Studies on the response of *Lactobacillus casei* to folate vitamin in foods

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1. Folate was measured microbiologically with *Lactobacillus casei* in extracts from a range of different foods at two incubation pH values, 6·2 and 6·8.

2. The values for folate content obtained at pH 6·2 were, in several instances, considerably higher than at pH 6·8. The 'positive drift' seen in the values for dilutions incubated at pH 6·8 were absent from results at pH 6·2.

3. A comparison was made of the ability of the two main sources (hog kidney and chicken pancreas) of deconjugase enzyme to produce measurable folate and the hog-kidney enzyme was shown to produce consistently higher values.

4. The results presented here will have significance for studies of folate intake using calculations from food composition tables, in the light of the apparent discrepancy between calculated folate intake and the recommended daily allowances for the UK.

A microbiological assay method using *Lactobacillus casei* is widely used for the measurement of folate vitamin in foods. Most of the folate in foods exists as polyglutamate forms, which do not produce a growth response in *L. casei* comparable with that for the monoglutamate forms (Tamura *et al.* 1972). The standard assay uses a deconjugase treatment to convert any polyglutamates present to mono- or diglutamyl forms (Bell, 1974; Malin, 1974). The commonly-used sources for the deconjugase are hog kidney, producing monoglutamate (Bird & McGlohon, 1972), and chicken pancreas which produces the diglutamate forms (Leichter *et al.* 1977). The pteroyl moities of the polyglutamates in foods are in reduced and substituted forms, mainly as the 5-methyl-tetrahydro-pteroyl or the 5-formyl-tetrahydro-pteroyl derivatives (Scott & Weir, 1976).

A recent publication (Phillips & Wright, 1982) demonstrated a notably lower response in *L. casei* for the 5-methyl-tetrahydro-pteroylmonoglutamate than for pteroylglutamic acid, commonly used as standard in the *L. casei* assay procedure, or for 5-formyl-tetrahydro-pteroylmonoglutamate. This was demonstrated for the range 0–1 ng folate per assay, the range used in the compilation of the tables in *McCance and Widdowson's The Composition of Foods* (Paul & Southgate, 1978). The response was shown to be dependent on the pH at which the microbiological assay incubation was performed. By lowering the pH of incubation from 6·8 to 6·2 the 5-methyl-and 5-formyl-tetrahydro-pteroylmonoglutamates produced similar growth responses in *L. casei*, as did pteroylglutamic acid. In this paper we present results obtained when a range of foods was assayed at the two incubation pH levels, 6·2 and 6·8, and demonstrate a significant increase in the values obtained for all the foods when assayed at the lower pH value.

The relative ability of deconjugases from the two main sources, hog kidney and chicken pancreas, to deconjugate the polyglutamates from a range of foods to produce measurable folate has been compared, showing that higher values for folate content are obtained when the enzyme from hog kidney is used. In a recent publication Spring *et al.* (1979) showed that total food folate values calculated from the food composition tables yielded daily

intakes of between 130 and 300 μg/d for typical members of the UK population, which are lower than the amount recommended (Department of Health and Social Security, 1979). The evidence presented in the present paper demonstrates that a significant portion of this discrepancy may be due to the method used to obtain the folate values used in the calculations.

EXPERIMENTAL

Materials

An acetone preparation of hog kidney was obtained from Sigma (London) Chemical Co. Ltd, Poole, Dorset, and Darco G-60 charcoal was obtained from BDH Chemicals, Poole, Dorset; the chicken pancreas preparation, Folic Acid Casei Medium, and the Bacto-Lactobacilli Agar AOAC and Micro-Inoculum Broth, were obtained from Difco Laboratories, West Molsley, Surrey. Dextran, molecular weight 40000, and folic acid (pteroylglutamic acid) of 99–100% purity, were obtained from Sigma (London) Chemical Co. Ltd. A stock solution of folic acid was prepared by dissolving 20 mg in a few drops of 1 M-sodium hydroxide, neutralizing with an equivalent amount of 1 M-hydrochloric acid and making to 100 ml with 57 mM-ascorbic acid adjusted to pH 6·0 with NaOH. Before assay a working solution containing 4 ng/ml was prepared by diluting the folic acid stock solution with fresh 5·7 mM-ascorbic acid, pH 6·0.

