Preparation of Riboflavin (UL)-^{14}C

L. K. LOWRY, Y. F. HERMAN, H. E. SAUBERLICH

U. S. Army Medical Research and Nutrition Laboratory, Fitzsimons General Hospital, Denver, Colorado 80240.

Received August 18, 1969.

SUMMARY

Uniformly labeled riboflavin-^{14}C was synthesized by the organism, Ashbya gossypii. *Riboflavin with a specific activity of 79.0 mCi/mmole was obtained after extensive purification. Degradation of riboflavin with light and NaOH indicated that the ^{14}C label was present in both the isoalloxazine ring and in the side chain. Radioautographs of the chromatographed vitamin showed that a single radioactive band was found that coincided with authentic riboflavin.*

INTRODUCTION.

The nutritional importance of riboflavin has been known for some time [1, 2]. Riboflavin has been intensively studied by many workers and today is recognized as both a vitamin and a coenzyme important in many diverse biochemical reactions [3]. Although much is known about the biosynthesis and function of riboflavin and its nucleotides, little is known of its mode of action. The availability of labeled riboflavin is important to such future studies. Riboflavin-2-^{14}C is commercially available and its metabolism has been studied in the rat [4, 5], however, the results of these studies were not conclusive because only the fate of the number 2 carbon of riboflavin was studied. This paper presents a method for the biosynthesis of uniformly labeled riboflavin of sufficient specific radioactivity for further metabolic studies.

The biosynthesis of riboflavin in microorganisms and the labeling patterns obtained with different substrates has been reviewed by Goodwin [6]. Compounds such as sodium formate-^{14}C, $H^{14}CO_3$, glycine-1,2-^{14}C, glycine-^{15}N, glucose-^{14}C, and adenine(UL)-^{14}C have been incorporated into specific positions of the riboflavin molecule. *Eremothecium ashbyii*, *Candida flareri* and *Ashbya gossypii* have been used most frequently in these biosynthetic studies. Of these, *Ashbya gossypii* was found to be the best organism for the production of uniformly labeled riboflavin. The requirements for uniformly labeling a

compound such as riboflavin are (*a*) the growth medium must be defined and (*b*) the specific radioactivity of each carbon atom in the medium must be the same. These criteria have been met.

The purification and characterization of the riboflavin produced was carried out using thin layer chromatography, column chromatography, paper chromatography, radioautography, and partial chemical degradation.

EXPERIMENTAL.

Source of Culture and Culture Conditions.

Ashbya gossypii, ATTC 10895, was maintained on agar slants containing 2.0 % glucose, 1.0 % peptone, 0.5 % yeast extract and 1.8 % agar. Cultures were incubated at 28° C ± 1° C for four days and then refrigerated. Seed cultures were prepared by serially transferring the highly pigmented areas so that cultures consistently produced pigmentation after 24-48 hours' incubation. After four days' growth these cultures were used to inoculate liquid media. Table 1 shows the defined minimal liquid medium initially used for growth and flavogenesis of *A. gossypii*.

TABLE 1. Defined minimal liquid medium for growth and flavogenesis of *Ashbya Gossypii*

A. Basal salts	
KH_2PO_4	0.1 %
K_2HPO_4	0.1 %
NaCl	0.05 %
$MgSO_4.7H_2O$	0.05 %
B. Glucose	2.0 %
C. Vitamin mixture (add per 100 ml medium)	
Inositol	3.0 mg
Thiamine-HCl	0.1 mg
D-biotin	2.0 μg
D. Amino acids (per 100 ml medium)	
L-Aspartic acid	210 mg
L-Arginine-HCl	10 mg
Glycine	30 mg
L-Histidine	33 mg
L-Leucine	25 mg

The liquid medium was inoculated as follows : Fifty ml of medium was added to each of two 250 ml erhlenmyer flasks which were set up with a compressed air circulation system containing a 10 % NaOH inlet trap to remove CO_2 from the compressed air, sterile cotton air filters, and two one liter outlet traps containing methyl cellosolve; monoethanolamine (2 : 1 v/v) to trap expired $^{14}CO_2$ [7]. The inoculum was prepared by aseptically transferring the pigmented mycelial mat with a sterile pipette. After centrifugation of the

cells, the slightly turbid, very yellow supernatant was used to inoculate the erhlenmyer flasks. The cultures were incubated in a 28° C shaking water bath for seven days in a darkened room with continuous aeration.

The Effects of Varying the Composition of the Media on Growth and Flavogenesis.

