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The threshold growth response of *Lactobacillus casei* to 5-methyl-tetrahydrofolic acid: implications for folate assays

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

AFRC Food Research Institute, Colney Lane, Norwich NR4 7UA

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1. The comparative and absolute growth response of *Lactobacillus casei* was measured nephelometrically for time periods of 17–23 h in the microbiological assay of folic acid and 5-methyl-tetrahydrofolic acid at concentrations of 0–8 ng/10 ml basal medium at pH 6·8.

2. At concentrations of 0–1 ng/10 ml the comparative growth response to 5-methyl-tetrahydrofolic acid was markedly depressed whereas growth was the same at 2 ng/10 ml and above. Comparative growth was unaffected by the length of assay incubation, depressed growth being due to differences in log-phase growth rates with the rate-plot for 5-methyl-tetrahydrofolic acid being sigmoidal and for folic acid being a rectangular hyperbola with linearity only in the 0–1 ng/10 ml range. The reciprocal rate-plot for folic acid was linear whereas that for 5-methyl-tetrahydrofolic acid was coincidental only in part, giving rise to the same estimate of maximum velocity and substrate concentration for half-maximum velocity, with the exhibition of a strong threshold at low concentration.

3. A previous observation (Phillips & Wright, 1982) that the *L. casei* growth response to 5-methyl-tetrahydrofolic acid may be significantly less than that to folic acid is confirmed as is the long-established view that the response to both folates may be equal. In the light of current knowledge regarding folate-binding, transport and metabolism by *L. casei*, it is argued that the intracellular oxidation of 5-methyl-tetrahydrofolic acid to 5, 10-methylene-tetrahydrofolic acid is a rate-limiting step at low substrate concentrations, subsequently giving rise to a threshold growth response peculiar to 5-methyl-tetrahydrofolic acid. Since the rate of *L. casei* growth with folic acid is not linear above 1 ng/10 ml, it is recommended that microbiological folate assays be conducted only in the 0–1 ng/10 ml range and at a pH that elicits the same growth response from *L. casei* to 5-methyl-tetrahydrofolic acid as to folic acid and other folate monoglutamates.

Microbiological assay in a basal medium of approximately pH 6·5–6·8 using *Lactobacillus casei* ATCC 7469 (NCIB 6375) is the most widely used method for the measurement of folate concentrations in foods and biological fluids. In a recent study, Phillips & Wright (1982) demonstrated that *L. casei* has a reduced growth response to 5-methyl-tetrahydrofolic acid at low concentrations (0–1 ng/10 ml assay), where the initial pH of the assay medium was 6·5–6·8, when compared with other folate monoglutamates such as folic acid which is commonly used as the calibration standard for the microbiological assay. Since 5-methyl-tetrahydrofolate (as polyglutamates) is one of the most important forms of folate in foods, it was argued that this reduced response of *L. casei* would result in an underestimation of total food folate. The present paper describes further studies of the microbiological assay of folic acid and 5-methyl-tetrahydrofolic acid with a view to establishing the cause and extent of the reduced *L. casei* growth response to 5-methyl-tetrahydrofolic acid.

EXPERIMENTAL

Folic acid (pteroylmonoglutamic acid) and DL-5-methyl-tetrahydrofolic acid were obtained from Sigma (London) Chemical Co. Ltd, Poole, Dorset. Stock solutions were prepared with ascorbic acid (10 g/l, 57 mM) adjusted to pH 6·0 with sodium hydroxide. When making up the stock solution of DL-5-methyl-tetrahydrofolic acid, consideration was given to the fact that *L. casei* responds only to the biologically-active L-form (Shane *et al.* 1980). Before assay, working solutions of 4 and 40 ng/ml of the two folates were freshly prepared from stock solutions by diluting portions with freshly-prepared ascorbic acid (1 g/l, 5·7 mM) adjusted to pH 6·0.

