M. Sc. Agricultural Chemistry

THE DETERMINATION AND SYNTHESIS OF RIBOFLAVIN IN CULTURES OF MICROORGANISMS

A Thesis

by

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INTRODUCTION

Research on growth factors essential to the metabolism of microorganisms and higher animals has proceeded with ever increasing strides in the past fifty years. The correlation of microbial growth factors with animal vitamins was a recent development. Indeed, as recently as 1936, B.C.J.G. Knight (15) stated, "It is not unlikely that some of the animal vitamins may be required by bacteria, but at present it has not been shown that any pure animal vitamin is required as an essential nutrient by any".

The work covered in this thesis concerns itself with one vitamin, namely, riboflavin. At first it was proposed to investigate the use of waste materials as substrates for the bacterial synthesis of riboflavin. However, four years elapsed before the work was completed and chemically synthesized riboflavin had become very cheap. The commercial production of riboflavin from waste materials was, therefore, considered unfeasible.

Nevertheless, the investigation was continued and many strains of riboflavin synthesizing bacteria were isolated. Methods for the fluorometric and microbiological assay of riboflavin were applied to bacterial cultures. Riboflavin concentrates have been prepared from some of these cultures. At the same time species of bacteria which required riboflavin for growth and some of the conditions for the synthesis of riboflavin by Vibrio perimastix were investigated.

The work included in this thesis was done during two distinct

periods. The first was during most of 1942 and the second was during the late spring and summer of 1946. The experiments on cellulose bacteria including Vibrio perimastix, the final fluorometric method for the determination of riboflavin, and the preparation of riboflavin concentrates were completed during the latter period. The remaining work was done during the former period.

DETERMINATION OF RIBOFLAVIN

Introduction

The microbiological method for the estimation of riboflavin (37) was originally chosen because it was recommended as a result of collaborative study (14).

The fluorometric method as outlined by Chapman and McFarlane (5) was originally chosen because it was well established in this laboratory and the authors were available to give advice on the method.

The fluorometric method as outlined by Rubin et al (34) was investigated for three reasons:

(a) it was applicable to a wide range of materials

(b) the use of immiscible solvents was avoided

(c) there was no adsorption and elution step

The authors give four steps in the procedure:

(a) acid extraction, (b) enzyme treatment, (c) permanganate oxidation, and (d) fluorometry. Of these steps, only the first and the last are essential and the other two steps may or may not be used depending on the nature of the sample as will be shown.

Experimental

Microbiological Assay

For the preliminary work, a culture of Lactobacillus casei was obtained from the American Type Culture Collection where it was listed

as culture number 7469. This culture was examined for purity by fermentation and staining methods and found to be pure. In the later work a culture of Lactobacillus casei was obtained from the Bacteriology Department of Macdonald College. Attempts were made to isolate fresh strains of Lactobacilli in order to investigate their use as assay organisms, but these were unsuccessful.

The method of assay was essentially the same as outlined by Snell and Strong (37). The authors kept their stock culture on yeast-water-glucose agar. However, little success was achieved with this medium and though the organism did grow, the growth was very scanty and mostly below the surface of the agar. The assay medium (37) to which about one microgram of riboflavin per 10 ml. had been added, proved to be satisfactory for transfers. The cultures remained pure as shown by repeated identical response to the same quantity of riboflavin.

In the preliminary work the enzyme extraction method of Chapman and McFarlane (5) was used in making up extracts of the materials to be assayed. This proved to be inadequate as will be shown (page 8).

In the later work the acid extraction procedure of Ruben et al (34) was employed. This procedure gave values which were in agreement with the fluorometric determination (page 13).

When the components of the medium had been mixed, the hydrogen-ion concentration of each tube was adjusted to pH 6.6 to 6.8 by adding approximately 2 N. sodium hydroxide or hydrochloric acid. Only one or two drops were needed for the adjustment, leaving the volume essentially the same.

To determine the acidity developed after 72 hours incubation, pH determinations were made instead of titrations with brom thymol blue as indicator. This was done for three reasons:

(a) The amount of time involved was reduced

(b) A Beckman pH apparatus was available

(c) The author was color-blind and had great difficulty in distinguishing the end point in titrations with the brom thymol blue indicator.

A graph of the change in pH produced by the assay cultures, with the change in their riboflavin concentrations was identical to the graph of the change in titration value of these cultures with their riboflavin concentrations.

For ease in pH determination the volume of each assay culture was doubled so that each test tube had twenty ml. of medium of the same concentration as used by the authors (37).

Nephelometric measurements were made on twenty-four hour cultures with the use of the Evelyn photoelectric colorimeter. Tests showed that the 420M filter gave slightly higher readings than the 540 filter used by Snell and Strong (37).

In an experiment to investigate the change of acidity with the incubation time of cultures with varying riboflavin concentrations, the following was noted. In general the acidity increased for five days and then remained relatively constant. The cultures with the higher riboflavin concentrations increased their acidity very slowly after five days while the cultures with the lower riboflavin concentrations reached their maximum acidity before the others.

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The acidity at the end of 72 hours, though not the maximum possible, approached this maximum very closely and results were constant enough for assay purposes.

A parallel experiment with turbidimetric measurements showed that the cultures increased in turbidity to a maximum within thirty-six hours and then slowly declined. An attempt was made to plot graphs of the logarithms of the riboflavin concentrations against the galvanometric deflection in the turbidity measurements. Though in some cases the graph approached a straight line, most of the graphs were not straight lines showing that though the riboflavin content of a culture had a pronounced effect on the number of cells in that culture the turbidity produced did not vary logarithmically with the riboflavin content.

