CEYLON J. MED. SCI. (D), VOL. VIII, PT. III (February, 1953).

Microbiological Assay of Vitamins
2—RIBOFLAVIN

BY

A. A. HOOVER and G. C. N. JAYASURIYA

(*Department of Biochemistry, Medical Research Institute, Colombo*)

Riboflavin has been assayed both by chemical and microbiological methods. Snell and Strong in 1939 (1) introduced *LACTOBACILLUS CASEI* for riboflavin assay and since then their original medium has been modified by various workers (2, 3, 4, 5, 6, 7). The Snell and Strong medium of 1939 is the one recommended by the Association of Official Agricultural Chemists while the U.S.P. XII medium differs from it only in the concentration of glucose which has been increased from 1 to 3 per cent. In the United Kingdom a Sub-Committee appointed by the Society of Public Analysts in 1946 recommended a medium which differs from the Snell and Strong medium in the following respects :—(*a*) no sodium acetate was added (*b*) the glucose concentration was doubled (*c*) sodium chloride was increased from 2 mg. to 1,000 mg. per cent. (*d*) ammonium sulphate was an additional inorganic salt (*e*) a pure vitamin supplement consisting of pyridoxine, calcium pantothenate, nicotinic acid and para amino benzoic acid was included (*f*) the purines adenine, guanine, uracil and the pyrimidine xanthine were added and (*g*) dl-tryptophane and xylose were also included.

Our medium described in this paper is based on the more recent knowledge concerning the nutritional requirements of *LACTOBACILLUS CASEI* especially in regard to its 'strepogenin' and folic acid requirements. The medium we propose does not materially differ from that of Rabinowitz, Mondy and Snell (8) for the assay of pyridoxal with *LACTOBACILLUS CASEI* except in the following respects :—(*a*) the glucose concentration has been doubled (*b*) sodium chloride concentration has been increased from 2 mg. to 1,000 mg. per cent. (*c*) riboflavin was omitted and instead 160 µg. of pyridoxine per 100 ml. were substituted and (*d*) the purines were also included.

Experimental

The organism, *LACTOBACILLUS CASEI*, obtained from the National Collection of Type Cultures, Central Public Health Laboratory, London, was maintained by fortnightly sub-culture on a marmite-dextrose agar. The culture was transferred from a stab to an enriched medium consisting of a liver-peptone broth, as described by Jones and Morris (9). This was incubated at 37° C for 22-24 hours and then

transferred from the liver-peptone broth back again to marmite-dextrose agar stabs which were incubated at 37° C for 22-24 hours and then kept in the refrigerator.

The liver-peptone broth was made up as follows:—

Liver Extract	.. ..	10 ml.
Peptone (Gurr)	.. ..	1·00 g.
Marmite	.. ..	0·25 g.
K_2HPO_4	.. ..	0·50 g.
Dextrose	.. ..	0·20 g.

The pH of the solution was adjusted to 7·0 and made up to 100 ml. with distilled water. Five ml. of this solution were measured into test tubes plugged with cotton-wool and sterilized by autoclaving at 15 lb. steam pressure for 15 minutes. This was allowed to cool and then stored in the refrigerator.

The liver extract was prepared according to Jones and Morris (9) but was not stored under toluene as described but without the preservative in 20 ml. ampules which were sterilized with the liver extract at 5 lb. steam pressure for 40 minutes. In this way the liver extract could be stored in the refrigerator without deterioration for at least 12 months.

The marmite-dextrose agar was made up as follows :—

Marmite ..	.. ..	1·50 g.
Dextrose	.. ..	0·50 g.
Agar ..	.. ..	2·00 g.
Peptone	.. ..	0·25 g
Salt solution (same as in basal medium)	..	4·0 ml.

The pH was adjusted to 6·8 and the volume made up to 100 ml. The suspension was steamed to dissolve the agar. Ten ml. portions of the hot solution were measured into tubes plugged with cotton-wool and sterilized at 15 lb. steam pressure for 15 minutes. The tubes were allowed to cool until the agar set and then stored in a refrigerator.

