Riboflavin Biosynthesis by *Candida robusta*

Part III. Effect of Metal Ion and Necessity of CaCO_3

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Metal ions had little influence on riboflavin production by *Candida robusta*, except that Ag, Cu and Hg were strongly inhibitory. The inhibiting action of iron on its production was comparatively small, so that any previous treatment for the removal of iron from the medium was not necessary. Addition of CaCO_3 was found to be essential in media containing sucrose or acetate as a carbon source, as pointed out in the previous paper.¹⁾ This effect of CaCO_3 was attributed to its neutralizing action. When the reaction of the medium was kept at the optimum pH, about 7.0, high yields of riboflavin could be obtained without the presence of CaCO_3 .

INTRODUCTION

In the previous papers,^{1,2)} it was reported that six strains of *Candida robusta*, isolated from fruits, had abilities to synthesize riboflavin in shaking culture, and acetate was much more effective as a carbon source than sugar. Amino acids, asparagine and peptone were unsuitable as nitrogen sources in the sucrose medium, but they were as effective as ammonium salts and urea in the acetate medium. No flavinogenic factor was necessary. However, the addition of excessive CaCO_3 was essential for the riboflavin production.

In this paper, the effect of metal ions on riboflavin production is reported and necessity of CaCO_3 is also explained.

MATERIALS AND METHODS

Microorganisms.

Candida robusta No. 6, selected as the best strain with riboflavin-producing ability (refer to the previous paper²⁾), was obtained from the Laboratory of Applied Microbiology, Hokkaido University.

Culture Procedure.

After growing on potato glucose agar slant at 30°C for

3 days, the yeast was inoculated into 50 ml of the following medium containing Ca-acetate or sucrose, as a carbon source, in 500-ml shake flasks, then cultured on a shaker at 30°C: 0.3% $(\text{NH}_4)_2\text{SO}_4$, 0.2% KH_2PO_4 , 0.1% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 5% CaCO_3 . Ten ml of the medium in shake tubes were also used in some additional experiments.

Analysis.

Riboflavin was estimated fluorophotometrically, in the same manner as described in the previous paper. Iron analysis of water was made by *o*-phenanthroline method.³⁾

RESULTS AND DISCUSSION

1. Effect of Metal Ions on Riboflavin Production.

Sixteen kinds of metal ions were tested in concentrations of 10, 100 and 500 $\gamma\%$. Each of them was added as a soluble salt to the medium containing Ca-acetate (corresponding to 6% of acetic acid). Iron was tested separately in another experiment, because it has been known as a very important factor for some flavinogenic microorganisms. The results obtained after 5 days' culture are shown in Table I. Bismuth in 500 $\gamma\%$, Li in 100% and Mn in 10% con-

1) S. Takao, This Journal, **28**, 559 (1964).

2) S. Takao, *ibid.*, **28**, 566 (1964).

3) L.G. Saywell and B.B. Cunningham, *Ind. Eng. Chem., Anal. Ed.*, **9**, 67 (1937); W.B. Fortune and M.G. Mellon, *ibid.*, **10**, 60 (1938).

TABLE I. EFFECT OF METAL IONS

Metal	Riboflavin, mg %		
	10	100	500 γ %
Al	11.8	14.2	12.2
Ag	12.3	3.5	0
Ba	11.0	12.2	12.4
Bi	13.7	14.4	17.0
Cd	12.4	13.6	12.8
Co	11.6	12.0	13.2
Cu	11.1	4.3	0.9
Hg	12.6	11.8	0
Li	12.4	15.3	14.1
Mn	15.3	12.2	10.6
Ni	12.0	12.9	14.3
Pb	12.0	12.2	12.0
Sb	12.5	12.9	12.1
Sn	12.8	12.4	12.6
U	12.4	12.8	12.3
Zn	12.1	12.6	12.8
None	12.6		

centrations, respectively, were somewhat effective in promoting riboflavin production. On the other hand, Ag, Cu and Hg strongly inhibited its production when present in the medium in increasing concentrations. However, none of the other metals appreciably influenced its biosynthesis.

