determination of vitamin A and β -carotene in the same sample. The application of the method for the determination of vitamin A in mouse and rat livers is briefly discussed.

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The Microbiological Assay of Riboflavin. The Influence of Inorganic Constituents and Unknown Growth Factors

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We are here concerned with the assay of riboflavin which has developed from the investigations of Snell & Strong [1939] and which showed the linear relationship between the acid production from glucose by *Lactobacillus casei* ϵ (Helveticus) and the concentration of riboflavin present in the medium. In this paper the effect of the addition of various inorganic constituents on the microbiological assay of riboflavin will be discussed, and suggestions made for the improvement of the method.

EXPERIMENTAL

The basal medium of Snell & Strong [1939] has been modified a number of times, notably by Snell & Wright [1941], Hutchings, Peterson & Shull [1942], and Lindner & Pollack [1942], mainly by the addition of substances which might function as growth factors or accessories. In our view the only real source of variation in the preparation of this medium is in the composition of the acid hydrolysate of casein. We have invariably prepared our hydrolysate according to the method used in J. H. Mueller's laboratory and described as Method 1 [Johnson & Mueller, 1941]. This hydrolysate is practically free from Fe and P and there is little, if any, Ca present.

The results which follow were obtained with a 20% acid digest of casein, whose free HCl, when neutralized, gives 3.44 g. NaCl/100 ml. (1.72 g. NaCl/l. of medium). It contains 19.46 g. N_2 /l., no P, and the Fe content is very small.

The basal medium first used was as follows: 50 ml. casein hydrolysate (20%); 2 ml. 20% cystine

solution in 2.5N HCl; 0.1 g. l-tryptophan; 10.0 g. glucose; 0.5 g. KH_2PO_4 ; 100 μ g. nicotinic acid; 50 μ g. pantothenic acid; 50 μ g. p-amino-benzoic acid; 2.5 ml. liver eluate; 0.4 mg. $FeSO_4 \cdot 7H_2O$; 2.0 ml. mineral solution.

This was adjusted to pH 6.8 and the volume made up to 1 l.; the medium was sterilized for 10 min. at 10 lb./sq. in.

The mineral solution contained: 22.5 g. $MgSO_4 \cdot 7H_2O$; 5.0 ml. $CuSO_4 \cdot 5H_2O$ (1% w/v); 4.0 ml. $ZnSO_4 \cdot 7H_2O$ (1% w/v); 1.5 ml. $MnCl_2 \cdot 4H_2O$ (1% w/v); 3.0 ml. conc. HCl; H_2O to 100 ml.

After sterilization in 20-ml. amounts in 125 ml. Pyrex flasks, riboflavin, or extracts of the food to be tested, were added in variable amounts and also 0.2 ml. of 0.5% (w/v) $CaCl_2$.

The $CaCl_2$ was added since it was soon observed that in the absence of such addition, growth and acid production were negligible.

It will be noticed that the medium contains p-amino-benzoic acid. In the examination of the partially purified liver fraction it became apparent that this compound was present in the active material. Tests revealed the fact that the compound was a growth stimulant for the *Lactobacillus* and it was consequently included in the basal medium.

Preparation of active liver extracts

In early assay methods [Snell & Strong, 1939] photolysed peptone and yeast extract were added to the food medium; subsequently it was suggested that nicotinic acid and pantothenic acid could replace the yeast extract. In our experience this was

