https://doi.org/10.1079/BJN19820026 Published online by Cambridge University Press

Studies on the response of *Lactobacillus casei* to different folate monoglutamates

By D. R. PHILLIPS AND A. J. A. WRIGHT

Agricultural Research Council, Food Research Institute, Colney Lane, Norwich, Norfolk NR4 7UA

(*Received 28 September 1981 – Accepted 2 October 1981*)

1. The response of *Lactobacillus casei* was measured for a number of the monoglutamyl forms of folate derivatives.

2. At the concentrations of folate commonly used in the assay of folate vitamin in foods the response of *L. casei* to folic acid, (pteroyl glutamic acid) and 5-formyl-tetrahydrofolic acid was similar, but 5-methyl-tetrahydrofolic acid gave as little as half the response of folic acid.

3. The response was modified by altering pH but not by concentration of ascorbate.

4. These results have implications for the assays of foods for folate where mixtures of folate derivatives are present.

5. A modified procedure is suggested in which the monoglutamates give similar responses.

One of the most widely used methods for measurement of folate in food is microbiological assay using *Lactobacillus casei.* The organism shows a reduced response to polyglutamate forms of folate with more than three glutamate residues (Tamura *et al.* 1972) and the standard assays therefore incorporate a conjugase treatment to convert the polyglutamates present to mono- and diglutamates (Bell, 1974; Malin, 1974). The response of *L. casei* to different monoglutamyl forms has been shown to vary (Stokstad & Oace, 1965; Bird & McGlohan, 1972) and the greatest differences were seen at concentrations of folate, up to 5 ng/10 ml assay (Ruddick *et al.* 1978). Some authors have drawn attention to problems in the interpretation of results from *L. casei* analyses on the mixtures of folates such as occur in foods (Bird & Robbins, 1946; Tamura *et al.* 1972) the major fault being described as 'positive drift', where the responses to serial dilutions of an extract are not proportional to each other.

During the course of the development of high performance liquid chromatography procedures for the separation and estimation of folates in foods, we had occasion to apply microbiological assay to the different monoglutamyl folates and this has focussed our attention on the lack of quantitative information on the response of *L. casei* to the different forms.

The response of *L. casei* to 5-methyl-tetrahydrofolic acid at the concentration of folate used (0–1 ng/assay) in several procedures (Waters & Mollin, 1961; Bell, 1974), and by Difco Laboratories (West Molesey, Surrey) in the instructions for use of their Folic Acid Casei Medium, was found to be low when compared with folic acid and 5-formyl-tetrahydrofolic acid. As 5-methyl-tetrahydrofolates are often the most important form in foods (Scott & Weir, 1976), reduced response in the procedures used would result in an underestimation of the total monoglutamyl form after conjugase treatment. This paper describes a study of the microbiological assay procedure undertaken in order to establish the cause of this reduced response to 5-methyl-tetrahydrofolic acid.

EXPERIMENTAL

Materials. Folic acid (pteroylglutamic acid), DL-5-methyl-tetrahydrofolic acid and DL-5-formyl-tetrahydrofolic acid were obtained from Sigma (London) Chemical Co. Ltd. The

stated purities were 99–100, 90 and 93% respectively. The purity was checked by comparison of the u.v. absorption *v*. the molar extinction coefficients quoted by Maruyama *et al.* (1978) which are 23400 at 283 nm for folic acid, 37200 at 285 nm for 5-formyl-tetrahydrofolic acid and 31700 at 290 nm for 5-methyl-tetrahydrofolic acid. A confirmation of purity was obtained using HPLC on a Partisil-10-SAX (Whatman) column using 0·05 M-phosphate pH 6·0 as eluting buffer at 1 ml/min and 1000 p.s.i. ($6{\cdot}894 \times 10^6$ Pa) and the folates used were shown to be homogeneous. Standard solutions of the folates were prepared in ascorbic acid (1 g/l, 5·7 mM) and the assay medium contained (1 g/l 5·7 mM) ascorbate at final concentration.

