium of glucose and inorganic salts. The detection of pyruvic acid in cultures of *A. niger* on a glucose medium containing sodium arsenite is also recorded. The significance of these findings lies in the fact that various hypotheses concerning the biogenesis of citric acid assign essential roles to these two acids.

Heinze (1903) reported the occurrence of acetic acid after 18 weeks in a culture of *A. niger* on a medium of glucose and inorganic salts, but he did not characterize it, and the literature contains no other reference to the detection of acetic acid in cultures of *A. niger* on glucose. Small quantities of pyruvic acid have been trapped by sodium sulphite in cultures of *A. niger* on sugar (Hida, 1935), but in these conditions formation of citrate is strongly depressed or inhibited, whereas in our experiments the arsenite did not depress the formation of citrate.

EXPERIMENTAL METHODS AND RESULTS

Formation of acetic acid

Four strains of *A. niger* (designated N1, B18, N.R.R.L. 67 and Ac.1) were used. All, in previous work, had given citric acid in glucose media, better yields (40–50%) having been obtained with N1 and N.R.R.L. 67 than with the other two strains. The medium was one favourable to the formation of citric acid (Johnson, Knight & Walker, 1937). When seeking for acetic acid the glucose concentration was 17.5% (w/v), and the pH was adjusted with HCl to 2.5 prior to inoculation. When working with strain N.R.R.L. 67 the medium was that recommended by Wells, Moyer & May (1936). The temperature of incubation was 30° in all cases. The melting points given later were checked by the use of a standard thermometer, but the correction for emergent stem was not applied.

Exp. 1; isolation of silver acetate. The glucose medium (5500 ml.) was divided between twenty-two conical flasks, each of 1500 ml. capacity, and inoculated with spores of strain N1. On the fifth day, a little citric acid was present in the flasks as shown by Denigés's (1899) test, whereupon the liquid contents of the flasks were combined and examined. Tests for the presence of volatile acids were negative. The bulk of the liquid was then extracted for 50 hr. with ether

and yielded a small quantity of syrupy material. A sample of the latter on treatment with H_2SO_4 and with a 5% (w/v) ethanolic solution of guaiacol gave a violet coloration, a positive test for glycollic acid (Denigés, 1909 a, b). Another portion of the syrup yielded a reddish violet coloration on treatment with naphthoresorcinol and fuming HCl, a reaction of glyoxylic acid (Neuberg, 1910). It had been shown already that acetate yields some glycollate and glyoxylate when exposed to the action of *A. niger* (Challenger, Subramaniam & Walker, 1927 a).

The mycelial felts which had been grown on the 5500 ml. of medium were washed with water, drained, ground with clean sand and extracted thoroughly, first with ether and then with hot water. The ethereal extract was evaporated and the slight residue was dissolved in the hot-water extract. This aqueous solution was then extracted continuously with ether during 50 hr. On evaporation of the ethereal extract a small quantity of liquid remained and had the smell of acetic acid. This residue was submitted to steam distillation until the liquid coming over was no longer acid, 950 ml. of distillate being collected. A 20 ml. portion was found to be equivalent to 8 ml. of 0.1074N-NaOH. The normality of the distillate was thus 0.043, representing, if due entirely to acetic acid, 2.45 g. in the 950 ml. of solution.

From this stage, the procedure followed for the isolation of acetic acid as its Ag salt was that of Neuberg & Hirsch (1919). The total distillate was made just alkaline with NaOH and evaporated to a small volume. The residue was just neutralized with HNO_3 and then treated with a concentrated solution of $AgNO_3$. The flocculent precipitate, presumably Ag acetate, was filtered off, washed until free from HNO_3 and dried *in vacuo*. (Found: Ag, 64.3. Calc. for $CH_3.COOAg$: Ag, 64.65%.)

Exp. 2; isolation of p-nitrobenzyl acetate. Strain N1 was again employed, being grown in six 2 l. conical flasks, each containing 400 ml. of medium, and in six 1 l. conical flasks each containing 250 ml. of medium.

On the sixth day citric acid had begun to accumulate in the medium, and a sample of the latter also gave a strong naphthoresorcinol colour reaction for glyoxylic acid. Acetic acid was not detected, however, in the medium. The mycelial felts were then collected, ground with sand and extracted as in Exp. 1. In this case, however, the mycelial matter after extraction was subjected to steam distillation and the small quantity of acid which passed over was combined with that obtained from the ethereal and aqueous extracts of the mycelium. The total volatile acid gave an Na salt equivalent in quantity to 2.601 g. of acetic acid. From this salt the p-nitrobenzyl ester was prepared. It melted at 77.5°, and

the mixed melting point with an authentic sample (m.p. 78°) prepared from British Drug Houses Ltd. acetic acid was 77-5°. (Found: N, 7.1. Calc. for $C_6H_8O_4N$: N, 7.2%.)

