STUDIES ON OXIDATIVE FERMENTATION

II. ON THE NEW γ -PYRONE COMPOUND, RUBIGINOL, PRODUCED BY *GLUCONO- ACETOBACTER-LIQUEFACIENS*

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INTRODUCTION

The formation of γ -pyrone compounds as the metabolic products of micro-organisms seldom occurs except for kojic acid, which was discovered by a Japanese investigator K. Saito⁽³⁾ in 1907, the constitution of which was decided by T. Yabuta⁽⁹⁾⁽¹⁰⁾⁽¹¹⁾ in 1924. It is well known that the *Aspergillus oryzae-flavus-tamarii* group produces a large amount of kojic acid from sugars. On the other hand, in 1933 Takahashi and Asai⁽⁴⁾⁽⁵⁾⁽⁶⁾ confirmed that kojic acid was also produced by the oxidative bacteria from nannitol and fructose but not from glucose. Kondō⁽²⁾ recently reported its formation from sorboe by the *Gluconobacter* species. Another γ -pyrone compound, comenic acid was found and identified by Takahashi and Asai⁽⁷⁾⁽⁸⁾ in the fermentation products of galactose by *Gluconobacter liquefaciens*.

The author, in the course of a study on the metabolic products of *Gluconobacter*, *Pseudomonas*, and *Serratia*, found that three γ -pyrone compounds were produced in the culture liquid of *Gluconoacetobacter liq.* with a medium of 0.8% yeast extract in water containing 9.3% glucose and 2.5% CaCO_3 by shaking culture but not by surface culture. Two of these compound were confirmed to be new γ -pyrone compounds, to which the names of "Rubiginol" and "Rubiginic acid" were given.

The present paper reports the experimental results dealing with the chemical properties of rubiginol and its chemical structure which has been decided as 3,5-dihydroxy-1,4-pyrone.

EXPERIMENTAL

1. *Properties of rubiginol.*

Rubiginol was obtained as colorless plate crystals. When its aqueous solution was cooled rapidly, it crystallized out as prismatic needles which contained one molecule of water of crystallization, but the water of crystallization was lost easily at room temperature. It melts at 203.5° (uncorr.). It is easily soluble in pyridine, dioxane, acetone, methanol and ethanol, soluble in water, isopropanol, butanol, galcial acetic acid, ethylacetate and

ether; and scarcely soluble in toluol, xylol and benzol. Its aqueous solution is slightly acidic and reduces Fehling's solution and ammoniacal silver nitrate solution. Light green copper salt precipitates out from the aqueous solution upon the addition of copper acetate solution. It decolorizes bromine water and potassium permanganate solution. From the above mentioned facts, it seemed to be a weak acid belonging to phenolic or enolic compounds which contains at least one carbonyl group and at least one double bond.

Fig. 1. Photographs of crystals.

2. *Elemental analysis.*

After it was dried by Abderhalden's apparatus, elemental analysis was conducted. It was freed from nitrogen and sulphur.

Analysis.	Found	C	46.92	H	2.96
			46.90		3.29
	Calcd. for C ₅ H ₄ O ₄	C	46.88	H	3.15

Accordingly, the empirical formula was given as (C₅H₄O₄)_n.

3. *Determination of molecular weight.*

Molecular weight was determined by Rast's method. Found 144 Calcd. for C₅H₄O₄ 128.

Molecular weight was calculated as 128 from potentiometric titration curve. Molecular formula was then decided as C₅H₄O₄.

4. *Infra-red absorption spectrum of rubiginol.*

Infra-red recording spectrophotometer, Baird model B No. 143 was used. The absorption spectrum of infra-red ray showed the existence of a secondary or phenolic OH group, possibly a conjugated carbonyl group in its structure, while the existence of primary or tertiary OH groups, lactone or carboxyl groups were negated.

5. *Ultraviolet absorption spectrum.*

Ultraviolet absorption spectra of diacetylrubiginol and diacetyl kojic

Fig. 2. Infrared absorption spectrum of rubiginol.

Fig. 3. Infrared absorption spectrum of kojic acid.

Fig. 4. Ultraviolet absorption spectra
 1: diacetylkojic acid 2: diacetylrubiginol

acid were compared. As shown in Fig. 4, both spectra were very similar. Maximum absorption was observed at 250 mμ with diacetyl kojic acid and at 255 mμ with diacetylrubiginol. Since the maximum absorption of furan derivatives generally occurs at longer wave lengths, it was concluded that rubiginol is more similar to kojic acid than to furan derivatives.

6. Function of oxygen atoms.

The substance contains four oxygen atoms in its structure, and it is certain that at least one of them exists as a carbonyl group.

To ascertain the behavior of the other oxygen atoms, the existence and the number of OH groups were examined.

(a) *Methylation.* 1 g of sample was dissolved in 8.4 ml of 10% NaOH solution and was shaken after the addition of 2 ml of dimethylsulfate. After a while the methylated compound crystallized out. This was recrystallized from methanol, and colorless plate crystals which melted at 143° were obtained, yield, 730 mg and it showed no color reaction with ferric chloride. It does not show an acidic reaction.

Analysis.	Found	C	53.91	H	4.85
			53.81		4.77
	Calcd. for $C_5H_2O_2(OCH_3)_2$	C	53.86	H	5.16
Analysis.	Found	OCH ₃	39.61		
			39.74		
	Calcd. for $C_5H_2O_2(OCH_3)_2$		39.74		

(b) *Acetylation.* 100 mg of rubiginol was dissolved in 0.5 ml of acetylanhydrid and heated at 100° under reflux cooler for 2 hours. After the reaction ceased, acetylanhydrid was decomposed by the addition of water, the acetic acid was neutralized with NaOH solution, and the acetylated compound was extracted with ether. After removal of the solvent, the extract was recrystallized from methanol and colorless crystals were obtained, mp 118°. However, after it was heated with NaOH solution and acidified with HCl it again showed reddish violet color reaction with ferric chloride.

