Some Effects of 2-Deaminopteroylglutamic Acid upon Bacterial Growth

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SUMMARY: Four analogues of pterioic acid and one of pteroylglutamic acid were tested for their ability to stimulate growth of *Streptococcus faecalis* R in the absence of pterioic acid or to inhibit growth in its presence. The four analogues of pterioic acid were inactive in either respect, but 2-deaminopteroylglutamic acid was inhibitory. Inhibition of the organism was most marked in the presence of pterioic acid, and became relatively more efficient as the concentrations of inhibitor and growth factor were increased. Inhibition was much less marked in the presence of rhizopterin or Leucovorin, the relative efficiency decreasing in comparable conditions. Pteroylglutamic acid had an intermediate position and showed a competitive relationship.

Lactobacillus casei was similarly inhibited when growth was supported by either pteroylglutamic acid or Leucovorin, but was less pronounced in the presence of the latter. On the other hand, the analogue could substitute for pteroylglutamic acid in stimulating the suboptimal growth supported by a mixture of thymine and a purine in the absence of added folic acid.

Several other organisms whose growth is independent of exogenous folic acid were unaffected by the analogue.

The synthesis of analogues and derivatives of pterioic acid and pteroylglutamic acid has been undertaken in several laboratories in order to throw light upon the functioning of the metabolically important folic acid group of substances (e.g. see reviews by Jukes, Franklin & Stokstad, 1950; Woolley, 1952). The synthesis of 4-hydroxy-6-methylpteridine (Albert, Brown & Cheeseman, 1952) made it possible for the first time to prepare analogues which lack the amino-group in the 2-position. Five compounds of this type have been synthesized (Brown, 1953), and their structures are indicated in Fig. 1.

METHODS

Organisms

Streptococcus faecalis R (American Type Culture Collection, ATCC no. 8043) was maintained by stab subculture in medium A at 37°.

Lactobacillus casei (ATCC No. 7469) was similarly maintained in medium B1.

Media

Medium A. For each 100 ml.: Marmite, 1 g.; glucose, 0.5 g.; sodium acetate trihydrate, 1 g.; agar, 1.5 g.; pH adjusted to 6.7-6.9.

Medium B1. For each 100 ml.: Tryptose (Difco), 1 g.; yeast extract (Difco), 0.2 g.; glucose, 1 g.; K_2HPO_4 , 0.5 g.; liver extract (Oxoid, 16% w/v), 10 ml.;

MgSO₄·7H₂O, 20 mg.; FeSO₄·7H₂O, 1 mg.; MnSO₄·4H₂O, 1 mg; CaCO₃, 1.5 g.; agar, 1.5 g.

Medium B2. As medium B1 but without CaCO₃ and agar; pH 6.7–6.9.

Medium C. This was the medium of Rabinowitz & Snell (1947) with DL-cysteine (200 mg./l.) instead of L-cysteine, with pyridoxin added at 1 mg./l. and with folic acid and *p*-aminobenzoic acid left out.

Medium D. The culture medium for *L. casei* was that modification of the Roberts & Snell (1946) medium previously described (Nimmo-Smith, Lascelles & Woods, 1948).

Fig. 1. The five compounds investigated were: 6-anilinomethyl-4-hydroxypteridine (R=—H); 6-*p*-anisidinomethyl-4-hydroxypteridine (R=—OCH₃); 4-hydroxy-6-*p*-sulphanilinomethylpteridine (R=—SO₃H); 6-*p*-carboxyanilinomethyl-4-hydroxypteridine (R=COOH) and 2-deaminopteroylglutamic acid

Materials

Pteric acid, pteroylglutamic acid and Leucovorin (5-formyl-5:6:7:8-tetrahydropteroyl-L-glutamic acid; 'folinic acid') were synthetic samples generously supplied by Lederle Laboratories Division. Rhizopterin (N¹⁰-formylptericoic acid) was kindly given by the Merck Co., Inc. Stock solutions of each at 10⁻³M in 10⁻²M-NaHCO₃ were stored in the ice-chest and diluted when needed.

Stock solutions (10⁻³M in sterile 2 × 10⁻²N-NaOH) of the five analogue compounds were similarly stored.

