CHAPTER I

INTRODUCTION
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Kojic acid (5-hydroxy-2-hydroxymethyl-γ-pyrone) is produced from carbohydrate sources in an aerobic process by a variety of microorganisms. This was first reported in 1907 by Saito, who isolated it as a crystalline substance from the mycelia of Aspergillus oryzae grown on steamed rice. He thought that it was identical with β-resorcylicarboxylic acid. Shortly thereafter, Yabuta (1913) undertook an extensive investigation of the substance, gave it the name kojic acid and finally definitely established its constitution in 1924. It's chemical synthesis from D-glucose was achieved in 1930. Since then, a considerable amount of study has been devoted to the biosynthesis of kojic acid and numerous publications have dealt with its chemical and biological properties.

CHEMICAL PROPERTIES

Kojic acid is a substituted γ-pyrone (I)

\[ \text{Kojic acid} \]
The activities of the different centres of the kojic acid molecule are influenced to a considerable extent by resonance between different forms of the molecule.

The Hydroxyl Groups:

The phenolic hydroxyl group at C5 gives kojic acid its weakly acidic character and enables it to form salts with a number of metals like Na, Ba, Ca, Sr, Co, Cu, Ni, Fe, Mn, Cd, Pb, Zn, Al, La etc. (3,4,5,6) Many derivatives typical of hydroxy compounds have been prepared from kojic acid. Some of the known ethers and esters (II) have been listed in the following table.

Table 1. Some kojic Acid Derivatives

![Diagram of kojic acid derivative](attachment:kojic_acid_derivative.png)

Esters and ethers of kojic acid
<table>
<thead>
<tr>
<th>R</th>
<th>R'</th>
<th>References</th>
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<tbody>
<tr>
<td>H</td>
<td>Acetyl</td>
<td>4,7,8</td>
</tr>
<tr>
<td>H</td>
<td>Benzoyl</td>
<td>8</td>
</tr>
<tr>
<td>H</td>
<td>Caproyl</td>
<td>7</td>
</tr>
<tr>
<td>H</td>
<td>Methyl</td>
<td>8</td>
</tr>
<tr>
<td>Allyl</td>
<td>H</td>
<td>7</td>
</tr>
<tr>
<td>Benzoyl</td>
<td>H</td>
<td>3,8,9</td>
</tr>
<tr>
<td>Acetyl</td>
<td>Acetyl</td>
<td>3,4,10,11,12</td>
</tr>
<tr>
<td>Benzoyl</td>
<td>Benzoyl</td>
<td>3,4,8,10,13</td>
</tr>
<tr>
<td>Methyl</td>
<td>Acetyl</td>
<td>7</td>
</tr>
<tr>
<td>Methyl</td>
<td>Benzoyl</td>
<td>8</td>
</tr>
<tr>
<td>Benzoyl</td>
<td>Acetyl</td>
<td>8</td>
</tr>
</tbody>
</table>

The Unoccupied Nuclear Positions:

Of the two unsubstituted positions 3,6 in the kojic acid molecule, position 6 is very reactive. Several 6-substituted derivatives are known whereas only one 3-substituted derivative has been prepared. This may be ascribed to the activating effect of the phenolic function which would be particularly marked in alkaline media, where kojic acid probably exists in the form of a phenoxide ion (III). The negative charge would be shared by C6 where it would create
The Heteroatom of the Nucleus:

The cleavage of many γ pyrones at the ring oxygen atom has been observed in mildly alkaline solutions. The primary products of this reaction are labile bishydroxy methylene compounds. The initiation of the basic cleavage at the ring oxygen atom was further indicated by the easy formation of pyridones, when kojic acid and several of its derivatives were condensed with ammonia, primary amines, difunctional primary amines and glycine.

Oxidation Reactions:

Heyns and Vogelsang attempted to achieve the oxidation of kojic acid catalytically. A current of air was passed through solutions of kojic acid and its 5 methyl ether at 60°C for several hours; the solutions were kept at a pH close to 5, and a special platinum activated carbon catalyst was used. Only traces of comenic acid demonstrated by paper chromatography, were obtained from kojic acid; the yields of comenic acid methyl ether from kojic acid methyl ether were close to 40%. Kojic acid is oxidized by Fehling's solution and by moist silver oxide. These reactions are probably accompanied by degradation.