Difco® Folic Acid Casei Medium (Difco Laboratories, West Molesey, Surrey) was prepared at single strength by addition of 47 g dried medium to 1 litre distilled water which was subsequently brought to the boil for 1–2 min and then allowed to cool. Ascorbic acid (1 g/l, 5·7 mM) was then added and the pH of the medium adjusted with 1 M-NaOH to pH 7·0 before dispensing 10 ml per assay tube, capping with Fincaps (Clark Scientific Ltd, New Malden, Surrey) and autoclaving for 5 min at 15 psi (103×10^3 Pa) at 121°: autoclaving caused a drop in pH to 6·8.

The maintenance of culture and inoculum preparation was as previously described (Phillips & Wright, 1982) using *L. casei* NCIB 6375 (equivalent to ATCC 7469 and NCIB 8010).

Folic acid or L-5-methyl-tetrahydrofolic acid (0·0, 0·2, 0·4, 0·6, 0·8, 1·0, 2·0, 4·0, 6·0 and 8·0 ng) were each added to four sets of triplicate tubes from freshly-diluted stock solutions (maximum volume added was 250 μl) by aseptic technique (Futterman & Silverman, 1957; Bakerman, 1961; Herbert, 1966). Assays were started by the addition of 100 μl dilute inoculum and incubated at 37° for 17, 19, 21 and 23 h to produce a range of growth. *L. casei* growth in the tubes was measured in a Nephelometer with digital readout (Diffusion Systems Ltd, Rosebank Road, Hanwell, London). For each incubation period, comparative measurements of growth between a set of folic acid and L-5-methyl-tetrahydrofolic acid standards were obtained by reading tubes against a scale set to between 0 and 100 units using incubated blank medium and 1 ng folic acid standard. As an index of absolute growth, tubes were also read against a scale set to 0 with a tube of distilled water and to 100 units with a ground glass standard tube. 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. *L. casei* growth was plotted on semi-logarithmic paper with the folate concentration as the logarithmic plot.

RESULTS

The comparative growth curves for *L. casei* in an assay medium containing either folic acid or L-5-methyl-tetrahydrofolic acid, for incubation periods of 17, 19, 21 and 23 h, are shown in Fig. 1. The point of coincidence of the *L. casei* growth for the two forms of folate appeared to be constant at approximately 2 ng folate/10 ml. Since *L. casei* growth markedly increased between 17 and 23 h the comparative relation is independent of the time of incubation.

Plotting absolute bacterial growth *v.* incubation time revealed that *L. casei* was in logarithmic growth phase for both folates at all concentrations, with 5-methyl-tetrahydrofolic acid showing depressed rates of growth at low concentration but comparatively equal rates of growth at 2 ng/10 ml and above. When the rate of log-phase growth was plotted against folate concentration the plot for 5-methyl-tetrahydrofolic acid was sigmoidal whereas the plot for folic acid was described by the equation of a rectangular hyperbola with the rate of growth being directly proportional to the substrate concentration only in the 0–1 ng/10 ml range. The double-reciprocal plot for folic acid was linear whereas that for 5-methyl-tetrahydrofolic acid was coincidental only in part, giving rise to the same estimate of maximum velocity and substrate concentration for half-maximum velocity (K_s 3·3 ng/10 ml assay, i.e. 0·75 nM), with the exhibition of a strong threshold at low concentration where the reciprocal growth rate diverged rapidly to infinity.