Fortnightly transfers to an enriched medium were necessary since otherwise poor growth resulted in the inoculum tubes as well as in the assay tubes. In fact, Snell (1) who first described the microbiological method for the assay of riboflavin has recently (8) modified his original method of maintaining the culture of *LACTOBACILLUS CASEI* and has employed an enriched medium described by Hunter (10). Enriched media containing liver extracts have been described by Nymon and Gortner (11) and also by Jones and Morris (9). The medium described in this paper is based on that employed by Jones and Morris (9) for Streptococcus Faecalis.

Composition of Basal Medium

	Amount for 100 ml. double strength medium
Casein, Tryptic digest ..	.. 1000 mg.
Casein, acid hydrolysate ..	.. 1000 mg.
Glycine	.. 40 mg.
l-Cystine	.. 40 mg.
l-Asparagine	.. 20 mg.

Composition of Basal Medium—(*Contd.*)

	Amount for 100 ml. double strength medium
dl-Tryptophane	20 mg.
Adenine	2 mg.
Guanine	2 mg.
Uracil	2 mg.
Dextrose	4000 mg.
Sodium acetate, anhydrous	1200 mg.
NaCl	1000 mg.
KH_2PO_4	100 mg.
K_2HPO_4	100 mg.
$MgSO_4 \cdot 7H_2O$	40 mg.
$MnSO_4 \cdot 4H_2O$	2 mg.
$FeCl_3$	0·4 mg.
Thiamine hydrochloride	40 μg.
Niacin	80 μg.
Pyridoxine hydrochloride	160 μg.
Calcium pantothenate	80 μg.
Para amino benzoic acid	40 μg.
Folic acid	2 μg.
Biotin	0·4 μg.

pH of solution 6·8

The tryptic digest and the acid hydrolysate of casein were prepared as described before (12). The amino acids, the purines and the inorganic salts were kept in stock solution as described in the microbiological assay of thiamine (12).

Stock Solutions

Stock solution *A* of riboflavin:—50 mg. riboflavin, accurately weighed were suspended in about 350 ml. of distilled water containing 0·6 ml. glacial acetic acid. The suspension was warmed to about 80° C till the riboflavin dissolved. When cool the solution was transferred quantitatively into a 500 ml. volumetric flask and the volume made up to the mark with distilled water. This solution was stored under toluene in a dark bottle in the refrigerator. This solution containing 100 μg. riboflavin per ml. keeps for a month.

Stock solution *B* of riboflavin :—10 ml. of stock solution *A* of riboflavin were accurately diluted to 100 ml. with distilled water. This was stored under toluene in a dark bottle in the refrigerator. The solution keeps for a week and contains 10 μg. riboflavin per ml.

Standard solution of riboflavin :—1 ml. of stock solution *B* of riboflavin was accurately diluted to 100 ml. with distilled water. This solution containing 0·1 μg. riboflavin per ml. is prepared only on the day of use.

Inoculum

The medium for growing the inoculum was prepared in 10 ml. centrifuge tubes. Into each centrifuge tube plugged with cotton-wool were added 5 ml. of the double

strength basal medium and 5 ml. of a solution of riboflavin containing 0·2 µg. per ml. so that each tube would receive 1 µg. riboflavin. The tubes were sterilized at 15 lb. steam pressure for 10 minutes. The organism was transferred from a 24 hour liver-peptone broth culture to one of these tubes and incubated at 37° C for 22-24 hours. The tube was then centrifuged aseptically, the supernatant decanted off and the bacterial cells suspended in 10 ml. sterile saline. This was centrifuged again, the supernatant decanted and the bacterial cells re-suspended in 10 ml. sterile saline.

Extraction

The material to be examined was first homogenised as in the assay of thiamine (12) and then suspended in at least ten times its weight of 0·1 N·HCl. The suspension was autoclaved at 15 lb. steam pressure for 15 minutes. On cooling the pH was adjusted to 4·5 with NaOH and the solution filtered to remove interfering fatty substances. The pH of the filtrate was adjusted to 6·8 with NaOH and an aliquot was then suitably diluted so that it contained approximately 0·1 µg. riboflavin per ml.

