

XXXIII. ON THE MECHANISM OF OXALIC ACID FORMATION BY *ASPERGILLUS NIGER*.

BY HAROLD RAISTRICK AND ANNE BARBARA CLARK.

From the Biochemical Laboratory, Cambridge.

REPORT TO THE MEDICAL RESEARCH COMMITTEE.

(Received September 4th, 1919.)

THE production of organic acids, as a result of the growth of micro-organisms in solutions containing sugars, is of great importance both from a theoretical and practical point of view. A comparison of the acids produced by different organisms reveals the fact that, whereas the *bacteria* as a class chiefly produce monobasic acids, *e.g.*, acetic acid, butyric acid, and lactic acid, the formation of polybasic acids, *e.g.*, oxalic acid, citric acid, and fumaric acid, is almost entirely limited to the *fungi*. Even of the fungi the only family known to possess this chemical activity is that of the Aspergillaceæ. Although there were previously in the literature a number of isolated references to the production of oxalic acid by *Aspergillus niger*, the proof that this micro-organism really forms oxalic acid from sugar was not satisfactorily established until Wehmer published his classical researches on the subject in a series of papers in 1891. In addition to investigating the production of free oxalic acid from glucose under a variety of different conditions, he also showed that salts of oxalic acid are produced by the same fungus from salts of a few organic acids—notably from tartrates, malates, and citrates. He made no attempt, however, to offer any chemical explanation of the steps involved in the process. More recently Emmerling [1903] investigated the production of oxalates, by *A. niger*, from amino-acids and other protein derivatives. In particular, although he found that ammonium oxalate was produced in good yield from aspartic acid, he was quite unable to obtain any from the chemically allied substances—the ammonium salts of succinic, malic and tartaric acids. Heinze [1903], in a paper singularly devoid of experimental details, states that in the decomposition of glucose by *A. niger*, acetic acid is produced in addition to oxalic acid.

Until recently, it has been generally accepted that the production of citric acid from sugar was characteristic of a group of fungi, to which Wehmer gave the generic name of Citromyces, and that oxalic acid fermentation was almost equally characteristic of *Aspergillus niger*. That this is not so was proved by Currie [1917]. Working with a number of different strains of *A. niger*, he

showed that all of them produced from saccharose both oxalic acid and citric acid, the proportions of which could be varied by varying the conditions of the experiment. He pictured the general equation of metabolism of *A. niger* as follows:

Carbohydrate \rightarrow citric acid \rightarrow oxalic acid \rightarrow carbon dioxide \rightarrow mycelium,

and says "The equation has been written in this order, because *Aspergillus niger* will readily form oxalic acid when given only citric acid as a source of carbon."

A short time ago, Wehmer [1918] isolated another member of the *Aspergillaceæ*, to which he gave the name *Aspergillus fumaricus*, which produces from sugar fumaric acid, together with a little citric acid. These three poly-basic acids, oxalic, citric, and fumaric, are not to be regarded as extraneous by-products of the degradation of sugar by these micro-organisms, as is, for example, the succinic acid produced during the alcoholic fermentation of sugar, which has been shown by Ehrlich [1909] to have its origin in the glutamic acid of the yeast protein. A consideration of the yield, which is in each case above 50 % of the sugar consumed, shows that these acids really represent definite stages in the degradation of the sugar molecule.

It will be noticed that, with the exception of Currie, no worker has attempted to offer any explanation as to the steps involved in the formation of these acids from sugar. The experiments in this paper were undertaken in the hope of elucidating the mechanism of the production of oxalic acid from sugar by *Aspergillus niger*. It is proposed to study the production of citric acid at some future time.

From a consideration of the literature, we came to the conclusion that a direct method of investigation, though admittedly more satisfactory, offered less hope of success than an indirect attack. The former method involves the isolation of intermediate products, in this case between sugar and oxalic acid, since it is certain that this action does not take place in one stage. The latter method involves the investigation of the production, or non-production, of oxalic acid from possible intermediate compounds, on the assumption that a failure to produce oxalic acid from the substance under investigation justifies the conclusion that that substance does not represent a stage in the reactions involved in the production of oxalic acid. Production of oxalic acid, however, does not necessarily indicate that the substance in question is an intermediate product.

For these reasons, the method employed was to estimate the oxalic acid formed (but present as a salt of oxalic acid) when *A. niger* was cultivated in solutions containing salts of organic acids as the sole source of carbon. We were led to use salts of organic acids, rather than the free acids themselves, since, according to Wehmer, free oxalic acid, in the absence of other available carbon, is readily decomposed by *A. niger* at 37°, whereas fixation of the acid, as a salt, prevents its further decomposition by the fungus to any appreciable extent.

EXPERIMENTAL.