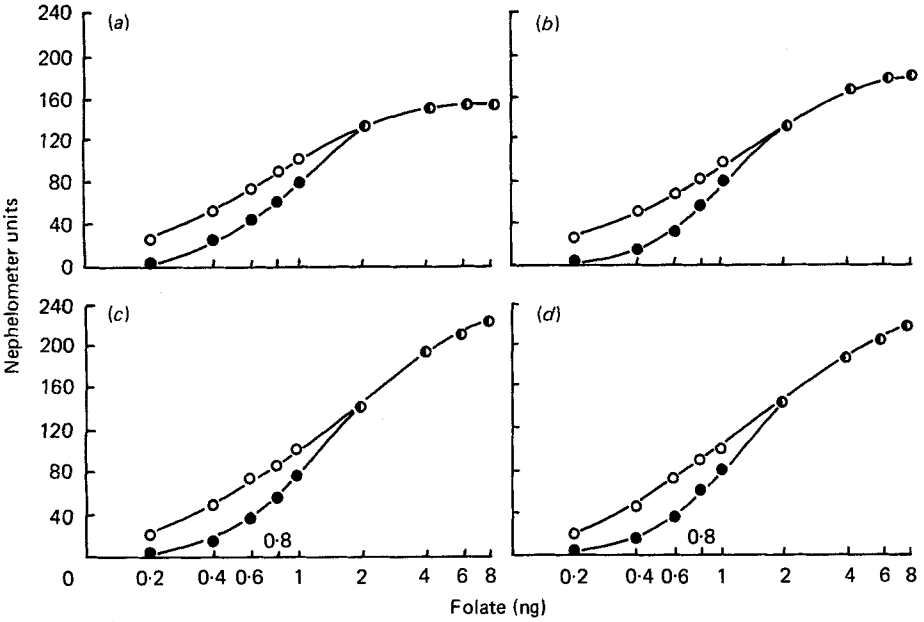

Fig. 1. Comparative *Lactobacillus casei* growth response to folic acid (○——○) and L-5-methyl-tetrahydrofolic acid (●——●) in 10-ml assay portions incubated for (*a*) 17, (*b*) 19, (*c*) 21 and (*d*) 23 h using basal medium at pH 6·8. Coincident points (◐——◐). Points are mean values of triplicate tubes. Nephelometer readings were taken from a scale set to 0 units with incubated uninoculated basal medium and to 100 units with the growth produced by 1 ng folic acid.

DISCUSSION

The possible connection between fetal neural tube defects and poor peri-conceptional folate status (Laurence *et al.* 1980, 1981; Smithells *et al.* 1980, 1981) has added to the growing concern over the apparent discrepancy between the recommended and the much lower calculated folate intakes for the UK population. It has been postulated (Bates *et al.* 1982) that one of the reasons for this discrepancy may be the underestimation of true dietary intakes.

In a recent study, Phillips & Wright (1982) demonstrated that where microbiological assays had a folate concentration of 0–1 ng/10 ml assay and an initial pH of 6·8, the growth response of *L. casei* to 5-methyl-tetrahydrofolic acid was poor in comparison with the growth response to folic acid, the folate commonly used to construct the standard calibration curve of *L. casei* assays. It is interesting to note that these assay conditions (Bell, 1974) were used to obtain values for the total folate content of foods listed in *McCance and Widdowson's The Composition of Foods* (Paul & Southgate, 1978). The disparity in *L. casei* growth to these two forms of folate conflicts with the long-established view that the response of *L. casei* to the biologically active forms of all folate monoglutamates is very similar (Stokstad & Oace, 1965; Bird & McGlohon, 1972; Stokstad & Thenen, 1972; Shane *et al.* 1980). The implication of this finding is that previously published food folate assays may have seriously underestimated total folate values, particularly in foods such as vegetables where the folate content is predominantly in the 5-methyl-tetrahydrofolate-polyglutamate form (Scott & Weir, 1976) which, after deconjugation with hog kidney conjugase (*EC* 3.4.22.12), would be assayed as 5-methyl-tetrahydrofolic acid.

The present study demonstrates that at concentrations below 2 ng/10 ml assay the reduced growth response of *L. casei* to 5-methyl-tetrahydrofolic acid is the result of a

decrease in the rate of log-phase growth in comparison with that shown for an equal concentration of folic acid. Double-reciprocal plots of rate of log-phase growth and substrate concentration show that this discrepancy is associated with an apparent threshold effect.

