THE SYNTHESIS OF "FOLIC ACID" BY *BACTERIUM COLI* AND *STAPHYLOCOCCUS AUREUS* AND ITS INHIBITION BY SULPHONAMIDES.

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PTEROYLGLUTAMIC acid (synthetic folic acid) was prepared by Angier and fifteen co-workers (1946), and shown to have the full biological activity of the growth factor for *Lactobacillus casei* present in liver. The pteroylglutamate (PtG) molecule contains a *p*-aminobenzoate (*p*-AB) residue; many organisms have now been studied with regard to the ability of PtG to replace *p*-AB both as a growth factor, and as an antagonist of inhibition of growth by sulphonamides. Such studies have been reviewed in detail by Woods (1950). Briefly, certain organisms which normally require *p*-AB as a growth factor (e.g., *Streptobacterium plantarum*, *Lactobacillus arabinosus*, *Clostridium acetobutylicum*) can grow when PtG is supplied instead, although the molar concentration necessary is 10 to 50 times higher; in the case of *Clostridium tetanomorphum*, however, PtG is rather more active than *p*-AB. With all these organisms inhibition of growth by sulphonamides is overcome by both *p*-AB and by PtG; the former shows the usual competitive relationship with sulphonamides, whilst PtG acts non-competitively and at growth factor levels; i.e., the organisms are essentially insensitive to sulphonamide inhibition in the presence of PtG (Nimmo-Smith and Woods, 1948; Lampen and Jones, 1947; Sims and Woods, 1949).

Pteroylglutamate, however, behaves quite differently with other organisms which require *p*-AB for growth. It will not, for example, replace either the growth factor or anti-sulphonamide function of *p*-AB with an induced mutant strain of *Bacterium* (*Escherichia*) *coli* (Lampen, Jones and Roepke, 1949). It is similarly ineffective with *Acetobacter suboxydans* 621, induced mutant strains of *Neurospora* (unpublished work of this laboratory) and a *p*-AB-requiring strain of yeast (Rainbow, 1948).

The anti-sulphonamide action of PtG has also been examined with several organisms which do not require *p*-AB as growth factor; it has been found by itself to be inactive (Lampen *et al.*, 1949; Winkler and de Haan, 1948).

Nimmo-Smith, Lascelles and Woods (1948) have shown that suspensions of *Sbm. plantarum* 5S synthesize *Lb. casei* factor (related if not identical with PtG) in a simple system in which growth does not occur. Such synthesis depended on the presence of *p*-AB and was inhibited by sulphonamides; this inhibition was overcome by *p*-AB in a competitive manner.

It thus seems clearly established that one of the effects of the competition of sulphonamides with *p*-AB is to prevent the synthesis of "folic acid." In some

* Exhibition of 1851 Overseas Scholar at the time of this work.

organisms, indeed, this is sufficient on its own to account for the effect of sulphonamides on growth. With others, however, the position appears to be more complex, at any rate in so far as can be judged from experiments with synthetic PtG. One method of approach to a solution of this problem is to determine if typical organisms with which PtG is inactive can synthesize "folic acid" from *p*-AB in simple systems, and if this synthesis is inhibited by sulphonamides. Such studies with strains of *Bact. coli* requiring *p*-AB and with strains of *Staph. aureus* and *Bact. coli* not requiring this factor are the subject of the present paper.

MATERIALS AND METHODS.

Organisms.

Bact. coli.—Strain 273–384 (American Type Culture Collection *Esch. coli* No. 9723a, hereinafter called *Bact. coli* 273, was an X-ray induced mutant requiring *p*-AB for growth. Some of its properties have been described by Lampen *et al.* (1949). The parent strain (15–9–518; A.T.C.C. No. 9723) was also used and is referred to as 518. Both were obtained from Dr. R. O. Roblin. A further strain (Y 44) which required histidine as well as *p*-AB was obtained from Dr. E. L. Tatum.

Staph. aureus.—A laboratory strain (2102) was used. A substrain (2102R), which was more resistant to sulphonamide, was obtained from it by subculture in increasing concentrations of sulphathiazole. In the medium described below it grew in the presence of 5×10^{-4} M sulphathiazole, whereas 2102 grew only if the drug was below 10^{-5} M.

All strains of *Bact. coli* and *Staph. aureus* were maintained by monthly subculture on tryptic meat agar slopes which were incubated 18 hr. at 37° and then stored at 4°. Working cultures were derived from these and transferred twice a week.

Other organisms.—Details concerning the strains of *Lb. casei* (A.T.C.C. No. 7469) and *Sbm. plantarum* 5S have been given previously (Nimmo-Smith *et al.*, 1948). *Leuconostoc citrovorum* (A.T.C.C. No. 8081) was maintained on the same medium as *Sbm. plantarum*, but was incubated at 37°.

Media.

Culture media used for the preparation of cell suspensions are shown in Table I. Medium A (*Bact. coli*) was essentially that of Roepke, Libby and Small (1944); for strain 273, *p*-AB ($2{\cdot}5 \times 10^{-8}$ M) was added, and also L-histidine (10^{-3} M) in the case of Y44. Sulphathiazole was added (*v. infra*) to medium B for *Staph. aureus*.

Medium C (*Sbm. plantarum*) was as described by Nimmo-Smith *et al.* (1948).*

Medium D for the assay of *Lb. casei* factor (*Lc.* factor) was modified from that described before (Nimmo-Smith *et al.*, 1948) by the omission of asparagine and tyrosine (which had no effect on growth), and by reducing the concentrations of adenine and guanine to 5×10^{-5} M and 10^{-5} M respectively.