A paper by Barton-Wright and Booth (2) indicated that asparagine, xylose, pantothenic acid, and nicotinic acid were required in addition to the ingredients used by Snell and Strong (37) in the microbiological assay of riboflavin. The use of glass distilled water was also recommended. The growth of Lactobacillus casei on the former medium (2) as determined acidimetrically was compared with the growth on the latter (37). The former medium gave a better response with minute amounts of riboflavin but there was no significant difference between the two media over most of the assay range.

Original Fluorometric Method

There was no essential deviation from the original method proposed by the authors (5). If the samples were extremely cloudy after

enzyme treatment, they were centrifuged and the supernatant liquid poured off into a 100 ml. volumetric flask and made up to volume. In the culture determinations, where there was little solid matter, the sample was filtered through Whatman number 40 paper into a 100 ml. flask and made up to volume, thus time was saved and filtering was just as efficient as centrifuging.

The above method was found to be slightly inaccurate since all the determinations were made on samples having very low riboflavin content and the range was at the limit of the sensitivity of the method. However, the results agreed quite well with the microbiological assay, in determinations of the riboflavin content of milk powder.

In each set of determinations the fluorescence of solutions of pure riboflavin was determined and the values obtained acted as standards. The standards were used because some slight deviation was found in the fluorometer and though the curve was the same shape, it varied slightly from time to time.

For the recovery of waste benzyl alcohol, an all glass distiling apparatus was used. Previously when corks and rubber stoppers were used, green or brown distillates were produced. This was found to be due to the benzyl alcohol attacking cork to produce a brown compound and rubber to produce a green compound. With an all glass apparatus, no trouble was experienced and it was found unnecessary to use trichloracetic acid as recommended by the authors.

When the method was tested on faeces, solid matter interfered and, for accurate determinations, comparatively clear extracts had to be

used. The solid matter of the faeces contained a water insoluble material which gave a red fluorescence when concentrated and a pink fluorescence when diluted in benzyl alcohol solution; the interfering substance was soluble in chloroform, precipitated by acetone and adsorbed by lead sulphide.

Recovery experiments of riboflavin in a clear faeces extract gave good results. Pure riboflavin added to the faeces gave the same reading as the same amount of riboflavin in benzyl alcohol solution. Another recovery experiment with a cloudy faeces extract showed that the faeces interfered. The interfering material in the faeces reacted with stannous chloride, therefore, the fluorometric method was satisfactory only for clear faeces extracts. Table I gives a comparison of the microbiological assay with the above fluorometric procedure. The results indicate that most of the cultures contained growth stimulants which showed their maximum effect at the lower part of the range of riboflavin concentrations for the microbiological assay. One of the cultures seemed to contain an inhibitor and the value obtained was less for the microbiological assay than for the fluorometric procedure.

Several investigators have reported the presence of stimulants in the extracts prepared for microbiological assay. Strong and Carpenter (38) reported that certain free fatty acids stimulated while others inhibited the growth of Lactobacillus casei. Sullivan et al (39) showed that the fat soluble fraction of the extracts gave much higher values in the microbiological assay than the fluorometric procedure. Isbell et al (12) showed that urea inhibited acid production by the test organism.

TABLE I

The Riboflavin Content of Some Cultures (Approximately Ten Weeks Old)

The original fluorometric method is compared with the Microbiological assay in which small samples are compared with large samples.

	Values Obtained γ per G.		
	Microbiological		
Culture	1 ml. Extract	5 ml. Extract	Fluorometric
xI	2.71	1.77	0.336
x2	2.25	0.827	0.452
x3	2.59	1.37	0.854
x4	1.57	0.86	0.78
x5	2.52	0.96	1.13
x6	2.74	0.87	1.11
x7	1.76	0.32	0.35
x8	1.20	0.573	1.245
x9	2.84	0.94	0.37
x10	4.24	2.02	1.03
x11	4.57	2.80	1.94
x12	2.66	0.89	-
116	0.67	0.48	0.42
2	3.62	0.997	0.453
34	0.87	0.74	0.19
81	2.63	0.74	0.84
44	1.45	-	0.61
H5	0.66	0.38	1.76
129	4.37	1.88	0.51

Loy and Kline (22) reported that both the fat soluble and protein fractions of the extracts caused a non specific stimulation in the microbiological assay for riboflavin.

The enzyme treatment used was evidently insufficient in eliminating the stimulants present in the materials tested.

Final Fluorometric Method

The procedure followed was essentially the same as that described by the authors (34). The following minor modifications were made:

To increase the accuracy, volumetric flasks were used instead of graduate cylinders wherever any solutions were to be made up to volume. For this reason the sample was made up to 200 ml. instead of 250 ml. in the acid extraction step and the final dilution of the 50 ml. of extract used in fluorometry was to 100 ml. This gave a dilution factor of 400 instead of 375 as used by the authors.

Since the riboflavin determinations were made on small samples which were liquid or had a relatively high riboflavin content, 100 ml. 0.1 N. sulphuric acid were found to be ample for digestion. Consequently, 100 ml. 0.1 N. sulphuric acid were used for acid digestion instead of 150 ml. as used by the authors.

In the actual measurement of fluorescence, instead of shaking the test solution in the air for fifteen minutes, an aeration apparatus constructed with a boiling tube 25 x 100 mm. was used. The apparatus was attached to a water pump and approximately 50 ml. of each solution were aerated for fifteen minutes.

