The Estimation of Kojic Acid

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Studies in the Biochemistry of Micro-organisms.

PART VIII.—*The Estimation of Kojic Acid.*

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In the estimation of the residual glucose in the metabolic products of *Aspergillus parasiticus* SPEARE, it was found that very much lower results were given by the copper reduction method of SHAFFER and HARTMANN (1921) than by the iodine absorption method of HINTON and MACARA (1924). In the latter method, which in all essential details is the same as that previously described by WILLSTÄTTER and SCHUDEL (1918), glucose is oxidized by iodine in alkaline solution, the only product arising from the glucose being gluconic acid (as the sodium salt) according to the equation:

$$CH_2OH \,.\, (CHOH)_4 \,.\, CHO + I_2 + 3NaOH = CH_2OH \,.\, (CHOH)_4\, COONa + 2NaI + 2H_2O.$$

It was noticed during the estimation of the glucose in the *A. parasiticus* experiment that an immediate turbidity and a strong smell of iodoform were produced after the addition of the iodine and sodium hydroxide to the metabolism solution, thus indicating that some other reaction was occurring besides the oxidation of glucose to gluconic acid. Since kojic acid was known to be present among the metabolic products of *A. parasiticus* on glucose it was considered that this might be the cause of the anomalous results. A test with pure kojic acid showed that this was the case.

As no method save a rough colorimetric one was available for the estimation of kojic acid, the reaction was further investigated with a view to its utilisation for this purpose, and it was found that, when the conditions of HINTON and MACARA's method were suitably modified, a quantitative relationship existed between the iodine used and the kojic acid present.

This was established as follows:—An aqueous solution of pure kojic acid was prepared containing 1·4538 gm. kojic acid per litre. Measured volumes of this solution were then treated with alkaline iodine solution (40 c.c. of N/10 iodine and 50 c.c. of N/10 sodium hydroxide) for different lengths of time, and the results of a series of these estimations are given in Table I.

The figures in column 4 give the weight in grams of kojic acid equivalent to 1000 c.c. of N/1 iodine, under the conditions of the test and are calculated from columns 2 and 3.

TABLE I.—Absorption of Iodine by different amounts of Kojic Acid Solution (1·4538 gm. per litre) after varying time intervals.

Time in minutes.	Cubic centimetres of kojic acid solution used.	Cubic centimetres N/10 iodine absorbed.	Gm. of kojic acid equivalent to 1000 c.c. N/1 iodine.	Atoms of iodine absorbed by 1 molecule of kojic acid.
120	25·02	23·80	15·28	9·29
60	20·10	19·67	14·86	9·56
90	20·10	19·82	14·74	9·63
120	20·10	19·74	14·80	9·60
60	10·02	10·09	14·44	9·83
90	10·02	10·21	14·27	9·95
120	10·02	10·21	14·27	9·95
120	5·00	5·12	14·20	10·00

The figures in column 5 give the number of atoms of iodine absorbed by one molecule of kojic acid (molecular weight 142), under the conditions of the test, and are calculated from column 4.

It will be seen that, provided a sufficient excess of iodine is present, and a sufficient time allowed for the reaction, kojic acid reacts quantitatively with iodine in alkaline solution, one molecule of kojic acid absorbing 10 atoms of iodine. The conditions necessary to secure this are:—

(1) To use at least four times the quantity of iodine theoretically required.
(2) To allow at least 90 minutes for the reaction.

These conditions were adopted as standard.

The following are the details of the method proposed for the estimation of kojic acid:—

An amount of solution containing 7 to 14 mgm. of kojic acid is pipetted into a 350 c.c. conical flask and the volume made up to 100 c.c. with distilled water from a measuring cylinder. 40 c.c. of N/10 iodine are added from a pipette followed by 50 c.c. of N/10 sodium hydroxide from a cylinder. The mixture is shaken and set aside for 90 minutes, the flask being stoppered. At the end of this time 10 c.c. of 2 N sulphuric acid are added and the excess of iodine is titrated with N/10 sodium thiosulphate solution. The difference between this figure and a blank on the 40 c.c. of iodine alone gives the amount of iodine absorbed, and from this the amount of kojic acid in the solution can be readily calculated since 1 c.c. N/10 iodine ≡ 0·00142 gm. of kojic acid.

The method is equally applicable to the estimation of kojic acid in the presence of glucose, since it has been shown by experiment that glucose, in pure solution, may be estimated accurately by following the details given in the above method. Hence, if it is desired to estimate kojic acid in the presence of glucose, an estimation is carried out on the solution containing the two substances, exactly as described in the method detailed above. The amount of iodine absorbed gives the iodine equivalent of the sum of the glucose and kojic acid present. The glucose is then estimated separately by any method which gives accurate results for glucose in the presence of kojic acid, and from this figure the iodine equivalent of the glucose may be calculated since 1 c.c. N/10 iodine $\equiv$ 0·0090 gm. glucose. If this figure is deducted from the iodine equivalent previously found for the sum of the glucose and kojic acid, the iodine equivalent, and hence the amount, of kojic acid may be calculated.