For the detection and assay of *L. citrovorum* factor activity the medium used and procedure was essentially that of Sauberlich and Baumann (1948). The vitamin B_6 derivatives were, however, replaced by pyridoxal only (sterilized by filtration; final concentration 2×10^{-7} M) which was added to the autoclaved

* Owing to a typographical error, not detected in proof, two vitamin components were omitted from the published composition of this medium; they were calcium pantothenate and nicotinic acid (400 μg. and 100 μg. per litre respectively).

medium. The cultures were incubated in an atmosphere of 5 per cent CO_2 in H_2 since this improved growth. A specimen of *L. citrovorum* factor concentrate of potency *c.* 4000 units/ml. was kindly provided by Dr. W. B. Emery of Glaxo Laboratories Ltd.

TABLE I.—*Composition of Media.*

	Medium A (*Bact. coli*).	Medium B (*Staph. aureus*).
Glucose	2 g.	7 g.*
Acid-hydrolysed casein (vitamin-free)†	—	3 g.
DL-tryptophan	—	40 mg.
DL-cystein	—	100 mg.
L-Asparagine	2 g.	—
$(NH_4)_2SO_4$	5 g.	—
$Na_2HPO_4.12H_2O$	—	5 g.
NaCl	5 g.	2 g.
KH_2PO_4	3 g.	350 mg.
$MgSO_4.7H_2O$	2 mg.	100 mg.
$FeSO_4(NH_4)_2SO_4.6H_2O$	—	6 mg.
$CaCl_2.6H_2O$	2 mg.	—
Iron-citrate solution‡	3 ml.	—
Thiamin hydrochloride	—	200 μg.
Nicotinic acid	—	1000 μg.
Biotin	—	1 μg.
Water to	1 l.	1 l.
pH adjusted to	7·4	7·4

* Added as a sterile solution to the autoclaved medium.

† Prepared after the method of Snell and Rannefeld (1945). The amount used was equivalent to 3 g. original casein.

‡ 236 mg. sodium citrate ($3H_2O$) and 168 mg. $FeSO_4$ $(NH_4)_2SO_4.6H_2O$ dissolved in 100 ml. H_2O.

General procedure for synthesis experiments.

Preparation of cell suspensions.—The same method was adopted with all strains of *Bact. coli* and *Staph. aureus*. The medium (25 ml.) in 7 in. × 1¼-in. Pyrex tubes was inoculated with cells taken from an agar slope to give an initial population of *c.* 4 × 10^6 cells/ml. The tubes were incubated in a sloped position for 14 hr. at 37°. The cells were centrifuged out and washed once with the culture volume of 0·02 M phosphate buffer, pH 6·8.

Synthesis system.—After any necessary further treatment to reduce the amount of *Lc.* factor initially present (*v. infra*) the cells were finally suspended to a concentration of *c.* 0·2 mg. dry weight/ml. in solution F which contained *p*-AB (10^{-5} M), glucose (0·01 M), L-glutamate (0·01 M) and phosphate buffer pH 6·8 (0·1 M), prepared from $Na_2HPO_4.12H_2O$ and KH_2PO_4. Such suspensions (2·5 ml. in 15 ml. centrifuge tubes) were usually incubated 5 hr. at 37°.

Preparation of material for assay.—Normally the total amount of *Lc.* factor present in both cells and suspending solution was assayed. After incubation the suspensions were diluted with an equal volume of water (*Bact. coli*) or of medium D (*Staph. aureus*) and autoclaved for 10 min. at 10 lb. pressure. The supernatant

fluid after centrifuging was used for the assays with *Lb. casei*. If the *Staph. aureus* suspensions were diluted with water, less *Lc.* factor was found.

RESULTS.

Nature and Assay of the Lb. casei *Factor.*

It must be emphasized that the material whose synthesis was studied was simply material which could replace PtG for the growth of *Lb. casei*. It is therefore referred to as *Lc.* factor; the evidence presented below indicates that it is probably related to PtG, though certainly not identical with the synthetic product. Thymine, in relatively high concentration and in the presence of purines, is known to replace PtG for *Lb. casei* (Lampen and Jones, 1946); it is shown below that thymine, even with PtG present also, cannot account for the activity of the present material.

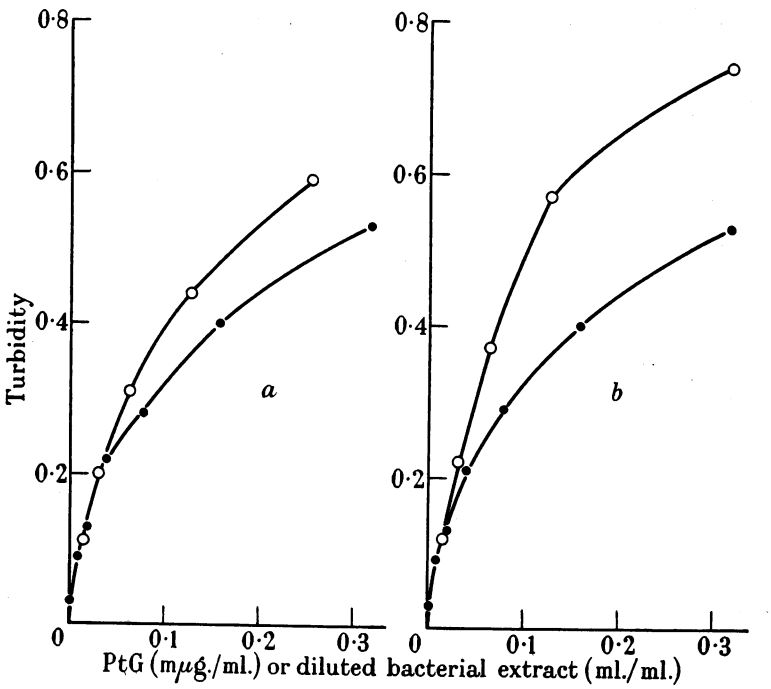

FIG. 1*a* and *b*.—Response of *Lb. casei* to pteroylglutamate and to bacterial extracts. Incubation was for 23 hr. with PtG (– ● –), or extract (– ○ –) from *Bact. coli* 273 (*a*) or from *Staph. aureus* 2102 (*b*).