Enzyme Treatment

To determine whether the materials tested required preliminary enzyme digestion, a series of determinations was made with and without enzyme treatment. Table II gives the results. There is no significant

difference between the values obtained with and without the enzyme treatment. The other samples being more easily digested than the three listed in Table II, were not treated with clarase.

TABLE II

Comparison of Values Obtained with and Without Preliminary Enzyme Digestion

Material	Riboflavin Content (γ per G.) With Clarase	Without Clarase	% Difference
Dehydrated Alfalfa	17.0	16.3	4.3
Distiller's Corn Solubles	12.16	13.01	7.0
Distiller's Wheat Solubles	24.99	25.36	1.5

Use of Permanganate

The permanganate oxidation step was investigated in an effort to reduce the high value for the non-riboflavin fluorescence. Distiller's wheat solubles was used and graded amounts of 4% potassium permanganate were added to a series of flasks, each containing an aliquot of the same sample. Table III gives the results.

This Table shows that the values obtained with permanganate oxidation were very erratic and the non-riboflavin fluorescence was still very high. Leviton (20) has shown that even though riboflavin is stable to the action of pure hydrogen peroxide, minute amounts of ferrous-ion will cause the hydrogen peroxide to break down the riboflavin very rapidly.

TABLE III

The Effect of Potassium Permanganate and Hydrogen Peroxide on the Fluorometric Determination of Riboflavin

(The fluorometry was carried out with 50 ml. aliquots of a 500 ml. dilution of an acid extract of 2.92 grams distiller's wheat solubles)

Potassium Permanganate (ml.)	Hydrogen Peroxide (Drops)	Calculated Riboflavin (γ per G.)	Non-Riboflavin Fluorescence (%)
0	1	5.65	95.5
0.1	2	9.07	91.0
0.2	2	11.4	85.8
0.3	2	17.3	84.0
0.4	3	14.9	84.5
0.5	4	4.1	93.0
0.6	4	14.2	82.0

Table III shows that lower values were obtained when the peroxide-permanganate ratio was relatively high.

In other attempts to use potassium permanganate the accuracy was not increased and the non-riboflavin fluorescence was lowered by a maximum of 30 per cent. The permanganate oxidation step was therefore omitted in all subsequent determinations and consequently the final determinations were made without enzyme digestion or permanganate oxidation.

A recovery experiment was run to check the results; 97.5% of the riboflavin added to distiller's wheat solubles was recovered. Since the

authors (Table IV) give the results of recovery experiments for a number of samples, further recovery experiments were considered unnecessary.

A number of materials were analysed by the above method and the values obtained were checked by nicrobiological assay. Table IV gives the comparison of the values obtained. In most cases duplicate samples were used and in a few, three or four samples were analysed.

TABLE IV

Comparison of the Fluorometric Method of Ruben et al with the Microbiological Assay of Snell and Strong

Material	Riboflavin (γ per G.)			Deviation (%)
	Fluorometric	Microbiological	Mean	
Dehydrated Alfalfa	16.3	15.7	16.0	1.9
Cod Liver Meal	19.6	20.7	20.2	2.7
Fish Solubles	8.64	7.73	8.19	5.6
Dehydrated Egg	10.6	8.9	9.7	8.8
Distiller's Wheat Solubles	25.2	14.2	19.7	27.8
Distiller's Corn Solubles	12.6	10.3	11.5	10.0
Brewers' Dried Yeast	28.0	38.2	33.1	15.4
Double Washed Yeast	32.4	34.5	33.5	3.3
Debittered Yeast	32.6	31.1	31.9	2.3
Animal Yeast	31.5	36.9	34.2	7.7

MICROBIAL GROWTH IN A RIBOFLAVIN FREE MEDIUM

Introduction

In recent years numerous papers have been published on the riboflavin requirements of microorganisms, (30), (16), (17), (13), and (32). Peterson and Peterson (30) reviewed the literature up to 1944 and Table I in this review shows that fifty-one organisms required riboflavin. Among these were included streptococci, staphylococci, a species of Rhizobium, propionic acid bacteria, Leuconostoc species, Lactobacilli, Clostridium tetani and Brucella species.

Lactobacillus casei required biotin (19), nicotinic acid (19), pantothenic acid (19), pyridoxine (19), p-amino benzoic acid (6), riboflavin (37), thiamine (3) and folic acid (25). The basal medium for the microbiological assay of riboflavin prepared according to Snell and Strong (37) supported the growth of Lactobacillus casei when riboflavin was added. It was, therefore, decided to use the above basal medium to determine which organisms required riboflavin.

Experimental

The riboflavin-free substrate was prepared according to Snell and Strong (37). The basal medium was transferred to test-tubes in 5 ml. amounts and was not diluted. The concentration of substrate materials was double that used in the microbiological assay of riboflavin.

For the riboflavin containing medium, 2 micrograms of riboflavin in

2 ml. distilled water were added to each tube of basal medium before autoclaving. The organisms tested were all from the Bacteriology Department of Macdonald College.

Inoculations of each organism were made into duplicate test tubes containing the riboflavin-free broth and the riboflavin-added medium. A loopful of bacteria from a nutrient agar slope was used as inoculum.

Three transfers of the organisms were made. Table V gives the results obtained. The criterion of response was the visible density of growth in the cultures.