If, as occasionally happens, there are present in solution along with kojic acid, alcohol, acetaldehyde, or other volatile compounds giving rise to iodoform when treated with alkaline iodine solution, it is essential to remove these from solution by distillation, before carrying out an estimation. The method is, of course, inaccurate in the presence of any non-volatile compounds which absorb iodine from alkaline iodine solution.

The method has been used with considerable success to follow the course of the production of kojic acid by certain moulds.

To complete the investigation and confirm the results so far obtained it was decided to investigate the reaction between iodine and kojic acid in more detail, in order, if possible, to obtain an equation for the reaction, and also to determine by what mechanism it arises. The work carried out with these purposes in view will be described under the headings :

(A) Equation of the Reaction between Iodine and Kojic Acid (p. 141).
(B) Mechanism of the Reaction between Iodine and Kojic Acid (p. 148).

A. *Equation of the Reaction between Iodine and Kojic Acid.*

Qualitative examination of the products of the reaction between iodine and kojic acid in alkaline solution showed that the following substances are formed :

(1) iodoform
(2) iodide
(3) formic acid
(4) oxalic acid
(5) glycollic acid.

The details of this qualitative examination follow, and it will be seen that the experimental method adopted was slightly different from that employed in the quantitative method. The following changes were made :—In order to keep the volume of liquid within reasonable limits and to avoid the addition of extraneous potassium iodide, a methyl-alcoholic solution of iodine was used in place of the usual aqueous solution of iodine in potassium iodide, and, in order to avoid the introduction of elements which could not be removed later, *e.g.*, sodium or potassium, barium hydroxide was used to dissolve the iodine.

1·42 gm. of pure kojic acid were dissolved in 2,700 c.c. of water, and to this was added a solution of 25·4 gm. of iodine in 150 c.c. of pure methyl alcohol. Some of the iodine was precipitated in a finely divided state, but was immediately re-dissolved on the addition of 40 gm. of barium hydroxide crystals ($Ba(OH)_2 . 8 H_2O$) dissolved in 300 c.c. of water. A yellow precipitate was formed, and after standing for three hours this was filtered off and recrystallised from acetone. It had the characteristic crystalline form (hexagonal plates), smell, and melting point (119° C.) of iodoform.

250 c.c. of N/1 sulphuric acid (approximately equivalent to the baryta) were added to the filtrate from the iodoform, the precipitated barium sulphate and iodine removed by filtration, and the iodine remaining in solution extracted by shaking out several times with ether. A test applied to a little of the extracted solution, *i.e.*, acidification with nitric acid and addition of silver nitrate, showed the presence in solution of iodides, and these, together with the small amount of free iodine still remaining in solution, were removed by treatment with an excess of freshly prepared and well-washed silver carbonate. The precipitated silver iodide was filtered off, and the excess of silver removed from the filtrate by treatment with sulphuretted hydrogen.

The filtrate from the silver sulphide was evaporated *in vacuo* for a short time to remove all sulphuretted hydrogen, neutralised with baryta to obtain as barium salts any organic acids formed from the kojic acid, and the solution evaporated *in vacuo* to small bulk, after the previous removal of a slight precipitate of barium sulphate. During the evaporation a barium salt separated, and was filtered off when the volume of the liquid had been reduced to about 300 c.c. This compound was dissolved in dilute hydrochloric acid, the barium precipitated with sulphuric acid, filtered, the filtrate made slightly alkaline with ammonia, and a little calcium acetate solution added. An immediate precipitate was formed which was insoluble in acetic acid but soluble in hydrochloric acid, indicating the presence in solution of oxalic acid, which was precipitated during evaporation as barium oxalate.

The filtrate from the barium oxalate, containing in solution any soluble barium salts arising from the kojic acid, was quantitatively precipitated with sulphuric acid to remove barium, and the solution of free acids evaporated *in vacuo* almost to dryness. The distillate was strongly acid, and, on treatment with lead carbonate and filtration of the hot solution, deposited characteristic crystals of lead formate, which on analysis gave :

	Found.	Calc. for $(HCOO)_2$ Pb.
	Per cent.	Per cent.
Lead	69·68	69·71

Formic acid is thus one of the products of the action of iodine on kojic acid.

The solution left in the distillation flask after the removal of the formic acid set to a mass of crystals on leaving in a desiccator over concentrated sulphuric acid. As no satisfactory solvent could be found for the recrystallisation of the acid, the latter was dissolved in water, neutralised with calcium carbonate, and the calcium salt crystallised from hot water. It had the characteristic appearance of calcium glycollate, a specimen of which was prepared and crystallised in the same way for comparison. Analysis after drying to constant weight at 110° C. confirmed the salt as calcium glycollate, and hence proved the formation of glycollic acid from kojic acid.