Assay procedure.

The method was only slightly modified from that described by Nimmo-Smith *et al.* (1948). Aliquots of the extracts, after suitable dilution, were added with a calibrated dropping-pipette to tubes of medium D before autoclaving. This second autoclaving of the extracts did not affect their content of factor.

Nature of the growth response.

Graded quantities of extracts of *Staph. aureus* gave a response with *Lb. casei*

(Fig. 1*b*) which diverged somewhat from that obtained with PtG. A similar though less marked difference was found with extracts of *Bact. coli* (Fig. 1*a*). The divergence increased with increasing quantities of the extract and occurred whether the growth time was 16, 24 or 40 hr. Extracts of *Sbm. plantarum* had previously been found to show this effect, but only when the growth period exceeded 20 hr. (Nimmo-Smith *et al.*, 1948). In the present assays incubation was for 20–24 hr.

A divergence of this type might be explained (*a*) by the presence in the bacterial extracts of non-specific factors which stimulate the growth of *Lb. casei* in the presence of *Lc.* factor, or (*b*) by the possibility that the *Lc.* factor present is not chemically identical with PtG, though having the same biological effect—in this case a more potent one since the optimum mass of growth is greater than with PtG. Evidence against (*a*) was found in experiments in which the response to

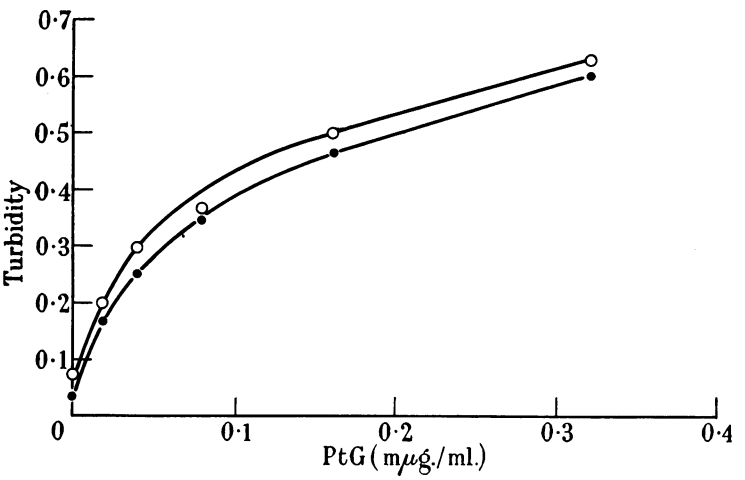

FIG. 2.—Effect of staphylococcal extract on response of *Lb. casei* to pteroylglutamate. Incubation was for 22 hr. with PtG (– ● –) or with PtG *plus* 0·05 ml./ml. staphylococcal extract (– ○ –).

PtG in the presence and absence of a constant amount of staphylococcal extract was compared. The extract was added in volume at least equal to or greater than that used for normal assay, and itself contained only minimal amounts of *Lc.* factor. These extracts were prepared either from cells grown in the presence of sulphathiazole, or from washed cells after incubation in solution F but with sulphathiazole present (*v. infra*). A typical result (Fig. 2) shows the only effect of the extract to be a small constant increase in growth at all concentrations of PtG, corresponding to the small amount of *Lc.* factor in the extract. It was therefore provisionally concluded that the extracts contained no non-specific stimulatory factors unless their production (as with *Lc.* factor) was prevented by sulphathiazole.

The following substances gave response curves which were in no case similar to those obtained with the staphylococcal extracts (those marked* were also tested in admixture with PtG): pteroyl-γ-γ'-diglutamylglutamic acid,* pteroyl-α-glutamylglutamic acid, pteroylglutamine, N^{10}-formylpteroylglutamic acid,* thymine* and thymidine.*

Relation of the factor to Leuconostoc citrovorum *factor (folinic acid).*

A growth factor (CF) for *Leuconostoc citrovorum* has been shown recently to be closely related to folic acid, and there is strong evidence that the substance is 5-formyl-5, 6, 7, 8-tetrahydropteroylglutamic acid. On mild acid treatment it no longer supports the growth of *L. citrovorum*, but is still active with *Lb. casei* (Bond, Bardos, Sibley and Shive, 1949; Brockman *et al.*, 1950; Broquist, Stokstad and Jukes, 1950; Pohland, Flynn, Jones and Shive, 1951).

Typical cell extracts of *Staph. aureus* were 100–1000 times as active for *Lb. casei* as for *L. citrovorum*, whilst with a CF concentrate the difference was only 2–3 fold (Table II). Both the concentrate and a specimen of the synthetic factor (kindly provided by Dr. T. H. Jukes) gave response curves with *Lb. casei* which

TABLE II.—*Activity of Staphylococcal Extracts and* L. citrovorum *Factor for* Lb. casei *and* L. citrovorum.