Discussion

The effect of the addition of riboflavin on the species of bacteria which showed only scanty growth, even with riboflavin added, cannot be deduced since some growth factor or factors were evidently missing or present in insufficient quantities for proper growth so that the organism could grow only with difficulty. However, the mere fact that most of these organisms grew in the riboflavin-free broth indicates that riboflavin is not absolutely essential to their growth.

Of the forty-three strains tested, twenty-eight come under the above classification with either a very slight increase in growth or no change due to the addition of riboflavin. These are as follows: Aerobacter aerogenes, Bacillus anthracis, Bacillus closteroides, Bacillus subtilis, Bacillus subtilis 85, Bacterium gossypium, Corynebacterium diphtheriae, Corynebacterium diphtheriae (Macdonald), Corynebacterium hoffmannii,

TABLE V

Growth of Bacteria in Riboflavin and Riboflavin-Free Media

Organism	Growth in Riboflavin-Free Medium (72 Hours)	Growth in Riboflavin-Added Medium (72 Hours)
Aerobacter aerogenes	Scanty	Scanty
Bacillus anthracis	Scanty	Scanty
B. cereus	Pellicle, medium cloudy	Precipitate formed
B. closteroides	Very Scanty	Scanty
B. mesentericus	Scanty	Slight deposit
Bacillus ruminatus	Slightly cloudy medium	Precipitate
B. subtilis	Scanty	Scanty
B. subtilis 85	Scanty	Scanty
B. subtilis Lister	Pellicle, medium cloudy	Heavy precipitate
Bacterium cloacae	Very scanty	Scanty
Bacterium gossypium	Very scanty	Scanty
Bacterium iophagum	Scanty	Small deposit
Cellulomonas biazotea	Scanty	Slight deposit
Corynebacterium diptheriae	Scanty	Scanty
" " (Macdonald)	Very scanty	Scanty
Corynebacterium hoffmannii	Scanty	Scanty
Corynebacterium xerose	Scanty	Scanty
Eberthella typhi	Very scanty	Scanty
Erwinia phytophthora	No growth	Cloudy medium deposit

Organism	Growth in Riboflavin-Free Medium (72 Hours)	Growth in Riboflavin-Added Medium (72 Hours)
Escherichia coli	Scanty	Slight deposit
Klebsiella pneumoniae	Very scanty	Scanty
Micrococus cereus	Scanty	Scanty
M. perflavus	Scanty	Scanty
M. roseus	Scanty	Scanty
M. tetragenus	Very slight deposit	Slight deposit
Mycobacterium berolinensis	Scanty	Scanty
M. phlei M	Scanty	Slight deposit
Myco. phlei R	Scanty	Slight deposit
Myco. stercusis	Scanty	Scanty
Myco. thamnopheos	No growth	Cloudy medium) Slight deposit)
Mycoplana bullata	Very scanty	Scanty
Phytomonas phaseoli	Very scanty	Scanty
Phytomonas tumefaciens	Scanty	Scanty
Proactinomyces globerulus	Cloudy medium	Granular deposit
Proteus vulgaris	Cloudy medium	Deposit
Pseudomonas aeruginosa R	Scanty	Scanty
Pseudomonas aeruginosa S	Scanty	Slight deposit
Pseudomonas indoloxidans	Very scanty	Scanty
Pseudomonas pictorum	Scanty	Slight deposit
Salmonella enteritidis	Scanty	Scanty
Salmonella paratyphi	Very scanty	Scanty
Serratia marcescens Roach	Very scanty	Scanty
Serratia marcescens 171	Very scanty	Scanty

Corynebacterium xerose, Bacterium Cloacae, Eberthella typhi, Klebsiella pneumoniae, Micrococcus cereus, Micrococcus perflavus, Micrococcus tetragenus, Micrococcus roseus, Mycobacterium berolinensis, Mycobacterium stercusis, Mycoplana bullata, Phytomonas tumefaciens, Phytomonas phaseoli, Pseudomonas aeruginosa R, Pseudomonas indoloxidans, Salmonella enteritidis, Salmonella paratyphi, Serratia marcescens Roach, and Serratia marcescens 171.

Of the above organisms, a review of the literature gives the following as able to synthesize riboflavin and lists none as requiring it; Bacillus subtilis (4), Eberthella typhi (33), Klebsiella pneumoniae (41), Micrococcus cereus (41), Micrococcus perflavus (41), Aerobacter aerogenes (40), Phytomonas tumefaciens (24), Corynebacterium diphtheriae (9), Pseudomonas aeruginosa (33), and Serratia marcescens (40). These findings do not contradict this work since the only conclusion drawn here is that riboflavin is not required for growth.

Two organisms required riboflavin and did not grow in its absence, i.e. Erwinia phytophthora and Mycobacterium thamnopheos. The literature makes no mention of either of these species requiring riboflavin.

Five species grew well both in the riboflavin-free and the riboflavin-added broths, and in all cases riboflavin proved to be a stimulant. These were Bacillus subtilis Lister, Bacillus cereus, Bacillus ruminatus, Proactinomyces globerulus and Proteus vulgaris. Of the above Bacillus cereus (4), Bacillus ruminatus (41), and Proteus vulgaris (4), (40), have been shown to synthesize riboflavin. Although these species could synthesize riboflavin evidently they grew more rapidly if this

factor was supplied.