Calcium.

(By sulphated ash.)	Found	= 20·76 per cent.
	Theoretical	= 21·06 ,,

Carbon.

(By wet combustion.)	Found	= 24·68 per cent.
	Theoretical	= 25·26 ,,

Methods were therefore devised for the estimation of each of these reaction products—iodoform, iodide, formic, oxalic, and glycollic acids—and are described in detail below.

(1) *and* (2) *Estimation of Iodoform and Iodides.*—10·02 c.c. of a M/100 solution of pure kojic acid were submitted to the action of alkaline iodine as in the quantitative estimation, but, in order to avoid introducing extraneous iodide with the standard iodine solution, a special solution of hypoiodite was made up equivalent to 4N/50 in respect of iodine and N/10 in respect of sodium hydroxide. 50 c.c. of this solution were therefore equivalent to the 40 c.c. of N/10 iodine and 50 c.c. of N/10 sodium hydroxide recommended for use in the actual estimation of kojic acid. At the same time, a blank experiment was carried out on exactly the same lines, using 10 c.c. of water in place of the 10 c.c. of kojic acid solution. Curiously enough, when the hypoiodite solution and the kojic acid solution with the addition of the appropriate quantity of water were mixed, no reaction took place and the titration after 90 minutes was the same as the blank. The reaction could be induced, however, by adding to the reaction mixture 5 c.c. of N/1 sulphuric acid, which precipitated the iodine, followed immediately by 5 c.c. of N/1 sodium hydroxide to restore the alkalinity and dissolve the iodine. The reaction was then normal. After standing for 90 minutes the solution was acidified and titrated with N/10 sodium thiosulphate, thus removing the free iodine.

Estimation of Iodoform.—The iodoform was then extracted from the solution by means of ether, the ether extract washed with water, treated with alcoholic potassium hydroxide, and heated on the water bath (15–20 minutes) to hydrolyse the iodoform and convert it into potassium iodide. The iodide was then estimated by VOLHARD's method and found to be equivalent to 2·44 c.c. of N/10 iodine. A similar experiment in which the iodide was estimated gravimetrically gave a result equivalent to 2·40 c.c. of N/10 iodine.

Estimation of Iodide.—The washings from the ether solution were added to the main aqueous solution and the iodide present estimated by VOLHARD's method. This was found to be equivalent to 37·32 c.c. N/10 iodine. This figure, of course, represents the sum of the iodide produced by the action of iodine on kojic acid, together with that produced in the back titration with thiosulphate of the excess of iodine present after the reaction.

This latter figure ≡ 29·71 c.c. N/10 thiosulphate or iodine (mean of duplicate estimations).

Therefore, the iodide produced in the reaction between kojic acid and iodine

≡ 37·32 c.c. — 29·71 c.c.
≡ 7·61 c.c. N/10 iodine.

The total amount of iodine absorbed by the kojic acid is now given by the difference between the thiosulphate reading in the blank estimation, using water alone,

≡ 39·86 c.c. N/10 thiosulphate or iodine,

and the thiosulphate reading in the actual estimation, using kojic acid,

≡ 29·71 c.c. N/10 thiosulphate or iodine.

Therefore, the total iodine absorbed by kojic acid

≡ 39·86 c.c. — 29·71 c.c.
≡ 10·15 c.c. N/10 iodine.

Thus, these experiments show that 10·02 c.c. M/100 kojic acid absorb 10·15 c.c. N/10 iodine, producing iodoform equivalent to 2·44 c.c. N/10 iodine and iodide equivalent to 7·61 c.c. N/10 iodine. The inference from these figures is that one molecule of kojic acid absorbs 10 atoms of iodine (thus confirming the results previously described) with the production of one molecule of iodoform (this would require a theoretical value of 3·0 c.c. N/10 iodine in place of 2·44 c.c. actually obtained), and 7 molecules of iodide (requiring a theoretical value of 7·0 c.c. N/10 iodine in place of 7·61 c.c. actually obtained experimentally). It is also obvious that no substituted iodine derivatives other than iodoform are produced. The divergence between the experimental and theoretical figures is probably due to the fact that the iodoform undergoes slight decomposition in the alkaline hypoiodite solution with the formation of a corresponding amount of iodide.