Throughout this work one strain of *Aspergillus niger* was used. This was obtained from Dr Paine of the Imperial College of Science and Technology, S. Kensington, and was identified by Mr F. T. Brooks of Cambridge. To both of them our thanks are due.

A culture solution was made up with the following composition:

Water	1000 cc.
NH ₄ NO ₃	25 g.
KH ₂ PO ₄	12.5 g.
MgSO ₄ ·7H ₂ O	6.25 g.

20 cc. of this, diluted to 50 cc., gave one of Wehmer's solutions, having the following composition:

Water	50 cc.
NH ₄ NO ₃	0.5 g.
KH ₂ PO ₄	0.25 g.
MgSO ₄ ·7H ₂ O	0.125 g.

This solution is subsequently referred to as "Culture Solution A." In some cases 1.5 g. of a salt of the acid under investigation was weighed out, dissolved in 30 cc. water, and to this 20 cc. of the undiluted culture solution was added. In other cases, where it was more convenient to use the free acid as a starting point, 1.5 g. of the acid was weighed out and neutralised with the necessary amount of normal alkali (*N* soda or *N* ammonia), as determined by titration. The volume was then made up to 30 cc. by adding the calculated amount of water from a burette, and, as before, 20 cc. of the culture solution was added. In each case the 50 cc. of solution was contained in a 200 cc. flask, autoclaved, inoculated with spores of *A. niger*, and incubated at 37° in the dark.

After a definite period of time, the oxalic acid content was estimated quantitatively by the usual method. The fungus was filtered off, and thoroughly extracted with hot dilute HCl, and hot water. The combined filtrate and washings were made distinctly alkaline with ammonia, and then acid with acetic acid. The oxalic acid was then precipitated from the boiling solution as the calcium salt, by the addition of calcium acetate and acetic acid. After standing overnight, the washed calcium oxalate was titrated with standard permanganate in the usual way.

This method was shown to be quantitative by adding a known weight of oxalic acid to 50 cc. of the culture solution A, and estimating as above.

	Oxalic acid added to culture solution	Oxalic acid estimated
(a)	0.1964 g.	0.1967 g.
(b)	0.1954 g.	0.1944 g.

The only difficulty met was with culture solutions containing tartaric acid. On standing overnight, a crystalline precipitate, probably consisting of calcium racemate, was found to be mixed with the calcium oxalate. The mixed calcium salts were filtered off, washed with hot water, re-dissolved in hot *N* HCl, made

alkaline with ammonia, and then made acid with acetic acid. After the addition of a little calcium acetate, and while the solution was still hot, the calcium oxalate was filtered off, washed, and estimated in the usual way. The calcium racemate remains in solution, and though a very small amount of calcium oxalate may also remain dissolved in the hot solution, the method is sufficiently accurate for our purpose.

Reference to the results given later shows that we were unable, under any conditions, to obtain any oxalate formation from salts of lactic acid. It was thought possible that the method might not be strictly quantitative in the presence of lactic acid. That this is not so was proved in the following way. To the filtrate and washings from sodium lactate C (Table IV, p. 335), to which had been added calcium acetate in the usual way, without any precipitation of calcium oxalate, was added 100 cc. standard oxalic acid solution (= 15.20 cc. potassium permanganate solution). The precipitated calcium oxalate required 15.46 cc. permanganate solution.

Preliminary Experiments.

In order to obtain some idea of the activity of *A. niger*, the amount of oxalic acid produced from the ammonium salts of a series of acids was determined. (In the case of glyoxylic acid, the calcium salt was used.) The results are summarised in the following table:

Table I.
1.5 g. acid as ammonium salt. Culture Solution A.

Acid	Incubation period in days	Growth	Oxalic acid produced, expressed as g. $C_2H_2O_4$	Percentage of theoretical yield
Butyric	27	—	—	—
Succinic	24	+++	0.3764	16.45
Fumaric	25	+++	0.2671	11.48
Malic	25	+++	0.3477	17.26
Tartaric	26	++	0.0609	3.39
Propionic	27	—	—	—
Pyruvic	26	+++	0.0115	0.53
Lactic	26	++++	0	0
Acetic	26	+	0.0201	0.93
Glycollic	26	+	0	0
Glyoxylic (Ca salt)	32	+	0	0
Culture solution A as control	32	trace	0	0

+++ indicates extremely heavy growth.

++ " heavy growth.

++ " fairly good growth.

++ " distinct growth.

— " no growth.

The oxalic acid produced is calculated as anhydrous $C_2H_2O_4$. The theoretical yield is calculated on the assumption that all the carbon of the acid

investigated (determined by titration) is converted into oxalic acid, and our results are expressed as a percentage of this value.