Eight species grew very slightly in the riboflavin-free broth and the addition of riboflavin had a measurable stimulatory effect. These were Bacillus mesentericus, Bacterium iophagum, Cellulomonas biazotea, Escherichia coli, Mycobacterium phlei M, Mycobacterium phlei R, Pseudomonas aeruginosa S, and Pseudomonas pictorum. Of the above, Bacillus mesentericus (4), Escherichia coli (4), and Pseudomonas aeruginosa (33) have been reported as capable of synthesizing riboflavin. Evidently, the organisms could synthesize their own riboflavin requirements but grew more rapidly if additional riboflavin was supplied.

The third transfers were kept for several weeks and at the end of four weeks a curious phenomenon was observed. A red pigment appeared in the riboflavin-free broth cultures of Bacillus cereus and Bacillus subtilis Lister. This pigment was not present in the riboflavin-added cultures. There is no mention in the literature of any similar finding.

The pigment was again produced when the experiment was repeated four years later. Evidently continued transferring on riboflavin-free broth is necessary for the pigment formation, it could not be produced after the second transfer.

STUDIES ON RIBOFLAVIN SYNTHESIZING BACTERIA

Introduction

Many papers have appeared on the synthesis of riboflavin by microorganisms. Peterson and Peterson (30) listed sixty-five organisms which have been reported to synthesize riboflavin. Intestinal bacteria seemed to be the most promising organisms as shown by Najjar and Barrett (27) in their review on the synthesis of members of the vitamin B complex by intestinal bacteria. An attempt was therefore made to isolate riboflavin synthesizing bacteria from various sources and to determine the riboflavin content of the cultures.

Experimental

The organisms were isolated in the following manner. For isolations made from faeces, a water suspension of the faeces was first made, about 1 g. in 10 ml. sterile water. A loopful of the suspension was inoculated into melted nutrient agar (Difco) and plating was done by serial dilution on three Petrie dishes. Rumen bacteria were isolated by directly inoculating a loopful of rumen contents into melted nutrient agar and plating as above. The contaminants were isolated by exposing Petrie dishes of nutrient agar to the atmosphere. The remaining isolations were made from colonies on Petrie dishes used by the Bacteriology Department of Macdonald College in routine bacteriological investigations.

When colonies appeared on the plates, they were transferred to slopes containing the basal medium for the microbiological assay of riboflavin (37) with 1.5% agar (B.D.H.). Subsequent transfers were made on riboflavin-free broth. Five transfers were made of each organism. Table VI gives the results.

TABLE VI

Isolations of Organisms on Riboflavin-Free Broth

Material	Total Number of Isolations *	Number which Grew Well	Number which Grew with Difficulty	Number which did Not Grow
Chick Faeces	34	19	8	7
Sheep Faeces	14	7	6	1
Horse Faeces	8	7	1	0
Pig Faeces	16	11	2	3
Rat Faeces	15	4	3	8
Cow Faeces	5	5	0	0
Guinea Pig Faeces	25	12	8	5
Cow Rumen Contents	2	2	0	0
River Water	28	15	5	8
Raw Milk	11	4	6	1
Water Filters	6	0	2	4
Tap Water	4	1	0	3
Pasteurized Milk	3	0	0	3
Tomato Can	2	0	2	0
Contaminants	10	9	0	1
	183	96	43	44

* In these isolations no attempt was made to classify the strains isolated.

The riboflavin content of some of the cultures which grew luxuriantly was determined by the original fluorometric method and by microbiological assay. The latter method only was used fo the other cultures tested. The values obtained are listed in Tables VII and VIII.

As was previously pointed out (page 8) the riboflavin values obtained by microbiological assay are much too high in most cases However, the values do indicate that riboflavin is produced by al of these organisms.

TABLE VII

The Riboflavin Content of Two Week Old Broth Cultures Determine by Microbiological Assay

Culture		Riboflavin Content (γ per G.)
143	Greater than	2.71
95	" "	2.74
142	" "	3.07
S1	" "	3.02
141 (agar)	" "	2.13
118	" "	2.90
144	" "	2.92
147	" "	3.04
145	" "	2.41
60 (agar)		0.59
69 (agar)		0.74
73		2.18
150 (agar)		1.04
92		2.77
-		1.22

TABLE VIII

The Riboflavin Content of Ten Week Old Cultures as Determined by Microbiological Assay

Organism	Riboflavin Content (γ per G.)
12	0.786
91	0.813
p1	0.805
H6	0.689
1	1.134
43	0.600
82	0.625
H5	1.098
8	0.670
p5	0.678
H9	0.757
67	1.475
G1	0.745
95	1.968
123	0.520
70	0.776
66	0.595
19	0.596

STUDIES ON RUMEN CONTENTS AND FAECES

Introduction

Originally, the riboflavin in faeces and rumen contents was investigated because it was found that ruminants fed on a diet deficient in the components of the vitamin B complex did not develop any deficiency disease. As far back as 1928 (11) fermented rumen contents were found to be rich sources of the vitamin B complex. McElroy and Goss (23) found that the dried contents of the sheep rumen contained 33 micrograms of riboflavin per gram when the ration contained only 0.3 micrograms per gram. Experiments with rats showed that the components of the vitamin B complex were synthesized in the intestines (10, 26). The riboflavin content of chick faeces, incubated for one week at room temperature, increased 300 per cent (18). The human intestine has also been shown to be the site of riboflavin synthesis (28). Najjar and Barrett (27) reviewed the literature up to 1945.

An attempt was therefore made to determine the riboflavin content of faeces and rumen samples.

Experimental

Fresh cow faeces were collected and placed in a refrigerator at -10°C. so that there would be little change before the sample was assayed.