Assay Procedure

Pyrex lipless test tubes, 16 × 125 mm. were used for the assay. Into 14 tubes were measured 0, 0·5, 1·0, 1·5, 2·0, 2·5 and 3·0 ml. of the standard solution of riboflavin in duplicate. The volume in each tube was made up to 5 ml. with distilled water. Five ml. of double strength riboflavin-free basal medium were added to each tube. Into 10 other tubes were measured 0·5, 1·0, 1·5, 2·0, 2·5 ml. of the test extract in duplicate. As before the volume in each tube was made up to 5 ml. with distilled water followed by the addition of 5 ml. of double strength riboflavin-free basal medium. The tubes were plugged with cotton-wool and sterilized at 15 lb. steam pressure for 10 minutes.

On cooling to room temperature each tube was inoculated with one drop of the inoculum, described above, by means of a Pasteur pipette. The tubes were incubated in a constant temperature water bath for 72 hours at 37° C. The acid produced in each tube was then determined by titrating against 0·1 N·NaOH to pH 6·8 using a bench type Cambridge pH meter incorporating a glass electrode and a silver-silver chloride element.

Results and Discussion

The riboflavin content of the sample was deduced as previously described (12) by the 'slope-ratio' method. Plant materials including green leaves, vegetables, fruits and cereals have been assayed by this method and in all cases the methods of extraction and assay were strictly adhered to. The correlation coefficient between the dose and response in each case was above 0·9. In addition the 'test line' and the 'standard line' intersected the 'response axis' at points which were closely situated. These two facts point to the validity of this method of riboflavin assay and also to the absence of interfering substances in the materials investigated.

We have compared the results obtained by Snell and Strong with their original medium of 1939 (1) and a later medium put forward by Roberts and Snell in 1946 (6) with that obtained by the use of (*a*) the medium described in this paper and (*b*) the same medium without the purines which were considered unessential by Snell and therefore deliberately omitted by him in his medium for *LACTOBACILLUS CASEI* (8).

TABLE I

Riboflavin added in µg/10 ml.	*ml. 0·1 N acid produced*			
	I	*II*	*III*	*IV*
0·00	0·50	0·30	0·30	0·60
0·02	1·7	4·0	—	—
0·04	2·8	6·2	—	—
0·05	—	—	2·4	3·1
0·07	4·4	8·3	—	—
0·10	6·2	10·0	4·5	5·5
0·13	7·8	11·5	—	—
0·15	—	—	5·6	7·1
0·16	9·0	12·9	—	—
0·20	9·8	14·3	6·7	9·1
0·25	—	—	8·2	10·6
0·30	—	—	11·3	12·4
Correlation Coefficient	0·96	0·75	0·96	0·99

Note— I obtained with Snell-Strong medium, 1939.
II obtained with Roberts-Snell medium, 1946.
III obtained with our medium but without purines.
IV obtained with our medium, 1952.

We have observed that the presence of purines in our medium has a beneficial effect on the acid production although the linearity of response is not affected very much. The acid production in both cases is low compared with the values obtained by Roberts and Snell in 1946 but is not very different from the values obtained by Snell and Strong in 1939. The correlation coefficients have been worked out from the data obtained by us with our media and those obtained by Snell and Strong (1) and Roberts and Snell (6) with their media. Roberts-Snell medium gave the lowest figure for the correlation coefficient although the highest acid production was obtained with this same medium. In this connection it may be mentioned that Snell (13) has observed that ' it should be emphasized that the principal objective of these methods is to secure accurate assays with a maximum of convenience, and not, necessarily to secure the maximum possible growth or acid production '.

While the Snell-Strong medium (1) as well as the Roberts-Snell medium (6) cover an assay range of 0·0·2 µg. riboflavin per 10 ml.; the proposed medium gives a linear response for a slightly wider range of 0·0·3 µg. riboflavin per 10 ml.

Riboflavin values obtained by this method for some local foodstuffs, including green leaves, vegetables, fruits and cereals are presented in Tables 2, 3 and 4. Since the moisture content of green leaves is easily affected by storage conditions and other factors, the riboflavin values are also expressed in terms of a moisture conten of 85 per cent., which is actually the average value for the water content of the green leaves examined.