Preparation of medium. In the initial stages the Folic Acid Casei Medium, supplied by Difco Laboratories, was used. In later studies on the composition of the medium the basal medium was prepared as described by Jukes (1955). The medium when prepared was dispensed into tubes and autoclaved before the aseptic addition of the folate standards (Herbert, 1966). Sterility was maintained by use of Clark Fincaps from Clark Scientific Ltd, New Malden, Surrey. During the autoclaving stage the pH of the medium dropped slightly (max 0·5 pH unit at pH 7·4) and the values of pH stated for the medium in the Figs. and text always relate to this lower pH value which was the true starting pH value for the incubation. Addition of standard solutions of folates containing 1 g ascorbic acid/l (5·7 mM) at pH 6·0 had no further effect on these values.

Maintenance of cultures. L. casei (NCIB6375) was maintained as a stab culture in Bacto-Lactobacilli Agar, from Difco Laboratories, according to the scheme of Cooperman (1960) in order to ensure stable and constant growth characteristics. The stabs were incubated at 37° for 18 h then stored at 4°. Inoculum for the assay was made by subculturing from stab-culture to 10 ml sterile Bacto-Micro-Inoculum Broth (Difco Laboratories) prewarmed to 37°. After incubation for 18 h at 37° the contents were thoroughly mixed and 100 μl transferred to 10 ml prewarmed sterile single strength folic acid basal medium to which 1 ng folic acid had been added aseptically from a freshly prepared 4 ng/ml solution. The inoculum tubes were incubated for 6 h at 37° then mixed and 100 μl transferred to 10 ml prewarmed sterile folic acid basal medium. This dilute inoculum (100 μl) was used to inoculate each assay tube.

Microbiological assay. The microbiological assay was performed, essentially by the method of Bell (1974), as follows. Single strength folic acid basal medium (10 ml) after pH adjustment where necessary, was measured into test-tubes which were then capped and autoclaved 5 min at 15 p.s.i. (103×10^3 Pa) (121°). The folates were then added to duplicate tubes from freshly-prepared solutions (maximum volume added was 250 μl) by the aseptic technique described by Herbert (1966). Consideration was given, when making up standard solutions of the DL-5-formyl-tetrahydrofolic acid and DL-5-methyl-tetrahydrofolic acid, to the fact that *L. casei* responds only to the L-form in those racemic mixtures (Shane *et al.* 1980). The assays were started by the addition of 100 μl dilute inoculum. The assay tubes were incubated for 18 h at 37°. The growth in the tubes was estimated using an EEL Nephelometer with a neutral filter. The duplicate tubes were mixed on a vortex mixer with a 20 s time-lapse before reading so that any birefringence had decayed but before sedimentation started. Care was taken to use scratch-free tubes.

Initially, the response of *L. casei* to folate monoglutamates was measured using Folic Acid Casei Medium from Difco Laboratories, at single strength with the ascorbic acid concentration at 0·25 g/l (1·4 mM) as recommended by Difco Laboratories. Thereafter the basal medium of Jukes (1955) was used. Following initial observations when the concentration of the ascorbic acid was varied between 0·25 g/l (1·4 mM) and 10 g/l (56·8 mM) a suitable concentration at 1 g/l (5·7 mM) was chosen and all further studies were performed at that concentration. In the studies of the effects of altering pH on *L. casei*