Two samples of approximately 85 grams each were assayed, suspended

in 500 ml. distilled water and incubated at room temperature in the dark. The riboflavin content of the samples was determined periodically by the original fluorometric method and microbiological assay. Table IX gives the results obtained.

TABLE IX

Change in the Riboflavin Content of Cow Faeces During Incubation

Time (Days)	Sample	Riboflavin Content (γ per G.) Microbiological Assay		Fluorometric	
1	A	0.403	0.354	0.358	0.456
	B	0.346	0.766	1.35	1.21
4	A	0.044	-	-	-
	B	0.167	0.188	-	-
5	A	0.113	0.148	0.149	0.176
	B	0.046	0.048	0.616	0.212
17	A	0.287	0.407	-	-
	B	0.359	0.489	-	-
Fresh) Undiluted)	A	1.97	1.69	2.28	-
"	B	4.23	2.41	0.68	-

The values show very little agreement and it is believed that the method of sampling was faulty; the suspension was not homogeneous and proper sampling was very difficult. The enzyme treatment used to prepare the extracts also accounts for some of the discrepancy, (page 8).

Human Faeces

A determination was made on two samples of human faeces by the original fluorometric method and by microbiological assay. The values obtained by the microbiological and the fluorometric methods were 0.473 and 0.188 γ riboflavin per g. respectively.

Rumen Contents

The contents of a cow's rumen showed a concentration of 0.15 γ riboflavin per ml. as determined by the original fluorometric method. These were exposed to sunlight for twenty-four hours until there was no trace of riboflavin as shown fluorometrically. The material was then incubated in the dark for two days and the original amount of riboflavin was found to be present.

STUDIES ON CELLULOSE BACTERIA

(A) Vibrio perimastix

Introduction

Since Vibrio perimastix, a soil organism which digests cellulose, isolated and described by Alarie (1) was known to produce a yellow pigment, this organism was used for experiments on riboflavin synthesis. At the same time it was decided to determine the effect of ferric-ion since Leviton (20) showed that ferrous-ion had a decided effect. The possibility of carbohydrate having an effect on riboflavin synthesis was also investigated.

Since riboflavin is a component of an enzyme system which is not poisoned by cyanide or azide, an experiment was designed to determine whether the amount of the riboflavin containing system would be increased at the expense of the enzymes which would be poisoned by the addition of cyanide or azide.

Several papers have described the effect of sodium azide on microorganisms. In yeasts, it prevented aerobic assimilation of glucose (44). Lichstein and Soule (21) showed that sodium azide inhibited catalase activity of bacteria and that the inhibition of growth was much less for organisms which did not produce catalase.

Numerous investigations have been made on the effect of cyanide on the growth of microorganisms. Pett (31) was able to almost double the riboflavin content of yeast by cultivation in a medium containing

about 0.001 molar potassium cyanide. Winzler (43) found that cyanide inhibited yeast respiration. Sevag and Shelburne (35) showed that cyanide inhibited the aerobic respiration of Streptococcus pyogenes and pneumococcus.

The literature contains no reference to riboflavin synthesis by soil bacteria which digest cellulose.

Experimental

Two cultures of Vibrio perimastix were provided by the Department of Bacteriology. One was a mixed culture containing an organism as yet unidentified and the other was a pure culture. The original experiment was done with the mixed culture and all later experiments with the pure culture. Transfers were kept on starch peptone agar slopes.

The medium (22) used for riboflavin production had the following composition:

Dibasic sodium phosphate (calculated as anhydrous)	0.75	g.
Monobasic potassium phosphate	0.25	g.
Magnesium sulfate heptahydrate	0.2	g.
Calcium chloride	0.1	g.
Sodium chloride	0.1	g.
Ferric chloride	0.02	g.
Sodium nitrate	1.0	g.
Cellulose or glucose	10	g.
Distilled water	1	litre

The sodium nitrate was soon replaced by ammonium nitrate (1.0 gram per litre) because the latter was found to be a better source of nitrogen (20).

An experiment using 50 ml. aliquots of medium in 250 ml. Erlenmyer flasks with glucose as the source of carbohydrate showed quite markedly that better growth was obtained if the components of the medium were autoclaved separately and mixed aseptically, than if the composite medium was autoclaved. The flasks were placed in a mechanical shaker and incubated at 30°C.

The ingredients of the medium were consequently autoclaved (15 lbs. pressure for 15 min.) in separate lots and mixed with the use of sterile technique. To facilitate this procedure, stock solutions of the various salts were prepared, i.e., magnesium sulfate heptahydrate 2%, calcium chloride 1%, sodium chloride 1%, dibasic sodium phosphate 5%, monobasic potassium phosphate 5%, ferric chloride 4%, and ammonium nitrate 10%.

The carbohydrate was autoclaved separately, being dissolved in a volume of distilled water such that when the salts were added, the desired volume was obtained. The phosphates were mixed in the proportion of one part monobasic potassium phosphate to three parts dibasic sodium phosphate, so as to give a buffer solution of pH 6.9 to 7.1. Two ml. of this mixture were used per 100 ml. medium. One drop of ferric chloride and one ml. of each of the remaining salts were added to each 100 ml. of medium.

The hydrogen-ion concentration of one sample of each batch of media

was checked with brom thymol blue as external indicator.

Each 50 ml. of culture was incubated on a mechanical shaker and all mass cultures 500 ml. or 750 ml. were incubated on a rotary shaker, both at 30°C.