The biosynthesis of uniformly labeled riboflavin-^{14}C requires a completely defined liquid medium of relatively simple composition. The defined minimal liquid medium described in Table 1 was simplified by varying the amino acid composition. Table 2 shows the effects of various additions and deletions of amino acids on growth and flavogenesis. The table shows that a mixture of L-aspartic acid, L-arginine · HCl and glycine provides average growth and good flavogenesis.

TABLE 2. Effect of amino acid combinations on growth and flavogenesis of *Ashbya Gossypii*

Medium	Growth	Flavogenesis
DMLM-Complete[a]	++ (average)	+ (slight)
DMLM-less histidine	++ (average)	+ (slight)
DMLM-less leucine, aspartic acid, plus 210 mg glutamic acid	+ (minimal)	0 (none)
DMLM-less histidine, leucine	+++ (good)	+++ (good)
DMLM-less histidine, aspartic acid	++ (average)	+ (slight)
DMLM-less histidine, leucine, arginine.HCl	++ (average)	++ (average)
Complex[b] (Control)	++++ (very good)	++++ (very good)

[a] DMLM is the defined minimal liquid medium of Table 1 composed of A. Basal Salts; B. Glucose; C. Vitamin mixture; and D. Amino acids.

[b] Complex control medium composed of A. as above; B. as above; C. 0.5 % yeast extract, and D. 0.5 % peptone.

Since sodium formate-^{14}C has been shown to be incorporated into the 2 position of riboflavin [8], and since folic acid is involved in one carbon metabolism, the effects of added sodium formate and folic acid on growth and flavogenesis was investigated. Table 3 shows that addition of both folic acid and sodium formate promoted flavogenesis. Table 3 also shows that *A. gossypii* can grow and produce riboflavin on 1 % glucose.

Since the complex medium always provided better growth and flavogenesis than the defined liquid media, the organism was grown in different concentrations of glucose with small amounts of peptone and yeast extract added. Table 4 shows that the best growth and flavogenesis was obtained with 1 % glucose with 0.1 % yeast extract added. The addition of 0.1 % peptone

TABLE 3. Effect of folic acid and sodium formate on growth and flavogenesis of *Ashbya Gossypii*.

Medium	Growth	Flavogenesis
DMLM-Complete [a] plus folic acid plus sodium formate	+++ (good)	+++ (good)
DMLM-less folic acid	+++ (good)	++ (average)
DMLM-less folic acid and sodium formate	+++ (good)	++ (average)
Complex [b] (Control)	++++ (very good)	++++ (very good)

[a] DMLM is the defined minimal liquid medium of Table 1 with changes in the composition as follows : A. Basal Salts; B. 1.0 % glucose; C. Vitamin mixture plus 0.1 mg folic acid; D. Amino Acids, total of 5 changed by deleting histidine and leucine. Aspartic acid decreased to 150 mg; E. 37.8 mg sodium formate/100 ml medium.

[b] Complex control medium composed of A. as above; B. as above, C. 0.5 % yeast extract; D. 0.5 % peptone.

had no effect. Apparently, the yeast extract contains some required factor that was not added in the synthetic vitamin mix. Since yeast extract at 0.1 % concentration was low and consequently could contribute little carbon for riboflavin synthesis, it was included in the medium.

TABLE 4. Effect of glucose concentration and traces of peptone and yeast extract on growth and flavogenesis of *Ashbya Gossypii*.

Medium	Glucose conc.	Growth	Flavogenesis
DMLM-Complete [a] plus peptone and yeast extract	1.0 %	+++ (good)	+++ (good)
DMLM-less peptone	1.0 %	+++ (good)	+++ (good)
DMLM-less peptone and yeast extract	1.0 %	++ (average)	++ (average)
DMLM-Complete [b] plus peptone and yeast extract	0.5 %	+++ (good)	++ (average)
DMLM-less peptone	0.5 %	++ (average)	++ (average)

[a] DMLM is the defined minimal liquid medium of Table 3, plus 0.1 % peptone and 0.1 % yeast extract.

[b] DMLM is the defined minimal liquid medium, as above, except glcuose concentration is 0.5 %.

Composition of the Medium for the Biosynthesis of Uniformly Labeled Riboflavin-^{14}C.

The improved defined liquid medium used for the biosynthesis of uniformly labeled riboflavin-^{14}C is shown in Table 5. The specific activity (μCi/mgC) of each carbon atom of the carbon sources is the same. Although other carbon is present in the medium, it was present in vitamins and other factors necessary for growth and flavogenesis of *A. gossypii* and would not logically be incorporated into riboflavin.

