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A Modified Medium for *Lactobacillus casei* for the Assay of B Vitamins

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Snell & Wright (1941) were the first to point out that their method for the assay of nicotinic acid could be applied to the determination of biotin and pantothenic acid. Shortly afterwards, Landy & Dicken (1942) published a general procedure for the estimation of six vitamins, using *Lactobacillus casei* as the test organism. Roberts & Snell (1946) devised a medium for microbiological assays with *L. casei*, which was an improvement on the original medium of Landy & Dicken (1942).

On investigating the various factors affecting the growth of *L. casei*, we have modified the medium of Teply & Elvehjem (1945) with the aim of developing a suitable medium which could be used for assays of various vitamins.

MATERIALS AND METHODS

Stock solutions

Unless otherwise mentioned, the stock solutions were prepared according to techniques described by Snell (1950).

Salt solution E (Kodicek & Pepper, 1948*a*).

$MgSO_4.7H_2O$, 60 g.; $FeSO_4.7H_2O$, 4 mg.; $MnSO_4.4H_2O$, 1·5 g.; $CuSO_4.5H_2O$, 4 mg.; NaCl, 0·5 g.; $ZnSO_4.7H_2O$, 4 mg.

were dissolved in 250 ml. of water and 3 drops of conc. HCl added.

Casein hydrolysate: H_2SO_4-hydrolysed, vitamin-free 'Labco' casein. 'Labco' casein (200 g.) with 1 l. 25% (w/w) H_2SO_4, was autoclaved for 15 hr., at 15 lb. pressure. A litre of water was added, sulphate precipitated with about 850 g. $Ba(OH)_2$, the mixture autoclaved for 10 min. at 10 lb. pressure, and filtered hot through a Büchner funnel. The $BaSO_4$ cake was thoroughly broken up in 1 l. water, autoclaved and filtered hot. The precipitate was washed once more as before. The combined filtrates were heated and the excess barium was carefully precipitated with 25% H_2SO_4. The suspension was filtered hot, and the filtrate concentrated on a water bath to about 2500 ml. Dry weight determination gave a recovery of a little over 90%.

The hydrolysate at pH 6·0 was stirred for 1 hr. with 100 g. Norite charcoal (British Drug Houses Ltd.), filtered and the pH adjusted to 3·8 with glacial acetic acid. The charcoal treatment was repeated, the solution filtered and brought to pH 6·8–7·0 with 40% KOH. The hydrolysate was finally treated with Norite, 40 g., for 1 hr. The residue from each filtration was washed with 50–100 ml. of water and the washings added to the filtrate. The filtrate was concentrated on a water bath to contain 10% (w/v) casein solids. Recovery was 73% (casein solids). Before use, new batches of hydrolysate were always tested at several levels and the optimum concentration determined. An amount of 50 mg./10 ml. medium was usually found adequate. We have found it necessary to restandardize the adsorption treatment when a different brand of charcoal was used.

Peptone treated with Norite charcoal. 'Difco' bactopeptone (10 g.) was dissolved in about 80 ml. water, pH adjusted to 3·0 with HCl and made up to 100 ml. The solution was stirred with 5 g. Norite for 1 hr., filtered, and the charcoal treatment repeated twice with 2 g. Norite.

Pteroylglutamic acid (PGA). During the early part of the work 'Folvite' (Lederle Laboratories) was used as the PGA standard. Later, when a 97% pure and aldehyde-free preparation (Lederle Laboratories) became available it was used instead.

A stock solution of PGA containing 100 μg./ml. was prepared in 1% K_2HPO_4. It was renewed twice weekly. From the stock solution weaker standards were prepared for immediate use.

Bacteriological procedure

The double-strength medium, 5 ml., the composition of which is shown in Table 1, was diluted to 10 ml. with distilled water after adding the test solutions. The test substances were assayed usually in triplicate at each level. The tubes were placed in Petri dish canisters and autoclaved for 6 min. at 15 lb. pressure. Autoclaving for a longer period resulted in caramelization and consequent deterioration of the medium. On autoclaving, a slight precipitate resulted, but this redissolved on shaking at room temperature.