Riboflavin determinations were carried out on 50 ml. samples, the extract being prepared as described for the fluorometric method finally adopted. This extract was used for both the microbiological and the fluorometric determinations, the latter being done by the method finally adopted.

To establish the required treatment for the extraction of the riboflavin, two 50 ml. aliquots of a cellulose culture were treated with 100 ml. 0.1 N. sulphuric acid. One was placed in a boiling water bath for forty-five minutes and the other was allowed to stand in the dark at room temperature. Fluorometry showed a value of 0.259 γ riboflavin per ml. of culture for the unheated sample and 0.291 γ per ml. of culture for the heated sample. The difference here was not significant and the results were interpreted as showing that most of the riboflavin in the culture was readily extracted.

The change in the riboflavin content of cellulose cultures with time of incubation was determined by using two mass cultures of 750 ml. and by doing determinations on aliquots removed aseptically. Table X gives the results as determined by the fluorometric method.

The inoculum used was a mixed culture and a different proportion of the two organisms in each inoculum was probably responsible for the striking difference in results. A microbiological assay was done on

on the 8-day sample. The values obtained were 0.333 γ per ml. and 0.059 γ per ml. of cultures A and B respectively. This gave mean values of 0.393 and 0.064 with deviations from the mean of 15.2 and 7.1 per cent respectively.

TABLE X

The Change in the Riboflavin Content of Cellulose Cultures of Vibrio Perimastix with Time

Days	Riboflavin Content (γ per ml.) Culture A	Culture B
6	0.291	-
8	0.452	0.068
11	0.619	0.131
14	0.506	0.121
18	0.428	0.140

Effect of ferric chloride

The broth medium (page 29) with glucose as carbohydrate was prepared in 50 ml. quantities in 250 ml. Erlenmeyer flasks with varying amounts of ferric chloride in each flask. The cultures were incubated on a mechanical shaker at 30°C. A pure culture of Vibrio perimastix was used as inoculum.

Table XI gives the results obtained. The cultures were six days old and the riboflavin content was determined by the fluorometric method.

TABLE XI

The Effect of the Concentration of Ferric Chloride on Riboflavin Synthesis by Vibrio Perimastix

Molarity of Ferric Chloride	Riboflavin Content (γ per ml.)
0	0.163
9×10^{-6}	0.186
9×10^{-6}	0.163
4.7×10^{-5}	0.138
9.5×10^{-5}	0.155
1.89×10^{-4}	0.106
2.85×10^{-4}	0.111
3.78×10^{-4}	0.132
4.75×10^{-4}	0.095

The results indicated that the minute amounts of ferric-ion present as impurities in the components of the medium would support growth. There appeared to be a slight decrease in riboflavin production with increase in ferric-ion.

Effect of carbohydrate

The media were prepared in 50 ml. portions in 250 ml. Erlenmeyer flasks as outlined (page 29). The following sugars were tested in duplicate cultures; cellobiose, cellulose, fructose, glucose, lactose, maltose, mannose, starch and xylose. A pure culture of Vibrio perimastix suspended in 10 ml. sterile water was used as inoculum, (one drop per flask).

The cultures were incubated on a mechanical shaker at 30°C. Riboflavin was determined after seven days by microbiological assay and by the fluorometric method finally adopted.

TABLE XII

The Effect of Different Carbohydrate on Riboflavin Production by Vibrio Perimastix

Carbohydrate	Riboflavin Content (γ per ml.) Fluorometric Method	Microbiological Assay	Mean	Deviation %
Cellulose	-	-	-	-
Cellobiose	0.153	0.181	0.167	8.4
Cellobiose	0.111	0.147	0.129	14.0
Fructose	0.093	0.119	0.106	12.2
Glucose	0.254	0.282	0.268	5.2
Glucose	0.132	0.163	0.148	9.5
Lactose	0.051	0.083	0.067	24.0
Lactose	0.184	0.212	0.198	7.1
Maltose	0.032	0.030	0.031	3.3
Maltose	0.106	0.151	0.129	14.0
Mannose	0.078	0.091	0.085	7.6
Starch	0.145	0.147	0.146	0.69
Starch	0.206	0.188	0.197	4.5
Xylose	0.076	0.078	0.077	1.3
Xylose	0.128	0.150	0.139	7.9

The results showed good agreement between the microbiological assay and the fluorometric determination and glucose appeared to produce the greatest response. The lack of growth in the cellulose cultures is unexplainable because other experiments with cellulose showed that riboflavin was produced in amounts equivalent to the quantities produced from the other sugars. The variation between duplicates of one sugar is also unexplainable since conditions were identical for all cultures.

The effect of sodium azide

A glucose-salts medium was used as substrate and was prepared as already described (page 29). The sodium azide was prepared in varying concentrations in 10 ml. distilled water. The various components were autoclaved separately (15 lbs. pressure for 15 min.) and mixed aseptically in sterile 250 ml. Erlenmeyer flasks so that the volume was 50 ml. Each flask was inoculated with one drop of a suspension of Vibrio perimastix in sterile distilled water and incubated on a mechanical shaker at $30^{o}C$. The final concentrations of sodium azide ranged from 1.85×10^{-5} to one of 3.63×10^{-3} molar.

The control culture grew in 36 hours, the lowest azide concentration showed growth after 96 hours, and the others showed no growth even after seven days. The turbidity and riboflavin content of the cultures which showed growth were determined after seven days incubation. The riboflavin content of the control was 0.127 γ per ml. and that of the culture containing 1.85×10^{-5} molar sodium azide was 0.076 γ per ml. The ratio of the turbidity of the latter to the former was

0.615 while the ratio of their riboflavin concentrations was 0.597 which was within three per cent of the other ratio.