These results indicate that the chemical constitution of the acids, given as a source of carbon to the fungus, is of primary importance for the production of oxalic acid. Thus, although the best growth was obtained with the three-carbon acids, pyruvic and lactic, the amount of oxalic acid produced was nil or negligible, whereas with the four-carbon dibasic acids—succinic, fumaric, malic and tartaric—good yields were obtained. Similarly with the two-carbon acids—acetic, glycollic and glyoxylic—although growth was approximately the same in each case, acetic acid was the only one from which any oxalic acid was produced. It will be noticed that, although *A. niger* grew slightly in the culture solution alone, there was no growth at all when the ammonium salt of propionic acid or butyric acid was present. This fact was also observed by Wehmer, who was unable to obtain any growth from these two acids, under any conditions. This was probably due to hydrolytic dissociation of the organic salts, with the consequent formation of a certain amount of the free acids, which, above a certain concentration, are known to have a deleterious effect on the fungus [Duclaux, 1889]. For this reason, in the subsequent work two other culture solutions were made up, containing, in place of KH_2PO_4 , the one Na_2HPO_4 , and the other Na_3PO_4 . It was hoped that this would have a beneficial effect in fixing any oxalic acid that might be formed.

Main Series of Experiments.

In the following series of experiments, the conditions of which are similar to those of the preliminary series, three culture solutions were used:

1. *Culture Solution A* has already been described.
2. *Culture Solution B*. A stock solution was made such that 20 cc. diluted to 50 cc. (as with A) had the following composition:

Water	50 cc.
NH_4NO_3	0.5 g.
Na_2HPO_4	0.25 g.
K_2SO_4	0.125 g.
MgCl_2	0.005 g.

3. *Culture Solution C*. A stock solution was made such that, on dilution, (as with A and B) it had the following composition:

Water	50 cc.
NH_4NO_3	0.5 g.
Na_3PO_4	0.25 g.
K_2SO_4	0.125 g.
MgCl_2	0.005 g.

The production of oxalic acid (as oxalates) from a large series of acids, present as sodium, ammonium, or calcium salts, was investigated. The results, which are summarised in the following tables, are expressed as in Table I. We have also incorporated with them, for comparison, the results given in Table I.

Table II.

Four-carbon dibasic acids.

Acid	Salt	Medium	Incubation period in days	Growth	Oxalic acid produced, expressed as g. $C_2H_2O_4$	Percentage of theoretical yield
Succinic ...	Na	A	38	+++	1.0503	45.90
	"	B	38	++	0.8113	35.46
	"	C	38	+	0.4227	18.47
	NH ₄	A	24	+++	0.3764	16.45
	"	A	38	+++	0.6360	27.80
Fumaric ...	Na	A	38	+++	1.050	45.11
	"	B	38	++	1.074	46.14
	"	C	38	++	0.7910	34.00
	NH ₄	A	25	+++	0.2671	11.48
Maleic ...	Na	A	34	+	0	0
	"	B	34	+	0.0049	0.28
	"	C	34	+	0.0105	0.62
	"	A	38	+	0	0
	"	B	38	+	0.0080	0.48
	"	C	38	+	0.0333	1.97
Malic ...	Na	A	38	++	0.6493	32.22
	"	B	38	++	0.5506	27.33
	"	C	38	+++	0.951	47.2
	NH ₄	A	25	+++	0.3477	17.26
	"	A	38	++	0.3170	15.73
Tartaric ...	Na	A	38	+	0	0
	"	B	38	+	0	0
	"	C	38	trace	0	0
	NH ₄	A	26	++	0.0609	3.39

Table III.

Four-carbon monobasic acids.

Acid	Salt	Medium	Incubation period in days	Growth	$C_2H_2O_4$ g.	Percentage of theoretical yield
Butyric ...	Na	A	38	—	—	—
	"	B	38	+	0.0080	0.33
	"	C	38	—	—	—
	NH ₄	A	27	—	—	—
Isobutyric ...	Na	A	38	—	—	—
	"	B	38	—	—	—
	"	C	38	—	—	—
β -Hydroxybutyric	Na	A	38	—	—	—
	"	B	38	—	—	—
	"	C	38	—	—	—
Hydroxyisobutyric	Na	A	38	—	—	—
	"	B	38	—	—	—
	"	C	38	—	—	—

Table IV.

Three-carbon acids.