Exp. 3; other strains. Strain N.R.R.L. 67 was employed, the volume of medium was 8000 ml. and the cultures were worked up in three equal lots, after 4, 5 and 6 days of incubation, respectively. Small samples from the final three steam distillates gave positive reactions when examined for the presence of acetic acid by the lanthanum nitrate colour test (Krüger & Tschirch, 1929, 1930). These three steam distillates required for neutralization 32.8, 56.2 and 52.1 ml. of 0.1 N-NaOH, respectively. Total alkali, 141.1 ml. of 0.1 N-NaOH; equivalent to 0.85 g. of acetic acid.

From the Na salt, presumably acetate, obtained by evaporation of the neutralized distillates, a *p*-nitrobenzyl ester was prepared. This had m.p. 78° and the mixed melting point with an authentic sample (m.p. 78°) prepared from pure Na acetate was also 78°.

Experiments similar to those described above were made with strains B18 and Ac.1, but though the presence of acetic acid could be detected in extracts of the felts by means of the lanthanum nitrate test, the acid was insufficient in quantity to be isolated.

Exps. 4 and 5; citric acid as substrate. These were controls in which strains of N1 and N.R.R.L. 67 were grown on media which contained citric acid (5% w/v) in place of glucose, the respective solutions of nutrient inorganic salts being unchanged. In each case 5 l. of medium were employed, and in 5 days strongly developed mycelial felts were obtained. They were ground with sand and treated as in the previous experiments. The N.R.R.L. 67 felts yielded no trace of volatile acid. The N1 felts afforded a very small quantity (0.0128 g.) of volatile acid which gave a positive lanthanum nitrate test for acetic acid. This yield (0.005% of the weight of the citric acid) is negligible by comparison with the yields (0.244 and 0.382%) obtained by the action of this mould on glucose. The metabolism solutions from the citric acid cultures were found to be free from acetic acid in all cases. These results render it improbable that the appreciable quantities of acetic acid isolated from the felts grown on the glucose media had been produced from preformed citric acid.

Experiments with sodium arsenite leading to the trapping of pyruvic acid in glucose cultures

Carbon balance experiments with *A. niger* in presence and in absence of arsenite, which have been carried out recently in this laboratory, have shown that arsenite hinders decarboxylation in *A. niger* cultures. This led us to try to isolate pyruvic acid from cultures of this mould on glucose media.

Exp. 6. The solution of inorganic salts which formed the basis of the medium had the same composition as that employed in Exps. 1 and 2, and glucose was added to this in a concentration of 10% (w/v). After inoculation with spores of N1 incubation was conducted for 4 days; the medium was then poured away and replaced by a solution of glucose (10% w/v) in water which contained also 0.01 M-Na arsenite (A.R.). After keeping for a further period of 2 days in the incubator the solution was drained from the felts, collected in one vessel and filtered. Arsenic was removed by treatment with H_2S followed by filtration and, after removal of excess H_2S by a current of N_2 , samples of the solution were examined for the presence of pyruvic acid. Both the Na nitroprusside

colour test (Caron & Raquet, 1942) and the test described by Lu (1939) were positive. The bulk of the metabolism solution (3 l.) was then mixed with 3 l. of a saturated solution of 2:4-dinitrophenylhydrazine in 2 N-HCl. Three hours later the precipitate which had formed was filtered, washed with dilute HCl and then with water and dried on a tile in air; wt. 2.20 g. This material was extracted with a 10% (w/v) aqueous solution of Na_2CO_3 and from the filtered alkaline extract 0.85 g. of solid matter was precipitated by addition of dilute HCl. This was washed, dried in air and crystallized from ethanol. When pure it had m.p. 217-218° (decomp.). The mixed melting point with an authentic sample of the 2:4-dinitrophenylhydrazone of pyruvic acid prepared from Kahlbaum's pyruvic acid was identical. (Found: C, 40.1; H, 2.9; N, 21.25. Calc. for $C_6H_6O_6N_4$: C, 40.3; H, 2.9; N, 20.9%.)

Exp. 7. The procedure of Exp. 6 was repeated using strain N.R.R.L. 67 in place of strain N1. After incubation of the felts in the solution containing glucose and arsenite had proceeded for 3 days the solution was treated with 2:4-dinitrophenylhydrazine and yielded 1.46 g. of crude product from which 0.52 g. of the 2:4-dinitrophenylhydrazone of pyruvic acid was obtained. This, after several recrystallizations, had m.p. 218°. The mixed melting point with the authentic sample was also 218°. Pyruvic acid could not be detected in cultures of *A. niger* on the glucose medium in absence of arsenite.

