A REVISED MICROBIOLOGICAL ASSAY METHOD FOR RIBOFLAVIN AND PANTOTHENIC ACID

Thesis for the Degree of M. S.
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A Thesis Submitted to
the
Graduate Faculty
of

Michigan State College

of

Agriculture and Applied Science

as partial fulfillment
for the
Degree of Master of Science

Department of Bacteriology and Hygiene

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ACKNOWLEDGEMENT:

The writer wishes to express his gratitude to Dr. W. L. Mallmann and to Dr. C. A. Hoppert for their advice and guidance.

Chemical and physicochemical methods for the quantitative estimation of riboflavin in biological products involve many errors.(1) Workers agree that at present biological assay provides the most reliable method for the estimation of this vitamin. Biological assay methods for riboflavin other than the microbiological method are expensive, time-consuming, and of limited applicability because of the quantities of the material required for assay. Further, the value of the Bourquin-Sherman unit in terms of pure riboflavin as reported in the literature varies from less than two to approximately five micrograms per unit.(1,2,3,4,5)

Riboflavin has been shown to be essential for growth of certain lactic acid bacteria, and the amount of growth has been shown to be directly proportional to the concentration of riboflavin in the culture medium.(6) Naturally occurring riboflavin proved more active in promoting growth than did any of its homologs or isomers. Degradation products produced from riboflavin by light were inactive.

Snell and Strong developed an assay method for riboflavin in natural materials on the basis of the above information.

The bacterium, Lactobacillus helveticus, which is used as the test organism in a microbiological assay procedure for riboflavin also requires pantothenic acid.(7,8) A medium adequate for Lactobacillus helveticus except for the absence of pantothenic acid may be used to assay this vitamin since the addition of graded amounts of synthetic calcium pantothenate results in a proportionate increase in lactic acid production.(9)

After an inspection of the media used in the microbiological assays for riboflavin and pantothenic acid, the author conceived that a simplification of the assay media would be a contribution in this field since such simplification would lessen

the time and effort necessary to run assays of these vitamins. This paper contains a description of the studies made and the results obtained.

Lactobacillus helveticus was used as the test organism throughout these studies. To carry stock sultures, the method used was that of Snell and Strong. Stab cultures of the organism are carried in yeast-water agar containing one per cent of glucose. To prepare and carry cultures for assay purposes the following procedure may be invoked: From the original culture make a series of stab transfers into yeast-glucose-agar, incubate at 37 C°. for 24 hours, and store in the refrigerator. Several tubes are reserved as stock cultures, and the others are used to prepare inoculum for assay. A transfer is made into 10cc. of the basal medium to which is added one microgram of riboflavin and five-tenths microgram of calcium pantothenate. After 24 hours incubation at 37° C. this culture is again transferred into the same medium. This second subculture is ready for use at the end of a twenty-four hour incubation period at 37° C. Preferably this culture should be used prior to 40 hours incubation. In preparation for seeding assay tubes, the cells are centrifuged out aseptically, resuspended in sterile .85% sodium chloride solution, and one drop of this cell suspension added to each assay tube. By this procedure, cells from the agar stab culture undergo one subculture in liquid medium before use in routine assay.

EXPERIMENTAL

A series of studies was made to determine the minimum food requirements of Lactobacillus helveticus. Experimentation showed that a tryptic digest of casein with members of the vitamin B complex added is not

sufficient to support growth of this organism. However, the same tryptic digest of casein plus a small amount of an aqueous concentrate of liver extract and egg albumen supports growth indifferently well.

Bacto peptone with added dextrose and nutrient agar supports growth exceedingly well. Bacto tryptone with the addition of dextrose and nutrient agar supports growth slightly better than peptone. Either of these media may be used to carry stock cultures if these cultures are being used frequently. Using yeast-glucose-agar for stock cultures proves to be better since the rate of growth is much slower than in either peptone or tryptone media and allows carrying cultures for a longer period without necessitating transfer.

Yeast extract plus dextrose supports growth fairly well. Autolyzed yeast with dextrose added is a better medium for this organism but appears to be toxic since initiation of growth occurs slowly.

Addition of asparagine, adenine, and uracil to the medium used by Snell and Strong for a standard riboflavin curve stimulated increased acid production.

A medium containing Bacto-peptone with the proteose fraction removed by precipitation with saturated ammonium sulfate did not support growth.