Stock culture. Cultures of *L. casei*, A.T.C.C. no. 7469, with characteristics reported by Kodicek & Pepper (1948*b*) were maintained on slopes of 'Difco' yeast-dextrose-agar supplemented with 'Hepamino' liver powder (Evans) 1% and glucose 0·5%. They were subcultured every 2–3 weeks. Each subculture consisted of two transfers from liquid to

liquid medium and incubation at 37° for 16–20 hr. The medium (see Table 1) supplemented with 1% 'Hepamino' liver powder was used. After the second transfer, slopes were prepared by loop transfers from the centrifuged bacterial sediment, incubated for 20–24 hr. at 37° and stored in the refrigerator at 4°.

Table 1. *Basal medium for* L. casei*

(The amounts refer to a concentration in 10 ml. of single strength medium. Normally a double strength medium was prepared and finally diluted with water or test solution. Norite-treated peptone is omitted for biotin and tryptophan assays. The pH was adjusted to 6·8.)

Casein (Labco), H_2SO_4-hydrolysed, hydrolysate	50·0 mg.
Peptone, Norite-treated	2·0 mg.
Glucose	300·0 mg.
Potassium acetate	300·0 mg.
DL-Alanine	1·0 mg.
L-Cystine	2·0 mg.
DL-Tryptophan	2·0 mg.
Adenine, guanine, uracil and xanthine (each)	0·1 mg.
Aneurin chloride hydrochloride	10·0 μg.
Riboflavin	10·0 μg.
Nicotinic acid	10·0 μg.
Pyridoxin hydrochloride	25·0 μg.
Calcium-D-pantothenate	10·0 μg.
Biotin (free acid)	0·04 μg.
p-Aminobenzoic acid	2·0 μg.
Pteroylglutamic acid	0·1 μg.
K_2HPO_4 and KH_2PO_4 (each)	25·0 mg.
Salt solution E	0·05 ml.

* The composition of the medium has been communicated to the Biochemical Society (Kodicek & Mistry, 1949*a*).

Preparation of the inoculum. For the inoculum, a procedure similar to that adopted for the subculturing was used, except that after the second transfer from liquid to liquid medium the centrifuged bacterial sediment was thoroughly washed two or three times and resuspended in saline.

The inoculum was standardized to contain 30 μg. dry weight bacteria per ml. and 2 drops were seeded aseptically into each assay tube. This inoculum contained about 2×10^6 viable cells as determined by a plate count.

Estimation of growth. The assay tubes were incubated in their racks at 37° for 72 hr. and growth was estimated by determining acid produced. The cultures were titrated with 0·1 N-NaOH, using bromothymol blue as internal indicator. The tubes were not disturbed during the incubation period.

RESULTS

Effect of peptone. Peptone appreciably improved growth in the presence of PGA. With all the levels tested the acid production in the blanks remained the same, indicating that the preparation was practically free from PGA. A concentration of 2 mg. was adopted for the medium.

The effect of peptone was finally investigated at several concentrations of PGA and the results are shown in Table 2. In the presence of peptone a better agreement between replicates was observed.

The other constituents, namely, salts, glucose, cystine, tryptophan, alanine, purine and pyrimidine solution and B vitamins, were tested individually in increasing amounts and a concentration allowing for optimal growth was adopted for the medium (Table 1). Asparagine was not included in the medium since it retarded growth (Kodicek & Mistry, 1949*b*).

Table 2. *Effect of Norite-treated peptone on PGA dose response*

PGA (mμg./10 ml.)	Final medium: No peptone	Final medium: With 2 mg. peptone/10 ml
	0·1 N-acid produced (ml./10 ml.)	
0·0	3·4	3·2
0·1	4·2	5·6
0·2	5·2	8·8
0·4	7·1	12·8
0·6	8·6	13·7
0·8	9·8	16·2
1·0	10·6	17·7
2·0	16·3	20·2
100·0	23·1	28·2
Liver control*	30·4	30·8

* Hepamino 100 mg./10 ml.

The assay of pteroylglutamic acid

The titrimetric dose response curve is shown in Fig. 1. When compared with the published results of Teply & Elvehjem (1945) and Roberts & Snell (1946), the medium gave a better response per unit of the vitamin added. The turbidimetric response curve after 42 hr. incubation is shown in Fig. 2. Shorter incubation periods were not satisfactory. However, the titrimetric method was preferred as it was simple and convenient.