TABLE 5. Composition of the defined liquid medium used for the production of uniformly labeled ^{14}C-riboflavin by *Ashbya Gossypii*.

Component	Wt/100 ml Medium (mg)	Total ^{14}C (μCi)	Specific Activity μCi/mg C
D-glucose-U-^{14}C	1000	10,000	25.0
Glycine-1,2-^{14}C	30	240	25.0
L-aspartic acid-U-^{14}C	150	1,350	25.0
L-arginine-U-^{14}C (mono-HCl)	11.9	1,000	25.0
Sodium formate-^{14}C	38	168	25.0
Yeast Extract, Difco	100	—	—
Inositol	3	—	—
D-Biotin	0.002	—	—
Thiamine.HCl	0.10	—	—
Folic Acid	0.10	—	—
KH_2PO_4	100	—	—
K_2HPO_4	100	—	—
NaCl	50	—	—
$MgSO_4.H_2O$	50	—	—

Isolation and Purification of Riboflavin-^{14}C.

Riboflavin was isolated from *A. gossypii* by a modification of the procedure used by Merck and Company [9]. At the end of the incubation period, 12.5 ml of 0.123 M sodium acetate buffer, pH 4.6, was added to each flask to stabilize the riboflavin. The flasks were autoclaved 30 minutes at 15 lbs pressure and the cells removed by filtration. The filtrate, which contained the riboflavin was concentrated to approximately 15 ml on the flash evaporator under subdued light. All procedures from this point on were carried out in a darkened room. One hundred fifty ml of boiling acetone was added to the concentrate and the proteins allowed to precipitate overnight in the freezer compartment of the refrigerator. The supernatant was concentrated and again treated with boiling acetone for removal of residual protein. The supernatant, containing riboflavin, was extracted with ethyl ether to remove lipids.

The aqueous phase was chromatographed on a 2 × 20 cm florosil column which was eluted with water, 2 % acetic acid, additional water and pyridine-water mixtures from 1 to 10 % pyridine [10]. Riboflavin was eluted with 5-10 % pyridine in water. After concentration of the eluate, pyridine was removed by extraction with chloroform. The aqueous phase, containing riboflavin was filtered on moistened filter paper to remove suspended chloroform and concentrated to about 30 ml. The preparation was placed in the freezer overnight and the resulting gel-like precipitate centrifuged off.

The clear riboflavin containing supernatant was chromatographed on a 1 mm thick layer of Silica Gel G in glacial acetic acid : acetone : methanol : benzene (5 : 5 : 20 : 70 v/v) according to Ganshirt and Malzacker [11]. Riboflavin with an R_f of about 0.3 was scraped off the plate and eluted with water. Two minor bands of higher R_f were later identified as lumiflavin and lumichrome by paper chromatography in butanol : acetic acid : water (4:1:5 v/v)[12]. The eluted riboflavin was concentrated and the $CaSO_4$ binder from silica gel G removed by centrifugation. The concentrated riboflavin was chromatographed on Whatman Number 3 mm paper in butanol : acetic acid : water as previously described.

The purified riboflavin band was eluted, concentrated to a small volume and crystallized from a water-acetone mixture. The crystals were redissolved in water and concentrated to 100.0 ml. One ml was removed for microbiological assay with *Lactobacillus casei*, ATTC number 7469 and radioactivity determination by scintillation counting in Bray's solution [13]. The remaining 99 ml was lyophyllized and sealed in vacuo.

In order to determine the approximate distribution of radioactivity in the purified riboflavin-^{14}C, a partial degradation of riboflavin was carried out according to the procedure described by Goodwin and Jones [14]. Riboflavin was dissolved in 0.5 N NaOH and the solution placed in a petri dish under ultraviolet illumination (two fluorescent type lamps with maximum output at 3 650 Å) for 12 hours. After 12 hours illumination, the riboflavin yellow disappeared. At this time a sample of the irradiated riboflavin was acidified with 2 N HCl and extracted with $CHCl_3$. Both the aqueous phase, which showed no fluorescence, and the $CHCl_3$ phase, which contained lumiflavin, were counted in the scintillation counter as described above.

To verify the radiochemical purity of the synthesized riboflavin, about 5 000 dpm of the lyophilized vitamin was chromatographed along with standards on Silica Gel G as described above and a sheet of no-screen X-ray film placed over the plate. The entire assembly was placed in the refrigerator for 12 days after which the X-ray film was developed.

