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

III. THE INFLUENCE OF IODOACETATE AND OF FLUORIDE ON THE FORMATION OF ACIDS

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THE action of *A. niger* in the presence of sodium iodoacetate [Johnson *et al.* 1937] has now been studied with sucrose as the substrate and the experiments have yielded results similar to those found with glucose. Thus *M*/450 iodoacetate inhibited anaerobic formation of ethyl alcohol by six different strains of *A. niger* which were able in the presence of *M*/400 iodoacetate to produce both citric and oxalic acids in quantities sufficient for quantitative estimation. Further, although *M*/400 iodoacetate restricted the average quantity of sugar metabolized in 11 days to about 30 %, little difference was noted in the yield of gluconic acid in g. per 100 ml. culture liquid, since the yield of the latter acid expressed as a percentage of the weight of sugar consumed was raised from an average normal value of 13 % to a value of 45 % in the presence of *M*/400 iodoacetate (Table I).

Also, experiments conducted simultaneously in the presence and in the absence of iodoacetate showed that the latter substance at concentrations of *M*/1000 and *M*/500 caused a large rise in the weight of citric acid and a considerable fall in the weight of gluconic acid produced in unit volume of the culture medium (Table II). After incubation for 20 days in presence of *M*/500 iodoacetate the sugar metabolized was found to be 80 % of the quantity present initially, as compared with a consumption of 66.75 % in absence of iodoacetate.

Experiments were also undertaken to ascertain the behaviour of *A. niger* towards sucrose in the presence of NaF. Using strain T1 it was necessary to employ NaF at a concentration of *M*/100 in order completely to suppress the formation of citric acid, whereas under anaerobic conditions the formation of ethyl alcohol was inhibited in the presence of *M*/200 NaF. In further experiments with strain T1 conducted aerobically in the presence of NaF at concentrations from *M*/200 to *M*/400 it was found in every case that in 13 days the total acid produced was less than in the "controls" without NaF. The amounts of citric acid formed were particularly low in comparison with those produced in the "control" flasks but, on the other hand, the formation of gluconic acid, which was negligible in the "control" flasks, was strongly stimulated by all concentrations of NaF and reached a maximum when the latter was *M*/250 (Table III). The effects of NaF on acid formation by *A. niger* are similar, in some respects, therefore, to those exercised by iodoacetic acid. Bernhauer [1928] has reported that in the presence of certain concentrations of the commercial germicide "Uspulin" the action of *A. niger* on sugar results in largely increased production of gluconic acid with simultaneous suppression of the formation of citric acid.

The increase of gluconic acid formation in cases such as these may be explained in two ways. The poison may selectively cripple the enzyme system or systems responsible for the formation of citric acid whilst leaving undamaged, or even stimulating, the glucose oxidase which gives rise to gluconic acid. This would leave the glucose oxidase free to transform into gluconic acid the sugar which normally would be converted into citric acid. Alternatively, it is possible that the poison cripples the enzyme system employed by the mould for the conversion of gluconic acid into citric acid. If such is the case the experimental results in question are evidence in favour of the view that gluconic acid is an intermediate in the formation of some, at least, of the citric acid produced from sucrose. Chrzęszcz & Tiukow [1930] suggested that the first phase of citric acid formation from sugar under the influence of mould fungi consisted of the processes of alcoholic fermentation, and that this phase was succeeded by one in which acetic acid (formed from acetaldehyde or ethyl alcohol) gave rise to 4-carbon dicarboxylic acids in the following sequence:

The authors presumed that citric acid was then produced by a final dehydrogenation whereby 1 mol. of malic acid was condensed with 1 mol. of acetic acid. Whilst the subsequent work of a number of investigators [Butkewitsch & Gajevskaya, 1935; Wells *et al.* 1936; Clutterbuck, 1936; Walker, 1936] has proved that alcoholic fermentation cannot be an initial phase in citric acid formation, the experiments of Chrzęszcz & Tiukow [1930], of Chrzęszcz *et al.* [1932] and of Bernhauer *et al.* [1934] have fully substantiated the formation of succinic, fumaric, malic and citric acids in cultures of various moulds in contact with salts of acetic acid or with very dilute ethyl alcohol. Hence, if it could be shown that mould fungi can transform 1 mol. hexose into 3 mol. acetic acid or into 1 mol. acetic acid together with 1 mol. of any one of the above 4-carbon acids, it would then be justifiable to regard the later reactions immediately preceding the formation of citric acid as consisting of dehydrogenations of the type suggested by Chrzęszcz & Tiukow. An explanation on these lines would, moreover, approximate very closely to the hypothesis of Raistrick & Clark [1919], who viewed the initial processes of citric acid formation as consisting of dehydration and oxidation of the hexose molecule followed by fission into 1 mol. of acetic acid and 1 mol. of oxaloacetic acid, the two latter substances constituting the immediate precursors of the citric acid to which, by an aldol condensation, they were presumed to give rise. Views similar to those of Raistrick & Clark were also expressed later by Virtanen & Pulkki [1930]. Whilst these assumptions are quite in harmony with the known properties of the several substances involved, neither acetic acid nor oxaloacetic acid has yet been isolated from cultures of *A. niger* on sugar. According to Bernhauer *et al.* [1932] small quantities of malic acid may sometimes be detected in cultures of this mould on sugar solutions, a statement which we can confirm from our own experiments, and possibly this malic acid arises by reduction of oxaloacetic acid. It is equally possible, however, that such malic acid is derived from succinic acid, and this view leads to consideration of the possibility mentioned above, namely, that fission of the sugar molecule under the influence of the mould may give rise to the production of equimolecular quantities of acetic and succinic acids. Support for the belief that a non-phosphorylated glucose molecule can, by reactions involving fission, give rise to succinic acid, is found in the phenomena observed by Virtanen [1925] when investigating cell-free propionic acid fermen-

tation, cf. Virtanen & Simola [1927]. Moreover, Scheffer [1928], working with Kluver, obtained quantitative data in favour of the conception that in the fermentation of glucose by *B. coli* some of the sugar is split into 4-carbon and 2-carbon compounds. These observations led one of us to suggest [Johnson, 1934] that formation of citric acid may take place through an initial conversion of a molecule of hexose into 1 mol. acetic acid and 1 mol. succinic acid, and that these two acids would then give rise to the final product in accordance with the scheme of Chrzęszcz & Tiukow or with one of the closely related schemes suggested by Bernhauer *et al.* [1934]. Alternatively and with equal reason the succinic acid could be assumed to undergo conversion into oxaloacetic acid when the final condensation would be that postulated by Raistrick & Clark.

Gudlet [1936], from consideration of Virtanen's work, has also arrived at the same opinion and has suggested that the conversion of sugar into acetaldehyde and succinic acid, without loss of CO_2 by decarboxylation, is a necessary step in the production of citric acid by moulds.

Facts which have been co-ordinated by Kluver [1935] would seem to provide a reasonable explanation of the mechanism of the formation of both acetic and succinic acids from glucose by microbiological agency. He has brought forward the view that the formation of these acids from glucose by *B. coli* occurs through an initial fission of the hexose to ethylene glycol and tartaric acid dialdehyde, followed by transformation of the former into acetaldehyde and of the latter into succinic acid. Further, the well known formation of equimolecular quantities of acetic and lactic acids from xylose by the action of *Lactobacillus pentosus* is a conversion which is readily understandable if considered as completely analogous to the case of the action of *B. coli* on glucose, for the xylose may be presumed to yield glycollic aldehyde and glyceric aldehyde which are then transformed into acetic acid and lactic acid, respectively.

Consideration of the citric acid problem from this standpoint has led us to devise further experiments to gain information respecting the processes involved in the production of this acid from sugar. If succinic acid is an intermediate it follows that any agent which serves to inhibit its oxidation might also be expected to exercise an inhibitory effect on the production of citric acid in cultures of *A. niger* on sugar. Accordingly, in view of the fact that we had already shown that $M/500$ iodoacetate does not interfere with the production of citric acid from sugar, we next examined the behaviour of citric acid-forming moulds towards Na acetate in the presence of iodoacetate, and found that at $M/500$ concentration of the latter poison the synthesis of both malate and citrate from acetate by two species of *Penicillium* was not prevented and, in agreement with this result, we also made the observation that $M/500$ iodoacetate did not interfere with the conversion of succinic acid into *l*-malic acid under the influence of *A. niger* (strain N1). In the presence of $M/450$ iodoacetate *l*-malic acid was still produced, though in diminished yield, from succinic acid. According to Quastel & Whetham [1924] NaF strongly inhibits succinodehydrogenase. Since we had found that NaF at concentration $M/150$ allowed the formation by *A. niger* of a little citric acid from cane sugar, whereas at concentration $M/100$ no citric acid was obtained, the effects of NaF were tried in cultures of *A. niger* on succinic acid. It was found that whereas malic acid was still produced (though only in traces) when the concentration of fluoride was raised to $M/150$, at $M/100$ the formation of malic acid could no longer be demonstrated. It appears, therefore, that the respective concentrations of iodoacetate and NaF which were found to be just sufficient to prevent the mycological conversion of sugar into citric acid are also the concentrations which are necessary to prevent