The reproducibility of the results are shown in Table 3. The standard deviation, calculated for different levels of PGA, varied from ±0·431 to ±0·856, and the coefficient of variation ranged from about 3 to 13%.

Tests with PGA conjugates and related substances. Pteroic acid, rhizopterin, Diopterin (Pteroyl-α-glutamylglutamic acid), Teropterin (pteroyl-γ-glutamyl-γ-glutamylglutamic acid), and vitamin B_c conjugate (pteroylhexaglutamylglutamic acid) were tested at several levels.

Pteroic acid and rhizopterin were essentially inactive for *L. casei* as was reported previously (Jukes & Stockstad, 1948). Compared with PGA, Diopterin and Teropterin had on a molar basis an activity of 0·5 and 38%, respectively. The activity of the former agreed with that reported by Semb, Boothe, Angier, Waller, Mowat, Hutchings & Subbarow (1949), but Teropterin was found to be about 60% less active. On the other hand, B_c conjugate was shown to have 4·6% activity compared to 0·9–1·8% reported by Waller *et al.* (1948).

4-Hydroxypteridine, 2-amino-4-hydroxypteridine, 2-amino-4-hydroxy-6-pteridine carboxylic acid and 2-amino-4-hydroxy-7-pteridine carboxylic acid were tested individually at a concentration of 100 μg./10 ml. medium in absence and presence of PGA. None of them showed any activity, nor did they have any stimulatory or inhibitory effect in the presence of PGA.

The assay of other B vitamins and tryptophan

The medium was used for the assay of other B vitamins and the results are given below. The vitamin or amino-acid to be determined was omitted from the medium and was tested in graded amounts. Biotin and tryptophan were assayed in absence of