After an examination of the amino acid constituents of an aqueous extract of yeast, whole autolyzed yeast, and various peptones, an attempt was made to grow Lactobacillus helveticus on a medium consisting of amino acids, vitamins, dextrose, sodium acetate, and inorganic salts. This medium included the following constituents: inorganic salts, dextrose, sodium acetate, cystine, asparagine,

glycine, alanine, arginine, tryptophane, riboflavin, pantothenic acid, and biotin. No growth resulted. The addition of guanine, adenine, and uracil to this medium resulted in growth, but it was slow in appearing.

Further addition of pyridoxine, thiamine hydrochloride, and para-amino-benzoic acid caused no increased stimulus of growth.

From the information gathered by the above experimentation, a medium was chosen for an assay procedure. The following section contains a description of the preparation and use of this medium.

Preparation of Medium:

The riboflavin-free, pantothenic acid-free basal medium used is a modification of that developed by others.(10) It contains photolyzed, sodium hydroxide-treated peptone, 0.5%; inorganic salts; cystine, 0.01%; glucose, 1%; sodium acetate, 0.6%; amino acids; biotin, 500 micromicrograms per 10 cc. medium; nicotinic acid, 0.5 microgram per 10 cc. medium. When riboflavin assays are made, the medium contains 0.2 micrograms of d-calcium pantothenate per 10 cc. of medium. When pantothenic acid assays are made, the medium includes one microgram of riboflavin per 10 cc. of medium.

Photolyzed Sodium Hydroxide-treated Peptone:

A mixture of 40 grams of Bacto peptone in 250 cc. of water and 20 grams of sodium in 250 cc. of water is exposed in a 25 cm. crystallizing dish to light from a 100-watt bulb equipped with reflector at a distance of 30 cm. for ten hours, and then is allowed to stand at room temperature for an additional 14 hours. The basicity

is neutralized with glacial acetic acid, 7 grams of anhydrous sodium acetate added, and the mixture is diluted to 800 cc. This solution contains the equivalent of 5 per cent peptone and 6 per cent sodium acetate, which is ten times the concentration of these materials in the final medium. The solution is preserved under toluene in the refrigerator. The described treatment destroys all of the riboflavin as demonstrated by the failure of the solution to show fluorescence when checked in an electrofluorometer. The above procedure destroys pantothenic acid also.

Inorganic Salts:

Solution 1 consists of 25 grams of dipotassium hydrogen phosphate and 25 grams of potassium dihydrogen phosphate dissolved in 250 cc. of water. Solution 2 contains 10 grams of magnesium sulfate heptahydrate, and 0.5 gram of manganese sulfate tetrahydrate dissolved in 250 cc. of water, and 0.5 gram $FeSO_4 \cdot 7H_2O$ plus 0.5 gram NaCl.

Cystine:

A solution of cystine hydrochloride containing 1 mg. of cystine per cc. is prepared and stored under toluene.

Amino Acid Solution:

A stock solution containing 500 micrograms per ml. of adenine, 500 micrograms per ml. of guanine, and 500 micrograms per ml. of uracil is prepared and stored under toluene in the refrigerator.

Glycine, alanine, tryptophane, asparagine, and arginine are weighed, dissolved in hot water and added when needed.

Chemically pure anhydrous glucose is added dry as required.

A stock solution containing nicotinic acid and bio[illegible]

is prepared and refrigerated under toluene.

Standard Riboflavin Solution:

As a stock solution, pure crystalline riboflavin is weighed and dissolved in warm 0.02 N acetic acid. One hundred micrograms per cc. is a convenient concentration. For daily assaying, a more dilute solution is prepared from the stock solution by dilution with 0.02 N acetic acid. These solutions are stored under toluene in the refrigerator and protected from light. Under such conditions riboflavin is stable. No deterioration could be detected by periodic examination in an electrofluorometer over a period of several months. Synthetic d-riboflavin of at least 99% purity obtained from Merck and Company, Inc., was used as the primary standard for this vitamin during the entire course of the investigation.

A stock solution of pure d-calcium pantothenate, obtained from Merck, is prepared in 0.05 M phosphate buffer and preserved under toluene in the refrigerator.