Acid	Salt	Medium	Incubation period in days	Growth	C ₂ H ₂ O ₄ g.	Percentage of theoreti- cal yield
Lactic ...	Na	A	38	++++	0	0
		B	38	+++	0	0
		C	38	+++	0	0
		A	7	++	0	0
		B	7	++	0	0
		C	7	++	0	0
	NH ₄	A	26	++++	0	0
		A	38	++++	0	0
		B	38	+++	0	0
		C	38	+++	0	0
Pyruvic ...	Na	A	38	+++	0.0134	0.61
		B	38	++	0.1628	7.44
		C	38	++	0.1478	6.75
	NH ₄	A	26	+++	0.0115	0.53
Glyceric ...	Na	A	31	+++	0	0
		B	31	+++	0.0213	1.81
		C	31	+++	0.0258	2.19
		A	38	+++	0.0120	1.02
		B	38	+++	0.0838	7.11
		C	38	+++	0.0181	1.54
	NH ₄	A	38	+++	0	0
		B	38	+++	0	0
		C	38	++	0	0
Malonic ...	Na	A	38	+	0	0
		B	38	+	0	0
		C	38	+	0	0
	Ca	A	38	—	—	—
		B	38	—	—	—
		C	38	—	—	—
Propionic	Na	A	38	—	—	—
		B	38	++	0.0825	4.64
		C	38	trace	—	—
	NH ₄	A	27	—	—	—

Table V.

Two-carbon acids.

Acid	Salt	Medium	Incubation period in days	Growth	C ₂ H ₂ O ₄ g.	Percentage of theoreti- cal yield
Acetic ...	Na	A	38	+++	0.3869	38.98
		B	38	+++	0.4534	45.70
		C	38	+	0.0111	1.12
		A	38	+++	0.4211	42.44
		B	38	+++	0.4534	45.70
		C	38	trace	—	—
	NH ₄	A	26	+	0.0201	0.93
Glycollic ...	Na	A	38	+	0	0
		B	38	+	0	0
		C	38	+	0	0
Glycollic (a differ- ent sample of glycollic acid)	Na	A	38	++	0.0034	0.20
		B	38	++	0.0045	0.27
		C	38	++	0.0036	0.22
		A	26	+	0	0
	NH ₄	A	26	+	0	0
Glyoxylic ...	Ca	A	32	+	0	0

Table VI.

One-carbon acid.

Acid	Salt	Medium	Incubation period in days	Growth	C ₂ H ₂ O ₄ g.	Percentage of theoretical yield
Formic	Na	A	38	++	0	0
	"	B	38	++	0	0
	"	C	38	+	0	0

Control cultures containing only culture solution A, B, or C were inoculated with *A. niger* and grown for 38 days at 37°. No oxalic acid was formed.

In each experiment a control flask, containing the salt of the acid under investigation and culture solution, was incubated without inoculation. In no case was any oxalic acid formed.

INFLUENCE OF THE SOURCE OF NITROGEN ON OXALATE FORMATION.

In the literature on oxalic acid formation from sugar by *A. niger*, frequent references are made to the importance of the source of nitrogen supply. Thus Wehmer, in Lafar's handbook [1910], says "A decisive influence is also exerted by the inorganic bodies present, especially the source of nitrogen for the growing fungus, the liberation of acid (oxalic) being absent (in spite of good growth), when ammonium chloride or sulphate is substituted for potassium, calcium, or ammonium nitrate (even in the presence of sugar),—and, in fact, these additions will prevent the formation of acid in cultures that would otherwise acidify at once." This he explains as follows: The assimilation of nitrogen from a nitrate by the fungus results in the liberation of a base from the nitrate, *i.e.*, NH₄OH, NaOH, KOH or Ca(OH)₂, which would fix a portion of the oxalic acid formed, thus preventing its decomposition. The assimilation of nitrogen from ammonium chloride or sulphate, however, would result in the production of a mineral acid—HCl or H₂SO₄—which would tend to favour the decomposition of the oxalic acid, which is known to be much more easily decomposed by *A. niger* than are its salts. This explanation seems a little difficult to accept, in view of the following facts:

1. Only a relatively small amount of the nitrogen supplied is metabolised as protein, as we have proved.

2. Currie [1917] has shown that oxalic acid, along with citric acid, is produced from sugar in culture media made acid to P_H 3.4–3.5 with HCl.

It seemed to us desirable, because of the importance that we attached to acetic acid as a probable precursor of oxalic acid, to prove whether the nitrate ion has any specific importance in the production of oxalates (as distinct from free oxalic acid) from sodium acetate. With this end in view, *A. niger* was cultivated on solutions containing sodium acetate as the sole source of carbon, the nitrogen being supplied as ammonium nitrate, sulphate, chloride or phosphate.

Otherwise the conditions of the experiment were as before. The results given in Table VII are expressed in the same way as in the other tables.

Table VII.

1.5 g. crystalline Na acetate (contains 0.904 g. $C_2H_3O_2 Na$).