Methods of cultivation

The same general precautions were observed as in conducting folic acid assays (Nimmo-Smith, Lascelles & Woods 1948). Samples of the media (at 70% of final volume) were distributed in 5 × ½ in. hard-glass test-tubes. Suitable additions of diluted growth factor were made and, where necessary, distilled water was added so that subsequent additions of analogue solution and inoculum brought the contents of each tube to the same final volume.

The tubes were autoclaved for 10 min. at 10 lb./sq.in. This treatment, at pH 6.8, causes no detectable destruction of even the relatively unstable Leucovorin (unpublished observation by Dr June Lascelles). After autoclaving, different concentrations of the analogues to be tested were added and the tubes inoculated.

Strep. faecalis was grown for 22–24 hr. at 37° in a final volume of 5.0 ml. medium C and an atmosphere of air. The inoculum was prepared by growing the organism for 18 hr. in medium B2 and diluting the culture 1/100 in sterile water. To each tube 0.5 ml. of the dilute suspension was added.

L. casei was grown for 24–26 hr. at 37° in a final volume of 2.0 ml. of medium D and an atmosphere 5% CO₂ + 95% H₂ (Nimmo-Smith *et al.* 1948).

The inoculum was 0.2 ml. of a 1/100 dilution in water of a 24 hr. culture of the organism in medium D supplemented with 1.5×10^{-8} M-pteroylglutamic acid. This had been inoculated with about 0.02 ml. of a 16 hr. culture in medium B2, inoculated in turn from a fresh 24 hr. stab culture in medium B1.

In both cases growth was assessed with a portable colorimeter (Evans Electroselenium, Ltd.), uninoculated medium being used to adjust to zero the reading on the instrument scale. A calibration of dry weight against colorimeter reading was made for each organism and the relationship shown to be linear within the range encountered. Half-maximal growth of *Strep. faecalis* in our conditions corresponded to an instrument reading of 14 (0.69 mg. dry wt./ml.) and of *L. casei* to a reading of 19 (0.65 mg. dry wt./ml.).

In the quantitative experiments growth determined in this way was plotted against the logarithm of the concentration of either growth factor or analogue, whichever had been varied. For comparative purposes the molar ratio between analogue and growth factor obtaining at the point of half-maximal growth (or half-maximal inhibition) was used. In most cases serial 1/5 dilutions of growth factor were added to groups of tubes. Within each group the concentration of analogue was fixed, but varied by a factor of 5 from one group to the next. Less commonly the analogue was similarly titrated in the presence of fixed concentrations of the growth factor.

As a first step the five compounds were 'screened' with *Strep. faecalis* R in media (a) without folic acid, and (b) containing a suboptimal concentration (8.8×10^{-8} M) of pterioic acid. This technique allows the detection of compounds which can (i) replace folic acid, (ii) stimulate growth in the presence of low concentrations of folic acid or which are (iii) inhibitory.

RESULTS

Tested over a concentration range from 10^{-7} up to 10^{-4} M none of the compounds stimulated growth either on its own or in the presence of suboptimal pterioic acid. However, 2-deaminopteroylglutamic acid showed an interesting type of inhibition; experiments were carried out to determine the behaviour of this compound; the other four were not further investigated.

Effects of 2-deaminopteroylglutamic acid

Effect on growth of Strep. faecalis R. When pterioic acid was acting as source of folic acid the analogue showed itself to be a rather efficient inhibitor. The analogue/growth factor relationship was not a strictly competitive one, since the relative efficiency of the inhibitor increased markedly as the concentration of both was increased. This was shown by the amount of 2-deaminopteroylglutamic acid which had to be added to produce half-maximal inhibition in the presence of different concentrations of pterioic acid (Table 1). In Fig. 2 the logarithm of the molar ratio at half-maximal inhibition has been plotted against the logarithm of the analogue concentration. The position was further complicated by the fact that pterioic acid above about 8.8×10^{-8} M was itself slightly inhibitory.

In contrast, growth of the organism supported by rhizopterin was much less readily inhibited by the analogue, and the relative efficiency of the latter declined as concentrations were increased. When the concentration of the analogue was increased 25-fold the analogue : growth factor ratio at the half-maximal point rose by a factor of about 5 (Table 1).

