FOLIC ACID DERIVATIVES SYNTHESIZED DURING GROWTH OF *DIPLOCOCCUS PNEUMONIAE*

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Received for publication 25 October 1962

ABSTRACT

SIROTNAK, F. M. (Sloan-Kettering Institute for Cancer Research, New York, N.Y.), GLORIA J. DONATI, AND DORRIS J. HUTCHISON. Folic acid derivatives synthesized during growth of *Diplococcus pneumoniae*. J. Bacteriol. **85**:658–665. 1963.—Under cultural conditions permitting synthesis of folic acid in an amount greatly in excess (20- to 30-fold) of that required for maximal growth of *Diplococcus pneumoniae*, 85 to 90% of the growth factor accumulated as polyglutamates. Approximately equal amounts of mono- and diglutamates made up the remaining 10 to 15% found in culture material. Most of the polyglutamates occurred intracellularly, in a proportion of triglutamates to higher glutamates of about two to one. Only 10 to 15% of all folic acid derivatives (mono-, di-, and polyglutamates) found had folinic acid (5-formylfolate-H_4) activity for *Pediococcus cerevisiae*. Practically all synthesis of the glutamyl-peptide moieties of folate seems to occur at an enzymatic step prior to folic acid, since no appreciable peptide formation occurred under conditions blocking folate synthesis. Sulfanilamide inhibition of growth by a block in folate synthesis was reversed by the addition of dihydrofolic acid, but not folic acid. The examination of two genetically distinct amethopterin-resistant mutant strains has revealed no gross differences in folate accumulation during growth when compared with the wild strain.

Concurrent with studies of resistance to folic acid antagonists exhibited by various mutant strains of *Diplococcus pneumoniae*, we have examined the wild strain for folic acid derivatives biosynthesized during growth. Of particular interest was the fraction accumulated as polyglutamyl conjugates.

The biosynthesis of polyglutamate forms of folic acid has been shown to occur in a number of microorganisms. For example, derivatives have been found in yeast (Bird et al., 1946; Pfiffner et al., 1946; Doctor and Couch, 1953), in several lactic acid bacteria (Hendlin, Koditschek, and Soars, 1953), and in *Neurospora crassa* (Swendseid and Nyc, 1958). A triglutamate form with activity for *Pediococcus cerevisiae* was identified as a major constituent in cells of *Bacillus subtilis* (Hakala and Welch, 1955), in cells of *Streptococcus faecalis* (Zakrzewski and Nichol, 1955), and, along with a heptaglutamate derivative, in *Clostridium cylindrosporum* (Wright, 1955), and in a number of clostridia by Rabinowitz and Himes (1960).

In the following report, evidence is presented for the accumulation in cultures of *D. pneumoniae* of significant quantities of at least three polyglutamyl forms of both folic acid and the reduced coenzyme form. Additional data are also given which suggest the manner in which these derivatives originate during growth.

MATERIALS AND METHODS

The organism used as a source of folic acid derivatives for most of this work was a rough variant of *D. pneumoniae* strain R_6 (obtained through the courtesy of Rollin D. Hotchkiss, The Rockefeller Institute, New York). Two genetically distinct amethopterin-resistant mutants, R_6Am_b and R_6Am_d, were also used. Each has a single genetic marker, but at a different chromosomal locus (*unpublished data*), determining 100- and 500-fold increases in resistance, respectively, and derived from mutant strains described previously (Sirotnak, Lunt, and Hutchison, 1960*a*).

Inocula and cells used for the preparation of extracts were grown at pH 7.4 in a modification (Sirotnak et al., 1960*b*) of the partially synthetic medium of Adams and Roe (1945) in which the

