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Factors affecting the Requirement of *Tetrahymena pyriformis* (*geleii*) for Folic Acid

BY VIRGINIA C. DEWEY AND G. W. KIDDER

Biological Laboratory, Amherst College, Amherst, Massachusetts, U.S.A.

SUMMARY: In the use of an organism for assay of folic acid those compounds which affect the utilization of folic acid should be determined. With *Tetrahymena pyriformis* (*geleii*), it is shown that thymine (or thymidine), glycine, *p*-amino-benzoic acid, creatin and vitamin B₁₂ diminish the requirement for folic acid. Pterotic acid, *p*-aminobenzoylglutamic acid, 5-methylcytosine, *p*-hydroxybenzoic acid, carbamylaspartic acid and ribonucleic acid have no effect on the requirement, while cholesterol and cortisone increase the requirement.

For some time the efforts of this laboratory have been turned toward the formulation of a technique by means of which the ciliate *Tetrahymena pyriformis* (*geleii*) (Corliss, 1952), could be utilized as a means of assay for folic acid and its conjugates. For this purpose the organism possesses a number of advantages: (a) it is able to utilize either free folic acid or its polyglutamic acid conjugates with equal facility (Kidder & Dewey, 1947); (b) it does not utilize either pterotic acid or the SLR factor (Kidder & Fuller, 1946) in place of folic acid; (c) folic acid cannot be replaced by a mixture of purines plus thymine (Kidder, Dewey, Parks & Heinrich, 1950). The organism shares with some bacteria the ability to grow in a medium of chemically-defined composition (Dewey, Parks & Kidder, 1950) and the ease with which measurements of growth may be made (Kidder & Dewey, 1948). However, in order to assay materials of plant or animal origin, it must first be determined whether or not such materials contain substances which will spare the requirement for folic acid or give non-specific stimulation. Since such materials undoubtedly contain many substances of unknown nature, one can only test a series of compounds which seem to be involved in folic acid metabolism. The presence of another member of the folic acid group, i.e. the citrovorum factor (Sauberlich & Baumann, 1948) must also be taken into account. It has been shown that this material has an activity for *T. pyriformis* somewhat inferior to that of folic acid (Kidder, Dewey & Parks, 1951*a*). It has recently been reported that the naturally occurring citrovorum factor has an activity double that of the synthetic material (Keresztesy & Silverman, 1951). This difference appears to be due to the fact that the synthetic material consists of a racemic mixture of which only one isomer has appreciable biological activity (Cosulich, Smith & Broquist, 1952). On this basis it would appear that natural citrovorum factor has an activity for *T. pyriformis* about equal to that of folic acid. It has been suggested that all of the folic acid, within certain cells at least, can be accounted for as citrovorum factor (Swendseid, Bethell & Ackermann, 1951), although later work shows that there is a fairly large amount of free folic

acid in tissues and that both folic acid and citrovorum factor occur naturally as conjugates with glutamic acid (Wieland, Hutchings & Williams, 1952).

The present communication deals with the ability of many compounds to diminish the requirement of *T. pyriformis* for folic acid.

MATERIALS AND METHODS

The organism used was *Tetrahymena pyriformis* (geleii) W in pure bacteria-free culture. It was grown in medium A (Dewey *et al.* 1950) containing 10 mg. Tween 80/ml. in place of Tween 85. Folic acid (pteroylglutamic acid) was omitted from the medium and used as a variable component as has been described previously (Kidder, Dewey & Parks, 1951*b*). The range of concentrations tested was from 0.1 to 1.0 $\mu\text{g./ml.}$ To the basal medium additions were made as noted in the various experiments. All the experiments were done in triplicate and repeated a number of times.

The inoculum was prepared by transferring organisms grown in 2% proteose peptone into basal medium containing 0.5 $\mu\text{g.}$ folic acid/ml. This procedure is necessary to eliminate the 'carry-over' effect (Kidder, 1946). In certain experiments the age and size of the inoculum were varied to determine the effects of these factors on growth. A standard inoculum 7–9 days old at a dilution of 1/200 was used in most of the experiments. On a few occasions an inoculum grown in the complete absence (except for carry-over) of folic acid was used.