unsatisfactory; growth and acid production sometimes occurred, but the latter was so far short of an optimum that it was clear that additions made to a medium, when extracts of food or tissue fluids were added, modified the results. Because of deficiencies in source of supply, irregularity of result, etc., we began to use liver fractions which we were using in our studies on the nutrition of *C. diphtheriae* types. The properties of these active fractions have been described by Chattaway, Happold, Lythgoe, Sandford & Todd [1942]. Residues from the preparation of the liver anti-anaemic principle were adsorbed on Fuller's earth at pH 3, the bulk of the activity being in the filtrate. This was next adsorbed on norite. Activity was mainly found on the charcoal which was eluted with dilute NaOH until the eluate was slightly alkaline (pH 7.2) when elution was continued with water. After further acidification to pH 3, the eluate was extracted 5-8 times with amyl alcohol (in which the active component was insoluble). The aqueous phase was then concentrated and used instead of the yeast extract. It was free from riboflavin, and from all but traces of pantothenic and nicotinic acids. It should be noted that activity decreases on standing in weak solutions and this accounts for differences in total acidity in the series presented. The optimum activity of such a concentrate having been determined, it was added to the basal medium in the appropriate amounts.

RESULTS

Effect of Fe concentration on acid production

Twenty-four flasks each containing 20 ml. of medium were prepared in four series of 6. Series 1 contained no added $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, series 2 had 4 $\mu\text{g.}$, series 3, 8 $\mu\text{g.}$ and series 4, 16 $\mu\text{g.}$ $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ added to each 20 ml. medium.

The six flasks in each series had 0, 0.2, 0.4, 0.6, 0.8 and 1.0 $\mu\text{g.}$ riboflavin added respectively; 0.2 ml. 0.5% solution CaCl_2 was added to each flask. On incubation for 72 hr. the results in Table 1 were obtained.

Table 1. *Influence of addition to the medium of Fe and riboflavin on acid production*

Riboflavin added $\mu\text{g.}$	Acid production (ml. N/10)			
	FeSO ₄ ·7H ₂ O added			
	None	4 $\mu\text{g.}$	8 $\mu\text{g.}$	16 $\mu\text{g.}$
Nil	Nil	Nil	Nil	Nil
0.2	6.0	6.4	8.1	3.5
0.4	8.8	10.1	10.1	4.1
0.6	11.5	11.6	11.7	6.4
0.8	11.0	11.9	12.3	9.6
1.0	3.5	9.4	9.8	7.5

It will be observed that the greatest acid production was with 8 $\mu\text{g.}$ $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ /20 ml. but that

the straight-line relationship which existed up to 0.6 $\mu\text{g.}$ riboflavin at this level was extended up to the 0.8 $\mu\text{g.}$ level with the higher concentration of the ferrous salt. It is not suggested that these levels will be the same for each batch of medium and they are almost certainly affected by the concentration of iron added in the liver eluate, but it is clear that the amount of Fe added with any food or tissue extract which is being assayed for riboflavin content should be known and suitable addition of Fe salt made to the control riboflavin series. Fortunately, tables of the mineral contents of foods and tissues are available and suitable additions can be made. That this is important will be realized by comparison of the above figures where 0.2 $\mu\text{g.}$ in series 1 or 2 is approximately equal to 0.6 $\mu\text{g.}$ riboflavin in series 4. Examination of the actual curves makes other differences apparent.

A further series of four flasks containing 0.8 $\mu\text{g.}$ riboflavin and 5, 10, 15, 30 $\mu\text{g.}$ $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ was tested (Table 2).

Table 2. *Influence of addition of Fe on acid production*

FeSO ₄ ·7H ₂ O $\mu\text{g.}$	Acid produced (ml. N/10) in		
	72 hr.	96 hr.	120 hr.
5	3.30	12.70	22.80
10	3.30	12.40	22.50
15	2.80	11.50	20.70
30	2.50	11.10	16.60

The Ca concentration in this experiment was double that used in the first, and, as will be seen later, was nearer to the optimum. These results agree well with those of the first series in showing a decrease in acid production with increasing concentration of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$.

In a further series in which $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ was added to the optimum of 400 $\mu\text{g./l.}$, in 250 $\mu\text{g.}$, 500 $\mu\text{g.}$, 1 mg., 2 mg., 3 mg., 4 mg., and 5 mg. amounts, there was a decrease in acid formation down to the 3 mg. level, after which stabilization occurred. Thus the higher level of Fe in the Snell-Strong medium is sound for assay work, provided that it be realized that this medium does not give optimum acid production.

