The Effect of Medium Redox Potential on the Folate-limited Growth of *Lactobacillus casei* var. *rharnosus*

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SUMMARY

The medium redox potential (E_h) influenced the folate-limited growth of *Lactobacillus casei*; the growth response was maximal at an E_h of +120 mV (pH 6.35). At raised E_h serum folate would support less growth than pteroylglutamic acid, and the response to N^5 -methyl tetrahydrofolic acid was intermediate between them. Pteroylglutamic acid was not destroyed during 24 h incubation at 37 °C in medium with E_h values between +40 and +440 mV. Destruction of N^5 -methyl tetrahydrofolic acid occurred within 24 h when the medium E_h was greater than +125 mV. Folate was taken up rapidly by *L. casei* with an E_h optimum at +270 mV.

INTRODUCTION

Ascorbic acid has been used to prevent oxidation of the folate activity of serum during assay procedures using *Lactobacillus casei*, since the work of Toennies, Frank & Gallant (1953). Herbert (1961) noted that ascorbic acid stimulates the growth of *L. casei* cultures containing pteroylglutamic acid (PteGlu) as folate source. Whole serum assay techniques using aseptic addition of serum (Herbert, 1966) or chloramphenicol-resistant strains of *L. casei* (Davis, Nichol & Kelly, 1970) have eliminated the oxidation problems associated with the extraction of folate activity. Published methods of folate assay with chloramphenicol-resistant strains of *L. casei* use ascorbate concentrations in the assay medium ranging from 0.05 g/l (O'Broin, Scott & Temperley, 1973) to 1.0 g/l (Chanarin, Kyle & Stacey, 1972; Scott, Ghanta & Herbert, 1974). The present work seeks to define the effect of ascorbate on the medium redox potential (E_h) and relate this to the growth response of *L. casei* to folate.

METHODS

Redox control. A range of medium E_h values was obtained by adding hydrogen peroxide ['20 volume' (i.e. 6 %, w/v) H_2O_2 in water] to the medium at 0.8 ml/l, together with doubling dilutions of a reducing agent. Sodium ascorbate was used in most experiments, over a concentration range of 0.025 to 25 g/l. Other reducing agents used were sodium erythorbate (sodium D-iso-ascorbate), over the range 0.5 to 50 g/l, and sodium thioglycollate, over the range 1.56 to 50 g/l.)

Measurement of redox potential. This was done with a Vibron 39A pH meter (Electronic Instruments Ltd, Chertsey, Surrey) using a platinum electrode and a calomel reference cell with a saturated KCl salt bridge. The correction voltage was +242 mV at 25 °C (Glasstone, 1956). The E_h measurements were made on uninoculated blanks which had been left at room temperature to equilibrate for 1.5 to 2 h.

Organisms. Several strains of *Lactobacillus casei* var. *rharnosus* were used (ATCC7469,

CRI, NCDO243, NCIB6375, NCIB8010, NCIB10463 and NCTC10302). The relationships of these strains to one another have been noted previously (Tennant *et al.* 1974), together with details of the maintenance procedures. All the strains were obtained as, or were made, chloramphenicol-resistant. Inoculum cultures were grown overnight at 37 °C in 10 ml volumes of assay medium containing PteGlu (250 ng/l) and chloramphenicol (20 mg/l). Organisms were not washed before use as the growth was folate-limited.

Culture procedure. The conditions under which cultures were grown were based on the serum folate assay procedure of Tennant & Withey (1972). They were similar to those described previously (Tennant *et al.* 1974) except that the phosphate buffer concentration in the assay medium was decreased to 0.075 M. The pH of the assay medium was 6.35.

Folate source. PteGlu (Sigma) solutions of 5.5 µg/l were freshly prepared from 1 g/l stock solutions stored at -20 °C. DL- N^5 -methyl tetrahydrofolic acid (5CH₃H₄PteGlu; Sigma) was found to be about half as effective as PteGlu in supporting the growth of *L. casei*. Working solutions of 11 µg/l were used. Sera with folate concentrations between 5.0 and 6.0 µg/l were pooled. Folate sources were diluted 1/40 into the culture medium.

Oxidative destruction of folate. PteGlu (50 µg/l) and 5CH₃H₄PteGlu (100 µg/l) were incubated at 37 °C for 24 h in uninoculated medium at a range of E_h values. The folate remaining was assayed after diluting 1/10 in 0.2 % (w/v) sodium ascorbate solution. The resultant E_h was +140 mV (pH 6.55) regardless of the E_h of the medium incubated.

Uptake of folate by *L. casei*. The rate of PteGlu uptake was measured by resuspending bacteria from overnight cultures in double strength assay medium ($E_{560nm}^{1cm} = 0.03$). Samples were diluted with equal volumes of PteGlu solution (10 µg/l) and incubated in centrifuge tubes at 37 °C. Tubes were withdrawn at intervals and the organisms sedimented. The supernatant folate was assayed.

The effect of medium E_h on the uptake of PteGlu was measured similarly. Bacteria were resuspended in four times strength medium and equal volumes of PteGlu, hydrogen peroxide and sodium ascorbate solutions added. Incubation was for 15 min.

RESULTS

Control of medium E_h

The E_h value of folate assay medium without redox control varied from day to day within the range +150 to +220 mV. The effect of adding ascorbate is shown in Fig. 1. An extended E_h range of about +40 to +440 mV was obtained by adding reducing agent to medium containing hydrogen peroxide. None of the strains showed any catalase-like activity when hydrogen peroxide was added to dense concentrations of the bacteria (Whittenbury, 1964).