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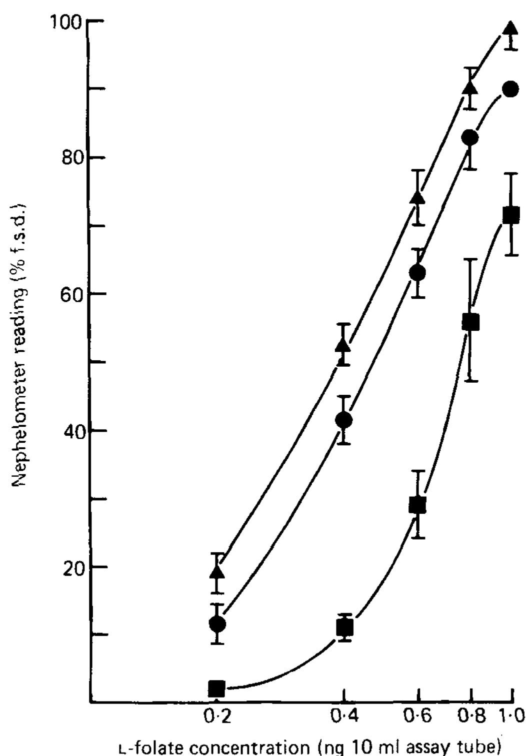

Fig. 1. Calibration curve for *Lactobacillus casei* response to folic acid (●——●), 5-formyl-tetrahydrofolic acid (▲——▲) and 5-methyl-tetrahydrofolic acid (■——■). The curves were produced using Difco Laboratories Folic Acid Casei Medium at pH 6·8. Points are mean values of duplicate tubes and standard deviations represented by vertical bars are for six separate assays. Values on the nephelometer were read off a scale set at 90% deflection for the growth produced by 1 ng folic acid.

response, the media of different pH were prepared by adjusting the pH with 1 M-acetic acid or 1 M-NaOH before dispensing into the assay tubes. The tubes were then autoclaved, and the pH values, checked in control tubes, gave the actual starting pH of the incubation. To study the effect of pH on the relationship between the response curves for folic acid and 5-methyl-tetrahydrofolic acid, medium at three pH values was prepared, i.e., pH 6·2, 6·8 and 7·4. After autoclaving the actual pH of the latter two had dropped to pH 6·5 and 6·8 respectively. For the pH optimum curves for folic acid and 5-methyl-tetrahydrofolic acid a range of media of different pH values ranging from 5·6 to 8·0 was prepared. After autoclaving this range dropped to 5·6–7·1.

RESULTS

Response of L. casei *to different folate monoglutamates.* The range of 0–1 ng/10 ml tube (Waters & Mollin, 1961; Bell, 1974 and Difco Laboratories) gives a near linear response to folic acid and gives good sensitivity (Fig. 1). It is also a convenient range for the assay of the frequently low concentrations in extracts from foods which can be assayed without the inherent difficulties of concentration procedures.

The response curves in this range, using the Difco Laboratories medium at pH 6·8, were measured for folic acid, 5-formyl-tetrahydrofolic acid, and 5-methyl-tetrahydrofolic acid (Fig. 1). With the latter two, these were corrected to allow for the fact that *L. casei* responds only to the L-form in the DL-racemic mixtures commercially available (Shane *et al.* 1980).

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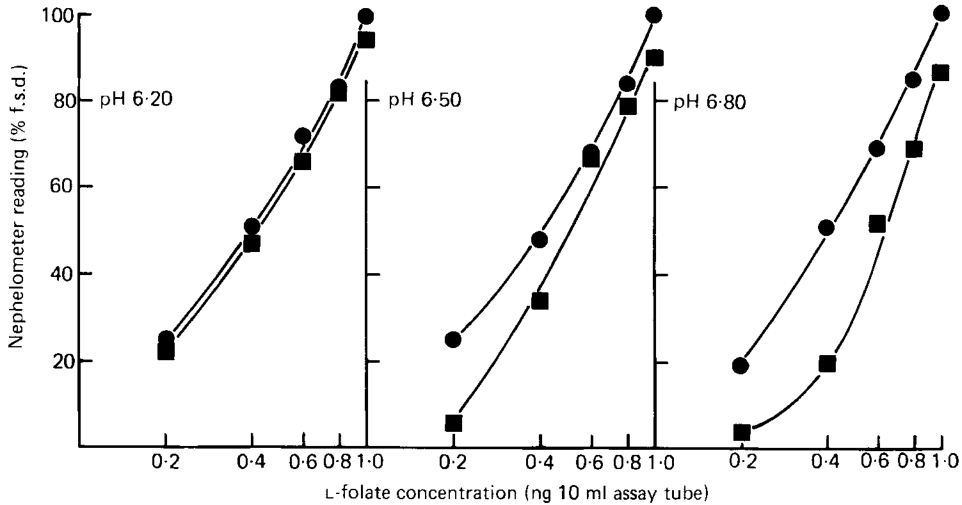

