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The Folic Acid Group of Growth Factors

1. THE COMPLEXITY OF GROWTH FACTORS FOR *STREPTOCOCCUS LACTIS* R

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Considerable confusion has arisen with regard to the various preparations which have been reported as having activity for *Streptococcus lactis* R. In the main this has been due to a desire on the part of many workers to over-simplify the question by claiming that preparations obtained by different methods from a variety of sources contain identical substances. The complexity of the subject was obvious, however, as soon as it became possible to compare the activity of the liver fractions of Bohonos, Hutchings & Peterson (1941) and of Stokstad (1943), with a yeast-derived sample obtained by the last worker, and one isolated from fermentation residues by Bohonos, Hutchings, Slobodkin & Stokstad (1944). Comparative data for these preparations, and for other active materials whose preparation and properties have since been reported, are given in Table 1.

Recently Johnson (1946) published data for *Lactobacillus casei* and *Strep. lactis* R, claiming to show that folic acid, Vitamin B₆ and the *L. casei*

factor (both synthetic, i.e. pteroyl-glutamic acid and natural, i.e. the preparation from liver) are identical, a conclusion which does not appear to be completely justified in view of the data of Table 1. Since synergism has been observed between various fractions isolated from liver (Chattaway, Dolby & Happold, 1944), it was decided to examine the effect of the simultaneous addition of pairs of various growth factor preparations to culture media, using *Strep. lactis* R as test organism. This organism was chosen in preference to *L. casei* because, using a medium based on an acid-hydrolysate of casein as generally employed in these laboratories, it has not been found possible to stimulate good growth of *L. casei* by the sole addition of one of the 'folic acid group' of factors. Simultaneous addition of a pair of growth-factor preparations, separately apparently identical as tested by the growth response of *Strep. lactis* R, might indicate differences in composition, whether the individual growth-factor preparations were single or complex

Table 1. *Comparative data for the folic acid group of factors*

Name of preparation	Source	Activity for		Ultra-violet spectrum	Other properties	Reference
		<i>L. casei</i>	<i>Strep. lactis</i> R			
		(Amounts for half max. growth $\mu\text{g./ml. medium}$)				
Norite eluate factor <i>L. casei</i> factor	Liver	0-000055	0-00025	Not like xanthopterin	Contains pteridine nucleus but apparently not the same structure as xanthopterin	Bohonos <i>et al.</i> (1941)
Do.	Yeast	0-000055	0-00050	Not like xanthopterin	—	Stokstad (1943)
Do.	Fermentation residues	0-000061	0-0042	Not like xanthopterin	—	Bohonos <i>et al.</i> (1944)
Pteroyl-glutamic acid synthetic <i>L. casei</i> factor	Synthetic	0-00007	0-0003	Not like xanthopterin	Claimed as sole requirement of <i>L. casei</i> ; not substantiated by Chattaway <i>et al.</i> (1944, 1945)	Angier <i>et al.</i> (1945, 1946)
Folic acid	Spinach	0-000072	0-000055	Xanthopterin-like	Claimed by Mitchell <i>et al.</i> to be like but not identical with xanthopterin	Mitchell <i>et al.</i> (1941)
Vitamin B ₆	Liver	0-00005	0-00025	—	Suggested as identical with 'norite eluate factor' and folic acid	Binkley <i>et al.</i> (1943)
Vitamin B ₆ conjugate	Yeast	0-01	0-12	—	—	Binkley <i>et al.</i> (1944)
<i>Strep. lactis</i> factor	—	0-8	0-000034	—	Gives unstable folic acid-like material when incubated with <i>Strep. lactis</i> . Foster <i>et al.</i> (1944) and Larsen & Stokes (1945)	Keresztesy <i>et al.</i> (1943)
<i>L. casei</i> factors	Liver	Certain of these factors alone show activity for <i>Strep. lactis</i> but none for <i>L. casei</i> . The addition of two or more produces a medium capable of supporting the growth and stimulating the acid production of <i>L. casei</i>				Chattaway <i>et al.</i> (1944, 1945)

In view of the observations of Mitchell & Snell (1941), Stokes (1944), and Kreuger & Peterson (1945) that thymine could replace folic acid and vitamin B₆ in the growth of *Strep. lactis* R and the claim of Chattaway (1944) that orotic acid was active for *L. casei*, these compounds have also been tested for synergism with the other naturally occurring factors.