In this connection, riboflavin production by *Eremothecium ashbyii* is stimulated by Zn;⁴⁾ that of *Candida guilliermondii* is also promoted with Zn^{5,6)} and Co.⁷⁾ In the case of *Clostridium acetobutylicum*, however, riboflavin production is extremely inhibited by the presence of small quantities of Co^{8,9)} and Ni.⁹⁾

2. Effect of Iron on Riboflavin Production.

Ferrous sulfate was added in concentrations ranging from 0 to 100,000 γ % as iron to the same medium as that previously used. The basal medium itself already contained 10 γ % of iron,

TABLE II. EFFECT OF VARIOUS CONCENTRATIONS OF IRON

Iron γ %	Rate of pigmentation					Riboflavin, mg% (5 days)
	1	2	3	4	5 days	
0	±	++	+++	+++	+++	16.62
50	±	++	+++	+++	+++	12.34
100	±	++	+++	+++	+++	8.22
500	±	++	+++	+++	+++	6.14
1000	±	+	++	++	++	2.99
5000	—	±	+	+	+	0.16
10000	—	±	±	+	+	0.13
50000	—	—	—	±	±	0.06
100000	—	—	—	—	±	0.06

because no treatment was made for the removal of this metal from the tap water employed. The results obtained after a period of 5 days are shown in Table II. It may be observed that riboflavin production was sharply reduced with increasing concentrations of iron. However, it appeared that there were no great differences in

FIG. 1. Effect of Iron on the Rate of Riboflavin Production.

4) H. Naganishi and R. Saruno, *Vitamins (in Japanese)*, **2**, 133 (1949).

5) F. Knüsel, *Arch. Mikrobiol.*, **27**, 219 (1957).

6) W.H. Schopfer and F. Knüsel, *Schweiz. Z. allgem. Path. u. Bakt.*, **19**, 659 (1956); *Chem. Abstr.*, **51**, 3732 (1957).

7) T.M. Enari, *Ann. Acad. Sci. Fennicae Sec. A*, II, No. **90**, 8 (1958); *Chem. Abstr.*, **53**, 2361 (1959).

8) C.F. Artzberger, *U.S. Patent*, 2,326,425 (1943).

9) K. Imai, *J. Agr. Chem. Soc. Japan*, **29**, 567 (1955).

respect to the rate of riboflavin production during the early stages of incubation, when using iron concentrations of 0 to 500 $\gamma\%$.

The results of detailed experiments are summarized in Fig. 1. Some flavinogenic microorganisms are well-known to be strongly inhibited in respect to riboflavin production when iron is present in the media; for example, two genera, *Clostridium* and *Candida*, are quite sensitive to this metal. In the case of *E. ashbyii*, riboflavin production is not markedly affected by the presence of iron. A level as high as 0.04% of iron is reported to be inhibitory,⁴⁾ but 0.02% of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ is sometimes added to the medium for its production.¹⁰⁾ Yamasaki¹¹⁾ reported that riboflavin was scarcely produced by *Cl. acetobutylicum* when the level of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ was 0.5 mg% (100 $\gamma\%$ as Fe) in the medium and no riboflavin was found in a 1 mg% concentration (200 $\gamma\%$ as Fe). Leviton¹²⁾ showed that only ferrous iron was inhibitory, but Katagiri¹³⁾, Obata and Tomoeda,¹⁴⁾ and Imai¹⁵⁾ stated that both ferrous and ferric irons were similarly antiriboflavinogenic. Tanner et al.¹⁶⁾ indicated that the optimum concentration of iron was about 100 $\gamma\%$ for this bacterium and this was 100 times greater than the optimum concentration for the *Candida* species. Van Lanen¹⁷⁾ also reported that 100 to 300 $\gamma\%$ of iron was optimum.

Tanner et al.¹⁶⁾ observed that the addition of 10 $\gamma\%$ of iron sharply reduced riboflavin synthesis by *C. guilliermondii* and *C. flarerii*, and the highest yields were obtained in the concentrations of 0.5 to 1 $\gamma\%$. Levine et al.¹⁸⁾ obtained high yields of riboflavin by these strains when the iron content of the medium was held to a minimum 4 to 6 $\gamma\%$. However, a previous treatment for

removal of iron from the medium is sometimes necessary to get such low levels of this metal. These yeasts, therefore, appear to be unfavorable for use in the commercial production of this vitamin.

Ten $\gamma\%$ of iron, which was extremely inhibitory to the synthesis of riboflavin by the above-named *Candida* species, was already contained in the medium without the addition of iron in this experiment. *C. robusta* was able to produce large amounts of riboflavin in this medium, so that any consideration for the removal of iron is not necessary. This fact, in addition to its high ability to synthesize riboflavin, suggests the possibility that *C. robusta* may be used for commercial purposes.