Preparation of food extracts

Diced food samples, approximately 1–10 g, were dropped into boiling 57 mM-ascorbic acid, pH 6·0, for 5 min. After cooling, the samples were homogenized, made up to 100 ml volume with 57 mM-ascorbic acid, pH 6·0, and centrifuged at 40000 ***g*** for 30 min. Supernatant fractions were stored at −20°.

Preparation of deconjugase enzymes

The hog-kidney preparation was made by homogenizing 10 g of the acetone powder with 100 ml L-cysteine hydrochloride (10 g/l) which had been adjusted to pH 4·6 with NaOH. The homogenate was incubated for 4 h at 37°, then centrifuged at 40000 ***g*** for 30 min. To the supernatant fraction was added 20 ml of Darco G-60 charcoal and dextran mixture, prepared by adding 1 g dextran to 10 g charcoal in a final volume of 100 ml distilled water. After 30 min at room temperature this was centrifuged and the supernatant fraction made up to 100 ml with L-cysteine hydrochloride (10 g/l), pH 4·6, and stored at −20°. The enzyme from chicken pancreas was prepared as described by Bell (1974).

Deconjugation of food extracts

The hog-kidney deconjugation system consisted of 3·74 ml 57 mM-ascorbic acid, pH 4·6, 0·25 ml food extract with 1 ml enzyme. The chicken pancreas system consisted of 3·75 ml 57 mM-ascorbic acid, pH 6·0, 0·25 ml food extract with 1 ml enzyme. These systems were incubated for 6 h at 37° and then stored at −20° until assayed.

Preparation of medium

Folic Acid Casei Medium was prepared at single strength with added 5·7 mM-ascorbic acid. The pH of the medium was adjusted to pH 6·2 before dispensing 10 ml per assay tube, capping with Fincaps (Clark Scientific Ltd, New Malden, Surrey) and autoclaving for 5 min at 15 p.s.i. (103×10^3 Pa) at 121°. The medium at pH 6·8 was prepared by adjusting the pH of the whole medium with 1 M-NaOH to pH 7·2 before autoclaving. The autoclaving caused the slight drop in pH to 6·8.

Microbiological assay

The maintenance of the cultures of *L. casei* (NCIB 6375) and the preparation of inoculum was as previously described (Phillips & Wright, 1982). Deconjugated food extracts and deconjugase enzyme blanks were added to duplicate medium tubes, maximum addition 250 μl, by the aseptic addition technique described by Herbert (1966). Assays were started by the addition of 100 μl of the diluted inoculum and incubated for 22 h at 37°. Pteroylglutamic acid, 0–1 ng/10 ml assay tube, was used for the calibration curve which was run simultaneously with each group of samples. Growth of *L. casei* was measured using an EEL Nephelometer with zero set against an incubated uninoculated medium blank and 90% full scale deflection set with the calibration standard containing 1 ng pteroylglutamic acid. Tubes were thoroughly mixed on a vortex mixer with a 20 s time-lapse before reading, so that any birefringence had decayed, but before sedimentation started. The calibration curve was plotted on semi-logarithmic paper with the standard concentration on the abscissa, as the logarithmic plot.

RESULTS

Comparison of assays at pH 6·2 and 6·8

After deconjugation using the enzyme preparation from hog kidney, serial dilutions of extracts from a range of foods were assayed at two incubation pH values. The assays were performed at pH 6·8 as recommended by Bell (1974), Waters & Mollin (1961) and by Difco in the instructions for use of their Folic Acid Casei Medium, and at pH 6·2 as recommended by Phillips & Wright (1982). The results are shown in Table 1. In every case the results at pH 6·8 showed marked 'positive drift' where the values obtained with the lower sample volume appear to show a significantly lower concentration of folate in the extract than that seen at the higher volumes. For several extracts the folate concentrations determined at pH 6·2 were consistently higher than at pH 6·8. Table 1 also lists the values given in *McCance and Widdowson's The Composition of Foods* (Paul & Southgate, 1978) for the level of total folate in these foods where the values quoted were obtained using the method described by Bell (1974) at pH 6·8 with chicken-pancreas deconjugase enzyme.