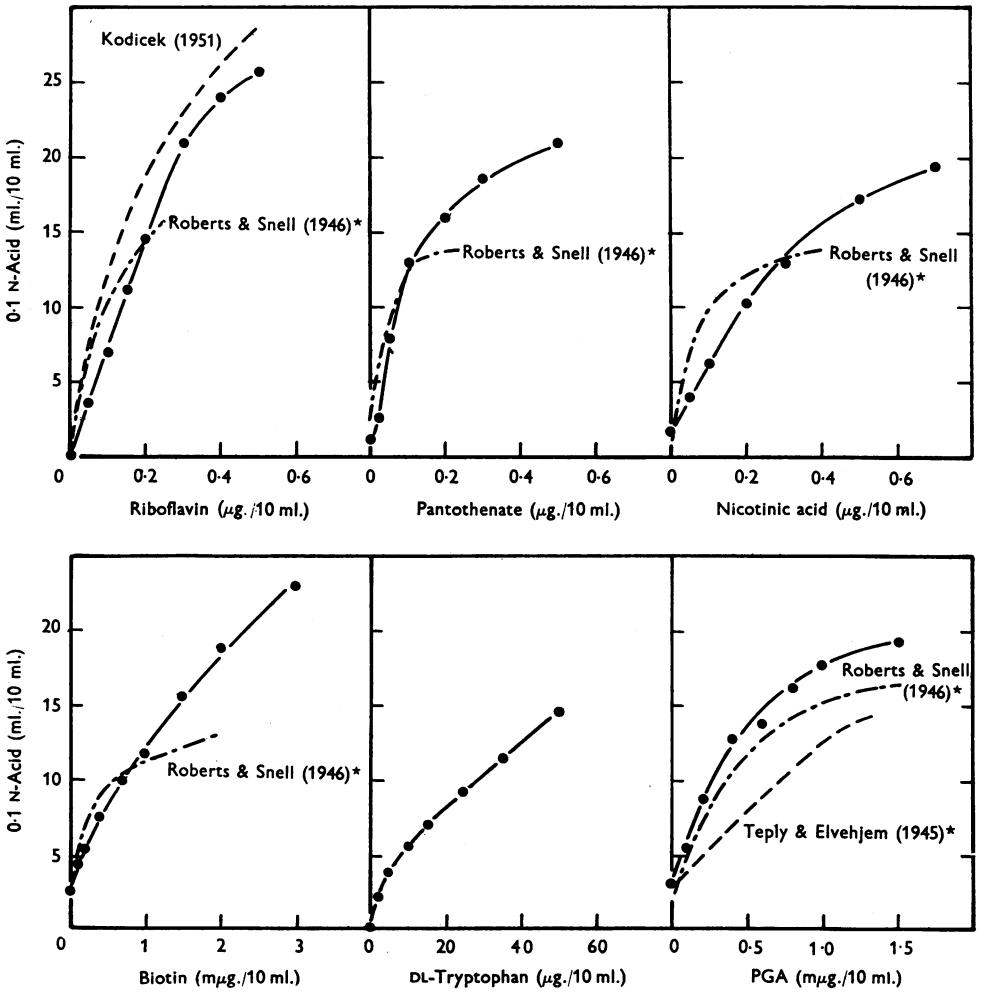

Fig. 1. Microbiological assays with *L. casei* (72 hr. titrimetric assay).
* Reproduced from original publications.

Table 3. *Response of* L. casei *to increasing concentrations of PGA*

Concn. of PGA (mμg./10 ml. medium)	No. of tubes	0·1 N-acid produced (ml.) Mean	Standard deviation	Coefficient of variation (%)
0	11	3·2	±0·431	13·4
0·2	11	8·8	±0·856	9·7
0·6	11	13·7	±0·702	5·1
1·0	11	17·7	±0·451	2·6

Norite-treated peptone. The results are shown in Fig. 1. For comparison, the dose-response curves from the published data of Roberts & Snell (1946) and Kodicek (1951) are also shown.

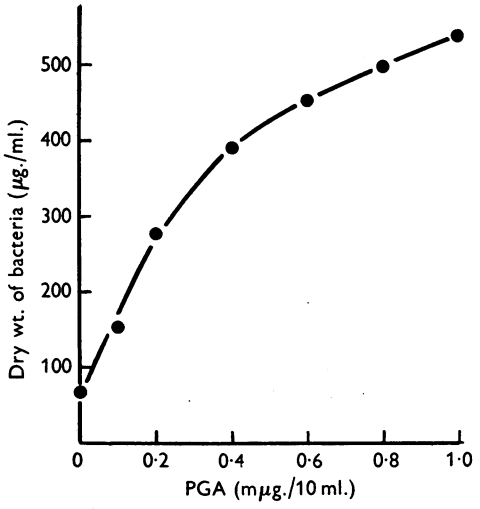

Fig. 2. PGA dose response curve with *L. casei* (42 hr. turbidimetric assay).

Nicotinic acid

As will be seen from Fig. 1, small amounts of nicotinic acid gave lower responses than those obtained with Roberts & Snell's (1946) medium. However, with the present medium the vitamin could be assayed over a much wider range of concentrations and the acid production using *L. casei* was higher than that reported by Kodicek & Pepper (1948*a*) with *L. arabinosus*. The medium has been repeatedly tested and the dose response curves showed little variation from experiment to experiment. The agreement between replicates at various levels of nicotinic acid was satisfactory (Table 4).

Extraction of nicotinic acid from test materials. A suitable extraction procedure for most cereals was found to be hydrolysis with N-NaOH for 45 min. on a water bath, and for other foodstuffs, treatment with N-H_2SO_4 at 15 lb. pressure for 1 hr., as described by Kodicek & Pepper (1948*b*). All extracts were washed with $CHCl_3$. This technique ensured a satisfactory extraction of active materials, and liberation of free nicotinic acid which was especially important with cereals which are known to contain a bound form of nicotinic acid (Krehl & Strong, 1944). For the assay of bound nicotinic acid, the material was extracted with 0·1 N-HCl for 1 hr. at 100°. This procedure did not liberate nicotinic acid from its bound form.