The medium was adjusted to pH 7.0 by means of a Beckman pH meter in order to protect the folic acid (the presence of Tween in the medium does not permit the accurate use of indicator dyes). Every possible effort was made to protect the media from light in order to minimize the destruction of folic acid. After inoculation the cultures were incubated in the dark at 25° in a sloped position for 96 hr. Readings of the turbidity produced by the growth of the organisms were made by means of a Lumetron photoelectric colorimeter with red filter no. 650.

RESULTS

In view of the many factors which affect the response of *T. pyriformis* to folic acid and produce variability in the results, typical experiments have been chosen to illustrate the effects of materials added to the medium. The same experiment done at different times may give somewhat different results quantitatively, although they have been repeated frequently enough to be certain of the qualitative responses.

Data obtained by varying the age of the inoculum indicate that the folic acid requirement decreases to a minimum when the inoculum is 7–9 days old, being greater with either older or younger inocula. When inocula are less than 7 days old this may be due to the fact that the inoculum is smaller. Inocula of 1/500 or 1/1000 also exhibit a larger folic acid requirement. This is not attributable to carry-over of folic acid as such in the medium, since essentially the same results are obtained using first transplant inocula grown in the absence of added folic acid (Kidder, 1946). Occasionally the older inocula failed to grow when transplanted into media containing even supra-

optimal amounts of folic acid (1 $\mu\text{g./ml.}$), although organisms from the same cultures grew rapidly when transplanted into proteose peptone. Folic acid has a large effect on the rate of growth of a culture of *T. pyriformis*. Sub-optimal concentrations of folic acid permit only slow growth, but the maximum yield of organisms eventually reaches nearly that attained in higher concentrations (Fig. 1). Thus it is apparent that the time at which the amount of growth is determined will have an influence on the apparent magnitude of the requirement for folic acid.

Fig. 1. Influence of folic acid and thymine on growth rate of *T. pyriformis*.

It was shown some years ago that folic acid could be replaced in the nutrition of *Lactobacillus casei* and *Streptococcus faecalis* by a mixture of thymine and purines (Stokstad, 1941; Snell & Mitchell, 1941). It has already been demonstrated that *T. pyriformis* requires folic acid in addition to purines and thymine (Kidder *et al.* 1950), although thymine stimulates growth. Table 1 shows the degree of stimulation given by thymine in the presence of low concentrations of folic acid. It can be seen that the addition of thymine to the medium greatly increases the blank reading. This blank is quite variable and in some cases higher than half-maximal growth. Nevertheless, media which produce such growth are incapable of supporting a second transplant; this must be attributed to the sparing of folic acid carried over in the inoculum. Thymidine is even more effective than thymine in sparing folic acid and for *Leuconostoc citrovorum* it can replace folic acid entirely (Sauberlich, 1949). In contrast, 5-methylcytosine has no influence on the folic acid requirement of *T. pyriformis*. This compound, like cytosine, is apparently not deaminated. It seems clear that one of the functions of folic

acid in the nutrition of *T. pyriformis* is in the synthesis of thymine or of thymidine. One of the suggested intermediates in the synthesis of pyrimidines by micro-organisms is ureidosuccinic acid (carbamylaspartic acid; Wright, Valentik, Spicer, Huff & Skeggs, 1950). For this reason carbamylaspartic acid was tested for its ability to replace either uracil or thymine in the growth of *T. pyriformis*. It was found to be entirely without activity. This was not entirely unexpected in view of the inactivity of orotic acid, of which ureidosuccinic acid is a precursor with *Lactobacillus bulgaricus*.

Table 1. *The influence of thymine on the folic acid requirement of Tetrahymena pyriformis*

Addition ($\mu\text{g./ml.}$)	Folic acid required for half-maximal growth ($\text{m}\mu\text{g./ml.}$)	Blank reading, optical density
None	0.320	0.056
Thymine		
10	0.190	0.102
20	0.180	0.117
30	0.175	0.128
40	0.170	0.123
Thymidine		
5	0.075	0.194

While *p*-aminobenzoic acid (*p*-AB) forms a part of the folic acid molecule, there have been suggestions (Lampen & Jones, 1947; Sarrett 1947) that *p*-AB has a function of its own. The effect of adding *p*-AB to media low in folic acid was tested, and it was found to increase growth slightly over that of controls

Table 2. *The effect of nucleic acid derivatives on the folic acid requirement of Tetrahymena pyriformis*