Reduction Reaction:

The catalytic hydrogenation of kojic acid has been studied repeatedly. Traetta-Mosca and Wijkman used
palladium and platinum catalysts and found the uptake of 4 and 6 atoms of hydrogen per molecule respectively. The non-catalytic reduction of kojic acid has not been reported, but 6-benzoylkojic acid and 6-acetyl kojic acid have been reduced to 6-benzyl kojic acid and 6-ethyl kojic acid respectively.\(^{(26,27)}\)

**PHYSICAL PROPERTIES**

Kojic acid crystallizes in the form of colourless, prismatic needles.\(^{(2,10)}\) Data on its crystal structure derived from X-ray investigations were provided by Fox and by Mckinstry et al.\(^{(28,29)}\) It is readily soluble in water, ethanol, acetone and is sparingly soluble in ether, ethyl acetate, chloroform and pyridine and difficulty soluble in most other liquids.\(^{(3,17)}\) It has been purified by recrystallization from acetone,\(^{(17)}\) ethanol-ether\(^{(30)}\) and methanol ethyl acetate and also by sublimation under diminished pressure at 150° to 200°C.\(^{(4,11,31)}\) The melting point has been reported variously as 151 to 152, 152°.\(^{(2,4,12)}\) 152.6\(^{(33)}\) and 154°C.\(^{(10,11)}\) The U.V. absorption spectra of kojic acid and its 5-7 diacetate derivative exhibit characteristic absorption maximum at 315 nm and 255 nm respectively.\(^{(34)}\)

Paper chromatographic data have been reported by Heyns and Vogelsang.\(^{(25)}\) Kojic acid had an Rf of 0.80 in butanol (saturated with water), glacial acetic acid:
water(18:2:5).

BIOLOGICAL PROPERTIES

Antibiotic Activity:

Yabuta\(^{(2)}\) noted that bacterial growth generally stopped in the presence of more than 0.5% of kojic acid, but interest in its antibacterial activity was only renewed quite incidentally, when the discovery of penicillin led to an intensified search for new antibiotic substances among mold metabolites. The promisingly high activity of some mold culture media against various bacteria was found to be due wholly to the relatively high concentration of kojic acid produced by these molds, \(^{(36 \text{ to } 40)}\) but the inhibitory power of pure kojic acid and some of its derivatives proved to be disappointingly low. \(^{(12, 38, 41 \text{ to } 46)}\) The potency of kojic acid was unaffected by the number of bacteria present or by incubation in 50% serum at 37°C. Foster and Karow\(^{(47)}\) also noted a slight inhibition of pure cultures of various bacteria by kojic acid. They observed that gram negative bacteria were more sensitive to sodium kojate than gram positive ones. With most other antibiotic substance the reverse is true. Kavanagh\(^{(48)}\) compared the activity of kojic acid with those of several of the well known potent antibiotic substances. Kojic acid showed by far the lowest activity in the whole group against seven out of nine species of bacteria. Lee and Coworkers\(^{(39)}\) found that
Kojic acid is active against human tubercle bacilli in vitro under a variety of conditions. Complete inhibition of a surface growth of the bacilli being caused by 45 mg of kojic acid per 100 ml of liquid medium. However, many other active substances produce similar effect at much lower concentration. The effect of kojic acid at such inhibitory levels is bacteriostatic and not bactericidal.

Kojic acid is not active against viruses. It was tested, with negative results, against one strain of polymyelitis virus and one strain of St. Louis encephalitis virus in mice \(^{(49)}\) and also against sixty races of bacteriophages. \(^{(50)}\) Klein and Olsen \(^{(51)}\) studied the action of kojic acid on the enzymatic oxidation of amino acids by rat liver and kidney tissue slices. Low concentration of kojic acid inhibited the in vitro oxidation of a number of D-amino acids, L-phenyl alanine and few related compounds. Kojic acid was found to compete with D-amino acid oxidase for its substrate. Buu Hoi and Ratsimamanga \(^{(40)}\) found that kojic acid protected the adrenal ascorbic acid in test animals during the reversible period of scurvy without itself showing Vitamin C activity.