the conversion of succinate (or acetate) into malate or citrate. Hence, none of the evidence provided by our experimental results disagrees with the suggestions made by one of us [Johnson, 1934] of a possible mechanism of citrate formation from sugar, *via* succinate plus acetate.

EXPERIMENTAL

The moulds employed were six pure strains of *A. niger*, designated N1, N2, B7, B12, B16 and T1, respectively, and cultures of *Penicillium citroenum* and of *P. Johannioli*. The two last named are citric acid-producing species which were kindly placed at our disposal by Prof. T. Chrzęszcz of Poznań. For experimental purposes they were cultivated in most cases on a sterilized medium containing 0.2% NH_4NO_3 ; 0.1% KH_2PO_4 ; 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and glucose or sucrose, 10 or 12.5%.

Action of A. niger on sucrose in presence of M/400 Na iodoacetate

Six strains of *A. niger* were grown at 30° from spores sown on the nutrient salt medium containing 10% of sucrose in 24 Erlenmeyer flasks of 1500 ml. capacity, each flask containing 300 ml. liquid. After 5 days the sugar solutions were poured away, the mycelia were washed as described [Johnson *et al.* 1937] and a fresh medium consisting of 10% sucrose containing M/400 Na iodoacetate was introduced under the mycelia in 12 of the flasks. The other 12 flasks were recharged with 10% sucrose without iodoacetate and were used as controls. All the 24 flasks were then returned to the incubator for a further period of 11 days. The analytical figures are shown in Table I.

Table I. *Iodoacetic acid (as Na salt) at concentration M/400 in 10% cane sugar solution*

Incubation at 30° for 11 days					
Strain of <i>A. niger</i>	Glucose consumed, % of initial wt.	Acidity, ml. 0.1N NaOH to neutralize 100 ml. medium	Acid produced, expressed as % by wt. of the sugar consumed		
			Citric acid	Oxalic acid	Gluconic acid
T 1	13	36.3	5.1	0.7	36.3
N 1	14	45.1	3.0	1.7	46.5
N 2	11	30.8	3.3	1.1	40.3
B 12	11	31.9	2.6	1.7	41.3
B 7	14	49.5	3.2	2.1	50.4
B 16	15	59.4	5.5	1.4	55.1
"Controls", in absence of iodoacetate					
T 1	43.5	110	10.7	—	17.0
N 1	66.6	271	9.7	9.1	17.4
N 2	55.6	150	12.6	2.2	5.9
B 12	62.5	213	13.6	1.5	19.3
B 7	56.0	147	11.0	0.6	15.0
B 16	47.8	117	14.2	0.4	2.9

In order to establish beyond doubt the identities of the three acids (presumed to be citric, oxalic and gluconic, respectively) produced in presence of M/400 iodoacetate (Table I), they were converted into derivatives. From the two first named, the *p*-nitrobenzyl esters were prepared; they melted at 102 and 204°, respectively, alone or in admixture with authentic specimens. The Ca gluconate was purified [cf. Butkewitsch, 1924] and dried at 100–110°. Found: CaO, 12.9%. Calc.: CaO, 13.02%. The phenylhydrazide of the acid was prepared and sintered

at 195°, M.P. (decomp.) c. 200° [cf. Butkewitsch, 1924; Fischer & Passmore, 1889]. Found: C, 50.2; H, 6.5; N, 10.0%. $C_{12}H_{18}O_6N_2$ requires C, 50.35; H, 6.3; N, 9.8%.

Action of A. niger on sucrose in presence of Na iodoacetate M/1000 and M/500

The mould T1 was cultivated in 20 flasks of 350 ml. capacity each charged with 100 ml. 10% sucrose saline medium. After growing for 4 days at 30° the mycelia were washed and the medium in 10 flasks was replaced by 10% sucrose and in the other 10 flasks by 10% sucrose together with M/1000 Na iodoacetate. This experiment was repeated with another series of 20 flasks, in which case M/500 iodoacetate was used. For the analyses, samples withdrawn from each of the 10 flasks which formed a series were mixed; thus the figures represent the averages of ten values in each case. The data are shown in Table II.