(3). *Estimation of Formic Acid.*—The method of estimation finally adopted was based on that described by FINCKE (1913). The details are :—

To 10 c.c. of a M/100 solution of kojic acid were added 120 c.c. of water, and 50 c.c. of the alkaline hypoiodite solution, prepared as described in the estimation of iodoform, followed by 5 c.c. of N/1 sulphuric acid and then 5 c.c. of N/1 sodium hydroxide. The mixture was allowed to stand for 90 minutes, acidified with 5 c.c. of N/1 sulphuric acid and the iodoform and liberated iodine removed by three extractions with ether. To the extracted solution was added silver carbonate, prepared from 25 c.c. of N/10 silver nitrate solution by the addition of sodium carbonate in slight excess and well washed by decantation. The mixture was well shaken in order to precipitate the whole of the iodine present as silver iodide, which was filtered off, and then 20 c.c. of N/10 hydrochloric acid were added to the filtrate to precipitate the excess silver as silver chloride, which, in its turn, was filtered off. The filtrate was then neutralised to phenolphthalein, evaporated *in vacuo* almost to dryness, the residue dissolved in water, and to this was added a solution containing 2 gm. sodium acetate, 2 gm. mercuric chloride, and 1·5 gm. sodium chloride. After standing overnight in the cold, the solution was filtered, made up to about 100 c.c., and heated for 2 hours in a boiling water bath. The precipitated mercurous chloride was filtered on a Gooch crucible, dried at 100° C. and weighed. From this weight the amount of formic acid was calculated according to the equation

$$\mathrm{H.COOH} + 2\,\mathrm{HgCl_2} \longrightarrow \mathrm{CO_2} + 2\,\mathrm{HCl} + \mathrm{Hg_2Cl_2}$$

In two experiments the weight of formic acid produced from 10 c.c. of M/100 kojic acid after treatment with iodine was 0·0048 gm. and 0·0044 gm. respectively. Theoretically, if one molecule of formic acid is produced from one molecule of kojic acid, this weight should be 0·0046 gm. Hence it follows that in this reaction one molecule of kojic acid gives rise to one molecule of formic acid.

(4) *Estimation of Oxalic Acid.*—The details of the preparation of the solution containing the products of reaction between kojic acid and iodine, for the estimation of oxalic acid, are the same as those described in the estimation of iodoform, except that ten times the usual quantities of reagents were used throughout and N/1 hydrochloric acid was used in place of N/1 sulphuric acid. Thus 100 c.c. M/100 kojic acid = 0·142 gm. of the acid, were used for the estimation.

When the reaction was complete the solution was acidified with 50 c.c. of N/1 hydrochloric acid and evaporated *in vacuo* to about 100 c.c. By this means the iodoform and liberated iodine were completely removed, and the solution was sufficiently concentrated to allow of the precipitation of the oxalic acid as calcium oxalate. This was carried out in the usual way, after making the solution alkaline with ammonia, and then slightly acid with acetic acid, by the addition of 5 c.c. of 20 per cent. calcium acetate to the boiling solution. The precipitated calcium oxalate was estimated by titration with N/10 permanganate.

In duplicate experiments the oxalic acid present was equivalent to 15·80 c.c. and 15·50 c.c. of N/10 permanganate respectively. Now, using 100 c.c. of M/100 kojic acid solution, for every molecule of oxalic acid produced by one molecule of kojic acid, the amount of N/10 permanganate theoretically required to oxidise the oxalic acid would be 20·0 c.c. Hence it follows that in the reaction between iodine and kojic acid one molecule of the latter gives rise to one molecule of oxalic acid. The somewhat low experimental results obtained may be readily explained by the appreciable solubility of calcium oxalate in the relatively concentrated solution of salts from which it was precipitated.

(5) *Estimation of Glycollic Acid.*—Since no specific method is available for the estimation of glycollic acid, advantage was taken of the fact that glycollic acid is the only product of the action of iodine on kojic acid which contains a hydroxyl group which can be acetylated by means of acetic anhydride and sodium acetate, to estimate the acid by this means. Preliminary experiments with pure glycollic acid had shown that this reaction provides a reasonably satisfactory quantitative method.

The experimental details (amounts of kojic acid, reagents, etc.) are the same as those used in the preparation of the reaction mixture for the estimation of oxalic acid. When the reaction was complete the solution was acidified with 50 c.c. of N/1 sulphuric acid and the iodine and iodoform extracted with ether. The iodide was precipitated by means of silver carbonate, the excess silver removed by hydrochloric acid, the solution filtered, neutralised and evaporated to low bulk *in vacuo.* The residual solution was washed into a 250 c.c. round-bottomed flask and evaporated to dryness in a gentle stream of hot air. The dry residue was then acetylated by means of acetic anhydride and sodium acetate in the manner of a glycerol analysis. After heating for one hour under a reflux the acetylation product was dissolved in cold water, and exactly neutralised with N/1 sodium hydroxide; 50 c.c. of N/1 sodium hydroxide solution were added from a pipette, the solution boiled under a reflux for fifteen minutes, cooled, and the excess of alkali titrated with acid. The alkali used up in hydrolysis is equivalent to the hydroxyl groups present, and was in this estimation

$$\equiv 0\cdot78 \text{ c.c. N/1 NaOH.}$$

Since it is evident by calculation that, for every hydroxyl group produced from one molecule of kojic acid, with the quantities of kojic acid used in this estimation, 1·0 c.c. of N/1 sodium hydroxide would be used up in hydrolysis, it is clear that in the action of iodine on kojic acid one molecule of kojic acid gives rise to a substance containing one hydroxyl group, *i.e.*, to one molecule of glycollic acid.