During the course of this work it was also noted that arsenite at 0.004 M increased the yield of citric acid in glucose media as compared with the yield in its absence. This matter is reserved for a further communication.

DISCUSSION

It is a well established fact that acetic acid is one of the products of the action of a number of bacterial species on citric acid or on citrates. Thus, it is formed in cultures of *Pseudomonas pyocyanea* on an ammonium citrate medium (Butterworth & Walker, 1929), and it is also a product of the action of certain species of *Aerobacter* on citric acid (Deffner, 1938). The species used by Deffner did not attack acetic acid. He considered it probable that under the action of these organisms citric acid undergoes hydrolytic fission to one molecule of oxaloacetic acid and one molecule of acetic acid. Brewer & Werkman (1939) using *Aerobacter indologenes* reached similar conclusions. Since *Aspergillus niger* forms citric acid from glucose, the question arises whether or not the acetic acid detected in the experiments described in the present communication was produced via such citric acid. However, apart from the fact that in the control experiments with citric acid as substrate no significant quantity of acetic acid could be detected, there are good reasons for considering it to be highly improbable that acetic acid is formed in appreciable quantities during the course of citric acid breakdown in mould fungi. For example, although thousands of tons of citric acid are produced annually from sugar by mycological agency the occurrence of acetic acid in the medium has not been reported.

Further, acetate is metabolized rapidly by *A. niger* and by many species of *Penicillium* which form citric acid, and it has been shown that such acetate is largely converted to succinate, fumarate, L-malate, oxalate and citrate (Chrzaszcz & Tiukov, 1930 *a, b, c*). Recently, also, two of the present authors (cf. Walker, 1949) obtained citrate in a yield of more than 21% by the action of *A. niger* N 1 on sodium acetate. Finally, in contrast to the breakdown of citrate to oxaloacetate and acetate under the influence of *Aerobacter* species, it was shown (Challenger *et al.* 1927 *a, b*) that *Aspergillus niger* can convert citric acid, in part at any rate, to acetone-dicarboxylic acid from which acetone and malonic acid are then formed. The only product which could be detected when the mould was allowed to act upon malonate was oxalate. Acetic acid was only once detected by Challenger *et al.* (1927 *b*) in a culture of *A. niger* on a citric acid medium and then only in very small quantity and in an old culture (21 days). Acetic acid never appeared in the metabolism solution in young cultures. Since we have established the production of pyruvic acid from glucose by *A. niger* in the presence of arsenite which latter, by suppressing oxidative decarboxylation, hinders the breakdown of the keto acid, it seems reasonable to assume that pyruvic acid was the immediate precursor of the acetic acid which was isolated in Exps. 1-3. Such pyruvic acid could have arisen either by the operation of the zymase system in the mould or as the ultimate product of a scheme of carbohydrate oxidation such as that suggested by Dickens (1936, 1938 *a, b*) and by Lipmann (1936), and in the cells of the mould the acid possibly exists as the phosphate of its enol form. The conversion of pyruvic acid to acetic acid in the mould could conceivably occur in one or more ways, for example: (a) by oxidative decarboxylation, (b) by decarboxylation to acetaldehyde and subsequent dehydrogenation of acetaldehyde hydrate, and (c) by the

action of pyruvic dehydrogenase on pyruvic acid phosphate.

The evidence brought forward in the present communication could therefore be considered to be in harmony with the hypothesis that in cultures of *A. niger* on glucose, pyruvic acid and acetic acid are precursors of citric acid. Since *A. niger* binds carbon dioxide (Foster, Carson, Ruben & Kamen, 1941) pyruvic acid could yield oxaloacetic acid which by union with acetic acid could afford presumably *cis*-aconitic acid and hence citric acid. This view receives support from a hitherto unpublished observation made recently by Mr G. Halliwell in this laboratory. He finds that addition of acetate to cultures of *A. niger* on glucose under certain conditions leads to a considerable increase in the yield of citric acid.

SUMMARY

1. Acetic acid has been isolated in yields up to 0.382% from mycelial felts of citric acid-forming strains of *Aspergillus niger* grown on glucose media. It was not present in the media at any time during growth of the moulds.

2. The felts of cultures grown on media containing citric acid as sole source of carbon contained merely traces of acetic acid (yield 0.005%) or were entirely free from the latter.

3. Pyruvic acid has been detected in glucose solutions exposed to the action of *Aspergillus niger* in presence of 0.01M-sodium arsenite.

4. These facts are discussed in relation to the question of the mode of formation of citric acid in moulds.

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