Effect of medium E_h on the growth response to folate

Comparison of the growth response of *L. casei* (NCIB6375) to PteGlu, 5CH₃H₄PteGlu and serum folate over a medium E_h range of +40 to +440 mV is shown in Fig. 2. The curves are the mean results of four experiments. Results fitted the same curves when ascorbate was added to medium without hydrogen peroxide, although the E_h values were obtained with different concentrations of ascorbate. The use of sodium erythorbate and sodium thio-glycollate as reducing agents also gave similar curves.

Twenty sera with folate values between 2.0 and 9.0 µg/l were used individually as folate sources and 16 showed identical percentage growth responses to changing E_h , regardless of serum folate value. The growth responses of these 16 sera showed greater sensitivity to raised E_h values than was found with 5CH₃H₄PteGlu as folate source. Three other sera

Fig. 1

Fig. 1. Effect of sodium ascorbate concentration on the redox potential of assay medium (pH 6.35). $\circ$, Not boiled; $\bullet$, boiled; $\square$, plus hydrogen peroxide.

Fig. 2

Fig. 2. Effect of redox potential on the growth of *L. casei* NCIB6375 limited by PteGlu ($\circ$), 5CH₃H₄PteGlu ($\bullet$), and serum folate ($\square$).

Fig. 3

Fig. 3. Effect of redox potential on the stability of PteGlu ($\circ$) and 5CH₃H₄PteGlu ($\bullet$) during 24 h incubation at 37 °C.

Fig. 4

Fig. 4. Effect of redox potential on the uptake of PteGlu by NCIB6375 during 15 min incubation at 37 °C.

showed responses similar to 5CH₃H₄PteGlu and one was intermediate between 5CH₃H₄PteGlu and PteGlu.

When excess PteGlu (1 mg/l) was added to the culture medium the growth response was not affected by medium E_h over the range +35 to +420 mV.

All the strains derived from ATCC7469 (i.e. ATCC7469, CRI, NCDO243, NCIB6375, NCIB8010 and NCIB10463) showed a maximum growth response at an E_h of +120 mV, regardless of the folate source. In contrast NCTC10302 showed a maximum growth response at +90 mV. When compared with the other strains, ATCC7469 showed a lower growth response to all forms of folate at E_h values greater than +120 mV. The growth response below +120 mV was similar to the other strains.

Oxidative destruction of folate

5CH₃H₄PteGlu was stable during 24 h incubation at E_h values below +125 mV, but it was destroyed at higher values (Fig. 3). There was no destruction of PteGlu under these

conditions. The rate of destruction of $5\text{CH}_3\text{H}_4\text{PteGlu}$ was measured in medium with an E_h of +481 mV. Loss of assayable folate was detectable after less than 15 min incubation. About 25 % of the activity was lost after 2 h incubation, and after 24 h only 5 % remained.

Uptake of folate by L. casei

The rate of uptake of PteGlu was estimated in medium with a ratio of *L. casei* organisms to folate concentration similar to a mid-curve standard at inoculation of a routine assay, but with the concentrations of organisms and PteGlu both increased 40-fold. The rate of uptake varied from strain to strain; NCIB6375 removed about 50 % of the PteGlu from the medium in 10 to 15 min.

The effect of medium E_h on the uptake of PteGlu by NCIB6375 was measured over a 15 min period so that changes in growth response related to E_h should not influence the results. Maximum uptake was shown when the medium E_h was between +220 and +340 mV (Fig. 4).

DISCUSSION

Folate-limited growth of *L. casei* showed an optimum response at +120 mV; this was not a reflexion of oxidative destruction of folate, as PteGlu was stable in medium at the E_h values studied. The growth response to serum folate was lower than to PteGlu at E_h values above +125 mV and this was probably due to oxidation of the serum folate. The folate activity of serum has been shown to be due to $5\text{CH}_3\text{H}_4\text{PteGlu}$ (Herbert, Larrabee & Buchanan, 1962) and the lower growth occurred at E_h values where $5\text{CH}_3\text{H}_4\text{PteGlu}$ was destroyed. The difference in growth response was relatively small because the rapid uptake of folate into the bacteria allows minimal time for the destruction of serum folate. Approximately half the serum folate activity in healthy subjects is protein bound (Markkanen *et al.* 1973). This may slow the rate of uptake into bacteria (Waxman, 1975) and thereby result in an apparently greater lability of serum folate than $5\text{CH}_3\text{H}_4\text{PteGlu}$. Markkanen *et al.* (1973) reported a wide range of percentage binding in a variety of clinical situations. These included some sera with no apparent binding, which may explain the behaviour of those samples where E_h sensitivity was similar to that with $5\text{CH}_3\text{H}_4\text{PteGlu}$.

The effect of medium E_h on the folate-limited growth response seems to find expression in the uptake and utilization of folate by the cell. The growth response of cultures containing excess PteGlu were not affected by changes in the medium E_h . Folate is taken up into *L. casei* by an active transport mechanism (Wood & Hitchings, 1959) which has an E_h optimum at +270 mV. Comparison of Figs. 2 and 4 shows that the effect of medium E_h on the PteGlu uptake is probably the main factor in changes in the growth response at E_h values greater than about +300 mV.

In the whole serum assay of folate a medium E_h between +90 and +150 mV (pH 6.35) would maintain an optimum growth response which is independent of the form in which folate is provided. Under our conditions of assay the addition of sodium ascorbate to the medium at a concentration of 0.1 g/l (0.5 g/l if the medium is boiled) would achieve this (Fig. 1).

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