(6) *Estimation of Total Acidity.*—The total acidity was estimated by titrating, with standard sodium hydroxide solution, the acid produced during the reaction between iodine and kojic acid, correction being made for acidity of reagents by carrying out a parallel blank experiment in which water was used in place of kojic acid solution. This procedure is rendered possible, since no acidity is produced during the titration of the residual iodine with sodium thiosulphate. The details of the estimation are :—

To 10·02 c.c. of M/100 kojic acid solution were added 40 c.c. of N/10 iodine solution and 50 c.c. of N/10 sodium hydroxide, and the mixture allowed to stand for 90 minutes. A parallel blank experiment, with 10 c.c. of water in place of the kojic acid solution, was carried out in exactly the same way. To each was now added 60 c.c. of N/10 hydrochloric acid solution to ensure an excess of acid, and the residual iodine titrated back with N/10 thiosulphate. The whole volume of thiosulphate solution used was :—

Blank experiment	39·95 c.c.
Kojic acid experiment	30·03 ,,
Iodine absorbed by kojic acid	9·92 c.c.

The two solutions were then titrated with N/10 sodium hydroxide, using phenolphthalein as indicator. The volume of sodium hydroxide used was :—

Kojic acid experiment	20·83 c.c. N/10 NaOH.
Blank experiment	9·73 ,, ,, ,,
Total acidity produced during the reaction between iodine and kojic acid	11·10 ,,

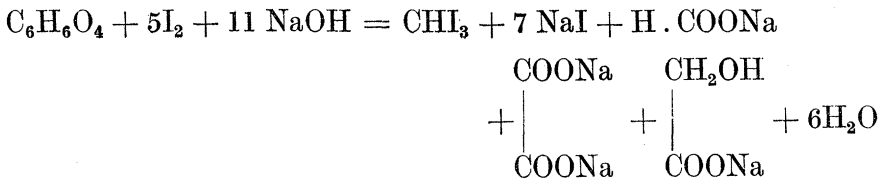
Thus, since 10·02 c.c. of M/100 kojic acid absorb 9·92 c.c. of N/10 iodine and produce acid equivalent to 11·10 c.c. of N/10 sodium hydroxide, it follows that during the reaction between iodine and kojic acid, one molecule of the latter absorbs 10 atoms of iodine (thus again confirming the previous results), and produces acid equivalent to 11 molecules of a monobasic acid. This is distributed as follows :—

(*a*) 7 molecules as hydriodic acid equivalent to the 7 molecules of alkaline iodide estimated (see p. 143).
(*b*) 1 molecule as formic acid (see p. 144).
(*c*) 2 molecules as one molecule of oxalic acid (see p. 145).
(*d*) 1 molecule as glycollic acid (see p. 146).

Hence it is clear that no other acid is formed during the reaction, and this is supported by the fact that, although carbon dioxide was tested for among the reaction products, its presence could not be demonstrated.

It is now possible, from a consideration of the results obtained, to construct an equation for the reaction. The results may be briefly summarized. It has been proved that one molecule of kojic acid in alkaline solution takes up 10 atoms of iodine (p. 140), and gives rise to one molecule of iodoform and 7 molecules of iodide (p. 143), one molecule of formic acid (p. 144), one molecule of oxalic acid (p. 145), one molecule of glycollic acid (p. 146), and a total acidity equivalent to 11 molecules of monobasic acid (p. 146).

Thus the equation for the action of iodine on kojic acid in alkaline solution is



$$C_6H_6O_4 + 5I_2 + 11\,NaOH = CHI_3 + 7\,NaI + H.COONa + \begin{array}{l}COONa\\ |\\ COONa\end{array} + \begin{array}{l}CH_2OH\\ |\\ COONa\end{array} + 6H_2O$$

B.—*Mechanism of the Reaction between Iodine and Kojic Acid.*

In considering the mechanism of the reaction between alkaline iodine solution and kojic acid it is well to bear in mind the fact that alkaline iodine solution can exercise a double function. On the one hand, it may act as an alkaline hydrolytic agent, and, on the other, it may act as an oxidising agent. Hence the final products of the reaction between alkaline iodine and kojic acid, *i.e.*, formic, glycollic, and oxalic acids, may possibly arise as a result either of hydrolysis, or of oxidation, or of a combination of the two processes. Iodoform must, of course, arise by a process of substitution from some intermediate compound.