Ammonium salt	Medium	Incubation period in days	Growth	$C_2H_4O_4$ g.	Percentage of theoretical yield
NH_4NO_3	A	38	+++	0.4211	42.44
	B	38	+++	0.4534	45.70
	C	38	trace	—	—
$(NH_4)_2HPO_4$	A 1	38	++	0.4204	42.37
	B 1	38	+++	0.4836	48.73
	C 1	38	+++	0.4873	49.11
NH_4Cl	A 2	38	+++	0.4067	40.98
	B 2	38	++	0.4739	47.77
	C 2	38	trace	—	—
$(NH_4)_2SO_4$	A 3	38	+++	0.4054	40.86
	B 3	38	++	0.4707	47.43
	C 3	38	trace	—	—

Culture Solutions:

A, B, C have the same composition as previously.

A 1, B 1, C 1 have the same composition as A, B, C, with the exception that NH_4NO_3 is replaced by the same weight of $(NH_4)_2HPO_4$.

A 2, B 2, C 2, NH_4NO_3 replaced by NH_4Cl .

A 3, B 3, C 3, NH_4NO_3 „ „ $(NH_4)_2SO_4$.

These remarkably concordant results show that the nitrate ion has no specific effect on oxalate formation, and confirm the previous observation that *A. niger* can oxidise acetic acid to oxalic acid very smoothly.

DISCUSSION OF RESULTS.

A consideration of the results obtained with the four-carbon dibasic acids shows that, in general, they give remarkably good yields of oxalic acid. In particular, this is especially true of succinic, fumaric, and malic acids. The failure of *A. niger* to produce any oxalic acid from the sodium salts of tartaric acid is probably due to the fact that the fungus only grew very slightly. The poor growth and poor yield of oxalic acid obtained with sodium maleate is in agreement with the observation made by Buchner [1892], who showed that *A. niger* could utilise fumaric acid, but not maleic acid, as a source of carbon. This he was led to expect from the fact that, whilst fumaric acid is a normal constituent of many plants, the isomeric maleic acid has not yet been found in nature. The small amount of growth we did obtain was probably a result of the molecular rearrangement of a portion of the sodium maleate, under the conditions of our experiment.

The outstanding feature of the experiments with the four-carbon monobasic acids is the almost entire absence of growth and failure to produce oxalic acid. The isolated case in which growth and oxalic acid formation was observed justifies the conclusion previously made as to the reason for the failure of the fungus to grow in a solution containing ammonium butyrate.

Most surprising and unexpected results were obtained from the series of acids containing three-carbon atoms. In almost all cases we obtained magnificent growths of the fungus, which were, on the whole, better than those obtained with the four-carbon dibasic acids. In spite of this, the yield of oxalic acid was either nil or very small. In particular was this the case with lactic acid, which is known to be an almost constant product of the *bacterial* degradation of sugar. A somewhat similar result was obtained by Wehmer [1891]. He cultivated *A. niger* on a solution identical with our culture solution A, and obtained the following results. In one experiment with free lactic acid he obtained no oxalic acid after 101 days; in two experiments with potassium lactate, after the same length of time, he observed the formation of 3.67 % of oxalic acid in the one case, and of no oxalic acid in the other. Finally with calcium lactate, in two experiments having an incubation period of 43 days, he obtained 0.76 % and 0.96 % respectively. That the oxalic acid is not first formed, and then decomposed, is indicated by our results, which proved its non-production after an incubation lasting only seven days.

A comparison of the results obtained with the two-carbon acids—acetic, glycollic, and glyoxylic—shows a sharp differentiation between acetic and the other two acids. With the exception of the one experiment with ammonium acetate, on which the fungus only grew very slightly, the yield of oxalic acid obtained from acetic acid was from 39 % to 49 %, whereas with glycollic and glyoxylic acids the yield was either nil or negligible. The behaviour of *A. niger* towards these three acids offers another example of the difference between biological and chemical reactions, for although it is a relatively simple matter to oxidise glycollic and glyoxylic acids to oxalic acid *in vitro*, it is difficult to obtain oxalic acid from acetic acid. The ease with which *A. niger* oxidises acetic acid seems to us to be a very significant fact.

No oxalic acid is produced from the one-carbon acid—formic acid—although the growth was relatively good. This suggests that oxalic acid formation is not a question of synthesis.

Three facts have been proved conclusively in the experiments described in this paper:

I. Acetic acid is the only one of the three possible two-carbon acids which is oxidised by *A. niger* to oxalic acid.

II. Taken as a class, the three-carbon acids are not oxidised to oxalic acid by the fungus to any appreciable extent.

III. The four-carbon dibasic acids are, as a class, smoothly oxidised to oxalic acid, whilst the four-carbon monobasic acids give no oxalic acid.