Although McGowan and Coworkers \(^{(52)}\) observed only a weak activity of kojic acid against three species of fungi, \(^{(53)}\) Kane and Morey recommended the use of its complexes with bivalent heavy metals against several fungal diseases of plants. They found copper, mercury cadmium, lead and tin complexes to be
superior to the well known Bordeaux mixture in the treatment of early blight (Alternaria solani). These complexes are easily suspended in liquids and these sprays adhere firmly to the foliage and do not injure the plants. Since the metal radicals are bound to kojic acid in a nonionizable form, these complexes are also much less toxic to human beings and safer to handle than Bordeaux mixture itself. Thus the activity of kojic acid against certain fungi may eventually find some practical application.

Mayer and Coworkers (54) discovered that kojic acid is a moderately effective activator for nicotine insecticides when treated against melonworm (Diaphania hyalinata L.) and southern army worm (Prodenia eridania cram.), kojic acid alone was not toxic, but the toxicity of a 5% nicotine sulfate-pyrophyllite dust was increased 35% and that of a 5% nicotine-bentonite spray by some 50% by the addition of 5% of kojic acid. These workers found kojic acid to be harmless to plants, but others reported it to be slightly toxic. (55) Bastin (56) investigated the effect of kojic acid on the growth of root cuttings. Further data on the antibiotic activity of kojic acid are reported in papers by Verona and Agelli, (57) Meyer and Coworkers (58) and Norton. (59)
Toxicity:

An indication of the toxicity of kojic acid to mammals was given by Friedemann (60) who observed a definite response in dogs after intravenous injection of 0.15 g of sodium kojate per kg of body weight and found the lethal dose to be about 1 g per kg of body weight. Practically the same symptoms were shown by rabbits and rats. For mice, toxicity of the same order was reported by Mortan and Coworkers (12) and by Jennings and Williams (38). The later workers also observed that human leucocytes are killed within three hours by a 1% solution of sodium kojate at pH 6.8. Twelve day chick embryos were also susceptible to kojic acid, the observed LD$_{100}$ being 12 mg per 100 g of egg weight (39). Moderate cardiotoxic and cardiotonic activities of kojic acid are also reported (61,62). Though the toxicity was not very great in all these instances, yet it was enough to discourage chemotherapeutic investigations and to contraindicate the medicinal use of kojic acid.

MICROBIAL FORMATION OF KOJIC ACID

Kojic Acid Fermentation:

Several molds of the genus Aspergillus have been found to possess the ability to produce kojic acid from suitable nutrient solutions. Yabuta (2) isolated kojic acid from the nutrient solutions of A. albus, A. candidus and
A. nidulans. Traetta Mosca (10) claimed to have found it in cultures of A. clavatus. Tamiya and Hida (63) reported its production by A. flavus, A. gymnosardae, A. awamori, A. clavatus, A. fumigatus and A. giganteus. Birkinshaw and Coworkers (30) showed that it is also formed by A. parasiticus, A. effusus and A. tamarii. Later on, A. luteovirens (12), A. lutescens (36), A. wentii (37) and A. alliaceus (37) were added to the list of kojic acid producers. Most members of the A. flavus-oryzae tamarii group were apparently capable of producing kojic acid and A. oryzae and A. flavus were possibly most widely used for this purpose. Ohara (64) used the production of kojic acid as an aid in the identification and classification of 111 strains of the A. tamarii-oryzae group. In addition, the formation of kojic acid was also observed in the cultures of Penicillium daleae (30), ten species of acetic acid bacilli (65) and the gluconic acid fermenters Gluconobacter opacus var mobilis (66) and Gluconobacter roseus (67).

Rice and other cereals were the prime carbon sources in the early studies, but soon they were replaced by solutions of pure compounds (1,2). Microorganisms were grown in media containing a great variety of carbohydrates and related substances and they produced kojic acid from compounds containing two to seven carbon atoms per molecule. Among them reducing sugars, sugar acids, sugar alcohols, di and polysaccharides were suitable substrates. Table 2 lists a number of such substrates.
Table 2. Carbon Sources for the Biosynthesis of Kojic Acid

