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

II. THE ACTION OF *ASPERGILLUS NIGER* ON GLUCOSE IN THE PRESENCE OF SODIUM IODOACETATE

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In Part I [Suthers & Walker, 1932] reference was made to the hypothesis advanced originally by Chrzaszcz & Tiukow [1930], that the conversion of hexoses into citric acid by mycological agency occurs in two distinct phases: first, one of alcoholic fermentation and oxidation of the alcohol to acetic acid; and secondly, one of dehydrogenation processes whereby three molecules of acetic acid are condensed in several steps to citric acid.

Butkewitsch & Gajevskaya [1935] drew attention to the fact that the maximum yield of citric acid which could be obtained from 100 g. of sugar metabolized by *A. niger*, assuming the sugar to undergo a preliminary process of alcoholic fermentation, could not be greater than 71.1 g., whereas, if the conversion were to be effected by a mechanism which involved no loss of carbon, the yield would be 106.7 g. In quantitative experiments with a selected strain of *A. niger* they obtained yields of citric acid as high as 89.1 g. from 100 g. of glucose and as high as 100.6 g. from 100 g. of sucrose utilized by the organism.

Wells *et al.* [1936] determined the carbon balance when a strain of *A. niger*, capable of giving consistently high yields of citric acid from glucose, was grown on glucose as the sole source of carbon. From the utilization of 100 g. of glucose the maximum yield of citric acid was found to be 88.6 g. They observed also that the ratio of the weight of citric acid produced to the weight of carbon dioxide liberated exceeded 1.45 : 1, which could not have been possible had the citric acid arisen via preliminary alcoholic fermentation. Finally, to this evidence may be added the statement of Clutterbuck [1936] that, during the investigations of Raistrick and his colleagues with *A. niger*, yields of citric acid as high as 87 g. per 100 g. of sugar consumed were obtained on numerous occasions.

Three years ago the present authors commenced to investigate the question as to whether citric acid production from sugar is dependent on an initial process of alcoholic fermentation by studying the behaviour of *A. niger* and of other moulds in the presence of iodoacetic acid [cf. Walker, 1936]. The evidence then obtained [Johnson, 1934] indicated that formation of citric acid could proceed in normal yield in the presence of iodoacetic acid at a concentration ($M/500$) which was sufficient to cause total inhibition of alcoholic fermentation by the various moulds in atmospheres of nitrogen or of carbon dioxide. During the course of our work we were informed by Prof. T. A. Bennet-Clark that he had observed the formation of citric acid from sugar by a species of *Penicillium* in the presence of a concentration of free iodoacetic acid which was sufficient to inhibit

the formation of alcohol by this particular mould when placed on a sugar medium in the absence of oxygen.

Hence, provided that the inhibiting action of *M*/500 iodoacetate on certain components of the zymase complex is exercised to an equal extent under both anaerobic and aerobic conditions, it follows from these results that we can legitimately conclude that alcoholic fermentation is not an essential phase in the formation of citric acid by moulds. Whilst we cannot offer direct proof that the mechanism of the action of iodoacetate on the enzymic processes of a mould is exactly the same in air as in nitrogen the following explanations, in our view, render it very probable that in the case under discussion the inhibiting activity is exercised in a manner which is independent of the presence or absence of oxygen. According to Meyerhof & Kiessling [1933; 1935] iodoacetic acid prevents the oxidation of triosephosphoric acid to phosphoglyceric acid, a reaction which constitutes one particular stage of alcoholic fermentation by enzyme preparations, and which is effected by acetaldehyde, the latter being reduced to alcohol. Now upon the completion of this coupled reaction depends the simultaneous phosphorylation of a second molecule of glucose by the inorganic phosphate which is present, followed by decomposition of the product, presumably into triosephosphate. Hence, iodoacetic acid directly inhibits one stage and, in so doing, it prevents at the same time the occurrence of another stage of the process.

