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Isolation and Determination of Riboflavin Produced by Tubercle Bacilli in Culture Media.

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Warburg and Christian[1] isolated from yeast the yellow ferment of which riboflavin is the active part. Adler and Euler[2] found riboflavin in *Thermobacter helveticum, Bacterium pasteurianum* and *Clostridium butyricum.* Pett[3] found it present in yeast, *Bacterium aerogenes* and *B. subtilis,* but absent in Aspergillus, Penicillium, *B. proteus vulgaris, B. coli* and *Staphylococcus albus.*

Several strains of tubercle bacilli produce a green pigment on Sauton's medium. Kolle[4] states that this was first observed by Lange and Piscatore at the time of the tragedy in Lübeck caused by contamination of BCG vaccine with the "Kiel" strain of tubercle bacilli. This strain could be recognized by its abundant pigment production after being recovered from the organs of children who had died from the administration of the contaminated vaccine. Kraus and Koref[5] denied that the production of pigment is an exclusive peculiarity of the "Kiel" strain and state that all tubercle bacilli produce a greenish yellow pigment which does not show any specific absorption. de Grolier[6] also found that all tubercle bacilli form some pigment but observed that only 3 strains produce large quantities of pigment and impart an intense green fluorescence to the culture medium, namely the "Kiel" strain and 2 attenuated human strains, R_1 of Trudeau and "Nathan Raw".

We have studied the pigment production of one human strain, H37, one attenuated human strain R_1, one bovine strain NJ, and one attenuated bovine strain BCG. We could confirm de Grolier's observation that the R_1 strain produces far more pigment than any of the other strains. An examinations of the fluorescence spectrum shows one band in the region from 494mμ to 614mμ, where the riboflavin band occurs and another band in the adjoining blue region. This latter band belongs to a different pigment which is

1 Warburg and Christian, *Biochem. Z.*, 1933, **266**, 377.

2 Adler and Euler, *Z. Physiol. Chem.*, 1934, **225**, 41.

3 Pett, *Biochem. J.*, 1935, **29**, 937.

4 Kolle, *Deutsche med. Wochensch.*, 1930, **58**, 304, 992.

5 Kraus and Koref, *Z. f. Tuberkulose*, 1933, **67**, 42.

6 de Grolier, *Compt. rend. d. l. Soc. d. Biol.*, 1933, **113**, 1506.

difficult to separate from the riboflavin. The following method was finally successful:

Ten liters of a 6-weeks-old culture of *Mycobacterium tuberculosis* R_1 on Sauton's medium are heated for 30 minutes to 90°C, brought to pH 1 with HCl and filtered to remove bacilli and precipitated protein. The riboflavin is then absorbed on Fuller's earth, which is separated from the liquid by centrifuging, washed free from HCl, and eluted with 200 cc of a mixture of 1 pyridine, 1 methanol and 4 water. After centrifuging, the pyridine and alcohol are removed by distillation under diminished pressure and the process of absorption and elution repeated twice more.[7] After the pyridine and alcohol have been removed for the last time, the solution is diluted to 100 cc with water and extracted twice with 20 cc portions of 88% phenol. The riboflavin goes into the phenol which is then removed by ether in the presence of water.[8] By repeating this process several times the riboflavin is obtained free from the blue fluorescing pigment. By repeated precipitation from water with acetone and recrystallization from water the pure riboflavin is obtained as orange crystals that melt and decompose at 280°C. The fluorescence spectrum produced by ultraviolet light in a watery solution is identical with that of pure synthetic riboflavin, and extends from approximately 494mμ to 614mμ.

For the quantitative determination of riboflavin in culture media the media are deproteinized with trichloracetic acid and the riboflavin absorbed on Fuller's earth and eluted as described above. The Fuller's earth sometimes causes difficulties by forming a colloidal solution in the eluent. This difficulty can usually be overcome by adding methyl alcohol after part of the pyridine and methyl alcohol have been removed by distillation under diminished pressure. In some cases it may be necessary to add salt to precipitate the colloidal earth, which does not affect the fluorescence. After complete removal of the pyridine and alcohol the solution is brought to a volume of 10 cc with water, and to a pH of 7.0. This solution is then placed in a quartz cell in front of a spectrograph and the fluorescence excited by ultraviolet light. A narrow beam of the light of a carbon arc filtered through a Wratten filter No. 18A, at right angles with the spectrograph slit, is focused on the cell by a quartz lens. The intensity of the fluorescent band produced on the photographic plate is then compared with the intensity of bands produced on the same photographic plate by riboflavin solutions of known strength. It is

[7] Kuhn, Gyorgy, Wagner, Jauregg, *Berichte*, 1933, **66**, 317, 576, 1034.

[8] Greene and Black, *J. Am. Chem. Soc.*, 1937, **59**, 1820.

necessary to determine the approximate concentration of the unknown in a preliminary experiment, so as to have the standard and the unknown of nearly the same concentration. The amount of fluorescent light excited depends both upon the concentration of the riboflavin and on the intensity of the exciting light. Since the riboflavin solution absorbs part of the exciting wavelengths, their intensity becomes less in high concentrations of riboflavin. By having standard and unknown at approximately the same concentration this source of error is avoided.

The following concentrations of riboflavin were observed:

Strain of tbc bacillus	Amount of riboflavin in culture medium, gamma per cc
R_1	2.86
BCG	1.07
Bovine	0.85
Human H37	0.50

The bovine strain is the most virulent of the four and the BCG the least virulent, but both produce less riboflavin than the R_1 strain. de Grolier[6] observed greatest pigment production in 2 human strains of reduced virulence, but also in the Kiel strain which was unfortunately virulent. Apparently there exists no close relationship between virulence and pigment production.

Summary. Riboflavin was isolated from Sauton medium on which R_1 tubercle bacilli had grown and the amount of riboflavin produced by 4 different strains of tubercle bacilli was determined by a method of fluorescence spectrography described in the paper.

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Comparison of Bacteriostatic Effects of Sulfanilamide and Sulfapyridine (2 Sulfanilyl Aminopyridine) on Bacteria in Broth Cultures.*

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Fleming[1] has recently reported that sulfapyridine—2(sulfanilyl aminopyridine)—in concentrations of from 1:256,000 to 1:8,000 inhibited the development of large inocula of type 23 pneumococci

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1 Fleming, A., *Lancet*, 1938, **2**, 74.