The effect of variations in PO_4^- and K^+ content of the medium

In the Snell-Strong medium PO_4^- is added as KH_2PO_4 (0.5 g./l.). This is the only source of K^+ and of phosphate in the medium. Since the importance of PO_4^- and of K^+ in carbohydrate metabolism is obvious, variations in their concentration have been tested. The first series of results presented below is with two separate fractions of liver extract; those appearing beneath the horizontal dividing line represent tests carried out some

weeks later than those above. The later tests contain 0.02 g. KH_2PO_4 /20 ml. medium; 0.25 ml. 0.5% (w/v) CaCl_2 solution was added to each 20 ml. medium after sterilization. This concentration of CaCl_2 does not represent an optimum. In the first series of results the optimum was obtained with a 0.1% (w/v) KH_2PO_4 concentration. Addition of further phosphate as the Na salt was without effect with fraction 'H', but with fraction 'S' there was a rise over a range of addition from 0.13 to 0.016 g. %. Fraction 'S' was obviously more active than fraction 'H' in encouraging growth, so that the phosphate present as the K salt was adequate with 'H', whereas with 'S' the further addition of Na phosphate to M/800 was necessary to give optimal results. It is encouraging that 8 times this concentration did not affect the result at 5 days, since this suggested that in assay work increased concentration of $\text{PO}_4^{=}$ due to that added with the material to be assayed, would not affect the result to an important degree (Table 3).

Table 3. *Influence of addition of phosphate on acid production from 20 ml. medium containing 0.8 μg . riboflavin*

KH_2PO_4 added g.	Medium + liver frac- tion 'S'. Acid produced (ml. N/10) in			Medium + liver fraction 'H'. Acid produced (ml. N/10) in	
	72 hr.	96 hr.	120 hr.	72 hr.	96 hr.
None					
+ 0.02	7.30	15.30	—	3.70	6.10
0.01	7.20	11.55	—	3.00	5.90
0.005	5.60	7.60	—	2.90	5.30
0.02	4.00	5.80	16.80	3.10	6.20
0.02 + Na phosphate buffer pH 7.2 to					
M/100	3.70	7.40	22.80	—	—
M/200	5.80	10.00	23.00	3.60	6.30
M/400	6.20	10.20	23.20	3.20	6.20
M/800	8.00	12.00	22.30	2.90	6.20

At this stage the concentration of KH_2PO_4 in the basal medium was doubled (0.04 g./20 ml. or 0.2%); this should give a safe optimum for phosphate. There was a pronounced rise in acid production which was attributed to the increased K^+ concentration; actually this latter was not yet at its optimum (Table 4).

Table 4. *Influence of addition of KCl on acid production*

Medium (20 ml.)	Added KCl mg.	ml. N/10 acid produced in	
		48 hr.	66 hr.
		2.5	4.85
"	17.0	3.0	5.30
"	13.6	3.2	5.60
"	10.2	2.9	5.30
"	6.8	3.0	5.60
"	3.4	3.2	5.50

This represented an additional K^+ optimum of 1.8 mg./20 ml. or 9 mg. %, though it is possible that for this medium it was less than this figure. The general stability of result with further increase in K^+ is again encouraging. Additions as KH_2PO_4 were also made and the results of a specimen experiment are given in Table 5. The optimum value was reached when the KH_2PO_4 was increased by 5 mg. KH_2PO_4 /20 ml. or 25 mg./100 ml.

Table 5. *Influence of addition of KH_2PO_4 on acid production*

(Reaction adjusted with N/10 NaOH after addition of further phosphate. Medium tested in 20 ml. lots.)