chief source of nitrogen was supplied in the form of a vitamin-free enzymatic digest of casein (General Biochemicals, Inc., Chagrin Falls, Ohio). The inoculum size was adjusted so that maximal growth (OD 0.75 as measured at 600 mμ in a Beckman model B spectrophotometer) occurred after approximately 10 hr of incubation at 37 C. Cell yield (dry weight) was about 400 mg/liter. Cells in the late exponential phase (around OD 0.6) or in the stationary phase of growth were harvested by centrifugation and resuspended in one-tenth the original volume in cold 0.1 M phosphate buffer (KH_2PO_4-Na_2HPO_4) at pH 6.5. Both the resuspended cells and the supernatant culture broth were autoclaved immediately, for 15 min at 121 C, and adjusted to their original volume with distilled water; the suspensions were centrifuged to remove cellular debris. The heat treatment releases growth factors from the cells and mediates the conversion of labile one-carbon substituted derivatives, such as N^{10}-formyl- and N^{5-10}-methenyl-folate-H_4, to the stable N^5-formylfolate-H_4. Folate-H_2 and folate-H_4, presumably, are oxidized to folic acid. When extracts were examined by the Bratton-Marshall (1939) procedure for the presence of *p*-aminobenzoylglutamic acid (PABG), a cleavage product of folate-H_2 and folate-H_4, none was detected. In addition, when synthetically prepared folate-H_2 was added to cell extracts prepared by sonic treatment and then heated, no Bratton-Marshall positive material could be detected. Such material, however, was detected almost immediately in controls prepared in either distilled water or phosphate buffer; a commensurate loss of activity for *S. faecalis* or *Lactobacillus casei* was also noted for such controls. It appears, therefore, that materials in the cell afford considerable protection to the reduced folates and that under these conditions the result from heating is one of oxidation to folic acid with little or no cleavage of the molecule to PABG and a pteridine derivative.

The heat extracts and the similarly heated supernatant medium, were stored at 5 C under toluene. To convert polyglutamates to readily utilizable growth factors available for microbiological assay, a portion of each sample, along with a portion of the culture supernatant, was treated for 17 hr at 37 C with a conjugase preparation from chicken pancreas or hog kidney. After pH adjustment to 6.5, 0.5 mg of dried chicken pancreas (Difco) was added per 10 ml of extract, and 0.2 ml of fresh hog-kidney suspension, made according to the method of Bird et al. (1945), was added to 10 ml of extract after it was adjusted to pH 4.5. Dried hog-kidney preparation (Difco) was found to be inactive under the same conditions.

Prior to bioautographic analysis, samples were concentrated tenfold by lyophilization and then treated with an equal volume of absolute ethyl alcohol. The resulting precipitate was removed by centrifugation, and the supernatant was stored at −20 C until used.

Folic acid derivatives were measured microbiologically. *S. faecalis* ATCC 8043 and *L. casei* ATCC 7479 were used for assays of total folic acid, with synthetic pteroylglutamic acid as a standard; *P. cerevisiae* ATCC 8081 was used for determination of the substituted folate-H_4 derivatives, with synthetic N^5-formylfolate-H_4 (folinic acid) as the standard. Media and conditions of assay were essentially those of Flynn et al. (1951) for *S. faecalis* and *L. casei* and those of Sauberlich and Baumann (1948) for *P. cerevisiae*. Growth response was measured turbidimetrically at 600 mμ with a Beckman model B spectrophotometer.

For the preparation of bioautograms, usually 0.01 ml of the concentrated samples or a suitable dilution was used. This was spotted on filter paper strips (Eaton-Dikeman no. 613, 0.5 in. in diameter) for ascending chromatography with 1% K_2HPO_4 plus 0.2% ethylenediaminetetraacetic acid (EDTA) as the solvent, during a period of approximately 7 hr at room temperature. The paper strips were then air-dried and placed on the surface of the appropriate assay medium, solidified by adding 1.5% agar, and seeded with one of the three assay organisms shown above. After 20 hr of incubation at 37 C, the paper strips were removed and areas of growth traced to be used for R_F calculations. All folic acid compounds used as standards in assay procedures and as controls for bioautograms (see Fig. 1) were generously supplied by Thomas H. Jukes, Agricultural Division, American Cyanamid Co. Folate-H_2 was prepared by the method of Futterman (1957) as modified by Blakley (1960).

RESULTS

Heat extracts were prepared in the manner described above from a number of cell samples of

TABLE 1. *Accumulation of folic acid derivatives during growth of Diplococcus pneumoniae as measured by microbiological assay*

Assay organism	Sample	Folic acid derivatives[a] (mμg/100 ml of culture)[b]					
		Cells			Supernatant		
		No addition	+CPe[c]	+HKe[d]	–	+CPe[c]	+HKe[d]
Streptococcus faecalis	I	59	2,010	520	380	860	—
	II	62	1,840	669	310	740	390
	III	133	1,300	—	455	966	—
Pediococcus cerevisiae	I	5	148	—	30	60	—
	II	8	226	30	28	53	36
	III	21	177	—	16	26	—

[a] Measured as equivalents of pteroylglutamic acid with *S. faecalis* and as equivalents of the active stereoisomer of synthetic N^5-formyl-folate-H_4 with *P. cerevisiae*. Average of duplicate determinations done at several sample dilutions.