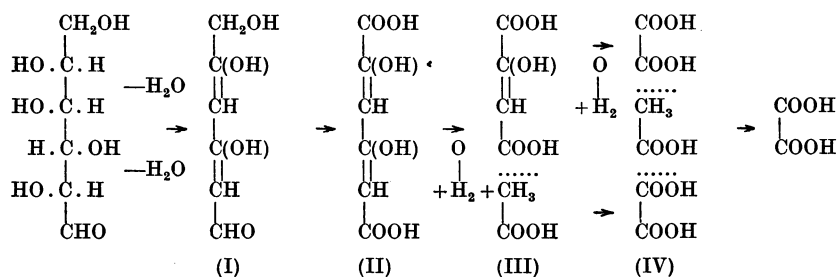
A consideration of these facts leads to the following conclusions:

(I) The breakdown of the sugar molecule by *A. niger* does not take place by a primary splitting of one molecule of sugar into two molecules of a three-carbon acid since, as was pointed out in the introduction, the non-production of oxalic acid from them, by *A. niger*, necessarily precludes the possibility of their being precursors of oxalic acid.

(II) The breakdown cannot take place carbon by carbon, leaving the last two carbon atoms in the chain to be oxidised to oxalic acid, since this would result in the production of one-carbon compounds, and it has been shown that *A. niger* is unable to *synthesise* oxalic acid. Also, if this hypothesis were correct, it would follow that the maximum yield obtainable would be about 33 %, whereas, in practise, the yield is usually about 50 %. It is also extremely unlikely on general grounds.

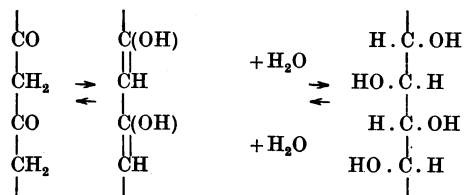
(III) The breakdown probably takes place in two stages, involving in the first stage the production, from one molecule of sugar, of one molecule of a two-carbon compound, and one molecule of a four-carbon compound. This is then broken down in the second stage into two molecules, each containing two carbon atoms.

To meet these conclusions the following scheme is suggested, since it seems to fit in with all the known facts:



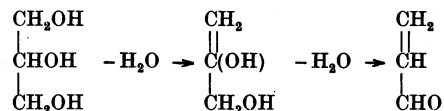
The first stage (I) suggested represents the formation, by simple dehydration, of the enol form of a "polyketide." So long ago as 1893 and, more recently, in 1907, Collie [1893, 1907], who proposed the term "polyketides" for the series of compounds containing $-\text{CH}_2 \cdot \text{CO}-$ groups, pointed out the importance for biological chemistry of this class of compounds. These observations do not seem to us to have received from biochemists the consideration that they deserve. As a class, the "polyketides" give rise to a large series of compounds which either occur in nature or are closely related to naturally occurring compounds. Moreover, the reactions involved are of a very simple nature, *e.g.*, hydration, dehydration, polymerisation, etc., and very often take place at the ordinary temperature. In particular, Collie

pointed out the close relationship between the sugars and the keten group—though at present we have no means of synthesising the sugars from keten or from any of its simple derivatives. Thus, if one takes two keten groups,

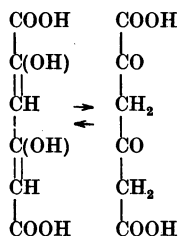


by simple hydration one arrives at a system of four-carbon atoms as present in the sugars.

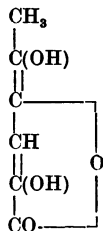
Conversely, the removal of two molecules of water from one molecule of sugar—a change which is probably very easily accomplished by the fungus—would immediately give rise to a very reactive compound. An interesting example of a similar type of change has recently been afforded by Voisenet [1918]. He isolated an organism, to which he gave the name *Bacillus amaracrylus*, which produces acraldehyde from glycerol. The changes involved are represented as follows:



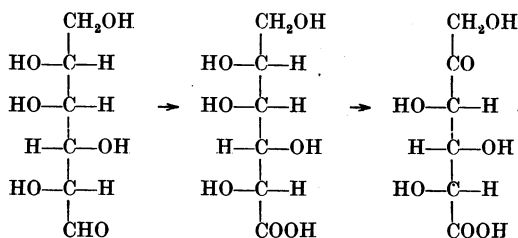
The second stage (II), involving the oxidation of the two terminal carbon atoms to carboxyl groups, is extremely probable in view of the oxidising powers which *A. niger* is known to possess. There is, of course, no evidence to show whether dehydration takes place before oxidation, or *vice versa*, but in either case there would result the formation of the enol form of a diketo-acid:



There are a number of examples of the production, by micro-organisms, of keto-acids from sugars—a fact which renders this change extremely probable. Traetta-Mosca [1914] isolated a fungus having the characteristics of *Aspergillus glaucus* which ferments sugars giving a hitherto unknown substance, which is in all probability the γ -lactone of a trihydroxyhexadienoic acid. For this acid, which is fermentable to alcohol by yeast, Traetta-Mosca suggests the following formula:



Also *Bacterium xylinum* [Adrian Brown, 1886] oxidises glucose first to gluconic acid, and then to keto-gluconic acid:



Weizmann's bacillus [1915], which produces acetone from the carbohydrates of maize, probably produces first aceto-acetic acid, which then loses CO_2 and forms acetone (Author's unpublished observations).