METHODS

Organism. The strain employed in these experiments, *Strep. lactis* R (that employed by Mitchell, Snell & Williams (1941) for the assay of folic acid preparations), was obtained from the National Collection of Type Cultures (No. 6459). The stock was maintained by weekly subculture on sucrose agar slopes fortified with liver extract. The inoculum for each 10 ml. tube of medium consisted of a 0-05 ml. drop of a sterile water suspension of a 24 hr. subculture also on liver-sucrose agar (approximately 10⁶ organisms/ml. of suspension).

Medium. The medium used was essentially that of Snell, Guirard & Williams (1942) for the assay of folic acid. The amino-acid complement was sup-

plied by the inclusion of an acid-hydrolysate of casein prepared by the method of Johnson & Mueller (1941), with the addition of cystine, tryptophan, leucine and isoleucine (cf. Dolby & Waters, 1944). The medium was prepared in double strength and all additions were made before the final volume was brought to 10 ml., thus ensuring identical conditions for each culture.

Growth-factor preparations used. The following samples of preparations having considerable growth activity for *Strep. lactis* were available:

(1) A sample of a 7% folic acid concentrate, isolated from spinach by Prof. R. J. Williams.

(2) The synthetic *L. casei* factor (pteroyl-glutamic acid) prepared in the Lederle laboratories (Angier, Boothe, Cosulich, Fahrenbach, Hutchings, Hultquist, Kuh, Mowat, Northey, Seager, Semb, Sickels, Smith, Stokstad, Subbarow & Waller, 1945).

(3) A sample of vitamin B₆ isolated by Binkley, Bird, Bloom, Brown, Emmett, Hogan, O'Dell & Pfflner (1943) and supplied by the Parke Davis laboratories.

(4) A liver fraction prepared by Chattaway, Dolby, Happold, McMillan & Waters (1945): the

alumina eluate fraction of a baryta soluble portion of a liver extract.

(5) An extract prepared from poppy-straw by Hoffmann La Roche Ltd. (Basle), and claimed to be equivalent to Williams's folic acid. These preparations were kept in aqueous solution containing 1 or 10 $\mu\text{g.}$ of solid/ml. at 0°, and added to the medium, after suitable dilution, before autoclaving. It was proved by control experiments in which the additions were made aseptically after sterilization that none of these preparations was adversely affected by steam sterilization in the presence of the medium. Orotic acid and thymine were similarly added before sterilization.

Measurement of the effects produced. Except on a few occasions growth *per se* was not measured, the acid produced by a culture being titrated with 0.05 N-NaOH to pH 7.4 instead. It has been shown (Luckey, Briggs & Elvehjem, 1944; Hall, 1947) that acid production is not strictly parallel to growth; therefore no claim can be made that the results quoted below indicate changes in growth. Qualitatively, however, with the preparations tested, parallelism existed between growth and acid production; no effect similar to that described by Dolby, Happold & Sandford (1944), in which growth could be entirely separated from acid production, was noticed. The rate of acid production decreased rapidly as the pH of the medium fell, and the glucose utilization in such cases was never optimal. Maintenance of pH at a constant level during long periods of incubation by the continuous addition of alkali was not practicable owing to the alterations in rate of acid production which are apparent during a long period of growth (cf. Hall, 1947). Therefore, as a compromise when longer periods of growth were studied, the pH value was adjusted to 7.4 by the aseptic addition of 0.05 N-NaOH every 24 hr. This enabled a progressive-record of the acid production to be obtained; in such cases the total amount of available glucose was ultimately utilized. The medium was sterilized by autoclaving for 10 min. at 10 lb./sq.in. The tubes of medium after inoculation were incubated at 30°.