[b] Cell yield is approximately 40 mg (dry wt)/100 ml.

[c] Treated with dried chicken-pancreas preparation at pH 6.5.

[d] Treated with fresh hog-kidney preparation at pH 4.5.

D. pneumoniae harvested at late exponential or stationary phases of growth. The results of microbiological assays of the conjugase-treated and untreated samples for total folic acid with *S. faecalis* and for the one-carbon substituted folate-H_4 derivatives with *P. cerevisiae* of three representative samples are shown in Table 1.

Total folic acid accumulating in the culture during growth was uniformly high (approximately 2,500 mμg/100 ml of culture containing an equivalent of 40 mg of dried cells). This represents approximately a 20- to 30-fold excess of the amount required for maximal growth of the organism, and was estimated by growing cells in an amount of sulfanilamide (*p*-aminobenzenesulfonamide, Merck and Co., Inc., Rahway, N.J.) just allowing maximal growth and comparing assay values with others obtained on cultures grown in the absence of the drug. Other assays, performed on cultures during various stages of growth, indicate a proportionate synthesis of folic acid derivatives, with a continual increase in total accumulation for several hours after cessation of growth.

Assay values with *P. cerevisiae*, expressed in terms of equivalents of "active" synthetic folinic acid, indicate that at most only 10 to 15% of the total amount present existed as a one-carbon substituted folate-H_4 (see Table 1).

From values obtained before and after treatment of samples with chicken-pancreas conjugase, it can be seen that no more than 15 to 20% of both folate and the one-carbon substituted folate-H_4 derivatives existed in a form utilizable by *S. faecalis* or *P. cerevisiae*, with the majority of this found extracellularly in the culture medium. The proportion of utilizable forms to nonutilizable polyglutamyl conjugates remained essentially the same during growth. Treatment with the fresh hog-kidney extract was considerably less efficient than the chicken-pancreas preparation in converting nonutilizable material to forms readily used by the assay organisms. In addition, treating samples consecutively with both conjugase preparations did not increase the value obtained with the chicken-pancreas preparation alone. Some heated samples were also

TABLE 2. *Intracellular content of folic acid derivatives as measured by microbiological assay*

Assay organism	Sample	Folic acid derivatives[a] (mμg/100 mg of dry weight)		
		No addition	+CPe[b]	Nonutilizable[c]
Streptococcus faecalis	IV	55	832	777
	V	89	1,230	1,141
	VI	202	1,790	1,588
Lactobacillus casei	IV	738	906	168
	V	708	1,180	472
	VI	1,500	1,760	260
Pediococcus cerevisiae	IV	20	193	173
	V	10	108	98
	VI	46	303	257

[a] Measured as equivalents of pteroylglutamic acid with *S. faecalis* and *L. casei* and equivalents of the active stereoisomer of synthetic N^5-formyl-folate-H_4 with *P. cerevisiae*. Average of duplicate determinations done at several sample dilutions.

[b] Assay values obtained after treatment of samples with chicken-pancreas preparation at pH 6.5.

[c] Calculated as that portion of the total not available for growth by the assay organism prior to treatment with chicken-pancreas conjugase.

treated at pH 4.5 and 6.5 with extracts prepared by sonic treatment to determine the presence of conjugase enzymes in cells of *D. pneumoniae*. Under these conditions, no increase in growth for assay organisms which could be due to conjugase activity was observed.