Analysis.	Found	C	51.39	H	4.16
			51.33		3.85
	Calcd. for $C_5H_2O_2(OCOCH_3)_2$	C	50.95	H	3.80

It agreed with diacetyl rubiginol.

From the results mentioned above, it was concluded that rubiginol has two hydroxyl groups in its structure which represent the acidic and ferric chlorid reactions.

(c) *Potentiometric titration of rubiginol.*

Potentiometric tiration curves of rubiginol and kojic acid are shown in Fig. 5. Rubiginol acted as a dibasic acid, pK value of the stronger acid group was 7.62 which was almost the same as that of kojic acid, 7.94 and the pK value of weaker acid group was 10.48. The substance was easily extracted from its $NaHCO_3$ solution with ether and could be set free by carbon dioxide. Therefore it is clear that the substance does not have a carbonyl group in its chemical structure and moreover the acidity and ferric chloride reaction are due to the existence of these two hydroxyl groups.

Fig. 5. Potentiometric titration curves of rubiginol and kojic acid

A : Rubiginol 59.4 mg in 100 ml H_2O

B : Kojic acid 61.7 mg in 10 ml H_2O

7. Existence of γ -pyrone ring.

Twenty seven chemical structures could be given for rubiginol from its molecular formula as ring compounds, i.e. α -pyrone, γ -pyrone, and furan derivatives. But in many respects rubiginol was found not to be an α -pyrone or furan derivative. As mentioned above, rubiginol is very similar to kojic acid with respect to its properties, the author assumed that it belongs to γ -pyrone compound. The proof of the existence of γ -pyrone ring in its chemical structure was conducted by transforming it into a pyridone derivative.

Diluted ammonia water was added to the dimethyl compound and heated on a water bath, the reaction product was obtained as colorless prismatic crystals when it was recrystallized from water. It melted at 270° and the results of its elemental analysis coincided with that of the corresponding pyridone derivative.

Analysis.	Found	C 53.68	H 5.87	N 9.24
	Calcd. for $\text{C}_7\text{H}_9\text{O}_3\text{N}$	C 54.19	H 5.85	N 9.03

Infra-red absorption spectrum of this compound also showed the existence of NH-group (Fig. 6).

Fig. 6. Infrared absorption spectrum of pyridone derivative produced from dimethylrubiginol.

8. Position of hydroxyl groups.

If rubiginol is one of the γ -pyrone derivatives, four different chemical structures should be given to it according to its molecular formula as shown in Fig. 7.

Among these, the fourth substance, viz. 2,4-dihydroxy-1,4-pyrone had been already reported by R. Kaushel⁽¹⁾ in 1940 and is clearly different from my substance according to its melting point (94°). In accordance

Fig. 7. γ -Pyrone derivatives having molecular formula $C_5H_4O_4$ and the decomposition products of their methylethers.

with the method adopted by previous investigators to decide the chemical structures of γ -pyrone derivatives, its dimethyl ether was hydrolyzed with barytha water in the hot state and then the decomposed products were subjected to analysis. The distilled fraction of the decomposition products of the second and third substances in Fig. 7, must be the same as that of kojic acid, i.e. acetolmethylether. The decomposition products of dimethyl-kojic acid was distilled and acetolmethylether was identified as its *p*-nitrophenylhydrazone, mp 111° , which coincided with Yabuta's investigation and 2,4-dinitrophenylhydrazone was also prepared which melted at 162° . On the other hand, the distilled fraction of decomposition products of dimethyl-rubiginol gave a different 2,4-dinitrophenylhydrazone, which melted at 121° , suggesting that only the first chemical structure might be possible for the substance. In fact, from the elemental analysis of this 2,4-dinitrophenylhydrazone, the carbon, hydrogen, nitrogen, and methoxyl contents agreed perfectly with those of dioxycetone dimethylether.

Analysis.	Found	C 44.17	H 4.40	N 19.26
		44.32	4.38	19.05
	Calcd. for $C_{11}H_{14}O_6N_4$	C 44.27	H 4.73	N 18.79

Found	OCH ₃	20.76
		20.68
Calcd. for C ₉ H ₈ O ₄ N ₄ (OCH ₃) ₂	OCH ₃	20.81

Residue of distillate was acidified with sulphuric acid and distilled in steam. The distillate was acidic and produced a white precipitate upon the addition of HgCl₂ solution in the hot state. The distillate was neutralized with CaCO₃ and Ca(OH)₂ and concentrated *in vacuo*. Ca salt was obtained by recrystallization as colorless plate crystals.

Analysis.	Found	Ca	30.61
			30.78
	Calcd. for (HCOO) ₂ Ca	Ca	30.80

It agreed with Ca formate. From 400 mg of rubiginoldimethylether, 20.6 mg of formic acid was found to be produced by titration and 21.4 mg of Ca formate was obtained, so it was concluded that 1 mol. of of dioxy-acetonedimethylether and 2 mol. of formic acid were produced from 1 mol. of dimethylrubiginol.

From the experimental results mentioned above, rubiginol was confirmed to having the structure formula [1] in Fig. 7, i.e. 3,5-dihydroxy-1,4-pyrone.

Fig. 8. Decomposition products of dimethylkojic acid.

SUMMARY

In the previous report, the authors found the formation of three substances which gave reddish violet color reactions with ferric chloride in the metabolic products of glucose by *Gluconoacetobacter liquefaciens*. One of them was found to be a new γ -pyrone derivative and was named as rubiginol. Its chemical properties was studied and the chemical structure was confirmed to be 3,5-dihydroxy-1,4-pyrone.

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