The staphylococcal extracts were prepared from three experiments with suspensions of *Staph. aureus* 2102*R* in solution F. For method of acid treatment see text.

Material assayed.	Amount (μl./ml. for half-maximum growth of—	
	Lb. casei.	*L. citrovorum.*
Extract (1) . .	0·1	230
Extract (2) . .	0·5	400
Extract (3) . .	0·7	100
Extract (3) acid-treated	0·8	500
CF concentrate . .	0·2	0·5
CF concentrate acid-treated . . .	0·2	2·5

were almost identical with that given by PtG. The characteristic divergent response curve of *Staph. aureus* extracts was not changed by treatment of the extracts with dilute HCl (pH 2) for 2 hr. at *c.* 18°, though there was an 80 per cent loss of the small CF activity (Table II). It is clear that the difference in behaviour of PtG and the *Lc.* factor in staphylococcal extracts is not due to the latter being CF either wholly or in part.

Assessment of the factor.

The range over which the response of *Lb. casei* to bacterial extracts coincided with that to PtG was narrow (Fig. 1); it was usually impossible to forecast the amount of an extract under test which would fall within this range. In practice therefore " unknowns " were assayed against a standard reference extract of the organism in question; the latter was in turn checked in each assay against PtG since there was some slight day-to-day variation in the relationship. Such reference extracts were obtained by pooling the residues of experimental extracts and storing at 0°; they retained full potency for at least four weeks.

Each complete assay therefore contained the following: (*a*) the " unknowns," each at two or three concentrations, (*b*) four levels of PtG in the range 0·01–0·08 mμg./ml., and (*c*) four levels of the appropriate reference extract. The latter had been previously assayed against PtG and was diluted so that the response to the

smallest quantity (24 μl.) was approximately equal to that with 0·01 mμg. PtG/ml. In plotting the results, the scale for the reference extract standard curve was determined in terms of PtG by equating the lowest level of reference extract to the concentration of PtG which gave the same turbidity. The potency of the "unknowns" (in terms of PtG) was then read off from the reference extract curve. If the response obtained was greater than that with 0·06 mμg. PtG/ml., the result was rejected and the assay repeated, using smaller quantities. The assessment of the *Lc.* factor in terms of mμg. PtG was adopted for convenience; it has of course no absolute significance, but this was immaterial in the present work since the interest was only in relative amounts of the factor produced under varying conditions. All results are given as mμg. *Lc.* factor/mg. dry wt. of cells.

Initial Lb. casei *Factor Content of Harvested Cells.*

As with *Sbm. plantarum* (Nimmo-Smith *et al.*, 1948) neither *Bact. coli* nor *Staph. aureus* synthesized *Lc.* factor in cell suspension systems unless the cells were initially low in their content of the factor. Such cells were obtained in the case of organisms requiring *p*-AB by growth in suboptimal concentrations of this factor, and with the others by growth in sub-inhibitory concentrations of sulphathiazole; when necessary a further reduction in content of the factor was effected by incubating the harvested cells with glucose in phosphate buffer (Table III).

TABLE III.—*Preparation of Cells Deficient in* Lc. *Factor.*

The figures given show the range found in at least five experiments with each organism. Incubation in buffered glucose was for 6 hr. in 0·1 M phosphate buffer (pH 6·9) containing 0·05 M glucose (20 ml.). The cells were then centrifuged out and stored as a paste overnight at 5°.

Organism.	Procedure for depleted cells.	*Lc.* factor (mμg./mg.) in harvested cells.	
		Normal.	Depleted.
Bact. coli 273	Grown in $2·5 \times 10^{-8}$ M *p*-AB; cells then incubated in buffered glucose	25–45	1–10
Bact. coli 518	Grown in 10^{-6} M sulphathiazole; cells incubated as above	30–80	5–10
Staph. aureus 2102	Grown in 5×10^{-6} M sulphathiazole	15–40	<1–2
Staph. aureus 2102R	Grown in 5×10^{-5} M sulphathiazole	100–300	1–8

Synthesis of Lb. casei *Factor by Cell Suspensions of* Bact. coli.

Bact. coli 273.

Substrates required.—Considerable synthesis took place in the simple reaction mixture (solution F). As judged by opacity measurements, some growth (never more than the equivalent of one cell division) took place during the usual incubation period of 4–5 hr. Analysis of the effect of the various components of solution F

(Table IV) proved that the only absolute requirement was for *p*-AB. A detailed study of the effect of the concentration of this substance (Fig. 3) showed a sharp rise in synthesis between 1 and 5×10^{-7} M *p*-AB; this concentration is rather greater than the optimum requirement for growth on medium A, which lies between 10^{-7} and 10^{-8} M. The omission of glucose resulted in reduction of synthesis by about 30 per cent.

TABLE IV.—*Synthesis of* Lc. *Factor by Cell Suspensions of* Bact. coli *and* Staph. aureus.

Cells were incubated for 5 hr. in solution F (see Methods), omitting components as required.