Table 4. *Variations between tubes in the assay of nicotinic acid*

Concn. of nicotinic acid (μg./10 ml. medium)	No. of tubes	0·1 N-acid produced (ml.)	Standard deviation	Coefficient of variation (%)
0	11	1·36	±0·13	9·6
0·10	14	4·88	±0·25	5·2
0·15	16	7·31	±0·27	3·7
0·20	16	9·06	±0·16	1·8
0·30	16	11·64	±0·22	1·9
0·50	16	15·50	±0·32	2·1

Table 5. *Comparison of microbiological and chemical estimations of nicotinic acid*

	Nicotinic acid					
	Microbiological method			Chemical method (Wang & Kodicek, 1943)		
Material	No. of estimations	Average result (μg./g.)	Standard error	No. of estimations	Average result (μg./g.)	Standard error
Yeast	8	221·0	±5·52	10	224·3	±13·74
Groundnuts	4	166·0	±3·89	6	167·7	±2·68
Kipper (dehydrated)	4	117·0	±3·19	6	122·0	±2·00
Muscle (beef, dehydrated)	8	103·0	±3·46	15	89·6	±4·83
Herring (dehydrated)	4	84·9	±1·79	6	81·1	±1·32
Malt extract, liquid	4	66·6	±1·85	6	64·3	±1·09
Whole wheat (ground)	4	46·8	±0·68	6	49·4	±0·50
Cocoa	4	18·2	±0·11	6	17·7	±0·65
Wheat meal flour (85% extraction)	7	17·0	±0·70	9	15·2	±0·56
Maize meal	4	13·1	±0·23	6	13·3	±0·20
Milk (dehydrated)	9	6·2	±0·14	8	4·8	±0·34
Tomatoes (fresh)	3	4·2	±0·07	5	3·6	±0·13

The results were calculated by using an 'internal standard' technique as described by Kodicek & Pepper (1948*b*).

The method has been applied to the estimation of nicotinic acid in a variety of foodstuffs and the results are shown in Table 5. It will be seen that the microbiological results are in good agreement with those obtained by a chemical procedure (Wang & Kodicek, 1943).

A number of brans has been assayed by this procedure to ascertain if *L. casei* responds to the bound form of nicotinic acid present in cereals (Table 6). It was found that the maximum response was obtained on alkaline hydrolysis, while 0·1N-hydrochloric acid extracts of wheat, rice and barley brans showed 10–20 % activity.

Table 6. *Microbiological assay of nicotinic acid in hydrolysed and unhydrolysed samples of various brans*

	Nicotinic acid (μg./g.)		
	Microbiological method		Chemical method (Wang & Kodicek, 1943)
Bran	Unhydrolysed	NaOH-hydrolysed	NaOH-hydrolysed
Rice	54·5	255	251
Wheat	42·9	219	204
Barley	36·9	202	233
Maize	19·1	31·8	29·6
Rye	9·8	19·1	20·4

On the other hand, when 0·1N-hydrochloric acid extracts of maize and rye brans were tested, more than 50 % of the total nicotinic acid activity was found. This may indicate either, that the bound form in maize and rye is different from the other brans tested, or that there was some free nicotinic acid in the extracts. The low activity for *L. casei* of the bound form of nicotinic acid was confirmed by testing a concentrate prepared according to Chaudhuri & Kodicek (1950) from wheat bran which contained by chemical estimation 35 mg. free nicotinic acid per gram of material. The microbiological results for the unhydrolysed and the hydrolysed material were 2·8 and 35·2 mg./g. respectively.

Riboflavin

The standard curve obtained with the present medium (Fig. 1), though quite satisfactory, was low compared to those of Roberts & Snell (1946) and Kodicek (1951). However, Kodicek's (1951) method was specially developed for the assay of riboflavin, and uses a semi-synthetic medium containing treated peptone and yeast extract which supplied additional growth stimulatory factors.

Calcium-D-pantothenate

The standard curve (Fig. 1) compared favourably with that of Roberts & Snell (1946). Except for a few assays on extracts of natural materials the medium has not been used for routine estimations of the vitamin.

Biotin and biocytin

The dose-response curve for biotin was considerably better (Fig. 1) than that of Roberts & Snell (1946). The linear portion of the curve extended up to 2 mμg. biotin/10 ml. medium.

Investigations of earlier workers indicated the existence of a complex of biotin in natural materials. Recently a bound form of the vitamin, termed biocytin, has been isolated in a crystalline form from yeast extract and its biological characteristics have also been described (Wright, Cresson, Valentik & Skeggs, 1950).