Fig. 2. Calibration curves for *Lactobacillus casei* response to folic acid (●——●) and 5-methyl-tetrahydrofolic acid (■——■). The curves were produced using Jukes' (1955) medium at initial assay pH values of 6·20, 6·50 and 6·80. Points are mean values of duplicate tubes. The values on the nephelometer were read off a scale set at 100% deflection for the growth produced by 1 ng folic acid.

Each series was run in duplicate on several separate occasions and the three curves consistently showed the relationships to each other seen in Fig. 1. The results were plotted on semi-logarithmic paper with the folate concentration, on the abscissa, as the logarithmic plot. It was noted that at concentrations of 1 ng folate, sufficient lactic acid was produced by *L. casei* growth to lower the initial pH from pH 6·8 to pH 5·4.

Effect of ascorbic acid concentration. Initial measurements were made with the medium containing 0·25 g ascorbic acid/l (1·4 mM) as recommended by Difco Laboratories, and later measurements using 1 g ascorbic acid/l (5·7 mM) (Herbert, 1966). The response curves at both concentrations were identical to those in Fig. 1. At the 1 g/l level the 10 ml assay medium contains 56·8 pmol ascorbic acid the purpose of which is to maintain the reduced state of 2·3 μmol 5-methyl-tetrahydrofolic acid at the upper end of the concentration range. A final series of incubations containing 10 g ascorbic acid/l (56·8 mM) in the standard solutions and in the medium, was performed for the three folate monoglutamates. These were again identical with the three curves seen in Fig. 1.

Effect of pH on L. casei *responses.* Basal medium at three different pH values was prepared, i.e. pH 6·2, 6·5 and 6·8. The folate standards in the range 0–1 ng/10 ml assay tube were then added aseptically and assayed.

The results seen in Fig. 2 show that as the starting pH is lowered so the response of the *L. casei* to 5-methyl-tetrahydrofolic acid moves closer to the response shown to folic acid. At pH 6·2 the response shown in the two curves is almost identical.

pH optima for growth response. A range of different pH values from 5·6 to 7·1 was prepared. Folate standards at concentration 0·5 and 1·0 ng/10 ml assay tube were added aseptically and the assays were performed as described.

The pH profiles seen in Fig. 3 demonstrate that the response of *L. casei* to 5-methyl-tetrahydrofolic acid falls off more rapidly at the upper end of the pH range than that for folic acid. The effect is shown most obviously at the 0·5 ng level, which is the middle of the accepted assay range.

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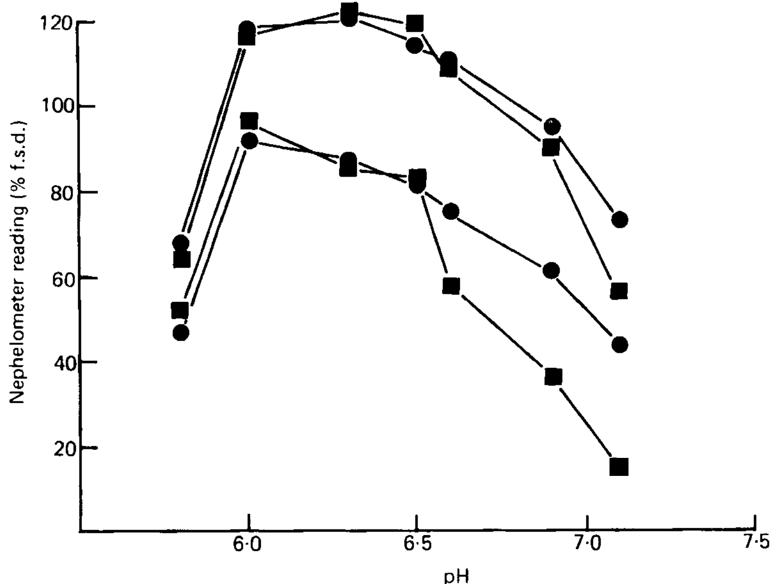