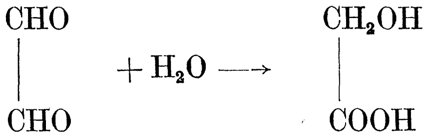
Let us consider these actions of hydrolysis and oxidation separately.

(1) *Hydrolysis.*—No information is available as to the products of hydrolysis of kojic acid by means of sodium hydroxide, but a close analogy may be found in the action of barium hydroxide on the dimethyl ether of kojic acid. YABUTA (1916) investigated this reaction, and found that the products of hydrolysis were equimolecular proportions of (1) methoxyacetone, (2) formic acid, and (3) methoxyacetic acid. If the same type of hydrolysis takes place as a result of the action of sodium hydroxide on kojic acid the products of hydrolysis should be (1) hydroxyacetone or pyruvic alcohol, (2) formic acid and (3) glycollic acid or some substance which gives rise to glycollic acid on hydrolysis.

(2) *Oxidation.*—Since formic and glycollic acids have already been shown to be formed as a result of the action of alkaline iodine on kojic acid, it was obviously desirable to investigate the products of the reaction between alkaline iodine and pyruvic alcohol. This investigation, details of which are given later (p. 150), showed that pyruvic alcohol is acted on by alkaline iodine, one molecule of the alcohol absorbing 6 atoms of iodine and giving rise to iodoform, glycollic acid, and hydriodic acid, but not to either formic or oxalic acids. Hence these two acids must arise from the other half of the kojic acid molecule. Referring again to YABUTA's results it is seen that formic acid, but not oxalic acid, is a product of the hydrolysis of the dimethyl ether of kojic acid with barium hydroxide. Hence, it seems reasonable to assume that formic acid is also a product of hydrolysis in the case of kojic acid, and it only remains now to obtain a satisfactory explanation for the production of oxalic acid.

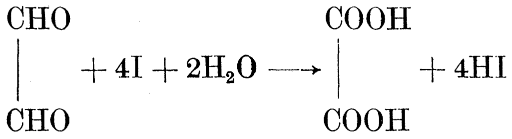
Up to the present only six atoms of iodine have been accounted for, *i.e.*, in the oxidation of one molecule of pyruvic alcohol. Hence, of the ten atoms of iodine shown in the equation, four remain to be considered, and it does not seem unreasonable to conclude that these are used up in an oxidative process resulting in the production of oxalic acid. It seems obvious at first sight that the oxalic acid arises by the oxidation of the glycollic acid, which, by analogy with YABUTA's results, should be formed along with formic acid and pyruvic alcohol. But if this were so, why should the same oxidising agent, which is thus supposed to oxidize glycollic acid to oxalic acid, not only produce glycollic acid and not oxalic acid from pyruvic alcohol, but also have no oxidising action itself on glycollic acid as was proved by direct experiment? The idea that the oxalic acid arises by oxidation of glycollic acid must, therefore, be abandoned.

It was previously pointed out that, if one may argue by analogy with YABUTA's results, kojic acid should give rise on hydrolysis to pyruvic alcohol, formic acid, and glycollic acid or some substance which gives rise to glycollic acid on hydrolysis. Hence a reasonable explanation of the origin of the oxalic acid seems to be that during the action of alkaline iodine on kojic acid, in addition to pyruvic alcohol and formic acid, a two carbon compound is momentarily formed which may give rise, on the one hand, by hydrolysis, to glycollic acid, or, on the other, by oxidation, to oxalic acid. Such a compound is glyoxal $CHO \cdot CHO$. Glyoxal, even in the cold, is converted by alkalies, by a CANNIZZARO reaction, into glycollic acid.



$$\begin{array}{c}CHO\\ |\\ CHO\end{array} + H_2O \longrightarrow \begin{array}{c}CH_2OH\\ |\\ COOH\end{array}$$

and is oxidized by alkaline iodine to oxalic acid according to the equation

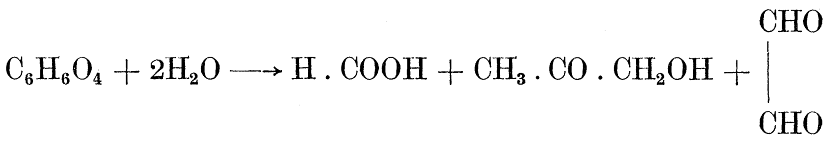


$$\begin{array}{c}CHO\\ |\\ CHO\end{array} + 4I + 2H_2O \longrightarrow \begin{array}{c}COOH\\ |\\ COOH\end{array} + 4HI$$

It seems, then, that in the presence of a large excess of alkaline iodine solution, oxidation of the momentarily formed glyoxal takes place in preference to hydrolysis, and hence, oxalic acid is the final product, and not glycollic acid, as would be expected if the action of alkaline iodine on kojic acid followed strictly the reaction occurring during the hydrolysis of the dimethyl ether of kojic acid by barium hydroxide. Glycollic acid is indeed a product of the reaction between alkaline iodine and kojic acid, but it is evident that it arises from the pyruvic alcohol and not from the compound (presumably glyoxal) corresponding to the precursor of the methoxyacetic acid found by YABUTA.