<table>
<thead>
<tr>
<th>Number of carbon atoms</th>
<th>Compound</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Ethanol</td>
<td>31, 64, 66</td>
</tr>
<tr>
<td></td>
<td>Glycine</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td>Sodium acetate</td>
<td>68</td>
</tr>
<tr>
<td>3</td>
<td>1,3 dihydroxy 2-propanone</td>
<td>69, 70, 71</td>
</tr>
<tr>
<td></td>
<td>Glyceraldehyde</td>
<td>71</td>
</tr>
<tr>
<td></td>
<td>Glycerol</td>
<td>11, 13, 17, 30, 66, 70, 73, 74</td>
</tr>
<tr>
<td></td>
<td>Sodium lactate</td>
<td>66</td>
</tr>
<tr>
<td></td>
<td>Sodium pyruvate</td>
<td>66, 68</td>
</tr>
<tr>
<td>4</td>
<td>Tartaric acid</td>
<td>73</td>
</tr>
<tr>
<td>5</td>
<td>Ribitol</td>
<td>74</td>
</tr>
<tr>
<td></td>
<td>Arabinose</td>
<td>30, 31, 64, 71, 73, 75</td>
</tr>
<tr>
<td></td>
<td>Xylose</td>
<td>30, 31, 64, 71, 73, 74, 77, 78</td>
</tr>
<tr>
<td>6</td>
<td>2 Deoxy glucose</td>
<td>31, 71, 73, 74</td>
</tr>
<tr>
<td></td>
<td>Fructose</td>
<td>10, 13, 30, 64, 65</td>
</tr>
<tr>
<td></td>
<td>Galactose</td>
<td>66, 67, 71, 73, 74</td>
</tr>
<tr>
<td></td>
<td>Gluconic acid</td>
<td>71</td>
</tr>
<tr>
<td></td>
<td>Gluconolactone</td>
<td>74</td>
</tr>
<tr>
<td></td>
<td>Glucose</td>
<td>3, 10, 11, 13, 30, 33, 64, 66, 69, 71, 72, 73, 74, 75, 77, 78</td>
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continued Table 2/

<table>
<thead>
<tr>
<th>Compound</th>
<th>References</th>
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<tbody>
<tr>
<td>Inositol</td>
<td>71,73</td>
</tr>
<tr>
<td>Mannitol</td>
<td>11,30,64,65,73,74</td>
</tr>
<tr>
<td></td>
<td>74,79</td>
</tr>
<tr>
<td>Mannose</td>
<td>64,71,73,74</td>
</tr>
<tr>
<td>Sorbitol</td>
<td>71,74</td>
</tr>
<tr>
<td>Sorbose</td>
<td>80</td>
</tr>
<tr>
<td>Quinic acid</td>
<td>72</td>
</tr>
<tr>
<td>Shikimic acid</td>
<td>72</td>
</tr>
<tr>
<td>Lactobionic acid</td>
<td>72</td>
</tr>
<tr>
<td>Lactose</td>
<td>30,64</td>
</tr>
<tr>
<td>Maltose</td>
<td>38,64,71</td>
</tr>
<tr>
<td>Sucrose</td>
<td>10,11,13,30,32,64,71,74,79,81</td>
</tr>
<tr>
<td>Trehalose</td>
<td>72</td>
</tr>
<tr>
<td>Raffinose</td>
<td>64</td>
</tr>
<tr>
<td>Dextrin</td>
<td>64</td>
</tr>
<tr>
<td>Inulin</td>
<td>64,71</td>
</tr>
<tr>
<td>Pectin</td>
<td>72</td>
</tr>
<tr>
<td>Starch</td>
<td>30,64</td>
</tr>
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</table>
The concentration of carbon sources varying from 5 to 30% have been used but the best results have been obtained with 20% glucose concentration. Media of different compositions have been used for kojic acid production, such as medium K(79) Czapekdox medium, yeast extract sucrose medium(35) and the medium used by Katagiri and Kitahari(71). It was shown that the yeast extract sucrose medium supported better growth of A. flavus than a glucose salts medium(82). Stationary cultures produced higher yields of kojic acid than shake flask cultures. Inorganic salts like KCl, NH₄NO₃, MgSO₄ 7H₂O have been used in different media. Peoptone, yeast-extract and cobaltamines have been found to be more satisfactory as nitrogenous sources. Recently Shohei and Coworkers(85) have reported that 50% of this complex organic nitrogen source i.e. yeast extract, can be substituted by urea or ammonium nitrate without sacrificing kojic acid yields.