Component(s) of solution F omitted.	*Lc.* factor formed (mμg./mg.).		
	Bact. coli 273.	*Bact. coli* 518.	*Staph. aureus* 2102.
None . . .	60	32	24
Glucose . . .	33	—	2
Glutamate . . .	44	—	18
Glucose and glutamate	24	—	—
p-AB	0	0	17

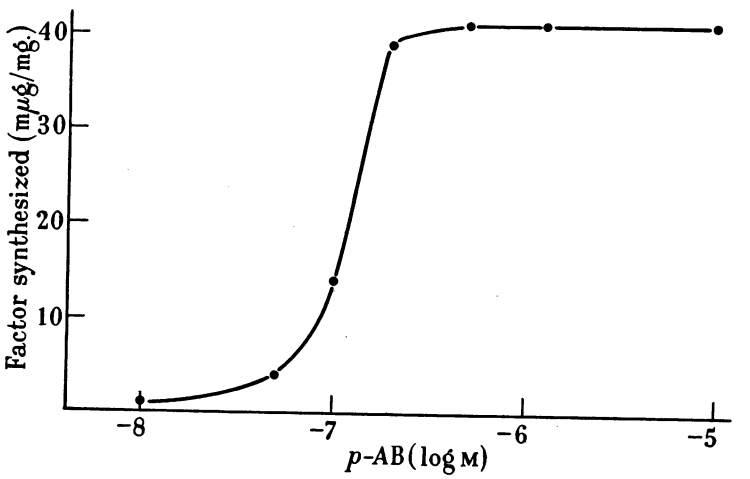

FIG. 3.—Effect of concentration of *p*-aminobenzoate on *Lc.* factor synthesis by *Bact. coli* 273. Incubation was for 5 hr. in solution F without *p*-AB, which was added to give the required concentration. Initial factor content, 2 mμg./mg.

The course of the synthesis with and without added L-glutamate is shown in Table V; its presence permitted a higher initial rate, and synthesis continued for a longer time. Substitution of DL-aspartate gave a slightly greater initial rate, but synthesis then ceased for a time; on prolonged incubation there was a further small rise. With both amino-acids present the initial rate was still greater, but again ceased temporarily; by 10 hr. however the value had risen to that found with glutamate alone. These results were observed consistently but the matter has not been investigated further.

Effect of pterin derivatives.—Solution F contains no preformed source of the pterin residue of folic acid, and in the above experiments this must have been present in, or synthesized from, cell constituents, or synthesized from the organic components of the solution. Formation of *Lc.* factor was not increased by the addition of any of the following possible precursors : 2-amino-4-hydroxy-6-methylpterin, 2-amino-4-hydroxy-6-carboxypterin and 2, 5, 6-triamino-4-hydroxypyrimidine ; all were tested at 10^{-3} M and 10^{-4} M. Another substance, 2-amino-4-hydroxy-6-formylpterin, was studied in more detail as it was found (*v. infra*)

TABLE V.—*Effect of Glutamate and Aspartate on* Lc. *Factor Synthesis by* Bact. coli 273.

Washed cells with an initial factor content of 2 mμg./mg. dry wt. were suspended in 10 ml. quantities of solution F without glutamate, and incubated in 6 × 1 in. tubes with the additions shown. Samples (2 ml.) were withdrawn at intervals.

Additions.		*Lc.* factor formed (mμg./mg.) after incubation for (hr.)—			
L-Glutamate (0·01 M).	DL. Aspartate (0·02 M).	1.	3.	5.	10.
—	—	29	45	48	45
+	—	45	53	66	84
—	+	56	51	52	63
+	+	73	67	63	84

TABLE VI.—*Effect of Sulphathiazole on* Lc. *Factor Synthesis by Strains of* Bact. coli.

Data obtained from a series of curves similar to those of Fig. 4 and expressed to the nearest 10 per cent. Molar ratio *p*-AB/sulphathiazole permitting half-maximum synthesis : strain 273 = 0·2 ; strain 518 = 1·0.

Strain.	*p*-AB/sulphathiazole (molar ratio).	Per cent inhibition of synthesis by sulphathiazole at (M)—			
		10^{-3}.	10^{-4}.	10^{-5}.	10^{-6}.
273	0·01	100	90	90	90
	0·1	70	60	60	60
	1·0	20	30	20	10
518	0·1	—	100	100	100
	1·0	—	40	50	40
	10·0	—	30	30	20

to be stimulatory with *Staph. aureus* 2102. With *Bact. coli* 273 it inhibited synthesis (*c.* 70–80 per cent) with concentrations from 10^{-4} M to 4×10^{-4} M and with either optimal or suboptimal *p*-AB. This pterin also inhibited the growth of *Lb. casei*, and in these experiments *Lc.* factor was assayed only on the washed cells after their removal from the reaction mixture by centrifuging.

The effect of sulphathiazole.—Synthesis was inhibited by sulphathiazole, and this was overcome when the concentration of *p*-AB was raised. Over a 1000-fold range of sulphonamide concentration the effect of *p*-AB was competitive (Table VI).

Since the curves obtained by plotting *p*-AB concentration against *Lc.* factor formed are parallel for all concentrations of sulphathiazole (Fig. 4), it follows that the critical ratio *p*-AB/sulphonamide is constant at all levels of *Lc.* factor synthesis. With a ratio of 0·2 synthesis was reduced to about one half; synthesis was almost maximal when the ratio was 1.

Synthesis of *Lc.* factor by the other mutant strain of *Bact. coli* (Y44) was also inhibited by sulphathiazole: the conditions were the same as those described for strain 273. The relation between *p*-AB and sulphathiazole was again competitive over a wide range of drug concentration.