Added KH_2PO_4 mg.	Acid produced (ml. N/10) in		
	48 hr.	72 hr.	96 hr.
Basal medium (0.2% KH_2PO_4)	1.90	4.0	8.5
"	5	2.30	5.10
"	11	2.25	4.90
"	30	2.15	4.75
"	50	2.20	4.85

The K^+ increase was thus 7.2 mg./100 ml. (9 mg. with KCl) and the total K^+ concentration added as KH_2PO_4 was 0.065 g./100 ml. Since the Na value (by neutralization of free HCl) was 0.079 g./100 ml., the K/Na ratio for additions was at least 0.82, a value which approximated to that obtained for optimal metabolism of stored carbohydrate in cells of *Escherichia coli* as shown by Evans, Handley & Happold [1942].

The effect of variation in the Ca^{++} concentration

The importance of this effect is easily observed in Table 6.

Table 6. *Influence of addition of CaCl_2 on acid production*

Added 0.5% CaCl_2 ml.	Acid (ml. N/10) produced/20 ml. medium in	
	72 hr.	96 hr.
Basal medium (0.2% KH_2PO_4)	Nil	Nil
"	0.1	6.35
"	0.2	4.85
"	0.3	12.05
"	0.4	6.75
"	0.6	4.00
"	0.8	4.10

A further series with a different liver fraction gave a CaCl_2 optimum of 0.35 ml. of 0.5% CaCl_2 solution. It will be noticed that there was a steep drop with excess of Ca. In view of the high concentration of Ca in milk it is important that the Ca curve for any medium which is being used to assay

milk products should be made and that the Ca equivalent of the milk should be added to the control riboflavin series in each assay. It will be noticed that each 0.1 ml. was equivalent to 0.18 mg. Ca^{++} . The Ca concentration of the medium before the addition of the CaCl_2 was 0.41 mg. Ca/100 ml., or 0.082 mg. Ca in each flask of test medium.

The effect of variation in the NaCl concentration

It was found that the basal medium prepared as described above did not contain an optimum concentration of NaCl. Preliminary experiments showed that the optimum lay between 90 and 100 mg. NaCl/20 ml. medium. The results (Table 7) show more exactly the value of this optimum concentration and also that addition of NaCl above this level does not cause a serious drop in acid production. Thus its addition in food or tissue extracts will not greatly affect riboflavin assay. All other mineral constituents and riboflavin itself (0.8 $\mu\text{g.}/20$ ml.) were at an optimum for this experiment.

Table 7. *Influence of addition of NaCl on acid production*

NaCl/20 ml. medium mg.	ml. N/10 acid produced in 72 hr.
54 (basal medium)	1.9
89	2.25
91	2.65
93	3.6
94	3.2
95	2.9
97	2.7
99	2.7
134	2.7

Application of assay method

Assays were carried out on cabbage, new potatoes, meat and garden peas (served in British Restaurants), and though the Ca and K concentration of the media used was not at the optimum the results obtained with the first two foodstuffs illustrate the type of difficulty which may arise from the addition of unknown growth factors contained in a food extract to the basal medium. The extracts

were prepared by refluxing the foodstuff for 2 hr. with an excess of $N/2$ HCl and then adjusting the reaction of the extract to pH 6.8. The extracts were added to two series of media which contained liver extract equivalent either to 12 g. or to 6 g. of raw liver. The results with the meat and the garden peas were useless because these contained either further growth factors, or more probably additional mineral constituents such as K, in significant amounts. The results with cabbage and new potato (Table 8), which do not add significant amounts of the important mineral constituents, are of interest in that they fit the curve of riboflavin variation at the higher concentration of liver extract but not at the lower one (Table 8 and Fig. 1).

It will be seen that the data of Table 8 fall on curve A (Fig. 1) rather than on curve B. The data derived from the addition of 0.5 ml. extract are probably less reliable than the other results in

Fig. 1. Relationship between concentration of riboflavin present in the medium, and titratable acidity produced by growth of *Esch. coli*. (A) basal medium + liver extract equivalent to 12 g. liver. (B) basal medium + liver extract equivalent to 6 g. liver.

