SYNTHESIS OF RIBOFLAVIN BY MICROORGANISMS

II. THE GREEN AND VIOLET FLUORESCENT COMPOUNDS PRODUCED IN THE CULTURE FILTRATE OF *CLOSTRIDIUM ACETOBUTYLICUM*

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The green and violet fluorescent compounds were found to be produced in the growing culture of microorganisms. The properties of these compounds are very similar to those of riboflavin and the periods of their production correspond also to the formation of the vitamin. These compounds were therefore tested as to whether they represent the precursors in the riboflavin synthesis.

EXPERIMENTAL

Microorganism — *Clostridium acetobutylicum* W-29 was used in this research.

Separation of the Green and Violet Fluorescent Compounds — Bacterial cells were cultured for four days in the synthetic medium described previously and were removed by centrifugation. The fluorescent compounds in the supernatant fluid were treated according to Crammer (1). The fluid was concentrated to about 1/6 volume under reduced pressure, to which ammonium sulfate was added, followed by extraction with phenol. Ether and water were then added to the phenol extracts and the aqueous layer was chromatographed on paper using butanol-pyridine-water (6 : 4 : 3 by volume) as a developing solvent system. The green fluorescent spot (R_F 0.22) and the violet fluorescent spot (R_F 0.15) were cut out and extracted with water.

These extracts were respectively rechromatographed with the same solvent system according to Crammer until green and violet fluorescent extracts were obtained in a pure state. The aqueous extracts were concentrated and the compound was crystallized from ethanol.

Cultures and Cultural Conditions — The medium, which was used to test the effects of green and violet fluorescent compounds in riboflavinogenesis, was a 3 fold diluted basal medium containing no amino acids. After 48-hour incubation in the synthetic basal medium, the cells were harvested and washed by centrifugation, then subcultured in the diluted medium mentioned above. After 24-hour culture, the cells were removed by centrifugation and the supernatant fluid was analyzed.

Analytical Procedure The changes of the fluorescent materials before and after incubation were traced in the following manners: definite amounts of the fluids were chromatographed on paper. The bands of green and violet fluorescent materials were eluted with hot water, and their amounts in the eluates were spectrophotometrically measured by their optical densities at 400 and 340 m μ . Riboflavin was determined at 450 m μ .

RESULTS

TABLE I
Paperchromatograms of Fluorescent Materials Produced in the Cultural Filtrate of Cl. acetobutylicum.

Solvent system	No. of spot							
	1	2	3	4	5	6	7	8
Butanol-acetic acid-water (4 : 1 : 5)	0.26	0.23	0.13	0.13	0.06	0.32	0.09	0.05
Butanol-pyridine-water (6 : 4 : 3)	0.35	0.30	0.22	0.15	0.14	0.42	0.08	0.06
Butanol-formic acid-water (4 : 1 : 1)	0.32	—	0.18	0.23	—	0.30	0.10	0.04
Butanol-ethanol-water (50 : 15 : 35)	—	—	0.22	0.25	0.14	0.40	0.21	0.13
Electrophoresis in acetate buffer pH 5.3 (mm)	—	—	-10	+12	-7.6	-6.0	+13	+25
Reaction of phosphate ion ^a	—	—	—	—	—	—	—	—
Fluorescence under ultra- violet lamp	violet	green (G ₁)	green (G)	violet	green (G ₂)	B ₂	FMN	FAD
Intensity on paper chromato- gram	±	+	+	+	±			

G Compound of spot No. 3 in Table I was decomposed by *N* NaOH at 100° for 30 min. When the decomposed products were chromatographed on paper, the existence of urea was identified by *p*-dimethylaminobenzaldehyde spray comparing with an authentic specimen.

^a Hanes, C. S., and Isherwood, F. A., *Nature.*, **164**, 1107 (1949).

The three green fluorescent compounds were named as follows: G₁ Compound for the spot revealing a rapid movement, G Compound for the spot No. 3 and G₂ Compound for the spot moving slowly (Table I and Fig. 2).