Procedure:

The assay fermentations are carried out in ordinary chemical or bacteriological test tubes containing the basal medium plus the assay sample in a volume of 10 cc. A metal rack may be used to segregate the tubes and may be autoclaved. If 50 assay tubes are to be set up from stock solutions of the concentrations given above, 50 cc. of photolyzed, sodium hydroxide-treated peptone solution, 50 cc. of of cystine hydrodhloride solution, 5.0 grams of glucose, 2.5 cc. of inorganic salt solution number 1, and 2.5 cc. of inorganic salt solution number 2 are mixed. Sufficient quantity of amino acids and vitamins are added

to give the following concentrations: glycine, .02%; alanine, .02%; tryptophane, .0075%; asparagine, .02%; adenine, 1 mg.%; guanine, 1 mg.%; uracil, 1 mg.%; biotin, 500 micromicrograms per 10cc. of medium; nicotinic acid, .5 microgram per 10cc. of medium. If a riboflavin assay is to be made, calcium pantothenate is added to give a concentration of 0.2 micrograms per 10cc. of medium. If a pantothenic acid determination is desired, riboflavin is added to give a concentration of one microgram per 10cc. of medium. The resulting solution is thoroughly mixed, adjusted to pH 6.8 with sodium hydroxide, and the mixture diluted to 250cc. Five cubic centimeters are then pipetted into each of fifty tubes, and a suitable aliquot of the riboflavin-containing or pantothenic acid-containing extract added, dependent on which vitamin is being determined. Where necessary the contents of each tube are diluted to give a resultant volume of 10cc. The tubes are sterilized by autoclaving at 15 pounds per square inch for fifteen minutes. Upon cooling the tubes are ready for seeding. The procedure described in the first section was used throughout for preparing inoculum and for seeding tubes with the assay organism.

Standard Curves:

With each set of riboflavin assays duplicate tubes are set up containing, per 10cc. of medium, 0.0, 0.05, 0.075, 0.1, 0.11, 0.15, 0.2, 0.3, and 0.5 microgram of riboflavin. Growth data obtained from these tubes permits the construction of a standard curve. To construct such a curve acid production is plotted against riboflavin concentration.

With each set of pantothenic acid determinations duplicate tubes are set up containing, per 10cc. of medium, 0.0, 0.025, 0.05, 0.075, 0.10, 0.125, and 0.15 micrograms of pure d-calcium pantothenate. A curve is drawn with calcium pantothenate plotted against acid production.

Determination of Bacterial Response:

Growth response of this organism to either riboflavin or pantothenic acid may be best measured by titration of lactic acid produced. Contents of the assay tubes are transferred to 125cc. Erlenmeyer flasks, 10cc. of distilled water added, and the suspension is titrated to pH 6.8 with 0.1 N sodium hydroxide. Bromothymol blue serves satisfactorily as an indicator. The end point is reproducible to $\pm$0.1cc.

Incubation Period:

All assays herein described were incubated for 48 hours at 37°C. Turbidity of cultures reaches a maximum within 24 hours, but acid production increases for at least three days. However, essentially the same results may be obtained by titrating at the end of 48 hours incubation as at the termination of a longer incubation period.

Evaluation of Bacterial Response in Terms of Riboflavin:

Response of Lactobacillus helveticus to riboflavin in a typical fermentation is illustrated in Figure 1. Within a limited range, acid production is directly proportional to the concentration of riboflavin; thus, the stimulus in growth caused by an unknown sample may be evaluated in terms of riboflavin by interpolation on

the standard curve obtained simultaneously with the growth data on the unknown sample. For the most accurate results the riboflavin content of assay cultures must fall on the more linear portion of the standard curve. Determinations are more valid when samples are assayed at several levels within the range indicated and the values obtained averaged.

When pure solutions are being analyzed, it is possible to determine a few hundredths of a microgram of riboflavin within a few per cent.

Preparation of Sample for Analysis:

For extraction of riboflavin from natural products, autoclaving the finely ground material at 15 pounds pressure per square inch for 15 minutes with a quantity of water may be used. Extraction with .1 N hydrochloric acid and subsequent neutralization effects the desired result. Liquids which are readily soluble in water may simply be diluted, neutralized if necessary, and assayed without autoclaving.

A similar procedure is feasible in preparing materials for pantothenic acid determinations.

Data for a typical analysis of riboflavin is presented in the accompanying table. Values taken from the literature as given by other investigators are included as a comparison. The milk assay was made directly. The yeast was suspended in water and autoclaved at 15 pounds pressure per square inch for 15 minutes. A hydrochloric acid extraction of the beef liver was made by autoclaving, and the insoluble residue was centrifuged out.

