Ten eyes were reimplanted in the orbit in *Rana clamitans* larvæ about 65 mm in length. Three died immediately after operation. Two eyes were severely injured at operation and were slowly resorbed by one month before the hosts metamorphosed. Five cases were sacrificed from 150 to 227 days after operation. Two of these were carried to metamorphosis and 3 were killed from 53 to 80 days after metamorphosis. All eyes and their pupils were slightly smaller than normal at the end of the experiment.

Histological studies showed that no degeneration was taking place in the eye when the hosts were killed. All retinæ were slightly thinner than normal. They showed a reduction in the number of ganglion cells and the inner nuclear zone was not as deep as the normal. In every case the small optic nerve stump at the bulb did not penetrate further than the choroid coat. The optic nerve was completely absent from the bulb to the chiasma. The lens was slightly smaller but normal histologically. All other structures appeared normal in both the living state and in histological preparations. The results in these older larvæ seem to be about the same as in those of the much younger *R. pipiens*.

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Occurrence of Riboflavin in Tubercle Bacillus.*

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It has been known for several years that aqueous extracts of the tubercle bacillus exhibit yellow fluorescence in ordinary light. We have attempted the purification and characterization of this yellow pigment. Extracts of partly defatted tubercle bacilli made with 25% alcohol showed a yellow fluorescence in ordinary light and a beautiful blue fluorescence in ultraviolet light. The formation of lumiflavin upon alkaline irradiation indicated the presence of a

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flavin-like pigment. This pigment was partially purified, as described below, and fed to rats receiving a diet lacking riboflavin. The resulting growth response indicated that the bacterial flavin was the well known water-soluble vitamin, riboflavin.[1]

At the time this work was done in 1937, there were no published reports indicating the chemical nature of this yellow pigment of the tubercle bacillus. However, since that time, Boissevain, Drea and Schultz[2] have announced the isolation of riboflavin from the tubercle bacillus, identified by melting point and absorption spectrum. Our work on the biological activity of the pigment may be considered to confirm its identity as riboflavin.

The several strains of tubercle bacilli used in this investigation had been grown on the Long[3] Synthetic Medium in the Biological Laboratories, Sharp and Dohme, at Glenolden, Pennsylvania. The material represented the dried cell residues after extraction with alcohol, ether and chloroform.

The cell residues from tubercle bacilli, Strain A-14, had been extracted in 1934 as described by Crowder, Stodola, Pangborn, and Anderson.[4] For the present examination 522 g of the dried cell residues were digested and extracted repeatedly with 2-liter portions of warm 25% alcohol. The combined extracts were filtered through a Chamberland filter and the clear filtrate was concentrated *in vacuo* to a volume of 1076 cc. This extract contained 54.8 g of solids, which were mostly polysaccharides and inorganic salts, but a small amount of protein was present, together with other undetermined constituents.

The extract showed a yellowish-green fluorescence in ultraviolet light. At pH 2 the fluorescence was green, but in the range of pH 3 to 5 it was blue when irradiated with ultraviolet light. Extraction with chloroform or other solvents either at neutral, acid or alkaline reaction did not remove any fluorescent pigment.

The amount of flavin was estimated in 2 cc of the extract by conversion to lumiflavin by irradiation in alkaline solution according to the procedure of Warburg and Christian.[5] The lumiflavin thus produced was extracted by chloroform from the acidified solution and estimated by means of the Pulfrich spectrophotometer according

[1] Kuhn, György, and Wagner-Jauregg, *Ber.*, 1933, **66**, 576, 1034.

[2] Boissevain, Drea, and Schultz, PROC. SOC. EXP. BIOL. AND MED., 1938, **39**, 481.

[3] Long, *Am. Rev. Tuberc.*, 1926, **13**, 393.

[4] Crowder, Stodola, Pangborn, and Anderson, *J. Am. Chem. Soc.*, 1936, **58**, 636.

[5] Warburg and Christian, *Biochem. Z.*, 1933, **266**, 377.

to Kuhn, Wagner-Jauregg and Kaltschmitt.[6] On this basis, it was calculated that the original dry bacilli contained 36.6 mg of flavin per kilo.

Portions of dry defatted human strain A-12 and the avian tubercle bacillus, Hygienic Laboratory No. 531, both grown on the Long[3] synthetic medium, were also examined for flavin pigments. The extraction and the estimation as lumiflavin were carried out as above. The values found corresponded to 13.0 mg of flavin in the human strain A-12 and 19.3 mg in the avian bacilli per kilo of original dry bacteria. These values can only be regarded as minimal. Living bacilli would undoubtedly contain larger quantities of flavin.