RESULTS

The growth-factor preparations tested separately as promoters of acid production

All the preparations were tested independently for activity in stimulating acid production in growing cultures of *Strep. lactis* R. A range of concentrations of from 1 $\mu\text{g.}$ to $1 \times 10^{-9} \mu\text{g./ml.}$ of medium was examined, thus enabling differences unnoticed by Johnson (1946) to be observed. Fig. 1 shows a typical acid production/concentration curve, after 18 hr. incubation, for a culture containing pteroyl-

glutamic acid. The portion indicated by the arrows, is almost linear when plotted directly and not semilogarithmically; this is the portion of most use for assay purposes, and covers the concentration range examined by Johnson.

Fig. 1. The relationship between acid production and concentration of pteroyl-glutamic acid added to cultures of *Strep. lactis* R. Incubation 18 hr. at 30°. The portion between the arrows is almost linear if not plotted as a logarithm.

Table 2 shows a series of typical acid production figures for 24 hr. growth of cultures stimulated by a variety of factors. It will be seen that over the region of steepest slope, the acid production curves bear a marked resemblance to one another, differing only in respect of the concentration of the factors, which is affected only by the purity of the individual preparations. The figures for the extremes of concentration, however, do not show such a marked similarity. The upper limit of acid production towards which the figures asymptotically approached was not a constant figure, but varied between 3.90 and 6.15 ml. The concentration at which the point of inflexion occurred was not a fixed value either, the required concentration for half maximum acid production occurring at 3×10^{-6} , 1×10^{-4} , 3×10^{-6} , 6×10^{-4} , and $10 \mu\text{g./ml.}$ of medium for pteroyl-glutamic acid, folic acid, vitamin B₁₂, the poppy-straw extract, and the liver fraction respectively.

There were differences at the lower extreme as well, folic acid always showing a step at the lowest concentrations. The activity in no case decreased gradually on the horizontal portion of the curve as with the synthetic material.

Table 2. *Comparison between the acid production induced in cultures of Strep. lactis R by the inclusion of various growth-factor preparations*

(Incubation period 24 hr. at 30°. Acid production is measured in terms of ml. of 0.05 N-NaOH needed to bring to pH 7.4, 10 ml. of culture. Blank for 10 ml. of medium without addenda = 0.30 ml. of 0.05 N-NaOH.)

Concentration of factor (Cols. 1-4, 1×10^{-x} μ g./ml. medium) (Col. 5, 1×10^{-x} mg./ml. medium)	Acid production				
	Pteroyl-glutamic acid	Folic acid	Vitamin B ₉	Poppy-straw extract	Liver fraction
	ml. 0.05 N-NaOH				
(x)					
0	—	3.90	—	6.15	—
1	5.90	3.40	—	1.30	3.90
2	5.95	3.10	3.95	0.50	3.60
3	5.85	2.20	3.65	0.25	2.80
4	5.80	1.90	3.00	0.20	2.10
5	5.25	1.50	1.90	—	1.40
6	1.45	1.30	0.90	—	1.20
7	0.70	1.20	0.65	—	0.90
8	0.40	1.20	0.65	—	—
9	0.20	1.10	—	—	—

The examination of the activity of pairs of growth-factor preparations

In the examination of pairs of growth-factor preparations, care was taken in the choice of concentrations in order that there might be no possibility of the composite activity producing an effect in excess of the direct sum merely because of the increased gradient of the middle portion of the curve. With this in view the concentrations were so chosen that the acid production figures associated with them lay on either the upper or lower horizontal portions of the curve.

whereas when such an addition was made to lower concentrations of folic acid, synergism occurred (see 0.0001 and 0.00001 μ g. levels of folic acid). The addition of much smaller concentrations of pteroyl-glutamic acid, however, had an effect which was at least additive over the whole range, and definitely synergistic in certain levels. The reciprocal effect which is indicated by the last two columns in Table 3, shows that the presence of large quantities of folic acid fixed the relative degree of acid production, and that alterations in the concentration of pteroyl-glutamic acid merely superimposed a slight variation on this. With lower concentrations

Table 3. *The effect of folic acid and pteroyl-glutamic acid when added to cultures simultaneously*

(Acid production is measured in terms of ml. of 0.05 N-NaOH/10 ml. medium needed to bring to pH 7.4 after 18 hr. incubation at 30°. Blank titration for 10 ml. medium without addenda = 0.20 ml.)