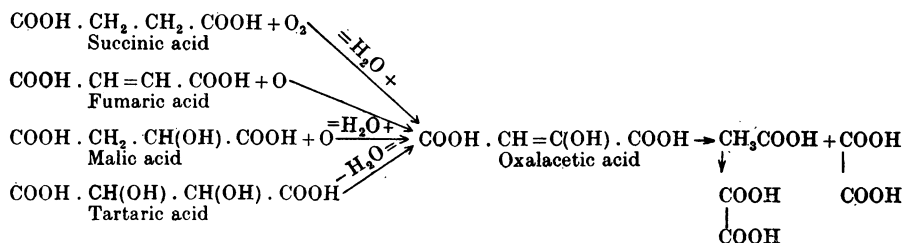
The subsequent breakdown of the diketo-acid II is readily explained. It contains a β -keto-linking, and (disregarding the δ -keto-linking for the moment) is thus a β -keto-acid. The β -keto-acids, as a class, are readily hydrolysed, giving an acid containing two carbon atoms less and acetic acid. This change is represented in stage III. The acetic acid is then oxidised to oxalic acid, a change which our results have shown to be easily accomplished by *A. niger*. The residual part of the molecule III is the enol form of the β -keto-acid—oxalacetic acid—which, undergoing a similar hydrolysis to II, gives rise to one molecule of oxalic acid and one molecule of acetic acid, which is then oxidised, as before, to oxalic acid.

The scheme suggested involves the intermediate formation of acetic acid, and so it might be objected that one ought to be able to identify this acid, as one of the degradation products of sugar by *A. niger*. It must be confessed at once that we have quite failed to do so. Heinze [1903], in a paper previously referred to, states that he obtained acetic acid, in very good yield, along with oxalic acid by the action of *A. niger* on sugar. Unfortunately the lack of experimental details in his paper is so marked—the production of acetic acid being stated as a fact, without any indication as to the method of identification—as to render the statement practically worthless. Obviously the accumulation of acetic acid, and hence the possibility of isolating it, is governed by the relative rates of two reactions:

- (a) The reaction by which it is produced from sugar.
- (b) The reaction by which it is decomposed.

Thus if reaction (a) proceeds more rapidly than (b), acetic acid will accumulate, and one ought to be able to isolate it. If, on the other hand, reaction (b) proceeds more quickly than (a), then it is obviously impossible, at any stage, to isolate any acetic acid. In other words, in order to explain the non-occurrence of acetic acid, one must be able to show that it is decomposed at least as quickly as is glucose. Fortunately there is perfectly satisfactory evidence to prove that this is the case. W. Pfeffer [1895] in a large series of experiments cultivated *A. niger* in solutions containing glucose, together with one of a number of other substances. He was thus able to compare the relative stability of these substances compared with glucose. His conclusions with regard to acetic acid may be given in (a translation of) his own words. "Although a sufficient amount of glucose is quite capable of more or less protecting glycerol or lactic acid from utilisation, this is not true of acetic acid, which is more freely used by the fungus (*A. niger*) than is glucose itself, when simultaneously present."

It was pointed out in the introduction that, although the non-production of oxalic acid from the substance under investigation justifies the conclusion that that substance does not represent an intermediate stage between sugar and oxalic acid, the production of oxalic acid in quantity from the substance under investigation does not necessarily prove that that substance represents an intermediate stage between sugar and oxalic acid. Nevertheless, such a production of oxalic acid justifies the conclusion that the substance in question probably gives rise, in its decomposition, to a substance which is intermediate between these two compounds. Thus, although succinic, fumaric, malic, and, to a less extent, tartaric acid give rise to good yields of oxalic acid, it is not claimed that each of these substances represents an intermediate stage in the degradation of the sugar molecule. If, however, the scheme suggested is accepted, the production of oxalic acid from these dibasic acids is readily explained. Thus, by a simple reaction, the enol form of oxalacetic acid may be supposed to arise from each acid--and hence oxalic acid:



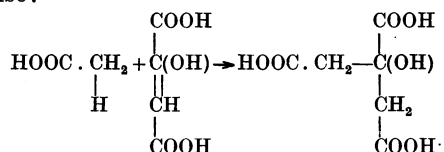
There is experimental evidence, in at least two of these cases, that this change takes place. Wohl and Oesterlin [1901] showed that, by reactions taking place at ordinary temperatures, tartaric acid may be converted into oxalacetic acid. Fenton and Jones [1900] obtained oxalacetic acid from malic acid by oxidation with H_2O_2 and a ferrous salt in the cold.