The course of the reaction may therefore be summed up as follows :—

(1)

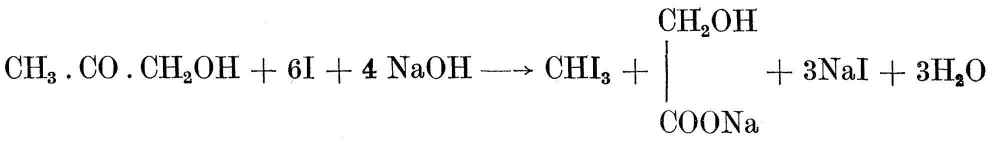


$$C_6H_6O_4 + 2H_2O \longrightarrow H . COOH + CH_3 . CO . CH_2OH + \begin{array}{c}CHO\\ |\\ CHO\end{array}$$

Kojic acid. Formic acid. Pyruvic alcohol. Glyoxal.

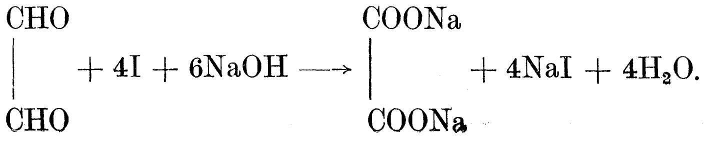
(2) $$H . COOH + NaOH \longrightarrow HCOONa + H_2O.$$

(3)



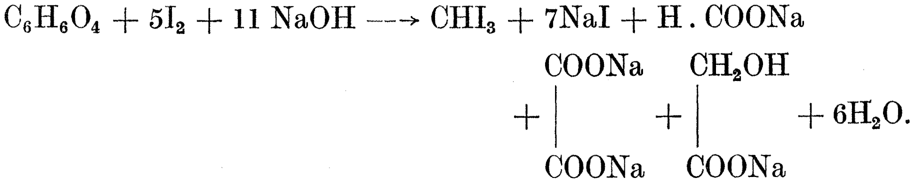
$$CH_3 . CO . CH_2OH + 6I + 4\ NaOH \longrightarrow CHI_3 + \begin{array}{c}CH_2OH\\ |\\ COONa\end{array} + 3NaI + 3H_2O$$

(4)



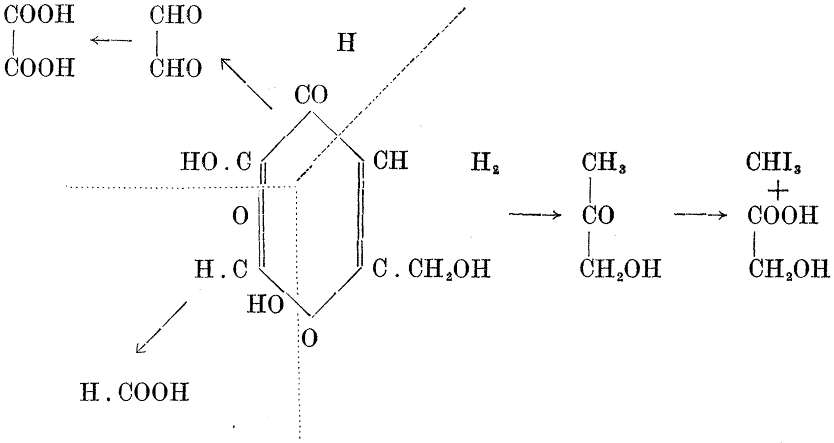
$$\begin{array}{c}CHO\\ |\\ CHO\end{array} + 4I + 6NaOH \longrightarrow \begin{array}{c}COONa\\ |\\ COONa\end{array} + 4NaI + 4H_2O.$$

Substituting equations (2), (3), and (4) in equation (1) we get the equation previously given on p. 147.



$$C_6H_6O_4 + 5I_2 + 11\,NaOH \longrightarrow CHI_3 + 7NaI + H\,.\,COONa + \begin{array}{c}COONa\\|\\COONa\end{array} + \begin{array}{c}CH_2OH\\|\\COONa\end{array} + 6H_2O.$$

The mechanism of the breakdown of the kojic acid may be represented graphically as follows :—



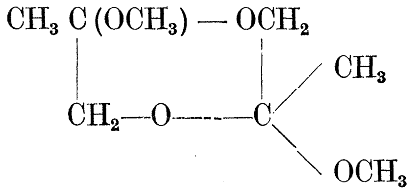
The Action of Alkaline Iodine on Pyruvic Alcohol.—Pyruvic alcohol was prepared from monobromoacetone by NEF's method (1904) and subjected to the action of alkaline iodine under the same conditions as in the kojic acid reaction. Very variable results were obtained, depending on the amount of pyruvic alcohol employed, but the relative amount of iodine used was found to increase as the quantity of pyruvic alcohol was cut down, until, finally, a maximum and constant value was attained when the iodine was present in quantity amounting to about twenty times that theoretically required.