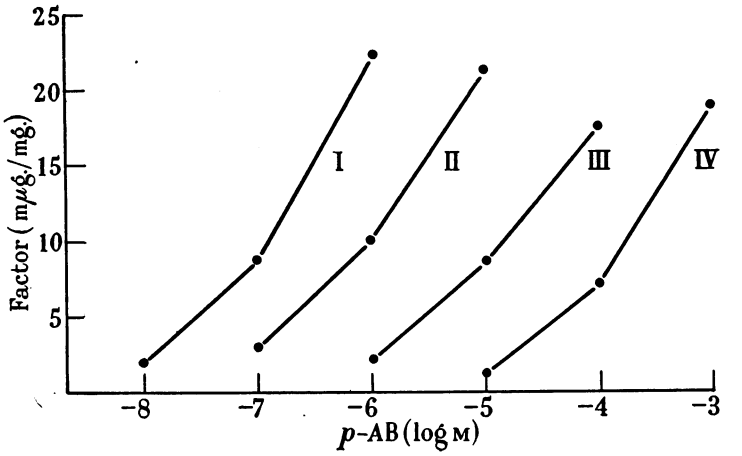

FIG. 4.—Effect of *p*-aminobenzoate concentration on the inhibition by sulphathiazole of *Lc.* factor synthesis by *Bact. coli* 273. Incubation was for 5 hr. in solution F with varying concentrations of *p*-AB and with sulphathiazole at (I) 10^{-6} M, (II) 10^{-5} M, (III) 10^{-4} M and (IV) 10^{-3} M. Initial factor content, 2 mμg./mg.

Bact. coli 518.

Requirement for p-*AB.*—The parent strain (518) did not require *p*-AB for growth. Nevertheless, cell suspensions synthesized little or no *Lc.* factor when *p*-AB was omitted from solution F (Table IV). Apparently the treated cells did not contain sufficient *p*-AB, or were unable to synthesize it in the required amount from the components of the solution.

Effect of sulphathiazole.—The results obtained were essentially similar to those with the two mutants except for one quantitative difference. Reference to Table VI shows that for any one strain the percentage inhibition of *Lc.* factor synthesis with a given *p*-AB/sulphonamide ratio is fairly constant over a 1000-fold range of drug concentration. With strain 518 however the critical ratio permitting synthesis was about five times greater than with strain 273, i.e., more *p*-AB was required. This is rather the opposite to what might have been expected, since an organism potentially able to synthesize *p*-AB might, if anything, have contributed to the amount of *p*-AB present. It is possible that the normal organism may catabolize this substance and thus reduce the amount available for synthesis of *Lc.* factor.

Synthesis of Lc. *factor by Cell Suspensions of* Staph. aureus.

Normal strain (2102).

Substrates required.—Synthesis occurred in solution F, but glucose proved to be the only essential constituent of this mixture (Table IV). Omission of *p*-AB resulted in a slower rate of synthesis which also decreased more rapidly: after 10 hr. almost twice as much factor had been formed in the presence of *p*-AB as in its absence (Fig. 5*a*). Since this organism can presumably synthesize *p*-AB it is possible that some was present preformed in the cells, or was produced by them from endogenous material or from the components of solution F.

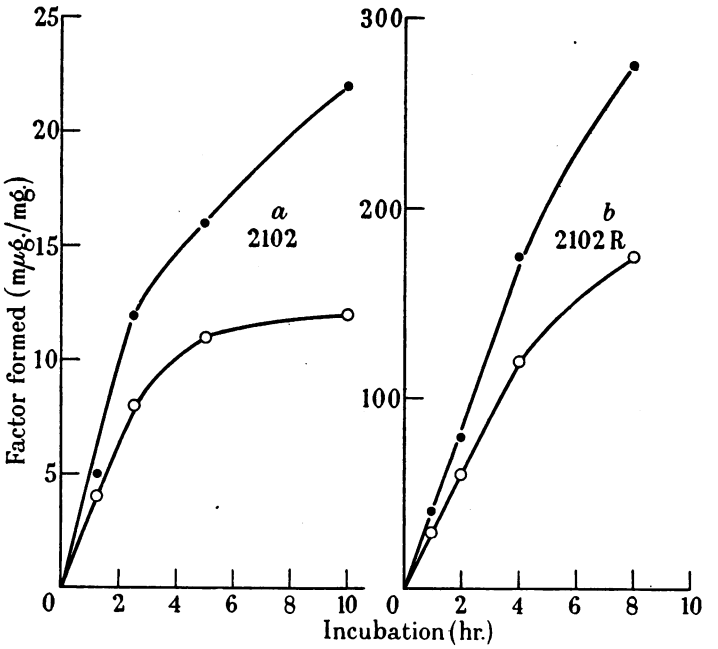

FIG. 5*a* and *b*.—Rate of *Lc.* factor synthesis by strains of *Staph. aureus*. Incubation was in solution F without *p*-AB (– ○ –) and with 10^{-5} M *p*-AB (– ● –). Initial factor content: 2102, <1 mμg./mg.; 2102R, 1 mμg./mg.

Omission of glutamate did not always decrease synthesis; in a few experiments when it did the effect was small and not increased by longer incubation. The cells were probably rich in free glutamate, since the growth medium contained acid-hydrolysed casein (Gale, 1947*b*). Attempts were made to diminish this by the methods used by Gale with *Streptococcus faecalis* R (1947*a*) and *Staph. aureus* (1947*b*). Cells incubated first in buffer and glucose formed very little *Lc.* factor whether or not glutamate was present. Similar treatment of cells of *Sbm. plantarum* also impaired their synthetic powers (Nimmo-Smith *et al.*, 1948). With cells harvested from young (6-hr.) cultures the stimulation by glutamate was again slight and variable. It is doubtful whether these cells were depleted of glutamate, since to obtain them deficient in *Lc.* factor it was necessary to add

sulphathiazole to the growth medium; Gale (1947*b*) had found that the drug increased the concentration of free glutamate in young staphylococcal cells.