Fig. 3. *Lactobacillus casei* response to 0·5 ng and 1·0 ng folic acid (●——●), and 0·5 ng and 1·0 ng 5-methyl-tetrahydrofolic acid (■——■). The pH response curves were produced using Jukes' (1955) medium with initial assay pH values over the range shown. Points are the mean values of duplicate tubes. The values on the nephelometer were read off a scale set at 90% deflection for the growth produced by 1 ng folic acid at pH 6·8.

DISCUSSION

Several authors have commented on the responses of *L. casei* to different monoglutamate forms of folate. In particular Stokstad & Oace (1965) publish a table of relative activities using plus or minus signs designating 70–100% activity with the plus and less than 5% with the minus sign, and both folic acid and 5-methyl-tetrahydrofolic acid are given plus signs. Bird & McGlohan (1972) quote in their table of specific growth activities of folates with *L. casei* values of 100% for 5-formyl-tetrahydrofolic acid and 5-methyl-tetrahydrofolic acid, and 120% for folic acid. Also, a very marked reduction in the response to 5-methyl-tetrahydrofolic acid compared with folic acid has been shown (Ruddick *et al.* 1978) as much as 55% lower, but in this instance the folate concentrations in the incubations rose over five times higher than the 1 ng/10 ml assay stated here.

The Bell (1974) method was used as a basis for the assay procedure except that the standards were not autoclaved but added later by aseptic addition (Herbert, 1966), and the ascorbate concentration was increased from 0·25 to 1 g/l in the standard solutions and in the incubation medium. These changes were made to ensure that the folate did not deteriorate due to heat or by atmospheric oxidation. The presence of 1 g ascorbate/l has been found to have marked stabilizing effect on 5-methyl-tetrahydrofolic acid (Chen & Cooper, 1979), only 20% of folate activity being lost after 3 h at 100°.

The formulation of the media generally accepted for the *L. casei* microbiological assay, described by several authors (Jukes, 1955; Baker *et al.* 1959; Waters & Mollin, 1961), are almost identical in every respect, and therefore, all share the same low buffering capacity at pH 6·8. Growth of *L. casei* on 1 ng folate causes the production of enough lactic acid to produce a significant drop in pH from an initial pH 6·8 to pH 5·4 at the end of the incubation period. Although it can be seen (Fig. 3) that they share a common pH optimum range (approximately pH 6·0–6·2), the pH response curves for the different folate forms

are not the same. For an initial assay pH higher than the optimum range (pH 6·8) (Fig. 1) the discrepancy in *L. casei* growth response is greater at low than at high concentrations. For example, to produce an equivalent growth to 0·2 ng folic acid, approximately 0·5 ng 5-methyl-tetrahydrofolic acid (+150%) is needed whereas, for an equivalent growth to 0·7 ng folic acid, only 1·0 ng 5-methyl-tetrahydrofolic acid (+40%) is required. The 'positive drift' phenomenon, seen in assays with initial pH values greater than 6·2 (e.g. pH 6·8, Fig. 1) can be explained as follows. When the folate concentration is at the lower end of the standard range (0–1 ng) the growth response of *L. casei* to 5-methyl-tetrahydrofolic acid is poor in comparison to folic acid, little lactic acid is produced and the final pH of assay is little different from the initial pH. However, at intermediate folate concentrations the *L. casei* growth response to 5-methyl-tetrahydrofolic acid is enough to cause sufficient lactic acid production such that the initial assay pH starts to fall, giving better *L. casei* growth. Moreover, at concentrations of folate slightly greater than 1 ng, *L. casei* growth to 5-methyl-tetrahydrofolic acid is sufficient to cause a rapid fall in initial pH such that absolute growth after 18–22 h incubation appears little different to that recorded for folic acid. By increasing the buffering capacity of the medium and reducing its starting pH to a point nearer the optimum (pH 6·2) for 5-methyl-tetrahydrofolic acid, a series of folate response curves is produced (Fig. 2, pH 6·2) which are almost identical, so that the relative proportion of the different derivatives of folates in a foodstuff becomes of minor importance.