The pH range of 2 to 5 and the temperature range of 30° to 35°C have been found to be optimum for kojic acid fermentation. The fermentation generally requires 9 to 20 days for completion, depending upon the type of substrate, pH, temperature, and other factors. After the complete exhaustion of sugar in the medium, kojic acid has been reported to be metabolized further by the mold resulting in a decrease in its concentration.
A number of substances have been found to promote kojic acid production. In a survey of forty organic compounds, it was found that ethylene chlorohydrin (1 g/l) produced a marked increase in kojic acid yield, in a period of 10 days. (86) Besides this methyl alcohol, ethyl alcohol, propyl alcohol, isopropyl alcohol and palmitic acid were found to be stimulants for kojic acid production. (87 to 89)

Phosphate as \( \text{KH}_2\text{PO}_4 \) was also found to be necessary for both growth and kojic acid production (90). High phosphate levels resulted in rapid kojic acid synthesis and degradation while at lower phosphate levels both the formation and degradation of kojic acid were slow. (91) Sodium fluoride, iodoacetic acid, arsenate, malonate, potassium cyanide, sodium azide, pentachlorophenol, dinitrophenol and sodium thiosulfate inhibited kojic acid production. Though inhibition by fluoride and iodoacetic acid was reversed when an organic acid like citrate, pyruvate or succinate was added to the medium, the inhibition by arsenate could not be reversed. (92)

**Isolation of Kojic Acid:**

After completion of fermentation, kojic acid is recovered from the broth by one of the following methods:- Precipitation as copper salt, (2, 31) extraction with ethyl acetate, (17) continuous extraction with ether, (33, 69) evaporation to small volume leading to crystallization (20).
oxidation to a ketonic intermediate which is then dehydrated to give kojic acid. This was frequently called the carving out theory of the formation of kojic acid.

\[
\begin{align*}
\text{D glucose} & \xrightarrow{\text{Oxidation}} \text{Ketonic intermediate} \xrightarrow{-2\text{H}_2\text{O}} \text{Kojic acid}
\end{align*}
\]

Opposed to it was the fission theory, which could be dated from the first conversion of pentoses to kojic acid.\(^{(13, 71, 75)}\) Challenger and Coworkers\(^{(75)}\) had hoped to obtain pyromeconic acid from pentoses with \(A. \text{oryzae}\), by analogy with the formation of kojic acid from hexoses.

\[
\begin{align*}
\text{Pentose} & \xrightarrow{-\text{H}_2} \xrightarrow{-2\text{H}_2\text{O}} \text{Pyromeconic acid}
\end{align*}
\]
Instead, kojic acid was produced from L. arabinose and D. Xylose. Corbellini and Gregorinii (13) too observed the production of kojic acid by A. flavus from these two pentoses and from D-fructose. They noted that Haworth's scheme would require the formation of an isomer of kojic acid from D-fructose.

\[
\begin{align*}
\text{D-Fructose} & \quad \text{Isomer of Kojic acid} \\
\end{align*}
\]

It was therefore suggested that kojic acid was formed directly by the condensation of two triose molecules without prior formation of a hexose. Glyceraldehyde and glyceral dialdehyde, which could be produced from sugars by fission and from glycerol by oxidation were assumed to be the triose precursors.

\[
\begin{align*}
\text{Glyceric dialdehyde} & \quad \text{Glyceraldehyde} & \quad \text{Kojic acid}
\end{align*}
\]
source they have, to a reserve carbohydrate and later hydro-
lyze it to a hexose which is then converted to kojic acid.
(73) Tamiya reached a similar conclusion and consi-
dering the customary long culturing periods, it seemed
entirely reasonable. Their view was shared later by Kluyver
(78) and Perquin when these workers found that, with short
incubation periods in special replacement cultures, *A. flavus*
produced practically no kojic acid in media containing
pentoses, sugar alcohols or hexoses other than glucose. Under
identical conditions, production of kojic acid from glucose is
at optimum levels. Negative results obtained with other
carbon sources suggested that only glucose is converted
directly to kojic acid and that all other compounds are first
assimilated into a reserve carbohydrate.