Effect of other amino-acids.—Mixtures of amino-acids increased *Lc.* factor production when added to solution F (Table VII). No single member of stimulatory mixtures accounted for the activity. Furthermore, groups containing four to six different amino-acids all gave the same (*c.* two-fold) stimulation. Ammonia (0·004 M to 0·04 M) was inactive.

TABLE VII.—*Effect of 2-Amino-4-hydroxy-6-formylpterin and Amino-acids on Synthesis by* Staph. aureus.

Incubation was for 5 hr. in solution F with the additions shown. In Exp. 1 and 2 the factor in the washed cells only was assayed.

Experiment.	Additions.		*Lc.* factor formed (mμg./mg.) by strain—	
	Formylpterin (M).	Amino-acids.*	2102.	2102R.
1	—	—	11	67
	10^{-4}	—	16	117
	2×10^{-4}	—	28	155
	4×10^{-4}	—	25	147
2	—	—	9	—
	2×10^{-4}	—	16	—
	—	+	21	—
	2×10^{-4}	+	21	—
3	—	—	11	101
	—	+	19	207

* The amino-acid mixture contained alanine, aspartic acid, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, valine (all DL-), L-arginine, L-histidine and glycine; each was at 0·001 M (L-isomer).

Effect of pterin derivatives.—Formylpterin (2-amino-4-hydroxy-6-formylpterin) doubled the amount of *Lc.* factor produced (Table VII). Since the autoclaving of suspensions containing this substance produced substances inhibitory to *Lb. casei* these assays were carried out on the cells only. Parallel assays on the supernatants were done in some cases, and the results excluded the possibility that the formylpterin was merely preventing the escape of *Lc.* factor from the cells. Such assays were possible if the supernatants were sterilised by heating to 100° only for 3 min.; inhibitory substances were not then formed, at least in sufficient concentration to interfere.

The increased synthesis, even with optimal concentrations of formylpterin, was not as great as with a mixture of fourteen amino-acids. When both amino-acids and formylpterin were added the effect was not additive (Table VII); indeed, no more *Lc.* factor was formed than with the amino-acids alone. One interpretation of these results is that formylpterin may be converted by this organism (but not by *Bact. coli*) to some true precursor of the pterin residue, but that such conversion is less efficient than a *de novo* synthesis from amino-acids.

The following compounds, tested at 10^{-3} M and 10^{-4} M, had no effect on

synthesis: 2-amino-4-hydroxy-6-methylpterin, 2-amino-4-hydroxy-6-carboxypterin and 2, 5, 6-triamino-4-hydroxypyrimidine.

Effect of sulphathiazole.—Synthesis of *Lc.* factor by this organism was also inhibited by sulphathiazole, and the inhibition was overcome in the usual competitive manner by *p*-AB over a 100-fold range of drug concentration (Table VIII).

TABLE VIII.—*Effect of Sulphathiazole on* Lc. *Factor Synthesis by Strains of* Staph. aureus.

The data have been calculated as shown in Table VI. Molar ratio *p*-AB/sulphathiazole permitting half-maximum synthesis: strain 2102 = 4 and 2102R = 0·5.

Strain.	*p*-AB/sulphathiazole (molar ratio).	Per cent inhibition of synthesis by sulphathiazole at (M)—		
		10^{-4}.	10^{-5}.	10^{-6}.
2102	0·1	100	100	100
	1·0	60	60	60
	10·0	—	20	20
	100·0	—	0	0
2102R	0·1	90	90	80
	1·0	40	40	40
	10·0	0	0	0

The critical ratio *p*-AB/sulphathiazole required to permit synthesis was of the same order as that for *Bact. coli* 518, and therefore about five times greater than that for the mutant requiring *p*-AB for growth. A possible explanation of this finding, which is rather the opposite of what might have been expected, has already been discussed.

Sulphonamide-resistant strain (2102*R*).

It was found (Table III) that harvested cells of this organism contained more than five times as much *Lc.* factor as those of the parent strain grown under similar conditions. In washed suspension the difference in *Lc.* factor synthesis in solution F was of the same order, the resistant cells producing about ten times as much as those of the parent organism (Table IX).

TABLE IX.—*Synthesis of* Lc. *Factor by* Staph. aureus 2102 *and* 2102R. Incubation was for 5 hr. in solution F.

Experiment.	*Lc.* factor (mμg./mg.).			
	2102.		2102R.	
	Initial.	Increase.	Initial.	Increase.
1	<1	14	3	175
2	1	16	5	138
3	1	13	8	108

There appears to be no recorded instance of increased ability to form *Lc.* factor occurring simultaneously with an increase in resistance to sulphonamide. This aspect will be discussed later.

Effect of p-*AB*.—Synthesis of *Lc.* factor occurred when *p*-AB was omitted from solution F, but as with the parent strain, the rate was not as great as in its presence (Fig. 5*b*).

Effect of amino-acids and formylpterin.—Addition of a mixture of amino-acids or of formylpterin to solution F increased *Lc.* factor synthesis: in each case the degree of stimulation was of the same order as that found with the parent strain (Table VII).

Effect of sulphathiazole.—*Lc.* factor synthesis by the resistant cells was inhibited by sulphathiazole, and *p*-AB overcame the inhibition competitively (Table VIII). The critical ratio *p*-AB/sulphathiazole for half-maximum synthesis was eight times less for the resistant strain than for the parent.

DISCUSSION.