The importance of the reduced response shown by *L. casei* to the 5-methyl-tetrahydrofolic acid is that in some foodstuffs important in the UK diet for their folate contribution, a very high proportion is present as 5-methyl-tetrahydrofolates. For example, in cabbage 96% is present, mainly as long chain polyglutamates (Chan *et al.* 1973), in milk 90%, mainly as monoglutamate (Shin *et al.* 1975) and in lettuce 72%, as monoglutamate and pentaglutamate (Batra *et al.* 1977).

If the assay is extended to measure the contribution to the diet from the polyglutamate folate present in the food, deconjugation by incubation with deconjugase enzyme is necessary prior to the assay. The contribution of the polyglutamate folate to the human diet has been the subject of considerable controversy over the years, but recent work has demonstrated that polyglutamates, ingested at normal dietary concentrations, have a similar availability to monoglutamates (Rosenberg *et al.* 1969; Tamura & Stokstad, 1973; Halsted, 1979). A recent publication clearly demonstrates the presence of two highly-active intestinal deconjugases in the human jejunal mucosa (Reisenaur *et al.* 1977).

The results shown in Fig. 1 for folic acid and 5-methyl-tetrahydrofolic acid give some explanation for the condition known as 'positive drift' shown in curves when three or four serial dilutions of a food extract are assayed by *L. casei.* The effect in food extracts could possibly have been attributed to the presence of a second growth-stimulating growth factor in the extract, but here the curve shape has been demonstrated using standards of known purity and under excessively stabilized conditions of incubation. If an extract contains a major proportion of the folate as 5-methyl-tetrahydrofolic acid the plot of serial dilutions will not be linear but will be closer to the curve shown by 5-methyl-tetrahydrofolic acid. Therefore it would be necessary to know the proportions of the different forms of folate in a foodstuff before its folate content could be assigned from the results of the microbiological assay as used at present. By exercising proper control on the pH and buffering capacity of the microbiological medium as shown in Fig. 2 i.e. by reducing the starting pH of the incubation to pH 6·2 this need is avoided since the two major forms expected in a foodstuff, i.e. 5-methyl-tetrahydrofolic acid and 5-formyl-tetrahydrofolic acid (Scott & Weir, 1976) can thus be quantified using the same calibration curve.

A number of factors are involved in the correct assessment of the folate activity in the

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diet. Our results indicate that the procedure used in the compilation of the new edition of *McCance & Widdowson's, The Composition of Foods* (Paul & Southgate, 1978) may be affected by one of these factors, because it appears that a major portion of the folates, in the foods from which we derive most of our dietary folate, is present as polyglutamyl forms of 5-methyl-tetrahydrofolic acid. Results based on *L. casei* assay using folic acid as standard will underestimate the dietary intake of folate unless care is taken to control the pH and buffering capacity of the medium. At present all values for folates in foods are in a sense based on arbitrary procedures and the final justification of any procedure for the measurement of folate activity can only be that the method predicts the biological activity in man (Paul & Southgate, 1978). Whenever a microbiological procedure is used it must be remembered that the actual results obtained may be profoundly affected by the precise conditions under which the assay is conducted.

The authors thank Dr D. A. T. Southgate of this Institute for his interest and helpful discussion, Miss Paula Noble for valuable technical assistance and Mrs J. Gee for expert advice and assistance in the initial setting up of the microbiological assay.

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Printed in Great Britain

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