3. Necessity of CaCO_3 for Riboflavin Production.

1) **Physical effect of CaCO_3 .** As reported in the previous paper,¹⁾ CaCO_3 was essential for riboflavin production by *C. robusta*. Since considerable amounts of organic acids were produced in the sucrose medium containing CaCO_3 during the early stages of incubation, CaCO_3 seems to act as a neutralizing agent. On the other hand, the addition of CaCO_3 was required even in Ca-acetate medium. Therefore, CaCO_3 may,

TABLE III. EFFECT ON RIBOFLAVIN PRODUCTION OF THE REPLACEMENT OF CaCO_3 BY SOME MATERIALS

Material (5%)	CaCO_3 %	Riboflavin mg %
Sand	0	0
	0.5	0.08
	1.0	0.15
Quartz sand	0	0
	0.5	0.18
	1.0	0.42
Diatomaceous earth	0	0
	0.5	0
	1.0	0
Activated earth	0	0
	0.5	0
	1.0	0
Activated alumina	0	0.53
	0.5	1.77
	1.0	2.74
None	0	0
	0.5	0.28
	1.0	0.52
	5.0	5.72

10) B. Tabenkin, *U.S. Patent*, 2,493,274 (1950).

11) I. Yamasaki, *Biochem. Z.*, **307**, 431 (1941).

12) A. Leviton, *J. Am. Chem. Soc.*, **68**, 835 (1946).

13) H. Katagiri, *Symposia on Enzyme Chemistry (in Japanese)*, **6**, 3 (1951).

14) Y. Obata and M. Tomoeda, *J. Agr. Chem. Soc. Japan*, **27**, 867 (1953).

15) K. Imai, *ibid.*, **29**, 570 (1955).

16) F.W. Tanner, C. Vojnovich and J.M. Van Lanen, *Science*, **101**, 180 (1945).

17) J.M. Van Lanen and F.W. Tanner, *Vitamins and Hormones*, **VI**, Academic Press, New York (1948).

18) H. Levine, J.E. Oyaas, L. Wasserman, J.C. Hoogerheide and R.M. Stern, *Ind. Eng. Chem.*, **41**, 1665 (1949).

19) R.A. Steinberg, *J. Agr. Research*, **51**, 413 (1935).

also, possess some physical advantage such as the absorption of inhibiting substance. In this connection, Steinberg¹⁹⁾ reported that CaCO_3 was very effective for the removal of metal ions, such as Fe, Zn, Cu and Mn, from the medium. In order to examine the possible physical effect of CaCO_3 , several substitutive materials were added to the medium containing 3% of sucrose. The results obtained after 5 days' shaking culture are shown in Table III. It appears that CaCO_3 has a certain physical effect, because activated alumina was somewhat effective in respect to riboflavin production in the medium containing no CaCO_3 .

2) Addition of activated alumina to acetate medium. Activated alumina was added in concentrations of 1 to 40% to the medium containing Ca-acetate (corresponding to 6% of acetic acid). The resulting data are shown in Table IV. High concentrations of activated alumina were as effective as CaCO_3 on riboflavin production in the acetate medium.

TABLE IV. EFFECT OF ACTIVATED ALUMINA ON THE PRODUCTION OF RIBOFLAVIN IN ACETATE MEDIUM

Activated alumina %	Riboflavin after 5 days mg %
1	0
3	0
5	0.03
7	0.08
10	0.36
13	0.42
16	1.91
20	20.92
30	22.00
40	19.29

3) Addition of small amounts of CaCO_3 to acetate medium. In the Ca-acetate medium, a neutralizing agent did not appear to be needed for promoting acid production. It is, however, necessary to neutralize the broth when the optimum reaction for riboflavin synthesis by this yeast is approximately neutral, because the pH drops to some extent when the sulfate ions are liberated from $(\text{NH}_4)_2\text{SO}_4$ during fermentation even

TABLE V. EFFECT OF SMALL AMOUNTS OF CaCO_3 ON RIBOFLAVIN PRODUCTION

CaCO_3 %	Riboflavin mg %
0	0
0.25	20.00
0.5	21.13
0.75	20.53
1.0	20.80
5.0	21.60

in the acetate medium. Aqueous suspensions of activated alumina are slightly alkaline, by the way. Hence, the alumina possesses some ability to neutralize the broth. If the above consideration is appropriate, much riboflavin must be produced in the acetate medium by the addition of small amounts of CaCO_3 to neutralize the liberated sulfate ions. As 5% of CaCO_3 has been used in the previous experiments, concentrations of 0.25 to 5% were tested in the Ca-acetate medium (containing 7% acetic acid). The results obtained after 6 days' culture are summarized in Table V. As little as 0.25% of CaCO_3 produced a yield of riboflavin similar to those obtained with higher concentrations. Based on these results, CaCO_3 and activated alumina are considered to be useful, almost exclusively, as a neutralizing agent.