Comparison of deconjugases from hog kidney and chicken pancreas

Partially-purified enzyme preparations from freeze-dried hog kidney and chicken pancreas were used to compare the ability of enzymes from these sources to produce deconjugated folate. Samples of an extract from frozen garden peas were deconjugated with a range of concentrations of deconjugase enzyme from both sources. The deconjugated products were then assayed for folate content with *L. casei* at pH 6·2. The results are shown in Fig. 1. The rate of production of assayable folate with chicken pancreas was faster at lower enzyme concentrations, probably due to a slightly higher activity. However, at higher concentrations of enzyme the hog kidney preparation produced significantly higher values and both curves reached a plateau. The evidence of a markedly-greater response of *L. casei* to the deconjugation product using hog-kidney enzyme is further reinforced by the values in Table 2, which shows the results obtained when extracts from a range of foods were deconjugated with the 1 ml quantity of enzyme, where the response in the enzyme concentration curve had levelled off for both sources of enzyme. In every case the use of hog-kidney enzyme resulted in a higher value for the folate content of the food. In the case of the Marmite® sample the value obtained was nearly double that obtained using chicken-pancreas enzyme. This very much greater value may possibly be due to the inhibitor of chicken-pancreas deconjugase known to be present in yeast extracts (Mims *et al.* 1947).

Table 1. *Folate contents of food extracts prepared in 57 mM-ascorbic acid at 100°, when conjugation was performed using the hog-kidney enzyme preparation and assays were performed using* Lactobacillus casei *at the incubation pH values of 6·2 and 6·8*

	Folate (μg/100 g)						
Incubation pH...	6·2			6·8			Values taken from Paul & Southgate (1978)
Deconjugated food extract (μl)...	25	50	100	25	50	100	
Garden peas (frozen)	—	156	158	—	72	112	78
Runner beans							
Frozen	—	152	160	—	68	114	60
Fresh	—	172	182	—	80	120	60
Brussels sprouts (fresh)	288	316	—	176	208	—	110
Marmite®	3200	3320	—	2400	2680	—	1010
Potato (raw)							14
Pentland Crown	—	41	42	—	28	43	—
King Edward	—	38	36	—	21	36	—
Desiree	—	46	45	—	30	45	—
Maris Piper	—	46	45	—	30	43	—

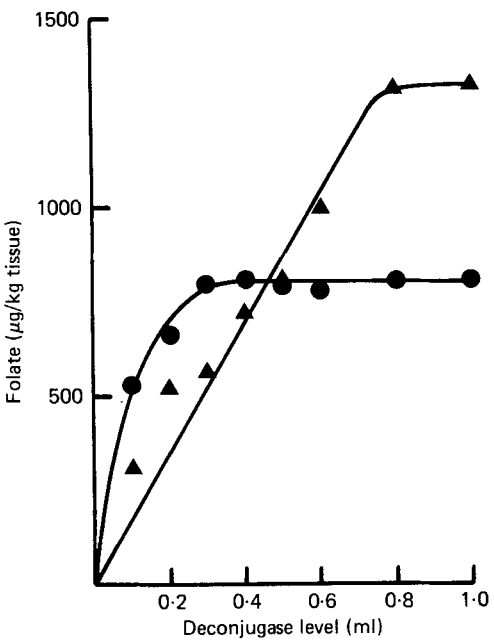

Fig. 1. Enzyme concentration curves for chicken-pancreas enzyme (●) and hog-kidney enzyme (▲). An extract from frozen garden peas was used as substrate.