Results and Discussion.

Uniformly labeled riboflavin-^{14}C with a specific activity of 79.0 mCi/mmole and a weight of 1.46 mg was prepared using *A. gossypii* and the medium

described in Table 5. The riboflavin prepared was purified extensively according to procedures described above. R_f values found for riboflavin-^{14}C chromatographed as indicated above were as follows :

Method	R_f *(found)*	R_f *(literature)*
Thin Layer Chromatography	0.30	0.35 [11]
Paper Chromatography	0.34	0.31 [12]

The radiochemical purity of the biosynthesized riboflavin was determined by radioautography as described in Experimental. The results are shown in Figure 1. Only one radioactive band appeared which corresponded to the R_f value of riboflavin. Only a faint haze was found at the origin which represented less than 5 % of the radioactivity applied to the plate.

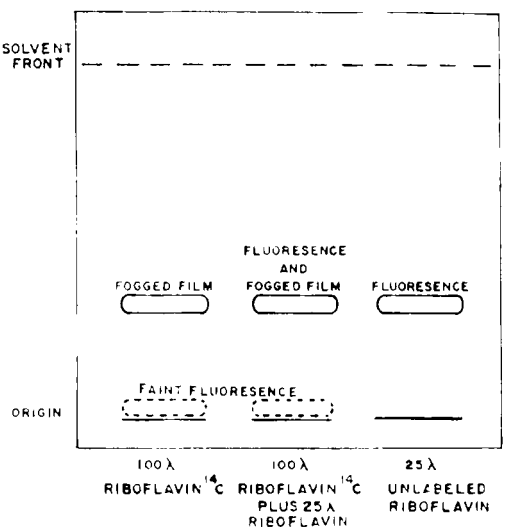

FIG. 1. Radioautograph of riboflavin-^{14}C.
Details of the chromatography are given in the text.

Since the specific activity of each carbon atom in the medium used was the same, the distribution of label in riboflavin-^{14}C should also be equal. In order to verify that ^{14}C was present in both the ribityl side chain and the isoalloxazine ring, the base catalyzed light degradation of riboflavin was carried out as described in Experimental. When the two phases, the aqueous and $CHCl_3$ phases were counted, radioactivity was found in both fractions which indicated that radioactivity was present in both the ribityl side chain and in the isoalloxazine ring.

The specific activity of the biosynthesized riboflavin-^{14}C was high enough to be used in future studies on the metabolism of riboflavin. Experiments are in progress on the metabolism of riboflavin-^{14}C in rats and in man.

REFERENCES

1. WARBURG, O. and CHRISTIAN, W. — *Biochem. Z.*, **254** : 438 (1932).
2. HORWITT, M. K., HARVEY, C. C., HILLS, O. W. and LIEBERT, E. J. — *J. Nutr.*, **41** : 247 (1950).
3. BEINERT, H. — in "The Enzymes" Edited by Boyer, P. D., Lardy, H. and Myrback, K. — Academic Press, New York, Volume 2, page 339 (1960).
4. AMOS, W. H., Jr., BALAGHI, M., RAMIREZ, O. Jr. and SAUBERLICH, H. E. — *Fed. Proc.*, **25** : 245 (1966).
5. RAICA, N. Jr., HERMAN, Y. F., AMOS, W. H. Jr. and SAUBERLICH, H. E. — *Fed. Proc.*, **25** : 245 (1966).
6. GOODWIN, T. W. — "The Biosynthesis of Vitamins and Related Compounds". Academic Press, New York, Chapter 2 (1963).
7. BEECHER, G. — Personal Communication.
8. PLAUT, G. W. E. — *J. Biol. Chem.*, **208** : 513 (1954).
9. Merck and Company, British Patent No. 621,401, April 8, 1949 (*Chem. Abst.*, **43** : 7189 (1949)).
10. LARDY, H. A., Editor. — "Biochemical Preparations", John Wiley and Sons, Inc., New York, Volume 7, page 51 (1960).
11. GANSHIRT, H. and MALZACKER, A. — *Naturwiss.*, **47** : 279 (1960).
12. KILGOUR, G. L., FELTON, S. P. and HUNNEKENS, F. M. — *J. Am. Chem. Soc.*, **79** : 2254 (1957).
13. BRAY, G. A. — *Anal. Biochem.*, **1** : 279 (1960).
14. GOODWIN, T. W. and JONES, O. T. G. — *Biochem. J.*, **64** : 9 (1956).