Concentration of folic acid or pteroyl-glutamic acid (μ g./ml.)	Acid production					
	Folic acid alone	Pteroyl-glutamic acid alone	Folic acid + pteroyl-glutamic acid		Pteroyl-glutamic acid + folic acid	
			0.00001 μ g./ml.	0.0000001 μ g./ml.	0.1 μ g./ml.	0.0001 μ g./ml.
0.1	2.56	—	3.05	2.95	—	—
0.01	2.28	2.55	2.88	3.15	3.95	2.74
0.001	1.92	2.45	2.59	2.06	—	—
0.0001	0.25	2.28	1.45	0.60	3.55	2.45
0.00001	0.00	0.76	0.94	0.25	—	—
0.000001	—	0.45	—	—	3.56	1.10
0.0000001	—	0.22	—	—	—	—
0.00000001	—	0.10	—	—	3.38	0.40

1. *Pteroyl-glutamic acid and folic acid.* Table 3 shows the figures for the acid production of a series of cultures containing both folic acid and pteroyl-glutamic acid at a series of concentrations, together with corresponding figures for the separate preparations. The addition of a relatively high concentration of pteroyl-glutamic acid to high concentrations of folic acid had no significant effect (column 4)

of folic acid, however, the two acted synergistically, while the relative concentration of pteroyl-glutamic acid was the deciding factor. The effects produced at intermediate concentrations tended to be suspect on account of the considerations mentioned in the introductory paragraph to this section, but it is quite definite that synergism at least as far as acid producing capacity is concerned occurred between

folic acid and pteroyl-glutamic acid at the lower concentrations.

2. *The relationship of the poppy-straw extract and the liver fraction to the other factors.* A pair of similar series were prepared containing the poppy-straw extract of Hoffmann La Roche Ltd. in addition to another preparation, either folic acid or pteroyl-glutamic acid. Tables 4 and 5 show the acid produced after 18 hr. incubation of cultures containing

Table 4. *The effect of the simultaneous addition of poppy-straw extract and folic acid*

(Acid production is measured in terms of ml. of 0.05 N-NaOH/10 ml. medium needed to bring to pH 7.4 after an incubation period of 24 hr. at 30°. Blank titration for 10 ml. medium without addenda = 0.30 ml. of 0.05 N-NaOH.)

Additions	Acid production			
	I	II	III	IV
10 μ g. poppy-straw extract I	6.60	—	5.61	6.15
0.01 μ g. poppy-straw extract II	—	0.50	—	0.95
0.1 μ g. folic acid III	—	—	2.34	—
0.001 μ g. folic acid IV	—	—	—	0.47

such additions. It can be seen from Table 4, that no synergism occurred between folic acid and the poppy-straw extract; in fact there was a slight inhibition when either was present in high concentrations. At lower concentrations summation occurred, but at no value was the combined activity greater than the sum of those of the separate factors. On the other hand there was slight inhibi-

tion when a high concentration of pteroyl-glutamic acid was added to the poppy-straw extract and less than the additive effect when a smaller concentration was given, but low concentrations of both preparations produced a synergistic effect. The composite activity in this case was nearly 50% higher than the sum of the two separate effects (see Table 5).