TABLE 2

Cereals	*Botanical Name*	*Moisture %*	*µg riboflavin/100 g.*
Rice, brown	Oryza Sativa	11	140
Kurakkan Flour	Eleusine Coracana	11	110
Maize Flour	Zea Mays	9	159
Sorghum Flour	Sorghum Vulgare	11	232

TABLE 3

Green Vegetables and Fruits	*Botanical Name*	*Moisture %*	*µg riboflavin/100 g*
Ash plantain	Musa paradisiaca	72	20
Bitter Gourd	Momordica Charantia	91	57
Cucumber	Cucurmis sativus	98	10
Drumstick	Moringa Pterygosperma	87	21
Pumpkin, Yellow	Cucurbita Maxima	92	40
Snake Gourd	Trichosanthes Anguina	96	20
Kolikuttu	Musa Sapientum	75	32
Papaya fruit	Papaya Carica	89	28
Tomato, ripe	Lycopersicum Esculantum	95	33
Wood apple	Feronia Elephantum	70	60

TABLE 4

Leafy Vegetables Common Name	*Botanical Name*	*Moisture %*	*µg riboflavin (a) per 100 g. material*	*(b) Calculated for 85% moisture*
Araikeerai	Amaranthus Viridis	86	250	268
Betel	Piper Betle	83	180	159
Cabbage	Brassica Oleracea	94	46	115
Carrot leaves	Daucus Carota	83	240	211
Erabadu	Erythrina Indica	80	430	322
Gotukola	Centella Asiatica	85	260	260
Kankun	Ipoemea Aquatica	85	320	320
Kathuru Murunga	Sesbania Grandiflora	82	660	550
Leeks	Allium Porum	89	60	82
Mint	Mentha Viridis	88	190	238

TABLE 4—(*Contd.*)

Leafy Vegetables Common Name	*Botanical Name*	*Moisture %*	*µg riboflavin (a) per 100 g. material*	*(b) Calculated for 85% moisture*
Mukunuwenna	Alternanthera Sessilis	86	340	364
Murunga leaves	Moringa Pterygosperma	79	720	514
Niviti	Basella Rubra	93	190	407
Peni-Thora	Cassia Occidentalis	76	873	544
Passion Fruit leaves	Passiflora Edulis	82	390	325
Sarana	Boerhavia Diffusa	91	160	267
Tamarind leaves	Tamarindus Indicus	65	340	146
Tampala, Suda	Amaranthus Gangeticus	93	300	643

Summary

1. A method for the microbiological estimation of riboflavin in plant materials is described.

2. A wider assay range is possible with our medium which is based on the more recent knowledge of the nutritional requirements of *LACTOBACILLUS CASEI*.

3. The riboflavin values of a number of plant materials of nutritional importance are reported.

References

1. SNELL, E. E. and STRONG, F. M. ; *Ind. Eng. Chem. Anal.* Ed. 1939, 11, 346.
2. LANDY, M. and DICKEN, D. M. ; *J. Lab. Clin. Med.* 1942, 27, 1086.
3. GREENE, R. D. and BLACK, A. ; *J. Am. Pharm. Assoc.* 1943, 32, 217.
4. BARTON-WRIGHT, E. C. and BOOTH, R. G. ; *Biochem. J.* 1943, 37, 25.
5. BARTON-WRIGHT, E. C. ; *Analyst*, 1945, 70, 283.
6. ROBERTS, E. C. and SNELL, E. E. ; *J. Biol. Chem.* 1946, 163, 499.
7. KENT-JONES, D. W. et al. ; *Analyst*, 1946, 71, 846.
8. RABINOWITZ, J. C., MONDY, N. I. and SNELL, E. E. ; *J. Biol. Chem.* 1948, 175, 147.
9. JONES, A. and MORRIS, S. ; *Analyst*, 1949, 74, 29.
10. HUNTER, G. J. E. ; *J. Dairy Res.* 1946, 14, 283.
11. NYMON, M. C. and GORTNER, W. A. ; *J. Biol. Chem.* 1946, 163, 277.
12. HOOVER, A. A. and JAYASURIYA, G. C. N. ; *Ceylon J. Med. Sci.* 1950, 7, 66.
13. *Vitamin Methods*, edited by PAUL GYORGY, 1950, 1, 343.