A difficulty in considering the results of the experimental work concerns the nature of the material whose synthesis was studied. It replaced folic acid for the growth of *Lb. casei*, but the response curve differed from that given by synthetic pteroylglutamate; this was especially the case with the material formed by the staphylococci. The material was intrinsically more active than PtG; it may therefore be more closely related than PtG to the functional form of folic acid in cell metabolism. One such substance is probably *L. citrovorum* factor (folinic acid), but conclusive evidence was obtained that only a small proportion of the activity of extracts of staphylococci could be due to the presence of this factor. The functional form of folic acid might be expected (provided that it can penetrate the cell) to render the growth of all organisms insensitive to inhibition by sulphonamides. Cell extracts and culture fluids (rich in *Lc.* factor) of the sulphonamide-resistant staphylococcus were inactive in this respect with the normal *Staph. aureus* and with *Bact. coli*; they were, however, active, as is PtG itself, with *Sbm. plantarum* (unpublished observations). The use of less rigorous methods of extracting the cells than autoclaving did not change these results.

Folic acid also occurs naturally in the more complex form of conjugates with glutamic acid, though there is no evidence that these have intrinsic biological activity. *Lb. casei* is able to use at least some of these conjugates as a source of folic acid. The response curve given by staphylococcal extracts was not duplicated by any of the conjugates of PtG available for testing, either alone or in admixture with free PtG. All possible combinations were not tested, but it is unlikely that conjugates are involved, since there was a similar divergence in the response curves when *Strep. faecalis R* (which does not respond to known conjugates) was used as the assay organism; furthermore, these assays gave the same value for folic acid as those with *Lb. casei* (unpublished observations).

Finally, it seemed possible that the greater activity of the extracts was due to the presence of material unrelated to folic acid, but which stimulated growth of *Lb. casei* when optimal amounts of this factor were present. The experiments showed that, if this were the case, the production of these materials was also prevented by sulphonamides. The assay medium contained, with one exception, those substances for which there is evidence that synthesis depends on *p*-AB and is inhibited by sulphonamides (amino-acids, purines). The exception was thymine, but its addition did not convert the response curve of PtG to that given by the extracts.

An obvious component missing from the reaction mixture for the synthesis of folic acid was a preformed source of the pterin nucleus. 2-Amino-4-hydroxy-6-formylpterin doubled synthesis of *Lc.* factor by both strains of *Staphylococcus*. It is unlikely to be a direct intermediate since very high concentrations were required; furthermore, it inhibited synthesis by *Bact. coli* and was ineffective with *Sbm. plantarum* (unpublished results). It is possible that *Staph. aureus* can convert this substance to a direct intermediate. Amino-acids may also lead to increased synthesis of such an intermediate since they also stimulate *Lc.* factor production. Nimmo-Smith *et al.* (1948) found that incubation in the presence of amino-acids was necessary to obtain active cell suspensions of *Sbm. plantarum*. It is possible that a free pterin is not an intermediate, but that *p*-AB or *p*-aminobenzoylglutamate is combined with the pterin precursor. Reductone forms a compound with *p*-AB, and it has been suggested on purely chemical grounds that this compound is an intermediate in the biosynthesis of folic acid (Forrest and Walker, 1948).

Increased production of *p*-AB by sulphonamide-resistant staphylococci is well established (Landy, Larkum, Oswald and Streightoff, 1943; Housewright and Koser, 1944; Oakberg and Luria, 1947). The present resistant strain had an increased capacity to synthesize folic acid from *p*-AB compared with its parent. Increased production of *p*-AB itself, if it occurred, could not account for the increase in folic acid, since even in the presence of excess added *p*-AB there was the same difference between parent and resistant strains. Synthesis of folic acid by cell suspensions of the resistant strain was eight times more resistant to sulphathiazole than that of the parent; this compared with a fifty-fold difference in sensitivity when growth was the criterion. However, such quantitative comparisons cannot be realistic until it is known that all the reactants necessary for the synthesis of folic acid by cell suspensions are present in optimal amount.

With the organisms used PtG was inactive either in overcoming inhibition of growth by sulphonamides or in replacing *p*-AB as a growth factor. Nevertheless, they all formed in cell suspension or during growth a substance which replaced PtG for *Lb. casei*. The synthesis by washed cells was either dependent on or stimulated by *p*-AB and was inhibited competitively by sulphathiazole. Assuming that some form of folic acid is ultimately required by all micro-organisms for growth, these effects would alone be sufficient to explain why growth is prevented by sulphonamides, though the possibility that *p*-AB is also utilised for the production of a substance dissimilar from folic acid cannot be excluded.

SUMMARY.

A study has been made of folic acid synthesis by cell suspensions of (*a*) *Bacterium coli* (strains requiring *p*-aminobenzoate and a normal strain) and (*b*) *Staphylococcus aureus* (normal strain and a sulphathiazole-resistant strain derived from it). Pteroylglutamate is inactive in replacing either the growth factor or anti-sulphonamide activity of *p*-aminobenzoate with these organisms.

Folic acid was synthesized by all organisms in a mixture containing buffer, glucose, glutamate and *p*-aminobenzoate; the latter was essential or stimulatory in every case. Sulphathiazole inhibited the synthesis, the relation with *p*-aminobenzoate being competitive as in growth.

The folic acid formed gave a response curve with *Lactobacillus casei* which

differed from that given by pteroylglutamate in that heavier growth was produced at optimum concentrations ; it was not identical with any member of the folic acid group tested.

The sulphathiazole-resistant strain of *Staph. aureus* synthesized about ten times as much folic acid as the parent strain under similar conditions.

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