Table II. *A. niger*, T1, incubated at 30° on 10% sucrose solution in presence and in absence of M/1000 and M/500 Na iodoacetate

Time in days	Acidity, ml. 0.1 <i>N</i> NaOH to neutralize	Acidity expressed as mg. acid per 100 ml. medium			Acidity, ml. 0.1 <i>N</i> NaOH to neutralize	Acidity expressed as mg. acid per 100 ml. medium		
	100 ml. medium	Citric acid	Oxalic acid	Gluconic acid	100 ml. medium	Citric acid	Oxalic acid	Gluconic acid
	In presence of <i>M</i> /1000 iodoacetate				In absence of iodoacetate			
3	30	+	—	—	62	+	—	—
6	58	+	—	—	95	39	—	1744
8	64	155	+	842	116	78	22	2000
10	72	227	+	725	125	115	46	2000
13	84	266	3	842	158	143	89	2646
15	96	305	5	960	166	127	115	2606
17	106	337	5	1038	174	95	156	2783
22	144	470	202	1352	204	82	237	3234
In presence of <i>M</i> /500 iodoacetate					In absence of iodoacetate			
6	58	216	—	666	86	93	—	1460
8	66	237	—	568	98	96	63	1489
10	74	258	Trace	666	110	98	73	1705
13	96	358	103	686	134	102	101	2097
15	108	340	155	744	158	132	125	2430
17	118	335	193	862	169	145	145	2556
20*	136	238	345	1192	152	102	163	2313

* On the 20th day sugar estimations showed that in the flasks containing M/500 iodoacetate the sugar consumption had amounted, on the average, to 80% of the quantity initially present, whilst in absence of iodoacetate 66.75% of the sugar had been consumed.

Action of A. niger on sucrose in the presence of NaF

The mould T1 was cultivated on a 10% sucrose medium as in the previous experiments, and on the 5th day from the time of inoculation this medium was poured away, the mycelia were washed, and fresh 10% cane sugar solutions, containing different concentrations of NaF, were introduced under the mycelia in the several flasks. The culture media were examined after further incubation at 30° for 6 days and 13 days, respectively. In Table III the analytical data obtained on the 13th day are given. Oxalic acid was not detected in any of the flasks, and no citric acid could be detected in presence of M/100 NaF.

Table III. *A. niger*, *T1*, incubated at 30° for 13 days on 10% sucrose solution in the presence of NaF

Conc. of NaF	Sugar consumed, % of initial wt.	Acidity, ml. 0.1N NaOH to neutralize 100 ml. medium	Citric acid: (a) mg. per 100 ml. medium; (b) % of wt. of glucose consumed		Gluconic acid: (a) mg. per 100 ml. medium; (b) % of wt. of glucose consumed	
			(a)	(b)	(a)	(b)
<i>N/150</i>	28	36.3	20	0.7	664	23.6
<i>N/200</i>	28.5	38.5	36	1.3	650	22.9
<i>N/250</i>	34	55.0	62	1.8	900	27.0
<i>N/300</i>	32	48.0	94	2.9	700	24.9
<i>N/400</i>	23	22.0	47	2.0	294	12.8
Control (no fluoride)	45	68.2	422	9.4	44	1.0

The action of Penicillium citrogenum and of P. Johannoli on Na acetate in the presence of M/500 Na iodoacetate

These two *Penicillium* species were chosen in preference to *A. niger* for this particular experiment since they had been found by Chrzęszcz & Tiukow to be particularly suitable for demonstrating the mycological production of citric acid from acetic acid. Moreover, previous experiments with *P. citrogenum* had shown that when it was placed on a 12.5% glucose medium production of citric acid was considerably curtailed in presence of *M/500* iodoacetate.

Cultures of the two moulds were grown in 24 boiling tubes (12 tubes for each species) from spores sown on a medium consisting of sucrose (5%) in nutrient inorganic salt solution. After incubation for 4 days at 25° the mycelia were transferred to 24 conical flasks (1500 ml.) each containing 330 ml. of the same medium and incubation at 25° was conducted for 6 days, after which the mycelial mats were washed thoroughly with sterile water and allowed to stand overnight over water. After this treatment they no longer diffused citric acid into the water. The mycelia were then floated on a sterile aqueous solution of Na acetate (5.6% equivalent to 2.5% acetic acid) containing also *M/500* Na iodoacetate. The metabolism solutions were collected for examination after the mycelia had been in contact with the acetate medium for 8 days at 25°.