Folate monoglutamates are taken up by a single, actively-concentrative transport system which is directly dependent on a high-affinity, membrane-associated, folate-binding protein. This protein exhibits the same binding capacity for folic acid and 5-methyl-tetrahydrofolic acid at pH 6·8, transports the two folates similarly well, and has a broad pH optimum, being reported as 5·0–6·5 and 4·5–7·5 (Henderson & Huennekens, 1974, 1977; Shane & Stokstad, 1975, 1976). Because *L. casei* appears to possess a similar capacity to bind and transport both folates at pH 6·8, the threshold growth response to 5-methyl-tetrahydrofolic acid (with complete failure of growth at low concentrations) must be a function of some facet of intracellular metabolism not shared with folic acid.

L. casei requires folate primarily for the generation of purines and thymidylate, their synthesis being thought to be the most rate-limiting steps governing growth (Shane & Stokstad, 1977*b*). Folic acid uptake by *L. casei* is independent of conversion to other folate forms (Cooper, 1970), and enters the folate metabolic cycle via reduction to tetrahydrofolic acid which can be subsequently metabolized to 5, 10-methylene-tetrahydrofolic acid, the one-carbon moiety of which is rapidly incorporated into thymidylate and purines. Significant metabolism of folic acid to 5-methyl-tetrahydrofolic acid does not occur, as indicated by radio-tracer experiments with tetrahydrofolic acid (Shane & Stokstad, 1977*b*), nor is it metabolized to 5-methyl-tetrahydrofolate-polyglutamates (Shane *et al.* 1983).

In mammalian metabolism, after first having its methyl group transferred to methionine by tetrahydropteroylglutamate methyltransferase (*EC* 2.1.1.13), 5-methyl-tetrahydrofolic acid-monoglutamate (like folic acid) also enters the folate metabolic cycle as tetrahydrofolic acid with the subsequent synthesis of thymidylate and purines as described previously. *L. casei*, on the other hand, possesses a tetrahydropteroylglutamate methyltransferase (*EC* 2.1.1.14) that is polyglutamate-specific which cannot metabolize 5-methyl-tetrahydrofolic acid-monoglutamate directly (Shane & Stokstad, 1977*b*) and, since 5-methyl-tetrahydrofolic acid-monoglutamate is also an ineffective substrate for *L. casei* polyglutamate synthetase (Bognar & Shane, 1983), it cannot metabolize it indirectly either. The metabolism of 5-methyl-tetrahydrofolic acid-monoglutamate by *L. casei* can only proceed through its direct oxidation by 5, 10-methylene-tetrahydrofolate reductase ($FADH_2$) (*EC* 1.1.99.15) to 5, 10-methylene-tetrahydrofolic acid and its subsequent utilization in thymidylate and purine synthesis (Shane & Stokstad, 1977*a*). However, the equilibrium of 5, 10-methylene-tetrahydrofolic acid reductase can be strongly against this reaction (Shane & Stokstad, 1977*b*) and, as this is the only step that 5-methyl-tetrahydrofolic acid metabolism does not share with folic acid metabolism, it would appear that when *L. casei* is in a medium at pH 6·8 with low substrate concentrations, the amount of transported folate is insufficient to activate this enzyme (thus trapping folate unmetabolized and causing a threshold effect on growth) and that oxidation only occurs when the intracellular concentration is sufficient to overcome the enzyme equilibrium.

The present study extends our previous observation (Philips & Wright, 1982) that *L. casei* growth at low concentrations of 5-methyl-tetrahydrofolic acid in a basal medium of pH 6·8 is significantly less than that towards similarly low concentrations of folic acid. The response at higher concentrations is in agreement with the view that the growth response to all folate monoglutamates is similar. It is suggested that the threshold growth response to 5-methyl-tetrahydrofolic acid reported in the present paper is consistent with published observations regarding the intracellular metabolism of folates by *L. casei* and that the rate-limiting step in the utilization of low concentrations of 5-methyl-tetrahydrofolic

acid-monoglutamate by *L. casei* in growth media at pH 6·8 is related to its ability to be oxidized to 5, 10-methylene-tetrahydrofolic acid. When using folic acid as the calibration standard, identical growth responses can only be obtained at pH 6·8 when concentrations are above 2 ng/10 ml assay. However, a plot of rate of growth *v.* folic acid concentration shows linearity only between 0–1 ng/10 ml, with a rapid negative drift thereafter towards a plateau of maximum velocity, indicating the advisability of conducting assays only within this lower range; Fig. 1 shows growth plateau effects at high concentration. Previous work has indicated that assays may be conducted with safety in the 0–1 ng/10 ml range when the basal medium is of pH 6·2 since at this pH 5-methyl-tetrahydrofolic acid, like other folate monoglutamates, elicits the same growth response from *L. casei* (Phillips & Wright, 1982, 1983).