Trioses, particularly dihydroxyacetone were again
considered to be one of the most important intermediates
in the formation of kojic acid, when Katagiri and Kitahari
(72) who worked with *A. oryzae*, reported a 55% yield of kojic
acid from dihydroxy acetone, traces from glyceraldehyde but
none from acetaldehyde or diethyl acetal. Gould (74) found
that the production of kojic acid from glucose by *A. tamarii*
is not affected by the presence of aldehyde trapping reagents
like dimedone or bisulphite in the growth medium and that
no kojic acid is produced by the fungus if mold substance
i.e., reserve carbohydrate is the only source of carbon.
Several contradictory theories were thus presented in some papers; however it may be stressed that the extreme sensitivity of this microbiological process to seemingly minor changes in fermentation conditions should be kept in mind. Gould further observed that the formation of kojic acid probably did not proceed through the phosphoric esters, as it took place readily in the phosphate free media containing glucose, xylose or glycerol.

Sakaguchi and Coworkers (66) succeeded in producing kojic acid from ethanol by means of A. oryzae and thereby provided the first experimental evidence supporting the diosio intermediate theory. Barnard and Challenger (31) devoted considerable attention to this problem. They obtained 12 to 17% yields of kojic acid at 32°C from a culture of A. oryzae grown on basal salts medium containing 1.3 to 2.1% of ethanol, but none at lower temperatures or higher ethanol concentrations. The carefully washed fungus was grown on the basal salts medium alone for six weeks, and its failure to produce kojic acid eliminated the possibility of kojic acid production from reserve carbohydrate material in the experiments with ethanol. All fungal cultures grown on ethanol solutions, were shown to contain acetaldehyde and the addition of dimedon, but not of bisulphite, reduced the yield of kojic acid to 5% and delayed its formations. They could get further evidence for 'fission theory' and
against 'carving out' theory as revealed by the fact that three derivatives of glucose which were not expected to undergo fission gave no $\gamma$-pyrone derivatives and that a 20% yield of kojic acid was obtained from 2-deoxy-glucose which by the carving out process should have given rise to

2-hydroxymethyl $\gamma$-pyrone. Clearly the accumulated information revealed many details, but the exact nature of the biosynthetic process remained a matter of conjecture.

Arnstein and Bentley (106) applied the isotopic tracer technique in their studies on kojic acid biosynthesis. As the first step, they investigated the mechanism of alkaline Cleavage and degradation of di-O-methyl kojic acid. Yabuta (3) had found that treatment of di-O-methyl kojic acid with barium hydroxide yields equimolar quantities of formic acid, methoxacetone and methoxyacetic acid. The same three compounds were obtained from di-O-methyl kojic acid labelled with $^{14}$C in one of the methyl groups. The formic acid was completely non-radioactive and all the
Radioactivity was distributed between the other two fragments, the share of methoxyacetic acid being about 20% higher than that of methoxyacetone. These results indicated that the cleavage occurred in two different ways.

Scheme A

Di-0-methyl rosig acid

Scheme B

Di-0-methyl rosig acid
According to scheme A, C2 and C3 give methoxyacetic acid and C4 C5 and C6 give methoxyacetone. According to scheme B, C5 and C6 appear in methoxyacetic acid and C2, C3 and C4 in methoxyacetone. The greater radioactivity of methoxyacetic acid pointed to a slight predominance of scheme B. Formic acid was formed in all cases.

Further Arnstein and Bentley (69) produced kojic acid with A. oryzae and A. flavus-oryzae grown on media containing D-glucose labelled with $^{14}\text{C}$ on C1, D-glucose labelled on C3 and C4 and dihydroxyacetone labelled on C2. Between 2 and 20% of $^{14}\text{C}$ was recovered in kojic acid. Kojic acid was converted to same ethyl methyl ether in each case and degradation of this unsymmetrical ether by hot aqueous barium hydroxide followed by the separation of the fragments, and their assay for radioactivity gave an exact measure of the amount of $^{14}\text{C}$ incorporated into each of the six carbon atoms of kojic acid. The schemes for this degradation is given in the next page...
After treatment of ethyl methyl ether of kojic acid with hot aqueous barium hydroxide the liquor contained formic, methoxyacetic and ethoxyacetic acids, methoxyacetone and ethoxyacetone, produced by the two modes of cleavage A and B. Formic acid was converted with red mercuric oxide to carbon dioxide; this was recovered as barium carbonate, the radioactivity of which was a measure of the $^{14}$C incorporated into C1 of kojic acid in the biosynthetic process. The alkoxyacetones were removed from the liquor by steam distillation and converted to iodoform and a mixture of ethoxy and methoxy acetic acids. The iodoform was recovered by filtration, its radioactivity indicated the proportion of $^{14}$C incorporated into C4. The alkoxy acetic acids were isolated by continuous extraction of the filtrate with ether, and those in the original liquor were removed in the same way. These acids were separated chromatographically and converted to their silver salts. Methoxyacetic acid from both the sources contained C5 and C6 of kojic acid, while the whole of the ethoxy acetic acid was composed of C2 and C3 of kojic acid. Assay of the radioactivity contained in the silver salts gave the amount of $^{14}$C incorporated in C2 + C3 and in C5 + C6 of kojic acid respectively. Degradation of the silver salts with bromine liberated carbon dioxide containing C3 from ethoxyacetic, and carbon dioxide containing C5 from methoxy acetic acid.
These portions of CO₂ were also recovered and assayed in the form of barium carbonate. The amount of $^{14}$C which was incorporated into C2 and C6 was finally calculated by difference.