(1) *P. citrogenum* cultures. The mixed culture fluid from the 12 flasks was filtered and evaporated to one-third of its volume after which sat. Pb acetate was added to it in slight excess and the mixture was allowed to stand for 3 hr. The white precipitate which had formed was filtered and washed at the pump until the washings were free from Pb. The filtrate (A) was preserved for examination. The washed precipitate was suspended in 1750 ml. water, 600 ml. 2% acetic acid were added and the whole was warmed for 2 hr. on a steam bath and then filtered. The undissolved solid matter was well washed and the washings were added to the filtrate (B) which was preserved for examination. The washed solid (C) was suspended in water and decomposed by H₂S. The PbS was removed by filtration and the filtrate was evaporated to one-third of its volume. In this liquid were found the largest proportions of acidic products and these were dealt with as follows. Qualitative tests having shown the presence of citric and oxalic acids, these were separated through their Ca salts in the usual manner. A portion of the fraction which was presumed to be Ca citrate after repeated crystallization from hot water and drying at 130° gave 0.2451 g. pure Ca salt. Found: CaO, 33.9%. Calc. for anhydrous tricalcium citrate: CaO, 33.74%. A further portion (1.5 g.) of the same material was treated with the calc. H₂SO₄ and, after removal of the CaSO₄, the solution was

evaporated to one-half of its volume, neutralized with 0.5*N* alcoholic KOH and finally evaporated to dryness. To the solution of the K salt in 10 ml. water were added 30 ml. alcohol and 2 g. *p*-nitrobenzyl bromide. The whole was heated under reflux for 2 hr. after which, on cooling, the product separated and was collected and recrystallized from aqueous alcohol. The purified material had m.p. 102° alone or in admixture with an authentic and pure specimen of the tri-*p*-nitrobenzyl ester of citric acid.

The Ca salt obtained from the fraction presumed to contain oxalic acid was purified in the usual manner and analysed. Found: Ca, 38.11%. Calc. for $C_2O_4Ca, H_2O: Ca, 38.36\%$. A small quantity of the acid was converted into its *p*-nitrobenzyl ester which, after purification, had m.p. 204° alone or in admixture with an authentic specimen of the di-*p*-nitrobenzyl ester of oxalic acid.

Filtrate (A) was evaporated to one-half of its volume and this concentrate was mixed with 3 vol. of alcohol, but only a faint cloudiness appeared and no substance could be identified. Filtrate (B) was treated with H_2S , the precipitate of PbS removed and the filtrate evaporated, when a small quantity of sticky solid remained. This was freed from the last traces of water and acetic acid by addition of a mixture of benzene and alcohol and subsequent evaporation *in vacuo*. About 0.5 g. solid material remained which gave a strong positive reaction for malic acid when submitted to Denigès' Hg acetate test. This was confirmed by conversion of the acid into acetaldehyde which in turn was characterized by condensation with dimethyldihydroresorcinol to yield aldomedon, m.p. 142°.

(2) *P. Johannioli* cultures. The metabolism solution from these cultures was treated in a similar manner. Citric acid and oxalic acid were both isolated and characterized as their respective *p*-nitrobenzyl esters, but the quantities of these acids were less than those obtained in the case of *P. citrogenum*.

*The action of A. niger on NaH succinate in the presence
of Na iodoacetate*

Seventeen conical flasks (1000 ml.) were each charged with 250 ml. sucrose (5%) in the nutrient salt medium of Wells *et al.* [1936]. After sterilization the solutions were inoculated with cultures of *A. niger*, strain N1, previously developed from spores in boiling-tubes containing the same medium. After incubation for 5 days at 30° the culture fluid was poured away and the mycelial mats were washed with sterile water and allowed to stand for 6 hr. over fresh sterile water. The latter was then poured away and seven of the flasks were each charged with 250 ml. of a sterile 4% aqueous solution of NaH succinate containing *M*/500 Na iodoacetate. Seven other flasks were treated similarly except that in these the concentration of iodoacetate was *M*/450. The remaining three flasks contained succinate without iodoacetate as controls. All the flasks were returned to the incubator at 30° and from time to time samples were withdrawn and tested for the presence of malic acid by Denigès' Hg acetate solution. Positive tests were obtained after 7 days, and after 14 days the solutions were combined into two lots and examined. Since the colour in both cases was too dark to permit direct polarimetric examination, aliquots (100 ml.) of each were made alkaline with strong NH_3 and treated with excess Pb acetate. After the addition of 50 ml. alcohol each solution was allowed to stand for 48 hr. The precipitates which had settled were then filtered and after washing with 30% alcohol they were ground with water to a fine suspension and treated with H_2S . The PbS was removed, the clear solutions were boiled to expel H_2S and