Table 1. *Relationship between concentrations of 2-deaminopteroylglutamic acid and of various growth factors at half-maximal growth of Streptococcus faecalis R*

Pt. = pteric acid; *RPt.* = rhizopterin; *PtG.* = pteroylglutamic acid; *LV.* = Leucovorin; *DPtG.* = 2-deaminopteroylglutamic acid.

Concentrations		Ratio <i>a</i> : <i>b</i>
<i>a</i> <i>DPtG.</i> ($\times 10^{-6}$ M)	<i>b</i> Growth-factor ($\times 10^{-10}$ M)	
	<i>Pt.</i>	
0	990	—
3.05	1,000	305
6.35	3,300	190
10.0	10,000	100
10.9	33,000	33
	<i>RPt.</i>	
0	15	—
4	15	26,700
20	20	100,000
100	71	141,000
	<i>PtG.</i>	
0	14	—
4	40	10,000
20	170	11,800
100	865	11,600
	<i>LV.</i>	
0	14	—
4	19	21,000
20	45	44,000
100	110	90,000

When pteroylglutamic acid was acting as source of folic acid, 2-deaminopteroylglutamic acid produced half-maximal inhibition at a molar ratio of about 1 : 10,000. The relationship between the two, at least over a 50-fold range of concentration, appeared to be a competitive one (Table 1).

Behaviour of 2-deaminopteroylglutamic acid in the presence of Leucovorin was very similar to that in the presence of rhizopterin. A 25-fold increase in concentration of the analogue was associated with roughly a five-fold increase in the analogue : growth factor ratio (Table 1).

Synthetic Leucovorin is a mixture of the +L and -L isomers and is approximately 50 % as active for *Strep. faecalis* R as is the -L isomer (Cosulich, Smith & Broquist, 1952), and less active than the natural material for this organism also (Sauberlich, 1952). On the basis that probably only the *l*L isomer is available, concentrations of Leucovorin in Tables 1 and 2 are expressed in terms of this isomer only.

A medium in which thymine and a purine take the place of folic acid will support adequately the growth of *Strep. faecalis* R (Stokes, 1944). In these

Fig. 2. Relationship between the 2-deaminopteroylglutamic acid : pteric acid ratio at half-maximal inhibition of *Strep. faecalis* R and the absolute concentration of the analogue (two separate experiments).

Table 2. Relationships between concentrations of 2-deaminopteroylglutamic acid and of growth factor at half-maximal growth of *Lactobacillus casei*

Symbols (DPtG., etc.) have same significance as in Table 1.

Concentration		Ratio <i>a</i> : <i>b</i>
<i>a</i> DPtG. ($\times 10^{-8}$ M)	<i>b</i> Growth-factor ($\times 10^{-10}$ M)	
	PtG.	
0	7	—
4	24	16,700
20	44	45,500
100	144	69,000
	LV.	
0	4.8	—
4	6.2	64,000
20	11.5	174,000
100	39	255,000

conditions, with both thymine and hypoxanthine present at a concentration of 10^{-4} M, 2-deaminopteroylglutamic acid, tested over the range 10^{-7} up to 10^{-3} M, was completely without effect upon growth.

Effect on growth of L. casei. Neither pteric acid nor rhizopterin support the growth of this organism. 2-Deaminopteroylglutamic acid was inhibitory when growth of this organism was supported by pteroylglutamic acid, half-maximal inhibition being reached when the concentration of analogue was 1 to 7×10^4 times that of the growth factor.

There is some suggestion of a competitive relationship between the two but, owing to the relatively low activity and solubility of the analogue, it was not possible to test it over an adequate range of concentration. From Table 2 it can be seen that, although the concentration of 2-deaminopteroylglutamic acid was increased 25-fold the concentration of pteroylglutamic acid had only to be increased fivefold to obtain the same amount of growth.

Growth supported by Leucovorin was more resistant to inhibition (Table 2) than growth in presence of pteroylglutamic acid. Nevertheless, the progressive increase in ratio between analogue and growth factor was precisely the same in each case.

A mixture of thymine and a purine supports also the growth of *L. casei* in the absence of added folic acid. In confirmation of other workers (e.g. Stokes, 1944; Krueger & Peterson, 1945) we found that growth in these conditions, as measured turbidimetrically, was consistently about half as good as with pteroylglutamic acid. Supplementation with a relatively low concentration of the latter, however, stimulated maximal growth.