The liver fraction prepared by Chattaway *et al.* (1945) and the poppy-straw extract are both very impure. No claim was made that the liver material was homogeneous, but it has been shown that the majority of the folic acid activity as measured by the promotion of growth of *Strep. lactis* R is concentrated in this fraction of the original liver extract. Table 6 shows the effect of mixtures of this preparation with various other growth-factor preparations. It can be seen that this liver material showed a marked similarity in effect to pteroyl-glutamic acid giving synergism with the poppy-straw extract and with folic acid at low concentrations. There appeared, however, to be a factor present which caused a marked inhibition when large concentrations of the liver preparation were present. This had the effect of depressing the acid production induced by the simultaneous presence of 0.1 μ g. of folic acid and 1 mg. of liver extract.

3. *Vitamin B_c.* Of the factors available from animal and plant sources, vitamin B_c, as a member of the former group, might be expected to show similar properties to pteroyl-glutamic acid and the

Table 5. *The effect of adding poppy-straw extract simultaneously with pteroyl-glutamic acid*

(Acid production is measured in terms of ml. of 0.05 N-NaOH/10 ml. medium needed to bring to pH 7.4 after an incubation period of 18 hr. at 30°. Blank titration for 10 ml. medium without addenda = 0.17 ml. of 0.05 N-NaOH.)

Additions	Acid production			
	I	II	III	IV
10 μ g. poppy-straw extract I	6.6	—	5.95	6.96
0.01 μ g. poppy-straw extract II	—	0.45	—	1.54
0.001 μ g. pteroyl-glutamic acid III	—	—	3.9	—
0.000001 μ g. pteroyl-glutamic acid IV	—	—	—	0.60

Table 6. *The simultaneous addition of liver extract (Chattaway et al. 1945) with other growth-factor preparations of the folic acid group*

(Acid production is measured in terms of ml. of 0.05 N-NaOH/10 ml. medium needed to bring to pH 7.4. Blank titration for 10 ml. of medium without addenda = 0.24 ml. of 0.05 N-NaOH. Incubation period 18 hr. at 30°.)

Additions	Acid production		
	Alone	With 1 mg. liver extract	With 0.001 mg. liver extract
0.01 μ g. pteroyl-glutamic acid	2.65	2.50	—
0.000001 μ g. pteroyl-glutamic acid	0.85	2.85	0.95
0.1 μ g. folic acid	2.35	3.68	—
0.0001 μ g. folic acid	0.32	3.39	0.98
10 μ g. poppy-straw extract	6.46	5.85	—
0.01 μ g. poppy-straw extract	0.55	2.96	1.30
1 mg. liver extract	2.80	—	—
0.001 mg. liver extract	0.55	—	—

liver fraction. Table 7 shows a set of typical figures for the simultaneous inclusion of vitamin B₆ and both folic acid and pteroyl-glutamic acid. Synergism was not so marked with folic acid as might be expected, and inclusion of pteroyl-glutamic acid produced a result, the implications of which are discussed below.

titration figures. As claimed by Stokes (1944) and others, thymine had considerable activity as a factor inducing acid production by *Strep. lactis* R, and a fact noticed by Stokes (1944), but apparently ignored in his discussion of results, is particularly apparent from these figures, i.e. there was a lag phase during which comparatively little growth or

Table 7. *The simultaneous addition of vitamin B₆ and other growth-factor preparations to growing cultures of Strep. lactis R*

(Acid production is measured in terms of ml. of 0.05 N-NaOH/10 ml. medium needed to bring to pH 7.4 after 18 hr. incubation at 30°. Blank titration for 10 ml. medium without addenda = 0.05 N-NaOH.)

Additions of vitamin B ₆ (μg.)	Alone	Acid production					
		Folic acid (μg.)			Pteroyl-glutamic acid (μg.)		
		0.01	0.0001	0.000001	0.0001	0.000001	0.00000001
0	—	1.58	1.05	0.75	1.40	0.75	0.55
0.01	2.08	1.35	1.25	1.30	—	—	—
0.0001	1.70	1.56	1.60	1.55	1.20	1.15	0.75
0.000001	0.60	1.75	1.10	0.74	1.20	0.95	0.75
0.00000001	0.45	1.24	0.75	0.75	1.15	0.90	0.75
0.0000000001	0.46	1.30	0.75	0.72	—	—	—

Examination of the effect of thymine and orotic acid on acid production

Both thymine and orotic acid were examined for independent activity as promoters of acid production by *Strep. lactis* R; Table 8 gives typical

Table 8. *The effect of the addition of orotic acid and thymine to cultures of Strep. lactis R*

(Acid production is measured in terms of ml. of 0.05 N-NaOH/10 ml. medium needed to bring to pH 7.4 after incubation for periods indicated at 30°.)