These results show that sodium azide is a strong inhibitor of Vibrio perimastix and concentrations as low as 2.50×10^{-4} molar will completely inhibit growth. The concentration of 1.85×10^{-5} did permit growth but the relative riboflavin content was not increased and seemed to vary with the number of cells in the culture.

The presence of hydrogen peroxide was tested with luminol (36). No hydrogen peroxide could be demonstrated.

Effect of cyanide

The cyanide culture medium was prepared in the same manner as the sodium azide medium. Sodium cyanide was used and nine concentrations varying from 4×10^{-6} to 6.35×10^{-3} molar were employed. The method of inoculation and incubation was the same as that used in the sodium azide experiment (page 34).

The control and the cultures containing sodium cyanide up to 2.37×10^{-4} molar grew within forty-eight hours. The flask with 6.45×10^{-4} molar sodium cyanide showed growth after ninety-six hours. The flask containing 1.79×10^{-3} molar sodium cyanide showed growth after five days, and the last two cultures with 3.4×10^{-3} and 6.35×10^{-3} molar sodium cyanide respectively grew after six days.

Riboflavin and turbidity determinations showed little difference between the cultures. Cyanide was tested for by the Prussian blue test and in all cases the test was negative.

Evidently the organism was inhibited by cyanide and overcame the inhibition by destroying the cyanide. Once the cyanide had been destroyed, the organism grew in a normal manner.

(B) Cellulose Digesting Bacteria Isolated from Rumen Contents

Introduction

Of a great number of cellulose digesting bacteria isolated from sheep rumen contents, four strains produced a yellow pigment (8). These four organisms were investigated to determine whether the yellow pigment was riboflavin. A review of the literature failed to reveal any previous mention of cellulose digesting organisms synthesizing riboflavin.

Experimental

The cultures were obtained from the Bacteriology Department of Macdonald College. They were designated as cultures number 24, 42, 82, and 103 respectively. Transfers were kept on starch-peptone-agar or nutrient agar slopes; they grew well on both.

The broth medium used for the production of riboflavin was the same as the cellulose medium used with Vibrio perimastix except that the ammonium nitrate was replaced by 0.02% bacto-peptone (Difco). For inoculum the growth on the surface of an agar slope was emulsified in a few drops of sterile distilled water. Table XIII gives the riboflavin content of these cultures after five days on the rotary shaker at 30°C.

The above cultures of 500 ml. volume and three 500 ml. cultures of Vibrio perimastix were removed from the rotary shaker after seven days incubation. Ten milliliters of concentrated hydrochloric acid were added to each culture and all were steamed in an Arnold steamer for forty-five minutes and cooled. Each culture was then treated as follows:

TABLE XIII

Riboflavin Content of Five Day Cultures of Cellulose Digesting Bacteria Isolated from the Rumen

Culture	Riboflavin Content (γ per ml.) Fluorometric Method	Microbiological Assay	Mean	Deviation %
24	0.040	0.036	0.038	5.3
24	0.038	0.032	0.035	8.6
42	0.068	0.064	0.066	3.0
82	0.062	0.063	0.063	0.8
103	0.131	0.104	0.118	11.4
103	0.053	0.058	0.056	4.5

Sufficient saturated silver nitrate was added to precipitate almost all the chloride-ion. The culture was centrifuged and the supernatant was brought up to pH 6.4 to 6.8 with concentrated sodium hydroxide and using the Backman pH meter.

The solution was then transferred to a freeze-dryer and evaporated to 20 ml. This phase generally took twenty-four to thirty-six hours. The hydrogen-ion concentration of the culture was not adjusted until the freeze-dryer was available so that the solution was acid in reaction during the entire procedure.

The absorption spectra of the concentrates were determined with the Backman spectrophotometer. Due to the presence of interfering substances the results were not conclusive. However, the concentrates did exhibit the characteristic fluorescence of riboflavin.

The samples had an extremely high absorption in the ultra violet below 290 m.μ. and one sample did give the characteristic peak at 267 m. μ.

SUMMARY

The fluorometric method for determining riboflavin as outlined by Ruben et al (34) was found to give results which compared favourably with the microbiological assay of Snell and Strong (37). In the fluorometric determination the use of potassium permanganate and the enzyme treatment were found to be unnecessary. The enzyme extraction method of Chapman and McFarlane (5) was also found to be unsatisfactory for preparing extracts for microbiological assay.

Two species of bacteria were shown to require riboflavin for growth. These are Erwinia phytophthora and Mycobacterium thamnopheos. Strains of organisms isolated from a number of sources were found to synthesize riboflavin.

The riboflavin content of cows' faeces, human faeces and rumen contents was determined. Rumen contents in which riboflavin had been destroyed, regained their riboflavin content when incubated in the dark.

Vibrio perimastix and four strains of cellulose digesting bacteria isolated from rumen contents were found to synthesize a yellow pigment resembling riboflavin. Concentrates rich in riboflavin were prepared from these cellulose cultures.

Sodium azide proved to strongly inhibit the growth of Vibrio perimastix, the inhibition being complete at a concentration of 2.50×10^{-4} molar. Lower concentrations of sodium azide did not affect the synthesis of riboflavin which appeared to vary according to the number of cells in the culture.

Sodium cyanide, (6.35×10^{-3} molar) slightly inhibited the growth

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