Data for pantothenic acid assays is not incorporated here as only a few assays were made. However, the results obtained indicate good agreement with values recorded in the literature.

RIBOFLAVIN ASSAY DATA

Sample	Amount Assayed Mgs.	Riboflavin Found per tube microgram	Riboflavin Found per gram sample micrograms	Literature Values micrograms per gram
Milk 1	50	0.096	1.92	
	60	0.123	2.05	
	70	0.135	1.93	
			Av. 1.96	
				1.68-2.0
Milk 2	50	0.079	1.59	
	60	0.102	1.70	
	70	0.128	1.83	
			Av. 1.70	
Fresh beef Liver	2.00	0.078	39.00	
	3.00	0.130	44.30	
	4.00	0.169	42.26	40-50
	6.00	0.248	41.30	
			Av. 41.71	
Yeast Extract	0.25	0.038	152	
	0.50	0.073	144	
	1.00	0.146	146	145-150
	2.00	0.294	147	
			Av. 147.25	

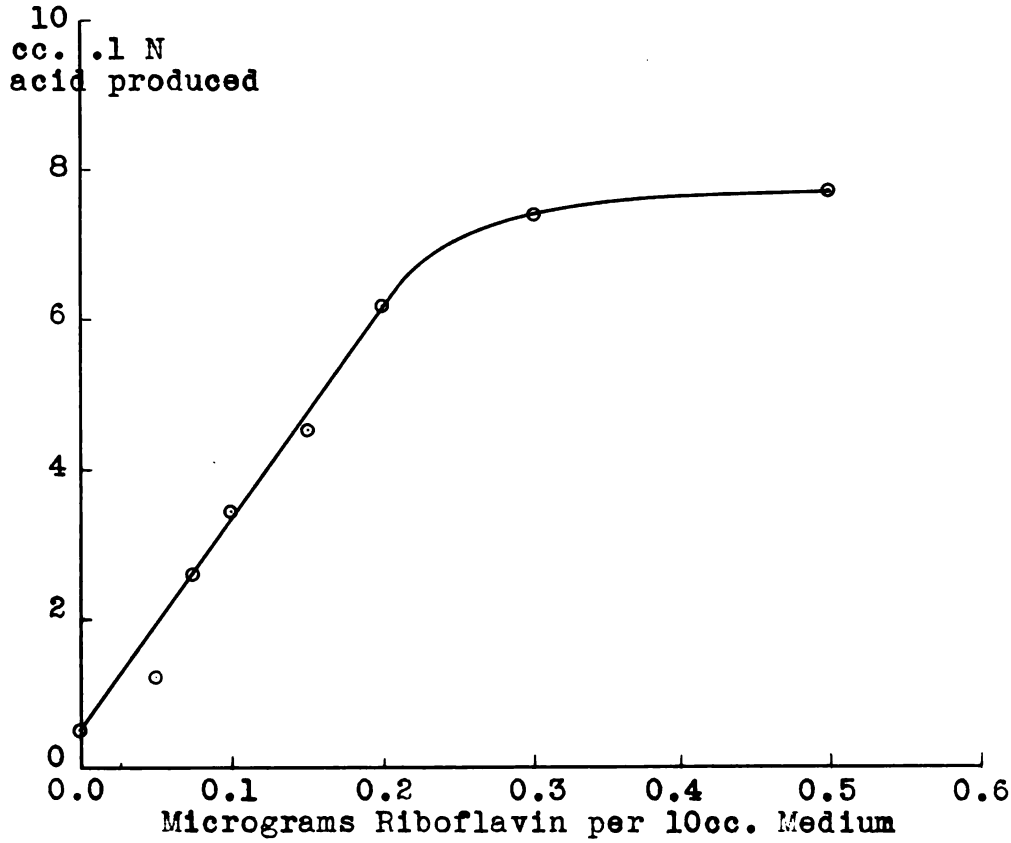

Figure 3

Standard curve obtained upon incubation of Lactobacillus helveticus at 37° C. for 48 hours with the addition of synthetic d-riboflavin to the basal medium used by Snell and Strong and with the further incorporation of the following materials: l-asparagine, 0.02%; adenine, 1 mg. %; and uracil, 1 mg. %.