The principles involved in the suggested scheme also offer an explanation

of the non-production of oxalic acid from the three-carbon acids. The production of a β -ketonic acid, giving rise by hydrolysis to acetic acid, and hence to oxalic acid, is the most essential part of the scheme. If one applies this suggestion to the three-carbon acids, it is seen that no β -ketonic acid, and hence no oxalic acid, can arise from them, since they only contain three carbon atoms. The occasional production of small amounts of oxalic acid from some of them—more particularly from sodium pyruvate in culture media B and C, which were alkaline in reaction—probably can be explained by the fact that secondary changes take place, during sterilisation, which give rise to small amounts of acetic acid.

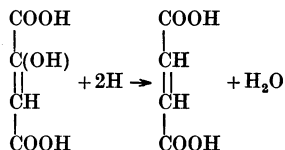
In conclusion, we may be allowed to point out that the scheme suggested for the breakdown of sugar by *A. niger* offers what may be a simple explanation of the production of citric acid by this fungus, and of fumaric acid by *A. fumaricus*.

In stage III of the reactions involved, we have produced one molecule of oxalacetic acid, and one molecule of acetic acid. By the simple union of these two compounds—both of which contain “keten” linkages, which as Collie pointed out readily undergo condensation with one another—a molecule of citric acid would arise:



Citric acid has indeed been synthesised by Lawrence [1897], by condensing oxalacetic ester with bromacetic ester in the presence of zinc. It seems inevitable, in order to explain the production of citric acid from sugar, to presuppose a primary splitting of the sugar molecule, followed by a condensation, because of the presence of a side chain in the citric acid molecule.

The production of fumaric acid might also be easily explained by the reduction of oxalacetic acid:



SUMMARY.

1. *Aspergillus niger* was cultivated on synthetic media containing, as the sole source of carbon, the salts (Na, (NH₄) or Ca) of various organic acids, and the amount of oxalic acid produced was estimated.

2. The following results were obtained:

(a) The four-carbon dibasic acids (succinic, fumaric, malic, tartaric) gave good growth, and good yields of oxalic acid.

(b) With the four-carbon monobasic acids (butyric, isobutyric, β -hydroxybutyric, hydroxyisobutyric) there was almost no growth, and no production of oxalic acid.

(c) The three-carbon acids (lactic, pyruvic, glyceric, malonic, propionic) showed, as a whole, remarkably good growth, but either entire absence, or very small yields, of oxalic acid.

(d) Of the two-carbon acids, acetic acid gave good growth, and good yields of oxalic acid. Glycollic and glyoxylic acids gave fairly good growth, but no oxalic acid.

(e) The one-carbon acid, formic acid, gave fairly good growth, but no oxalic acid.

3. From a consideration of the results obtained, a theoretical scheme is suggested to represent the breakdown of sugar to oxalic acid, involving the intermediate formation of β , δ -diketo-adipic acid. This undergoes hydrolysis into acetic acid and oxalacetic acid, which on further hydrolysis gives acetic acid and oxalic acid. The acetic acid, which is produced in each case, is then itself oxidised to oxalic acid. It is also suggested that the formation of citric and fumaric acids from sugar, by the *Aspergillaceæ*, may be referred to the intermediate production of oxalacetic acid.

We take this opportunity of thanking Professor F. Gowland Hopkins most sincerely for his kind encouragement and criticism during the progress of this work.

REFERENCES.

- Brown (1886). *J. Chem. Soc.* **49**, 172.
Buchner (1892). *Ber.* **25**, 1161.
Collie (1893). *J. Chem. Soc.* **63**, 329.
— (1907). *J. Chem. Soc.* **91**, 1806.
Currie (1917). *J. Biol. Chem.* **31**, 15.
Duclaux (1889). *Ann. Inst. Past.* **3**, 97.
Ehrlich (1909). *Biochem. Zeitsch.* **18**, 391.
Emmerling (1903). *Centralb. Bakt. Par.* (2 Abt.), **10**, 273.
Fenton and Jones (1900). *J. Chem. Soc.* **77**, 77.
Heinze (1903). *Ann. Mycologici*, **1**, 344.
Lawrence (1897). *J. Chem. Soc.* **71**, 457.
Pfeffer (1895). *Jahrb. wiss. Bot.* **28**, 214.
Traetta-Mosca (1914). *Annali Chim. Applicata*, **1**, 477.
Voisenet (1918). *Ann. Inst. Past.* **32**, 476.
Wehmer (1891). *Botan. Zeit.* **49**, 233.
— (1910). *Technical Mycology* (Lafar), **2**, ii, 356.
— (1918). *Ber.* **51**, 1663.
Weizmann (1915). *Brit. Pat.* 4845, Mar. 29th, 1915.
Wohl and Oesterlin (1901). *Ber.* **34**, 1139.