Concentration of Flavin. A considerable amount of polysaccharide and other solid matter was removed from the aqueous solution of the A-14 extract by precipitation with three parts acetone and one part alcohol. This precipitate also contained much of the nonflavin fluorescing pigments which have not yet been adequately investigated.

The supernatant solution was concentrated to 800 cc, adjusted to 0.1 N with H_2SO_4 and the flavin was adsorbed by shaking with fuller's earth. After elution with pyridine-methanol-water the concentration was continued by formation of the silver salt in the usual manner. Removal of the silver left 0.487 g of solid material containing 2 mg of flavin, determined by the irradiation procedure.

Biological Assay. The crude flavin concentrate was assayed as follows: A litter of eight 21-day-old albino rats were fed *ad libitum* a basal diet, complete except for the vitamin B complex, of the following composition: casein 18, sucrose 73, salt mixture, Osborne and Mendel, 4, Crisco 2, and cod liver oil 3 parts. In addition, each rat received daily a rice polish extract (tikitiki)[7] equivalent to 1 g of rice polish. We have shown[7] that this tikitiki contains all the members of the vitamin B complex required by rats except riboflavin, of which only traces are present.

When growth had nearly ceased, one group of rats was fed the bacterial flavin, one group was given crystalline riboflavin,§ and a third group was continued on the diet without further supplement. The growth curves are shown in Fig. 1 and indicate that the bacterial flavin possessed the same biological activity as crystalline riboflavin.

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[6] Kuhn, Wagner-Jauregg, and Kaltschmitt, *Ber.*, 1934, **67**, 1452.

[7] Street and Cowgill, *Am. J. Physiol.*, 1939, **125**, 323.

§ Obtained from the Borden Company, Bainbridge, N. Y., as crystalline lactoflavin, P X grade.

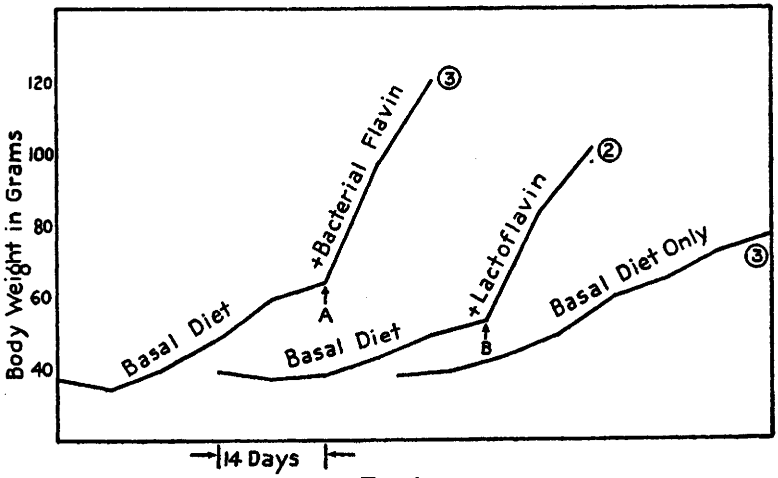

FIG. 1.

The effect of bacterial flavin and of crystalline riboflavin on the growth of rats fed a basal diet supplemented with rice polish extract. At point A the daily administration of the bacterial flavin preparation in amount calculated to supply 40 γ of flavin was begun. At point B the daily administration of 40 γ of pure crystalline riboflavin was begun. The curves are averages. The figure in the circle by each curve represents the number of animals in the group.

In view of the uniformity in growth response of the several animals, it appears justifiable to present the results in the form of curves based on averages. While the number of animals used was necessarily small, many other tests performed in this laboratory using the same basal diet, including rice polish extract alone or supplemented with crystalline riboflavin, have yielded results practically identical with the curves shown above.

The average daily gain in weight of 3.5 g daily observed in these experiments with a daily supplement of 40 γ of riboflavin, is essentially normal growth for this strain of rats and indicates that the basal ration supplies adequate amounts of all nutritional essentials required by the rat other than riboflavin. Under these circumstances, any increased gain over that of the negative controls produced by a vitamin bearing substance should be specific for riboflavin. Since this work was completed one of us (H.R.S.) has used a ration, containing tikitiki as a source of the B complex, for riboflavin assays involving the use of over 1000 rats, with entirely satisfactory results.

Summary. The tubercle bacillus cultivated on the Long synthetic medium is capable of synthesizing fluorescent flavin pigment. This pigment, when fed to young rats, promotes growth to the same extent as a corresponding quantity of crystalline riboflavin.