DISCUSSION

That riboflavin and pantothenic acid are essential for growth of Lactobacillus helveticus is evidenced by data gathered by a number of independent workers. Stimulus of growth by the addition of material to the riboflavin-free or pantothenic acid-free basal medium may be considered qualitative indication of the presence of riboflavin or pantothenic acid in that material. This viewpoint is supported by the structural specificity required for activity, the identity of flavins isolated from natural sources, and the inactivity of photolyzed biological extracts. (11)

It is likely that the bacteria are capable of utilizing riboflavin in its combined forms. Yeast and liver contain riboflavin in combined forms; the bacterial assay yields values comparable to those determined by other methods.

The presence of unknown growth factors in a sample might conceivably cause stimulus of the organism resulting in high values for riboflavin or pantothenic acid. Since the addition of a single pure substance, riboflavin or pantothenic acid, to the basal medium allows growth through repeated subculture, the medium appears to contain all of the essential growth factors for Lactobacillus helveticus. However, the existence of other stimulatory but non-essential substances not included in the medium must not be disregarded. In this work the assumption has been made that the growth effect of non-essential but stimulatory materials is negligible in a medium containing suboptimal amounts of an essential factor such as riboflavin or pantothenic acid.

Biotin was included in the medium since it has been shown to be essential for this organism. In fact, an assay method has been developed using Lactobacillus helveticus as the test organism for a determingtion of biotin in biological materials.(12)

SUMMARY

A description is given for a revised biological assay method for riboflavin and pantothenic acid which is based on the essential nature of these substances for Lactobacillus helveticus. The reliability of the mehtod is given support by agreement of the assay results at different levels, specificity of structure required for activity, and comparison of values obtained by other methods of biological assay of these vitamins on the same products.

The assay may be applied to crude extracts or to the whole ground sample in some cases. Thus, extensive preliminary purification involving destruction of riboflavin or pantothenic acid is avoided.

This method requires small sample amounts, and no elaborate equipment. A relatively short period of time is consumed in such a determination. The accuracy of the method is of the order of ±ten per cent.

BIBLIOGRAPHY

1. Ellinger, P., "Lyochromes in the Kidney. With a Note on the Quantitative Estimation of Lyochromes", Biochem. J., 32, 376 (1938)

2. Bessey, O.A., "Vitamin G and Synthetic Riboflavin", J. Nutrition, 15, 11 (1938)

3. Bourquin, A., and Sherman, H. C., "Quantitative Determination of Vitamin G (B_2)", J. Am. Chem. Soc., 53, 3501 (1931)

4. Hawk, Bergeim, and Cole, "Practical Physiological Chemistry", 11th ed., p. 902, Philadelphia, P. Blakiston's Son and Co., 1937

5. Snell, E. E., Strong, F. M., "A Microbiological Assay for Riboflavin", J. Ind. Eng. Chem., Anal. ed., 11, 346 (1939)

6. Snell, E. E., Strong, F. M., and Peterson, W. H., "Growth Factors for Bacteria. Fractionation and Properties of an Accessory Factor for Lactic Acid Bacteria.", Biochem. J., 31, 1789 (1937)

7. Pennington, D., Snell, E. E., Wielians, R. J., "An Assay Method for Pantothenic Acid", J. Biol. Chem., 135, 213 (1940)

8. Snell, E. E., Strong, F. M., and Peterson, W. H., "Pantothenic Acid and Nicotinic Acid as Growth Factors for Lactic Acid Bacteria", J. Am. Chem. Soc., 60, 2825 (1938)

9. Strong, F. M., Feeney, R. E., and Earle, "A Microbiological Assay Method for Pantothenic Acid", J. Ind. Eng. Chem., Anal. ed., 13, 566 (1941)

10. Snell, E. E., Strong, F. M., and Peterson, W. H., "Growth Factors for Bacteria. Pantothenic and Nicotinic Acids as Essential Growth Factors for Lactic and Propionic Acid Bacteria", J. Bact., 38, 293 (1939)

11. Snell, E. E., and Strong, F. M., "The Influence of Riboflavin and Certain Synthetic Flavins on the Growth of Lactic Acid Bacteria", J. Biochem., 123, 112 (1938)

12. Shull, G. M., Hutchings, B. L., and Peterson, W. H., "A Microbiological Assay for Biotin", J. Biochem., 142, 913 (1942)

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