Additions (μg./ml.)	Acid production		
	18 (hr.)	40 (hr.)	64 (hr.)
Orotic acid 10	0.41	1.15	1.55
1	0.33	0.35	0.70
0.1	0.27	0.29	0.50
Thymine 10	1.20	8.80	11.10
1	0.90	6.90	8.10
0.1	0.60	2.85	3.45
Nil	0.20	0.45	1.05

acid production occurred, followed by a period of intense activity during which the initial drop below the comparable figures for folic acid was completely obliterated. Orotic acid, on the other hand, did not show the same degree of activity, and the amounts of acid produced by cultures containing 10 μg./ml. were not significantly different from those produced by cultures, not containing addenda.

In the presence of other preparations, however, the results were very different. Table 9 shows the synergistic effects which were observed with orotic acid or thymine with folic acid, and the diminished if not altogether absent effect with pteroyl-glutamic acid. Orotic acid showed synergism with the higher level of folic acid tested, but appeared to be inactive, if not inhibitory, with the lower concentration. Thymine showed slight synergism at high levels, with the high concentration of folic acid, but not so pronouncedly at lower levels. With the highest concentration of pteroyl-glutamic acid there was no effect with orotic acid and inhibition

Table 9. *The synergistic effects produced by the simultaneous addition of folic acid or pteroyl-glutamic acid to media containing either thymine or orotic acid*

(Acid production is measured in terms of ml. of 0.05 N-NaOH/10 ml. medium needed to bring to pH 7.4 after 18 hr. incubation at 30°. 10 ml. medium in each culture.)

Additions of orotic acid or thymine (μg.)	Alone	Acid production					
		'Folic acid' (μg.)		Pteroyl-glutamic acid (μg.)			
		0.1	0.00001	0.0001	0.000001	0.00000001	
Orotic acid 0	0.00	2.90	1.05	2.65	0.50	0.35	
10	0.05	4.25	0.98	2.60	0.85	0.46	
1	0.00	4.06	0.68	2.50	0.55	0.38	
0.1	0.00	4.04	0.60	2.45	0.55	0.30	
Thymine 10	1.10	4.50	1.36	1.99	1.75	1.45	
1	0.85	4.00	0.96	2.45	1.40	0.55	
0.1	0.35	3.00	0.85	2.45	0.90	0.50	

at high levels of thymine; over the rest of the range the effects were either additive in the case of thymine, or very slightly synergistic in the case of orotic acid.

DISCUSSION

The use of different preparations of the 'folic acid group' as standards in assay procedures has led to considerable confusion, due to differences which have been apparent in the results of analyses carried out by these methods. This has caused many workers to doubt whether all the various preparations which have growth-promoting activity for the lactic acid bacteria contain identical growth-promoting factors. In an attempt to determine whether assay procedures based on this variety of standards were comparable, Johnson (1946) showed that over the ranges usually employed in assays, certain of these preparations give identical growth/concentration curves. The fact that at concentrations outside this range, the growth/concentration, or acid production/concentration curves are not identical, indicates the possibility that these observations, taken over a narrow range on the steepest portion of the curve, are not necessarily a true picture of the relationship between these various preparations. There is, however, much stronger evidence in favour of the non-identity of the active materials in certain of these preparations in the synergism which occurs between pairs of them.