In the transference of phosphoric acid groups during the processes which constitute alcoholic fermentation it is now considered probable that the main effect is produced by the cozymase, a complex diphosphodinucleotide containing the amide of nicotinic acid and adenine [cf. Warburg & Christian, 1936; Euler *et al.* 1935; 1936]. In the oxido-reduction reaction, previously mentioned, by means of which two atoms of hydrogen are transferred from triosephosphate to acetaldehyde, it is the pyridine nucleus of the cozymase system which functions as the hydrogen transporter. Now on grounds of molecular structure it is very probable that both the pyridine nucleus and the molecule of adenine can interact with iodoacetate, and possibly, also, interaction can occur between the latter substance and the specific protein carrier of the pyridine nucleotide. Presumably, it is by such chemical combinations that the prosthetic groups of one or more components of the cozymase are inactivated by iodoacetate, and since the occurrence of none of these condensations would be conditional upon either the presence or absence of air, it may be claimed that our experimental results afford reasonable justification for the view that the formation of citric acid by moulds does not depend upon a prior process of normal alcoholic fermentation, and our findings thus fall into line with the conclusions reached by others on the evidence of percentage yields. It will be understood, however, that this decision need not imply that no part of the mechanism of alcoholic fermentation is called into play in the formation of citric acid, for it is conceivable that here also phosphorylation of the hexose molecule may constitute one of the first stages of the transformation. Such a preliminary phosphorylation, moreover, need not be considered to be out of harmony with the subsequent occurrence of reactions such as those suggested by Raistrick & Clark [1919] as a possible scheme of citric acid formation, and a scheme which, it may be stated, would be capable of giving the aforementioned high yields.

EXPERIMENTAL

The strains of *Aspergillus niger* which were used in this work were all pure cultures obtained from different sources and they were designated N 1, N 2, B 7, B 7a, B 12, B 16 and T respectively. All were known to afford citric acid

from glucose when cultivated under normal conditions, but the yields of this acid from 10 % glucose medium had been found generally to be low, varying from about 6 to 30 % of the weight of sugar consumed, according to the particular strain and to the conditions of cultivation. All the strains were known also to be capable of giving rise to oxalic acid and to much gluconic acid in addition to citric acid when grown on glucose media. They were cultivated in sterile aqueous solutions containing NH_4NO_3 , 0.2 %; KH_2PO_4 , 0.1 %; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05 % and glucose, 10 or 12.5 %. Conical flasks of 1500 ml. capacity were each charged with 300 ml. of this medium and, after sterilization, they were given heavy sowings of spores, and incubation was then carried out at 30° for about 5 days. At the end of this time the medium in each flask was poured away and replaced by 100 ml. of sterile tap water at 30°, which was introduced under the mycelium by means of a pipette, care being taken not to wet the top of the fungal mat. The flasks were then returned to the incubator for a period of 3 hours, after which the washing process was repeated and the flasks were again allowed to stand for a further period of 2-3 hr. This treatment served to denude the cells of the moulds of absorbed sugar and to free the cultures from any traces of citric acid which may have been formed though, as a rule, very little citric acid was produced during the first 5 days of growth. After the removal of the second washing water the flasks were charged each with 300 ml. of a sterile 10 or 12.5 % aqueous solution of glucose containing a measured quantity of sodium iodoacetate but no inorganic salts. Similar flasks, but without iodoacetate, were prepared as "controls," and incubation of all of them was then resumed. In stating results, the number of days of incubation was reckoned in each case from the day on which the mycelium was washed and the flask recharged with sugar solution.

In every case, before withdrawing a sample, the flask was gently rotated to ensure uniform density of its liquid contents. Sugar estimations were carried out by the method of Schaffer and Hartmann or by the Bertrand method; determinations of total acidity were performed by titration against standard alkali, and the estimations of citric and oxalic acids were carried out by the method recommended by Butkewitsch [1922; 1924]. In those experiments in which it was necessary to estimate ethyl alcohol, this was done by repeated distillation and determination of the specific gravity of the final distillate by use of a pycnometer.

At the commencement, experiments were performed to ascertain the minimum concentration of iodoacetic acid (used in the form of its sodium salt) which was sufficient to prevent the normal germination of spores of *A. niger* sown on a medium containing inorganic salts together with 10 % of glucose. It was found in the cases of six different strains that the critical dose necessary for such suppression of growth was afforded by iodoacetate at a concentration of $M/500$, and in the subsequent work this concentration was found also to be the minimum which could be employed in order to suppress completely the formation of alcohol under anaerobic conditions, and to be the maximum concentration which permitted the formation of the normal yield of citric acid from a given weight of glucose submitted to the action of any one of the moulds.