Table 8, because this dose of extract contained too little added riboflavin. We may therefore assume that cabbage contains about 41.5 g./100 g. and potato between 160 and 195 g. riboflavin/100 g., the latter figure probably being closer to the real value.

Table 8. *Influence of addition of cabbage and potato extract on acid production*

Material extracted	Material equiv. to 100 ml. extract g.	Extract added to medium ml.	Acid produced ml. <i>N</i> /10	Riboflavin content (by ref. to curve <i>A</i> , Fig. 1) of		100 g. of material μg.
				Extract added to medium		
				μg.	μg. calc.*	
Cabbage	42.2	2.0	3.2	0.35	—	41.5
		1.5	2.5	0.26	0.26	41.7
		1.0	1.6	0.15	0.17	35.5
Potato	18.5	1.0	3.3	0.36	—	195.0
		0.5	1.6	0.15	—	163.0

* Calculated on the assumption that the value for 2.0 ml. is correct.

The riboflavin content of the cabbage is probably 41.5–41.7 $\mu\text{g.}/100\text{ g.}$ raw cabbage, the potato is probably nearer the higher value of the two presented.

DISCUSSION

It is of interest that Gillespie & Rubbo [1940] reported that biotin was not essential for *Cl. acetobutylicum* whereas the reverse was claimed by Park & Wood [1942]. There are marked differences in the mineral constituents in the media used by these two sets of investigators; in general the mineral content of the latter workers was 1/10 that of Gillespie & Rubbo. Park & Wood's concentrations would certainly be suboptimal for the *Lactobacillus* and it is again interesting to note that they report an enhancement of growth with ammonium phosphate instead of with asparagine. Assuming that both sets of results are correct it would appear that a change in the mineral balance of a medium has enabled the organism to dispense with biotin. Though this may seem unlikely, it does suggest that the amount of a growth factor necessary for optimum action is profoundly affected by the mineral balance. This lends additional emphasis to the main theme of this paper.

It should be added that a series of additions have been made to the basal medium by a variety of workers. We have tested many of these compounds and found them inactive. Thus, adenine, guanine,

uracil, thymine and adenylic acid were all tried separately and together, and found to be inactive. Since these components may be of natural origin it seems likely to us that their reported activity has been due to the adsorption on to the compound tested of small but adequate amounts of a growth factor or growth factors.

SUMMARY

Earlier work had revealed a number of irregularities in the application of the microbiological method of assay for riboflavin, using *Lactobacillus casei* ϵ . Evidence is presented to show that acid production can be altered by a variation in the concentrations of Fe^{++} , Ca^{++} , K^+ , PO_4^- and NaCl in the basal medium. Optimum concentrations for these constituents have been demonstrated and in addition it was shown that further increases in Fe^{++} , K^+ , PO_4^- and NaCl concentrations do not result in a great decrease in acid production. Further addition of Ca^{++} , however, results in a serious decrease. The necessity for, and the effect of, the addition of a liver eluate to the basal medium was shown. The bearing of these results on the assay of foodstuffs for riboflavin has been discussed.

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The State of Tyrosine in Egg Albumin and in Insulin as Determined by Spectrophotometric Titration

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Numerous studies on the reactions between protein and hydrogen and hydroxyl ions have yielded important information as to the number and character of ionizable groups in the protein molecule. The detailed interpretation of the titration curves is, however, in many cases ambiguous. The assumption is often implicitly made that the dissociation constants of the various polar groups in proteins are not very different from those found in amino-acids

or peptides. In this general form, such an assumption cannot be correct. Above and below the isoelectric point (I.E.P.) the protein carries a net positive or negative charge and the number and distribution of these charges will greatly influence the ionization of different groups. A considerable displacement of dissociation constants can therefore be expected as the net valency of the protein increases. Near the I.E.P. the electrostatic effect