Surprisingly, 2-deaminopteroylglutamic acid possesses a similar stimulatory activity. To obtain an increase of 50% above the basal growth, it must be present at a concentration about 100 times that of the concentration of pteroylglutamic acid needed to produce the same effect (Fig. 3). 2-Deaminopteroylglutamic acid produced its maximum growth stimulatory effect at about 10^{-6} M, and stimulated growth which was always slightly suboptimal; it had no further effect on being increased to 10^{-3} M.

Effect on growth of other organisms. In addition to the above two organisms which required folic acid as nutrient the analogue was tested for its effect upon the growth of four organisms whose growth in a defined medium is independent of an exogenous supply of folic acid, namely, *Bacterium coli*, *Lactobacillus arabinosus* 17-5, *L. plantarum* 10S and *Schizosaccharomyces octosporus*. Even at 10^{-3} M neither stimulation nor inhibition of growth of any of these organisms was induced by the analogue.

DISCUSSION

The results show that 2-deaminopteroylglutamic acid is a compound which can interfere with the metabolism of folic acid in two organisms which require this vitamin as a nutrient for growth. It is interesting to note, in the case of *Strep. faecalis*, how strikingly the relationship between analogue and growth factor alters with the nature of the latter. With pteric acid a 3.5-fold increase in the concentration of the analogue demands a 33-fold increase in growth factor concentration. Yet a 25-fold increase of analogue requires only a five-fold increase of rhizopterin to produce the same amount of growth. Both relationships are clearly not competitive, but deviate in opposite directions.

The relationship between the analogue and pteronic acid is similar to that between 4-aminopteroylglutamic acid and pteroylglutamic acid with this same organism. Oleson, Hutchings & SubbaRow (1948) found that a ninefold increase in concentration of inhibitor required a 100-fold rise in pteroylglutamic acid concentration to maintain half-maximal growth.

Fig. 8. Stimulation by pteroylglutamic acid (●) and by 2-deaminopteroylglutamic acid (○) of the suboptimal growth of *L. casei* supported by a mixture of thymine and hypoxanthine.

With *Strep. faecalis* the relationship between 2-deaminopteroylglutamic acid and pteroylglutamic acid, on the other hand, appears to be a competitive one. In the same test conditions pteroylaspartic acid (Hutchings *et al.* 1947) and several inhibitory pterins (Daniel, Norris, Scott & Heuser, 1947) showed a competitive relationship.

For the type of relationship of the analogue with rhizopterin and Leucovorin in *Strep. faecalis*, and with pteroylglutamic acid and Leucovorin in *L. casei*, no parallel can be found amongst the other reported analogues of folic acid. On the other hand, inhibition of growth of *Strep. faecalis* by 7-methylpteroylglutamic acid is overcome less efficiently by pteroylglutamic acid than by rhizopterin (Gordon, Ravel, Eakin & Shive, 1948). Similarly, natural *Leuconostoc citrovorum* factor overcomes the toxicity of 4-aminopteroylglutamic acid in the rat but pteroylglutamic acid does not (Nichol & Welch, 1950).

We can offer as yet no acceptable explanation for the stimulation of the growth of *L. casei* by 2-deaminopteroylglutamic acid in the medium which contained purine and thymine in place of folic acid. Two possibilities might be considered. The first is that *L. casei* is capable of aminating the compound and that this is a slow reaction which only proceeds at a significant rate when the population is large. It might be achieved where there is considerable growth independent of folic acid, but not where there is a small initial population and where the ratio between analogue and growth factor is an inhibitory one. The second and more remote possibility is that the thymine-purine medium contains the end-products of all but one of the reactions for which folic acid is required, and that this hypothetical reaction can be catalysed by a form of folic acid which lacks the 2-amino group. One of the difficulties of accepting this suggestion is that the organism grows in these conditions at an apparently maximal rate (although to a submaximal density) without the addition of folic acid. The last postulated function cannot therefore be regarded as essential, or else suboptimal traces of folic acid are (a) present in the medium, or are (b) synthesized by *L. casei* (cf. Rogers & Shive, 1948). All that can be said with certainty, however, is that the real explanation can only be found after further experiments.

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