In the experiments conducted under anaerobic conditions the moulds were first grown under normal aerobic conditions at 30° and then, after changing the medium, the flasks were fitted with rubber stoppers in which Bunsen valves were inserted, and the air in the flasks was replaced by sterile nitrogen. Subsequently, during the period allowed for fermentation, the flasks were kept at 34° and on two occasions fresh nitrogen was passed through them. The small quantities of acid produced by the various cultures under anaerobic conditions

Table I

Iodoacetic acid (as sodium salt) at concentration $M/500$ in 10% glucose solution.
Incubation in atmosphere of nitrogen at 34° for 12 days

Strain of <i>A. niger</i>	Glucose consumed, % of the weight present initially	Acidity, ml. 0.1 <i>N</i> NaOH required to neutralize 100 ml. of medium	Alcohol produced, % of the yield theoretically possible from the weight of glucose consumed
B 7	1.0	13.8	None
B 12	1.7	13.3	None
B 16	15.1	6.6	None
N 1	11.3	9.2	Mere trace detected
T	17.0	12.5	None
"Controls", in absence of iodoacetate			
B 7	11.0	35.9	24.3
B 12	13.9	28.3	34.3
B 16	27.7	10.6	46.9
N 1	18.6	16.8	35.4
T	23.9	18.4	35.9

(Table I) were examined and found to consist of oxalic acid, the formation of which was rendered possible no doubt by the small amount of air occluded in the mycelia at the commencement of the period of anaerobiosis.

In experiments carried out entirely under aerobic conditions it was found that, in the presence of iodoacetate at concentrations between $M/1000$ and $M/750$, the amount of glucose consumed and the titratable acidity reached in a given time were both greater than the corresponding values found in the absence of iodoacetate. In the presence of iodoacetate at a concentration of $M/500$ in a 12.5% aqueous solution of glucose, the results obtained were rather striking (Table II) for, in the cases of three out of four strains used, more citric acid was

Table II

Iodoacetic acid (as sodium salt) at concentration $M/500$ in 12.5% glucose solution.
Incubation at 30° for 10 days

Strain of <i>A. niger</i>	Glucose consumed, % of the weight present initially	Acidity, ml. of 0.1 <i>N</i> NaOH re- quired to neutralize 100 ml. of medium	Citric acid: (a) g. per 100 ml. of medium; (b) % of the weight of glucose consumed		Oxalic acid: (a) g. per 100 ml. of medium; (b) % of the weight of glucose consumed		Other acid, calculated as gluconic acid, % of the weight of glucose consumed
			(a)	(b)	(a)	(b)	
B 12	26.0	143	0.752	22.90	0.039	1.18	10.40
B 16	86.0	323	1.110	10.31	0.202	1.87	19.23
T	34.5	207	0.647	14.99	0.388	8.99	9.16
N 2	48.0	188	0.574	9.42	0.205	3.36	17.13
“Controls”, in absence of sodium iodoacetate							
B 12	67.8	272	0.820	9.66	0.041	0.48	30.71
B 16	70.8	169	0.543	6.13	0.134	1.51	12.25
T	64.0	175	0.561	7.00	0.032	0.40	19.76
N 2	66.7	170	0.509	6.10	0.019	0.24	20.45

formed than was produced in the respective control flask and, in the fourth case, the amount of citric acid formed was only a little less than the quantity which was formed in the corresponding control flask. Expressed as a percentage of the glucose actually consumed, the yield of citric acid was in every case considerably greater in the presence of $M/500$ iodoacetate than it was in the absence of the

latter, and another noteworthy feature was the stimulation of the production of oxalic acid in the presence of the iodoacetate. Both in the presence and in the absence of sodium iodoacetate a third acid was formed in relatively large proportions and was estimated, by difference, as gluconic acid. Subsequently, samples of this acid were isolated from the media containing iodoacetate and, on examination, were found to be identical in composition with an authentic sample of gluconic acid.