Since synergism occurs between folic acid and pteroyl-glutamic acid, it must be concluded that they are dissimilar. If it is conceded that folic acid is itself a mixture of factors it may still be held that one of these components is identical with pteroyl-glutamic acid. This is, however, unlikely since concentration considerations preclude there being any chance of a summation of effects between two portions of pteroyl-glutamic acid, one added in the folic acid and one already present, and their subsequent joint synergism with the remaining components of the folic acid preparation. The two impure preparations, from poppy-straw and from liver, apparently range themselves one on either side as far as their major constituents are concerned. Hence synergism is observed between pteroyl-glutamic acid and the poppy-straw extract, but not with the liver extract, and *vice versa* with folic acid. This division into two groups which can be classified with respect to the source of the preparation, i.e. one from liver, the other from plant sources, must also accommodate vitamin B_c which is obtained from liver. The effect of large concentrations of vitamin B_c with folic acid is to produce a marked inhibition, but as the concentration of vitamin B_c is decreased the composite

effect increases rapidly, until at a concentration of 0.000001 μ g./ml. of vitamin B_c an additive effect occurs, and at a concentration 100 times lower true synergism may be observed. Below this again the liver factor appears to be unable to stimulate the folic acid, and an average figure is produced. At lower concentrations of folic acid the peak occurs at a higher concentration of vitamin B_c, but does not reach a value high enough to constitute synergism. It appears, therefore, as if there may be some slight chemical difference between vitamin B_c and pteroyl-glutamic acid, which confers on the former the power of inhibiting folic acid to a certain extent while still maintaining its apparent identity with pteroyl-glutamic acid.

There is a significant difference between the absorption spectrum of folic acid and of pteroyl-glutamic acid, and hence it may be considered as likely that the above evidence is a true representation of the relationships between the various factors.

Stokes (1944) suggested that folic acid is not itself replaced by thymine, but that a thymine-like compound is the product of the reaction which utilizes folic acid. He also stated, however, that the activity observed after 40 hr. incubation with thymine was equivalent to that observed after 18 hr. when folic acid was used. This has been corroborated here, but by increasing the period of incubation it has been shown that the thymine-containing culture ultimately produced as much acid as the one containing folic acid, hence indicating that there is merely a lag in growth and acid production. It thus seems likely that the thymine may actually be a precursor of folic acid or that thymine is participating in some alternative metabolic path, since synergism is observed with folic acid, and there is no reason for synergism to occur between an enzyme and the product of its reaction. This is in agreement with the findings and conclusions of Hutchings, Falco & Sherwood (1945) who examined the effect of substituted uracils on the growth of *L. casei* and showed that there was a possibility that growth in the presence of folic acid does not entail the intermediary synthesis of thymine.

In view of the evidence advanced above, it may be better to consider the various members of the folic acid group of factors as independent entities bearing possibly close chemical relationship to one another, but having distinct physiological activities.

SUMMARY

1. A variety of preparations of the folic acid group of factors have been tested both separately and in binary mixtures for their effect on acid production by *Streptococcus lactis* R.

2. Evidence has been obtained which tends to show that folic acid (Mitchell, Snell & Williams, 1941) is not identical with pteroyl-glutamic acid (Angier *et al.* 1945, 1946) but that vitamin B₉ (Binkley *et al.* 1943) may possibly be more similar to the latter than the former.

3. From a consideration of the effects of two impure preparations, one from liver and the other from poppy-straw, there appears to be a connexion between physiological properties and source, since

the five preparations can be divided into two classes according as their sources are liver or plant tissue.

4. Synergism has been observed between folic acid and both thymine and orotic acid.

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The Folic Acid Group of Growth Factors

2. THE EFFECT OF FOLIC ACID ON ACID PRODUCTION BY GROWING CULTURES OF *STREPTOCOCCUS LACTIS* R

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It has been stated by Williams (1944) that folic acid, the growth factor preparation for *Streptococcus lactis* R and some other lactic acid producing bacteria, isolated by him from spinach leaves, is not a single entity but may consist of a group of compounds of similar chemical composition, but

differing sufficiently to possess distinct physiological properties. Since it has also been suggested (Hall, 1947a) that none of the components of this complex is identical with preparations of similar activity isolated from liver, it appeared to be of interest to consider folic acid separately to deter-