4) Riboflavin production without the addition of CaCO_3 . According to the above consideration, riboflavin should be produced without an addition of CaCO_3 by using nitrogen sources which

TABLE VI. EFFECT ON RIBOFLAVIN PRODUCTION OF VARIOUS NITROGEN SOURCES USED WITHOUT THE ADDITION OF CaCO_3

N source	Acetate	pH		Riboflavin after 6 days mg %
		Initial	Final	
Urea	{Ca-	6.4	7.0	4.55
	{Na-	7.2	8.6	0
Alanine	{Ca-	6.0	6.4	0
	{Na-	6.6	8.6	0
$(\text{NH}_4)_2\text{CO}_3 \cdot \text{H}_2\text{O}$	{Ca-	6.6	7.0	33.38
	{Na-	6.8	8.0	0
$(\text{NH}_4)_2\text{SO}_4$	{Ca-	6.0	6.4	0
	{Na-	6.6	8.2	0
	Ca-	6.5	7.0	21.63

(with 5% CaCO_3)

do not liberate any strong acid radical during the fermentation. Urea, alanine and $(\text{NH}_4)_2\text{CO}_3 \cdot \text{H}_2\text{O}$ were then supplied in a concentration corresponding to a nitrogen level of 0.3% $(\text{NH}_4)_2\text{SO}_4$ in Ca- or Na-acetate media (as 7% acetic acid). As shown in Table VI, riboflavin was produced in the absence of CaCO_3 in the Ca-acetate medium containing urea, or $(\text{NH}_4)_2\text{CO}_3 \cdot \text{H}_2\text{O}$. Actually, the latter gave a greater yield of riboflavin than $(\text{NH}_4)_2\text{SO}_4$ with CaCO_3 , although small amounts of CaCO_3 were present in the medium after sterilization, as a result of the combination of Ca-acetate and $(\text{NH}_4)_2\text{CO}_3$. On the other hand, the final reaction was pH 7.0 whenever riboflavin was found in the broth.

4. Optimum pH for Riboflavin Production.

The different pH values were obtained by adjustment of the media with HCl or KOH before sterilization. The results obtained with the medium containing Ca-acetate (corresponding to 7% acetic acid) and 0.25% urea in shake tubes after 7 days are shown in Table VII. A favorable pH for the riboflavin production by *C.*

robusta was found to be about 7.0; when it was 6.1, or below, the vitamin was not produced. Based on these results, it is believed that the effects of CaCO_3 and activated alumina on riboflavin biosynthesis are due to the neutralizing action, not only in sucrose medium but also in acetate medium.

The optimum pH ranges for riboflavin production by some flavinogenic microbes are as follows: *E. ashbyii*, pH 5.5 to 6.4;^{10,20,21} *A. gossypii*, pH 6.0 to 7.0;²² *C. guilliermondii* and *C. flareri*, pH 5.0 or below;^{16,18} and a flavin-producing mutant of *Saccharomyces cerevisiae*, pH 5.0.²³ In the case of *Cl. acetobutylicum*, CaCO_3 is usually added, but a pH 6.2 is sometimes used in the medium without the addition of CaCO_3 .²⁴

It thus appears that above organisms prefer relatively low pH for riboflavin production. Accordingly, *C. robusta* is somewhat different from these organisms with regard to the optimum pH.

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20) E. Deseive, *Milchwissenschaft*, **3**, 141 (1947); *Chem. Abstr.*, **42**, 2310 (1948).

21) K. Saito and R. Takata, *Jozogaku Zasshi* (in Japanese), **20**, 316 (1942).

22) F.W. Tanner, G. Vojnovich and J.M. Van Lanen, *J. Bact.*, **58**, 737 (1949).

23) K.V. Giri and P.R. Krishnaswamy, *ibid.*, **67**, 309 (1954).

24) Y. Obata and M. Tomoeda, *Vitamins* (in Japanese), **5**, 552 (1952).

TABLE VII. EFFECT OF pH ON RIBOFLAVIN PRODUCTION

Initial pH after sterilization	Final pH	Riboflavin after 7 days mg %
6.0	6.2	0
6.1	6.3	0
6.2	6.6	0.48
6.3	7.0	3.70
6.4	7.0	12.00
6.6	7.0	13.45
6.8	7.0	13.60
7.0	7.0	14.35
7.3	7.0	13.75
6.6	7.0	14.40

(with 5% CaCO_3)