The results of these experiments showed that in the case of kojic acid produced from D-glucose-1-$^{14}$C, 70 to 90% of the $^{14}$C was located in C1 and 6 to 16% in C5, in the case of kojic acid produced from D-glucose-3,4-$^{14}$C₂, 90% of the $^{14}$C was located in C3 and C4; and in the case of kojic acid produced from dihydroxyacetone-2-$^{14}$C, 60 to 70% was to found in C2 and C5 of kojic acid. These distributions of radioactivity very strongly indicate that kojic acid is formed from D-glucose, largely by direct conversion in which no splitting of carbon chain takes place. The distribution of $^{14}$C in the kojic acid produced from labelled dihydroxyacetone, moreover, showed clearly that if free dihydroxyacetone or dihydroxyacetone phosphate were an important intermediate, the conversion of D-glucose-1-$^{14}$C would have led to a more extensive incorporation of $^{14}$C in to C2 to C6 of kojic acid. The presence of 6 to 16% of the $^{14}$C in C6 nevertheless pointed to a minor pathway in the formation of kojic acid, which involves the splitting of the glucose molecule and the recombination of trioses thus formed.
The above results were also confirmed by Kitada and Fukimbara (107). They investigated the conversion of glucose to kojic acid, by *A. oryzae* in a submerged culture, using $^{1-14}C$, $^{6-14}C$, $^{3-3}H$, and $^{5-3}H$ glucose as substrates and examined the distribution of radioactivity in kojic acid formed. Between 80 and 90% of total radioactivity, contained in the isolated kojic acid, was found in carbon atoms corresponding to those labelled in glucose molecule. When $^{3-3}H$ glucose and $^{5-3}H$ glucose were used as substrates, the incorporation of $^{3}H$ into kojic acid was 3.5% and 1.35% respectively. These results indicate that major pathway of kojic acid formation is the direct conversion of glucose without any splitting of carbon chain.

In their subsequent studies, Arnstein and Bentley (91) demonstrated the presence of aldolase and triose phosphate isomerase in fungi, producing kojic acid. They also found that both production and destruction of kojic acid were rapid in media containing high phosphate levels and slow at lower phosphate levels. They preferred to consider kojic acid as a normal metabolite of fungi rather than as an end product.

Lastly Arnstein and Bentley (68) investigated the incorporation of small molecules into kojic acid. Buffered solutions of pyruvic acid, acetic acid, glycine and some other related compounds, all labelled with $^{14}C$ were...
added to *Aspergillus* cultures grown initially on medium containing unlabelled glucose. The recovery of $^{14}C$ in kojic acid was usually less than 1% and labelled carbon atoms were distributed over the whole molecule predominating however, in all cases in C4, C5 and C6. A similar predominance of radioactivity in the lower half of kojic acid molecule was observed during studies with labelled dihydroxy acetone in the presence of nonradioactive glucose. These observations might be explained in both instances by the condensation of a labelled three carbon intermediate e.g. pyruvic acid or dihydroxy acetone, with an unlabelled triose or triose phosphate derived from glucose. In neither case were major quantities of glucose converted to kojic acid by this pathway. Despite the above explanations, the exact mechanism of kojic acid biosynthesis is still not known. Hence it would be a very interesting problem to investigate.
REFERENCES