A concentrate of biocytin was tested on the basis of its biotin content in increasing concentrations from 0 to 5 mμg./10 ml. For comparison, crystalline biotin (free acid) was also tested at the same levels. At all concentrations crystalline biotin and the biocytin concentrate had essentially the same activity.

Tryptophan

A satisfactory linear response was obtained (Fig. 1) over a wide range of concentrations. Since only *L. arabinosus* has been used for the assay of this amino-acid by other workers, no comparative data can be given.

DISCUSSION

The medium, though primarily developed for the determination of pteroylglutamic acid with *L. casei*, was also found satisfactory for other B vitamins.

Most published media for the assay of nicotinic acid, pantothenic acid, and biotin, have preferred *L. arabinosus* to *L. casei* as the test organism (Snell, 1950). The choice of *L. arabinosus* was influenced by its simpler nutritional requirements. However, of the two organisms, *L. casei* has a greater acid-producing potential which increases the convenience and accuracy of titrations, and is also more sensitive to small increases in the vitamin under test, provided that the medium employed is optimal or at least near optimal. The proposed procedure is convenient in that a single basal medium and a single organism suffice for an assay of five vitamins and tryptophan. Furthermore, the same medium with minor modifications has been used for the assay of strepogenin activity (Mistry & Kodicek, 1951).

The medium showed good possibilities for the assay of biotin. Biocytin, a naturally occurring bound form of biotin, was active for *L. casei* which confirmed the findings of Wright, Cresson, Skeggs, Wood, Peck, Wolf & Folkers (1950).

Nicotinic acid has been almost exclusively assayed with *L. arabinosus* as the test organism. Of the more

recent methods, that described by Kodicek & Pepper (1948*b*) had been in use in this laboratory, but the present medium with *L. casei* has completely replaced the former procedure and was found to give more satisfactory results.

In the assay of pteroylglutamic acid the conjugates, with the exception of Teropterin, showed an activity in general agreement with the findings of other workers.

SUMMARY

1. An improved medium was developed for the assay of pteroylglutamic acid with *Lactobacillus casei* and was tested for some other B vitamins and tryptophan.

2. The titrimetric dose-response curves for riboflavin, nicotinic acid, calcium pantothenate, biotin and tryptophan were satisfactory.

3. The medium has been used in particular for the microbiological assay of total nicotinic acid, and the results were in good agreement with the chemical values. The bound form of nicotinic acid present in cereals was found to be less active than the free vitamin.

4. A number of conjugates of pteroylglutamic acid and other pteridines were tested.

We wish to thank Dr A. Albert for the synthetic pteridines, Dr T. H. Jukes for pteroylgutamic acid and conjugates, Dr J. J. Pfiffner for the vitamin B_c conjugate, Dr L. D. Wright for the biocytin concentrate, Dr K. Folkers for biotin and rhizopterin, and the Flour Millers Association for the supply of the different brans.

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Specificity of Glucose Oxidase (Notatin)

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The antibiotic 'notatin' isolated in a very highly purified state from culture medium filtrates of *Penicillium notatum* by Coulthard, Michaelis, Short, Sykes, Skrimshire, Standfast, Birkinshaw & Raistrick (1942, 1945) was shown by these authors to be identical with the glucose oxidase discovered by Müller (1928, 1941) in cultures of different moulds (for a summary of the literature, see Keilin & Hartree, 1948*a*). The enzyme is a flavoprotein (Coulthard *et al.* 1945) and the prosthetic group has been identified as alloxazine-adenine-dinucleotide (Keilin & Hartree, 1946).

A study of the catalytic activities of this enzyme revealed its very high specificity for glucose (Keilin & Hartree, 1948*a*) which is quantitatively oxidized to gluconic acid. Previous workers had shown that the reaction proceeds according to the equation

$$C_6H_{12}O_6 + O_2 + H_2O = C_6H_{12}O_7 + H_2O_2.$$

In presence of catalase, which decomposes the peroxide to oxygen and water, the overall reaction is

$$C_6H_{12}O_6 + \tfrac{1}{2}O_2 = C_6H_{12}O_7.$$