When the iodoacetate was employed at a concentration of $M/475$ (Table III) glucose consumption showed considerable reduction compared with "controls" but, in two cases out of the three, the yield of citric acid calculated on the sugar

Table III

Iodoacetic acid (as sodium salt) at concentration $M/475$ in 10% glucose solution.
Incubation at 30° for 9 days

Strain of <i>A. niger</i>	Glucose consumed, % of the weight present initially	Acidity, ml. of 0.1 <i>N</i> NaOH re- quired to neutralize 100 ml. of medium	Citric acid: (a) g. per 100 ml. of medium; (b) % of the weight of glucose consumed		Oxalic acid: (a) g. per 100 ml. of medium; (b) % of the weight of glucose consumed		Other acid, calculated as gluconic acid, % of the weight of glucose consumed
			(a)	(b)	(a)	(b)	
B 7	15.17	37	0.074	4.89	0.044	2.892	20.30
B 12	7.87	23	0.048	6.08	0.013	1.662	31.51
N 1	8.57	33.5	0.162	18.89	0.019	2.192	3.77
"Controls", in absence of sodium iodoacetate							
B 7	53.93	120	0.430	7.98	0.027	0.50	17.13
B 12	73.75	123	0.413	5.60	0.004	0.05	6.67
N 1	71.74	388	0.470	6.55	1.301	18.13	7.07

utilized was greater in the presence of the iodoacetate than in its absence. At concentration $M/400$ (Table IV) citric and oxalic acids were formed in quantities which, though very small, were quite sufficient for purposes of characterization and estimation.

Table IV

Iodoacetic acid (as sodium salt) at concentration $M/400$ in 12.5% glucose solution.
Incubation at 30° for 13 days

Strain of <i>A. niger</i>	Glucose consumed, % of the weight present initially	Acidity, ml. of 0.1 <i>N</i> NaOH re- quired to neutralize 100 ml. of medium	Citric acid: (<i>a</i>) g. per 100 ml. of medium; (<i>b</i>) % of the weight of glucose consumed		Oxalic acid: (<i>a</i>) g. per 100 ml. of medium; (<i>b</i>) % of the weight of glucose consumed		Other acid, calculated as gluconic acid, % of the weight of glucose consumed
			(<i>a</i>)	(<i>b</i>)	(<i>a</i>)	(<i>b</i>)	
B 7	15.13	57	0.021	1.11	0.047	2.49	44.86
B 7 <i>a</i>	22.26	67	0.009	0.33	0.036	1.29	40.57
B 12	19.70	71	0.012	0.50	0.049	2.00	46.29
N 1	16.11	60	0.087	4.33	0.079	3.95	27.97
"Controls", in absence of sodium iodoacetate							
B 7	44.08	193	0.492	8.93	0.050	0.90	37.66
B 7 <i>a</i>	40.03	181	0.087	1.75	Trace	—	65.75
B 12	53.92	328	0.522	7.74	Trace	—	71.93
N 1	56.63	608	0.722	10.21	2.09	29.54	8.39

SUMMARY

1. Iodoacetic acid (as sodium salt) when introduced at concentrations between $M/750$ and $M/1000$ into sugar solutions in contact with the fully developed mycelium of *Aspergillus niger* caused an increase both in the quantity of glucose consumed and in the degree of acidity produced in a given time as compared with control cultures in which iodoacetic acid was not present.

2. The presence of $M/500$ sodium iodoacetate in a medium of glucose and inorganic salts was just sufficient to prevent the development therein of the spores of six different strains of *A. niger*. The same concentration afforded the minimum quantity per unit volume which could be employed for suppression of the formation of ethyl alcohol by five strains of *A. niger* in contact with glucose solutions in atmospheres of nitrogen. The same concentration was found to afford the maximum quantity per unit volume which, in the average experiment, permitted the formation of a quantity of citric acid approximating in weight to the weight of citric acid obtained from a corresponding "control" experiment made without iodoacetate. The presence of $M/500$ iodoacetate was found, however, to diminish the weight of sugar metabolized in a given time as compared with the weight which was metabolized in a control experiment, hence the percentage yield of citric acid on the sugar used was greater in the presence of $M/500$ iodoacetate than in its absence.

3. $M/475$ iodoacetate caused considerable reduction in the quantity of glucose metabolized in a given time but did not result in a lowering of the yield of citric acid as calculated on the glucose utilized.

4. $M/400$ iodoacetate did not suppress entirely the formation of citric and oxalic acids by *A. niger*.

5. Attention is drawn to the bearing of these results on views which have been expressed regarding the mechanism of citric acid formation by moulds. The experimental data indicate that the formation of citric acid from glucose by *A. niger* does not involve a preliminary process of alcoholic fermentation.

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