REFERENCES

Bakerman, H. A. (1961). *Analytical Biochemistry* **2**, 558–567.

Bates, C. J., Black, A. E., Phillips, D. R., Wright, A. J. A. & Southgate, D. A. T. (1982). *Human Nutrition: Applied Nutrition* **36A**, 422–429.

Bell, J. G. (1974). *Laboratory Practice* **23**, 235–242, 252.

Bird, O. D. & McGlohon, V. M. (1972). In *Analytical Microbiology*, vol. 2, pp. 409–437 [F. Kavanagh, editor]. New York: Academic Press.

Bognar, A. L. & Shane, B. (1983). *Journal of Biological Chemistry* **258**, 12574–12581.

Cooper, B. A. (1970). *Biochimica et Biophysica Acta* **208**, 99–109.

Futterman, S. & Silverman, M. (1957). *Journal of Biological Chemistry* **224**, 31–40.

Henderson, G. B. & Huennekens, F. M. (1974). *Archives of Biochemistry & Biophysics* **164**, 722–728.

Henderson, G. B. & Huennekens, F. M. (1977). *Journal of Biological Chemistry* **252**, 3760–3765.

Herbert, V. (1966). *Journal of Clinical Pathology* **19**, 12–16.

Laurence, K. M., James, N., Miller, M. H. & Campbell, H. (1980). *British Medical Journal* **281**, 1592–1594.

Laurence, K. M., James, N., Miller, M. H., Tennant, G. B. & Campbell, H. (1981). *British Medical Journal* **282**, 1509–1511.

Paul, A. A. & Southgate, D. A. T. (1978). *McCance and Widdowson's The Composition of Foods*. London: H.M. Stationery Office.

Phillips, D. R. & Wright, A. J. A. (1982). *British Journal of Nutrition* **47**, 183–189.

Phillips, D. R. & Wright, A. J. A. (1983). *British Journal of Nutrition* **49**, 181–186.

Scott, J. M. & Weir, D. G. (1976). *Clinics in Haematology*, vol. 5, pp. 547–568, London: W. B. Saunders Co. Ltd.

Shane, B., Bognar, A. L., Goldfarb, R. D. & Le Bowitz, J. H. (1983). *Journal of Bacteriology* **153**, 316–325.

Shane, B. & Stokstad, E. L. R. (1975). *Journal of Biological Chemistry* **250**, 2243–2253.

Shane, B. & Stokstad, E. L. R. (1976). *Journal of Biological Chemistry* **251**, 3405–3410.

Shane, B. & Stokstad, E. L. R. (1977*a*). *Journal of General Microbiology* **103**, 249–259.

Shane, B. & Stokstad, E. L. R. (1977*b*). *Journal of General Microbiology* **103**, 261–270.

Shane, B., Tamura, T. & Stokstad, E. L. R. (1980). *Clinica Chimica Acta* **100**, 13–19.

Smithells, R. W., Sheppard, S. & Schorah, C. J. (1980). *Lancet* **i**, 339–340.

Smithells, R. W., Sheppard, S. & Schorah, C. J. (1981). *Archives of Disease in Childhood* **56**, 911–918.

Stokstad, E. L. R. & Oace, S. M. (1965). In *Newer Methods of Nutritional Biochemistry*, vol. 2, pp. 285–313 [A. A. Albanese, editor]. New York: Academic Press.

Stokstad, E. L. R. & Thenen, S. W. (1972). In *Analytical Microbiology*, vol. 2, pp. 387–408 [F. Kavanagh, editor]. New York: Academic Press.

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