G₁ Compound is rapidly decomposed into G and violet compounds (spots No. 3, No. 1 and No. 4). In the present paper, the properties of G (spot No. 3) and violet compounds (spot No. 4) were principally tested. They are readily soluble in water, sparingly soluble in methanol and ethanol, but insoluble in ether and chloroform. They are adsorbed by fuller's earth and active carbon. In addition, they are precipitated with the Hopkins-Cole reagent in the same manner as riboflavin.

The spectra of G and violet compounds in water, between 210 and 480 m μ , are shown in Fig. 1. It was clarified that G Compound is perfectly identical with the one found in the mycelium of *Eremothecium ashbyi*, as will be re-

FIG. 1 Absorption Spectra of Green Fluorescent Compound (R_F value 0.22 in butanol-pyridine-water, 6 : 4 : 3) and Violet Fluorescent One (R_F value 0.15) in Water.

FIG. 2 A Paper Chromatogram of the Fluorescent Compounds Produced by *Cl. acetobutylicum*.

FIG. 3 Production of Riboflavin, FMN, FAD and G Compound in the Basal Medium.

ported in the following paper.

The production of G Compound of spot No. 3 is parallel with the riboflavin biosynthesis as shown in Fig. 3. All the fluorescent compounds are never observed in iron-containing media (containing 2 mg of FeSO_4 per 100 ml), despite of the formation of flavin mononucleotide and flavin-adenine dinucleotide from riboflavin. Furthermore, the green fluorescent compound (spot No. 3) promotes riboflavin synthesis regardless of the presence or absence of inorganic iron, while the violet fluorescent compound (spot No. 4) seems to have little biological significance in riboflavinogenesis (Table II).

DISCUSSION

It has been reported in the previous communication (2) that various green and violet fluorescent compounds were found in the culture filtrate of *Cl. acetobutylicum* and the precursor of riboflavin might be present in these compounds. As the amounts of these compounds produced in the basal medium are so

TABLE II
Effects of G Compound (Spot No. 3 in Table I) and Violet Compound
(Spot No. 4) on Riboflavin Synthesis.

Addition	Amount of fluorescent materials		Riboflavin produced
	Before fermentation	After fermentation	
—	—	—	0.041
Violet compound	0.078	0.075	0.042
G Compound	0.130	0.048	0.052
G Compound plus FeSO ₄ (2 mg per 100 ml)	0.156	0.090	0.070
FeSO ₄ (2 mg per 100 ml)	—	0.050	0.046

Figures show the readings of spectrophotometer.

small, that the relationship to riboflavin biosynthesis could scarcely be observed in any of them.

The promoting effect on riboflavin synthesis by G Compound is observed, but for the ascertainment of this compound to be a precursor of riboflavin biosynthesis, the problem of permeability of this compound to the membrane of the intact cells must be solved, since the effect of G Compound is not so evident as was expected for an intermediate compound of riboflavin biosynthesis. No effect on riboflavin synthesis was observed with the violet compound and most of the violet compounds are suggested to be enzymatically or chemically decomposed products of the green compounds.

These fluorescent compounds are never accumulated in the culture medium nor in the bacterial cells in the presence of the following inhibitors at various concentrations; sodium arsenate, sodium fluoride, sulfanilamide and terramycin. It is interesting to point out that the same compound (G Compound) has been isolated from the mycelium of *Er. ashbyii*, (3), an aerobe, as well as *Cl. acetobutylicum*, an anaerobe.

SUMMARY

At least five fluorescent spots other than riboflavin substances were separated on the paper chromatogram of the culture filtrate of *Clostridium acetobutylicum*. Two of them had violet fluorescence and the remainders had green fluorescence (G, G₁ and G₂). The production of these compounds in the culture of this bacterium was parallel with the formation of riboflavin. One of the green fluorescent substances (G) promoted the synthesis of riboflavin by resting cells of this microorganism, while the violet fluorescent substance lacked such action.

REFERENCES

1. Crammer, J. L., *Nature.*, **161**, 349 (1948).
2. Imai, K., *J. Agr. Chem. Soc. Japan.*, **29**, 903 (1955).
3. Masuda, T., *Chem. Pharm. Bull.*, **4**, 375 (1956).