A stock solution of pyruvic alcohol was made up containing 0·5993 gm. per litre, and, to varying amounts of this, indicated in column 1 of the following table, 40 c.c. of N/10 iodine and 50 c.c. of N/10 sodium hydroxide were added in each case, exactly as was done with kojic acid. The amount of N/10 iodine used by the pyruvic alcohol and calculated in terms of c.c. N/10 iodine per 1 c.c. M/10 pyruvic alcohol is shown below.

Cubic centimetres of stock solution of pyruvic alcohol.	Cubic centimetres N/10 iodine equivalent to 1 c.c. M/10 pyruvic alcohol.
10	3·4
5	4·9
2	6·5
2	6·0
1	6·3
0·5	5·9

These figures indicate that, provided a sufficient excess of alkaline iodine is used, an approximately constant absorption value is obtained, corresponding to 6 atoms of iodine per molecule of pyruvic alcohol.

As pyruvic alcohol itself is rather unstable, it was considered advisable to confirm these results by utilising the rather curious compound, bis-pyruvic alcohol methyl alcoholate,



as a source of pure pyruvic alcohol. Bis-pyruvic alcohol methyl alcoholate is readily prepared from pyruvic alcohol and methyl alcohol (NEF, 1904) and has the great advantages as a standard for the purpose in view that it crystallises well, is quite stable, but is very easily hydrolysed by acids to give pyruvic alcohol and methyl alcohol.

A standard solution of the recrystallised alcoholate was therefore made up (M/200 equivalent to M/100 pyruvic alcohol) and aliquot portions were hydrolysed before use by heating at 60° C. in a closed flask for one hour with 10 c.c. of N/1 hydrochloric acid. After cooling, the acid was neutralised by the addition of 10 c.c. of N/1 sodium hydroxide, and N/10 iodine and alkali were added as with pyruvic alcohol. As before, a constant value for the iodine used up was not attained until the iodine employed was in very great excess of the theoretical.

Cubic centimetres of stock solution of alcoholate.	Cubic centimetres N/10 iodine equivalent to 1 c.c. M/10 pyruvic alcohol.
5	4·7
2	5·5
1	6·2
1	6·1
0·5	6·0
0·25	6·0

These results confirm those previously obtained and indicate that one molecule of pyruvic alcohol absorbs 6 atoms of iodine, behaving in this respect like acetone. The products formed during this reaction and the equation representing it were investigated as follows :—

0·9 gm. of bis-pyruvic alcohol methyl alcoholate was hydrolysed by heating for one hour at 60° C. with 20 c.c. of N/1 sulphuric acid. The mixture was then diluted with water to about 2700 c.c. and 25·4 gm. of iodine dissolved in 150 c.c. of pure methyl alcohol were added, followed by a solution of 43 gm. of crystalline barium hydroxide dissolved in warm water. The final volume was about 3 litres. After standing two hours, the mixture was investigated qualitatively by the same method as that described on p. 142 for the investigation of the reaction products of kojic acid and alkaline iodine.

The following compounds were tested for

(1) iodoform	present
(2) formic acid	absent
(3) oxalic acid	absent
(4) glycollic acid	present

and the last named was isolated as the calcium salt which gave the following analysis :—

	Found.	Calculated for $Ca(C_2H_3O_3)_2\ 4H_2O$
Water of crystallisation.	27·52 per cent.	27·48 per cent.
		Calculated for $Ca(C_2H_3O_3)_2$
Carbon in anhydrous salt.	25·77 per cent.	25·26 per cent.

The equation for the reaction between pyruvic alcohol and alkaline iodine is similar to that for the reaction with acetone except that glycollic acid is formed in place of acetic acid. It is therefore,

$$CH_3 . CO . CH_2OH + 6I + 4\ NaOH = CHI_3 + CH_2OH . COONa + 3NaI + 3H_2O.$$

Summary.

A method is described (p. 140) for the quantitative estimation of kojic acid (5-hydroxy-2-hydroxymethyl-γ-pyrone) which depends on the fact that kojic acid is quantitatively decomposed by alkaline iodine solution under experimental conditions which have been determined and of which details are given.

The equation of this reaction has been established (p. 141) and the mechanism by which the reaction takes place has been determined (p. 148).