Additions ($\text{m}\mu\text{g./ml.}$)	Nucleic acid derivatives*					
	None		None		Glycine and thymine†	
	<i>a</i>	<i>b</i>	<i>a</i>	<i>b</i>	<i>a</i>	<i>b</i>
None	0.410	0.056	0.440	0.062	0.315	0.123
<i>p</i> -Aminobenzoic acid						
0.1	0.360	0.057	0.385	0.058	0.270	0.128
0.5	0.320	0.053	0.296	0.065	0.250	0.114
1.0	0.325	0.058	0.296	0.058	0.200	0.127
10.0	0.330	0.061	0.290	0.064	0.175	0.128
100.0	0.350	0.059	0.290	0.068	0.160	0.138

* Nucleic acid derivatives used in the basal medium at tenfold concentration.

† Thymine, 40 $\mu\text{g./ml.}$; glycine, 200 $\mu\text{g./ml.}$

a = $\text{m}\mu\text{g.}$ folic acid/ml. required for half maximal growth.

b = Optical density readings of blank cultures.

(Table 2). This effect was additive to that obtained by the addition of thymine and glycine. While the addition of the nucleotides derived from nucleic acid had no effect alone, they improved growth when added in the presence of *p*-AB. The significance of these results is not yet clear. They suggest that if *p*-AB is required, it is obtained by the breakdown of the folic acid molecule or that

it is present as a trace contaminant of the folic acid used. It would appear that its function is not concerned with the metabolism of glycine or of the purines or pyrimidines. It is interesting that *p*-hydroxybenzoic acid is entirely inert under these conditions.

Pteric acid (*p*-aminobenzoylpteridine) has no sparing action on folic acid, nor has rhizopterin (SLR factor, formylptericoic acid), although both are capable of replacing folic acid in the growth of *Streptococcus faecalis* R (Stokes, Keresztesy & Foster, 1944). Pteric acid or SLR factor isolated from natural sources were, however, capable of replacing folic acid when used at sufficiently high levels, but this is undoubtedly due to contamination of such preparations with folic acid. On the other hand, *p*-aminobenzoylglutamic acid does not influence the folic acid requirement of *T. pyriformis*.

The work of Stekol & Weiss (1950) and of Stekol, Weiss & Weiss (1951) showed that the utilization of glycine as a source of labile methyl groups in mammalian nutrition is dependent on the presence of folic acid. That glycine also spares folic acid in the nutrition of *T. pyriformis* is shown in Table 3. This may be related to the fact that serine synthesis from glycine fails except in the presence of extremely high levels of folic acid (Kidder & Dewey, 1958). It would appear that conversion of serine to glycine is also under the control of folic acid. It may also be seen (Table 2) that the action of thymine, glycine and *p*-AB are additive.

Table 3. *The influence of glycine on the folic acid requirement of Tetrahymena pyriformis*

Additions ($\mu\text{g./ml.}$)	<i>p</i> -AB							
	None		Thymine		None		Thymine	
	<i>a</i>	<i>b</i>	None <i>a</i>	<i>p</i> -AB <i>b</i>	<i>a</i>	<i>b</i>	<i>a</i>	<i>b</i>
None	0.265	0.079	0.100	0.193	0.205	0.114	0.003	0.347
Glycine								
10	0.195	0.088	0.090	0.217	—	—	—	—
25	0.195	0.094	0.060	0.229	—	—	—	—
50	0.180	0.087	0.045	0.242	—	—	—	—
100	0.175	0.087	0.042	0.253	0.115	0.130	*	0.377
200	0.170	0.100	0.030	0.262	—	—	—	—

* Could not be determined because blank reading was greater than half-maximal.

a = Amount of folic acid required for half-maximal growth in $\text{m}\mu\text{g./ml.}$

b = Optical density readings of blank cultures.

Thymine concentration, 40 $\mu\text{g./ml.}$, *p*-AB, 1 $\mu\text{g./ml.}$

It has been reported that the sterols, dehydroisoandrosterone and cortisone (Gaines & Totter, 1950; Gaines & Broquist, 1951) can replace folic acid in the nutrition of *Lactobacillus casei*. The effect of the addition of either cortisone or cholesterol on the response of *T. pyriformis* to folic acid is shown in Fig. 2. While cortisone at 100 $\mu\text{g./ml.}$ is inhibitory, lower concentrations had no effect on growth. Cholesterol was somewhat less inhibitory, and in the presence of adequate folic acid was even slightly stimulatory. The addition of glycine and thymine to the medium did not overcome the inhibitory effect of cortisone, although it did that of cholesterol. Dehydroisoandrosterone was not tested.