DISCUSSION

Studies with authentic monoglutamates (Phillips & Wright, 1982) showed that 5-methyl-tetrahydrofolic acid produced a much-reduced growth response in *L. casei* compared with folic acid and 5-formyl-tetrahydrofolic acid and that the response was pH-dependent. The implications of the latter study were that in foods which contain a significant amount of

Table 2. *Folate contents of food extracts when deconjugation was performed using either the hog-kidney or chicken-pancreas enzyme preparation and assays were performed using* Lactobacillus casei *at the incubation pH of 6·2*

	Folate (μg/100 g)	
Source of deconjugase...	Hog kidney	Chicken pancreas
Garden peas (frozen)	134	80
Runner beans:		
Frozen	136	90
Fresh	164	96
Brussels sprouts (fresh)	304	200
Marmite®	3360	1720

5-methyl-tetrahydrofolate the values for food folate reported (Paul & Southgate, 1978) were probably underestimates. The results obtained in the present work have confirmed this suggestion. The shape of the growth-response curve of *L. casei* to 5-methyl-tetrahydrofolic acid also provided an explanation of the positive drift seen by workers assaying food folate. The present results also confirm this postulate because at pH 6·2 positive drift is no longer seen.

During the course of the present study we observed that the choice of deconjugase enzyme also affected the value obtained, and accordingly we made a detailed comparison. The increased growth response achieved by the products from the hog-kidney enzyme may reflect on the modes of transport employed by *L. casei* for the different deconjugation products. The hog-kidney enzyme forms a monoglutamate product (Bird & McGlohon, 1972) and the chicken-pancreas enzyme a diglutamate product (Leichter *et al.* 1977). It is widely assumed that the *L. casei* response to both forms is similar. The *L. casei* response to pteroylmonoglutamate, diglutamate and triglutamate is identical (Tamura *et al.* 1972). However, a similar response to the mono- and diglutamate forms of 5-methyl-tetrahydrofolic acid or 5-formyl-tetrahydrofolic acid has never been demonstrated. The mono- and polyglutamate forms of pteroylglutamic acid are actively transported and concentrated by a carrier-mediated system in *L. casei*, but the uptake of the tri- to octapolyglutamate forms of 5-methyl-tetrahydrofolic acid, whilst still carrier-mediated, is not subject to this process of active transport (Henderson & Huennekens, 1974; Shane & Stokstad, 1975, 1976). If the diglutamate form of 5-methyl-tetrahydrofolic acid or 5-formyl-tetrahydrofolic acid is not activity transported, its uptake would be a rate-limiting step in the growth of *L. casei* resulting in a lower growth response in *L. casei* to the product of deconjugation with chicken-pancreas enzyme seen in Fig. 1.

The evidence published in this paper has considerable implications for workers using the food tables compiled by Paul & Southgate (1978) to calculate dietary levels of folate from measured food intakes. The folate content of the foods published there were all based on assays performed by the procedure described by Bell (1974) using *L. casei* at an incubation pH of 6·8 and employing chicken-pancreas enzyme to effect the deconjugation of the polyglutamates in the foods. In a recent publication (Spring *et al.* 1979) the calcultions of the mean intake of folate in the UK, based on the National Food Survey's values for 1976 (Ministry of Agriculture, Fisheries and Food, 1977), and using the total food folate values from *McCance & Widdowson's The Composition of Foods* (Paul & Southgate, 1978), have yielded estimates of approximately 190 μg total folate intake/d. The official recommendations

for intake are currently very much higher than this estimated intake. The World Health Organization (1972) and the (US) National Research Council (1980) recommend 400 μg/d for adults rising to 800 μg/d during pregnancy. Using the presently-accepted values for food folate, very few of the UK population would achieve a folate intake of this level. In another publication (Bates *et al.* 1982) this discrepancy between folate intakes calculated and the folate recommended daily allowances has been discussed. The present study shows that the values for folates (Paul & Southgate, 1978) are underestimates. The extent of this underestimation will vary from food to food depending on the proportion of 5-methyl-tetrahydrofolate present and on the precise conditions of assay and the dilutions used. It is thus not possible to make corrections arithmetically, only by re-analysis.

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