their rotations were measured in 1 dm. tubes in the presence of ammonium molybdate [Williamson, 1918] with the following results: sample from *M*/500 iodoacetate flasks, $+1.6^\circ$ (Ventzke scale); sample from *M*/450 iodoacetate flasks, $+0.6^\circ$; sample from control iodoacetate flasks, $+0.85^\circ$. Since molybdate reverses the sign of rotation, these results represent *laevo* rotations caused by *l*-malic acid. A preliminary test had shown that *M*/450 iodoacetate did not affect the accuracy of Williamson's method. Since the separation of Pb salts was not quantitative, the values for the solutions incubated in presence of iodoacetate cannot be compared accurately with the "control".

The action of A. niger on NaH succinate in the presence of NaF

Preliminary experiments indicated that spores of *A. niger* (strain N1) would germinate in sugar solutions containing *M*/150 NaF but not in *M*/100. To each of a series of conical flasks (1000 ml.) containing 250 ml. of a 4% aqueous solution of NaH succinate, a different quantity of NaF was added and three flasks were left as controls without NaF. All were inoculated with spores of *A. niger* (strain N1) and were incubated at 30° . The mould grew freely in all the flasks and after 14 days Denigès' test for malic acid was positive in all cases, but the amounts of malic acid detected, including those found in the control flasks, were very small. After 28 days a mixed sample from the controls was treated with ammonium molybdate for polarimetry in a 2 dm. tube, when the rotation was found to be $+0.45^\circ$ (Ventzke scale) corresponding to a 0.015% solution of *l*-malic acid, while the culture liquid containing *M*/150 NaF on similar treatment showed $+0.3^\circ$, corresponding to 0.01% *l*-malic acid. A preliminary test had shown that *M*/150 NaF did not interfere with the polarimetric estimation of malic acid by this procedure.

SUMMARY

1. The effects of *M*/400 Na iodoacetate on the behaviour of *A. niger* (6 strains) in contact for 11 days at 30° with 10% sucrose solution, as shown by comparison with cultures incubated in absence of iodoacetate, resulted in (a) a restriction in the total acid formation; (b) a restriction in the quantity of sugar used to about 30% of that normally metabolized; (c) a decrease in the yield of citric acid expressed as a percentage of the sugar consumed; and (d) an increase in the yield of gluconic acid expressed as a percentage of the sugar consumed.

2. Iodoacetic acid (*M*/1000 or *M*/500 as Na salt) in 10% sucrose solution in contact with the fully developed mycelium of *A. niger* (strain T1) at 30° caused in a given time: (a) a diminished acid formation; (b) a large increase in the weight of citric acid formed; and (c) a large decrease in the weight of gluconic acid formed, as compared with a control experiment made in absence of iodoacetate.

3. NaF when added at concentrations from *M*/150 to *M*/400 to cultures of *A. niger* on cane sugar depressed the consumption of the sugar, the development of titratable acidity and the formation of citric acid. Spores of *A. niger* did not germinate in a nutrient salt-sugar medium containing *M*/100 NaF, and the same concentration of this poison was found to suppress completely the formation of citric acid by mycelia which had been grown normally before being brought into contact with fluoride.

4. Na iodoacetate (*M*/500) did not prevent the formation of malic and citric acids in cultures of *Penicillium citroenum* and *P. Johannioli* on a Na acetate solution.

5. Na iodoacetate, *M*/500 or *M*/450, did not prevent the formation of *l*-malic acid from succinic acid by *A. niger* (strain N1).

6. NaF (*M*/150) did not prevent the formation of *l*-malic acid from succinic acid by *A. niger* (strain N1).

The results summarized under headings 4, 5 and 6, when considered in conjunction with the other results described in this communication and in Part II [Johnson *et al.* 1937], provide no evidence against the view that succinic and acetic acids are intermediates in the mycological conversion of sugar into citric acid.

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