A discussion of the interrelationships involving the steroid hormones, folic acid and ascorbic acid may be found in Lloyd & Sinclair (1958).

In Table 4 are listed a number of other compounds which might possibly affect or be affected by folic acid. Betaine (Dubnoff, 1949) and formate (Sakami, 1948) are known to be sources of labile methyl groups in the mammal, but it is apparent that they have little, if any, effect on the folic acid requirement of *T. pyriformis*. It has been noted that excess creatin is excreted by

Fig. 2. Influence of sterols on response of *T. pyriformis* to folic acid.

the monkey in folic acid deficiency (Dinning & Day, 1949). Since creatin is a methylated compound it might spare folic acid in transmethylation reactions. This may, indeed, be the explanation of the slight sparing effect exhibited by creatin. Vitamin B₁₂ is believed to function in the synthesis of thymidine (Wright, Skeggs & Huff, 1948) or other deoxyribosides (Skeggs, Spizizen & Wright, 1950), and it is, therefore, to be expected that it might function also in the sparing of folic acid. This action was noted (Table 4).

The ability of preparations of ribonucleic acid (RNA) to spare folic acid and at high levels even to replace it, can be shown to be due to the presence of folic acid as a contaminant. Purification of commercial samples of RNA by

repeated reprecipitation with ethanol or with acid gave material which had no sparing effect on folic acid. Similar treatment of commercial deoxyribonucleic acid (DNA) was not successful in removing the activity, but purer samples isolated in the laboratory gave results which could be explained solely on the basis of the thymidine content of DNA. It seems probable that the reported ability of *T. pyriformis* to grow in the absence of certain vitamins (Elliott, 1950; Seaman, 1958) can be attributed to the use of contaminated RNA in the medium.

Table 4. Comparison of effects of various compounds on the folic acid requirement of *Tetrahymena pyriformis*

Addition ($\mu\text{g./ml.}$)	Folic acid required for half-maximal growth ($\mu\text{g./ml.}$)	Blank reading, optical density	Maximum reading, optical density
None	0.214	0.086	0.626
Betaine, 40	0.230	0.095	0.600
Cholesterol, 10	0.245	0.085	0.585
Haemin, 10	0.238	0.148	0.620
Formate, 100	0.190	0.102	0.581
Creatin, 40	0.155	0.110	0.591
Glycine, 100	0.140	0.100	0.623
Thymine, 40	0.065	0.238	0.629
Glycine, 100; thymine, 40	0.040	0.251	0.585
None	0.143	0.118	0.614
Vitamin B ₁₂ , 0.001	0.086	0.154	0.617

In order to perform assays for folic acid, its conjugates or citrovorum factor, by the use of *T. pyriformis*, it would be necessary to include in the medium all of the substances mentioned above which have shown a sparing effect, and to prepare an inoculum which would give a low blank reading without decreased vitality. On the other hand, since *T. pyriformis* exhibits a typical animal pattern in its folic acid nutrition, as well as in other respects, assays in the basal medium would give results indicative of the total folic acid activity of the material assayed. One major difference between the ciliate and the mammal lies in the inability of *T. pyriformis* to transmethylate homocysteine (Genghof, 1949), a reaction which is influenced by folic acid. Another major difference is the inability of the mammal to utilize exogenous thymine (Plentl & Schoenheimer, 1944). This discrepancy can be readily overcome by the inclusion of thymine in the assay medium. The main advantages of the use of *T. pyriformis* rather than the various lactobacilli for folic acid assays lies in the ability of the ciliate to utilize conjugates of folic acid without previous digestion. Bacterial assays would still be necessary in order to differentiate between folic acid and the citrovorum factor.

The only naturally occurring compound which will replace folic acid in the nutrition of *T. pyriformis* is the citrovorum factor (5-formyl-5:6:7:8-tetrahydropteroylglutamic acid) or the conjugates of these compounds. The earlier report (Kidder *et al.* 1951a) that aminopterin could replace folic acid for growth was due to gross contamination of the sample of aminopterin used

with folic acid (Heinrich, Dewey & Kidder, 1953). It seems probable that other folic acid analogues which promote growth of *T. pyriformis* are also contaminated.

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