For a more detailed estimation of polyglutamyl-conjugated derivatives present intracellularly, microbiological assays employing *L. casei* in addition to *S. faecalis* and *P. cerevisiae* were performed on several other samples. Data obtained with a representative group of three samples are shown in Table 2. Total folic acid values in both cases (i.e., with *S. faecalis* and *L. casei*) were nearly identical, indicating that unconjugated folic acid compounds stimulatory to only one of the two assay organisms (viz., pteroic acid, N^{10}-pteroic acid, prefolic A) do not accumulate to a significant extent in cells of *D. pneumoniae*. Of even greater interest are the values obtained in each case prior to treatment with the chicken-pancreas conjugase preparation. With *L. casei*, the assay value is nearly ten times higher than that obtained with *S. faecalis*. The proportion calculated as nonutilizable conjugated material in this case is considerably less and indicates the presence of large quantities of triglutamate derivatives utilized only by *L. casei*.

Assay values derived with *P. cerevisiae*, showing the proportion of free to nonutilizable conjugates, were surprisingly like those obtained with *S. faecalis*. Although both *L. casei* and *P. cerevisiae*, but not *S. faecalis*, were reported (Hakala and Welch, 1957) to utilize the triglutamate derivative of one-carbon substituted folate-H_4, it seems that the above data reflect a greater variation than that reported for the efficiency of utilization of this conjugate among the three organisms and that smaller amounts of higher conjugates also exist, since an increase in *L. casei* activity was also obtained after chicken-pancreas conjugase treatment.

We have attempted to differentiate chromatographically among the various folic acid conjugates. The results obtained with samples treated as described above are shown in Fig. 1, 2, and 3. Bioautograms obtained with heat extracts revealed several zones of growth with all three organisms. These gave R_F values of 0.3, 0.55, 0.7, 0.8, and 0.9, comparable to those obtained with mono-, di-, and triglutamate derivatives of both folic acid (not stimulatory for *P. cerevisiae*) and

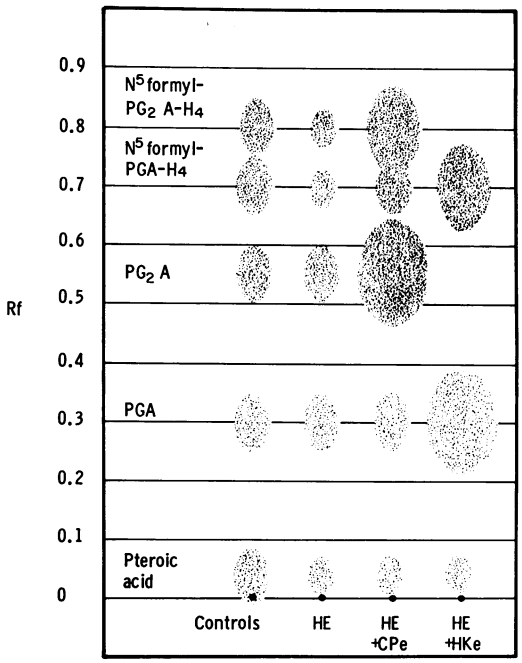

FIG. 1. *Bioautogram with Streptococcus faecalis. Controls, which were synthetic derivatives of folic acid, were spotted in the amount of 2.5 mμg each. They included pteroic acid, PGA (pteroylglutamic acid), PG_3A (pteroyltriglutamic acid), N^5-formyl-PGA-H_4 (N^5-formyltetrahydropteroylglutamic acid), N^5-formyl-PG_3A-H_4 (N^5-formyltetrahydropteroyltriglutamic acid). Synthetic preparations of PG_2A (pteroyldiglutamic acid) and N^5-formyl-PGA_2A-H_4 (N^5-formyltetrahydropteroyldiglutamic acid) were not available. However, R_F values for these two compounds are shown in the figure and are those reported by Hakala and Welch (1957). Extracts were spotted in the amount of 0.01 ml.* HE = *heat extracts of cells,* CPe = *chicken-pancreas conjugase,* HKe = *hog-kidney conjugase.*

N^5-formylfolate-H_4. In addition, a small zone occurred at an R_F less than 0.1 with *S. faecalis*, which is probably pteroic acid. Distinct zones at R_F 0.7 and 0.9 with *L. casei* were obtained only when dilution of the sample was carried out to the extent that other zones of growth did not appear. This confirmed findings shown above and again indicated the presence of large quantities of triglutamate derivatives of both folic acid (R_F 0.7) and a compound having an R_F (0.9) identical to the triglutamate of N^5-formylfolate-H_4. All of the control compounds shown, when stimulatory, gave equivalent growth zones, with

the exception of the triglutamate of N^5-formylfolate-H_4 for *P. cerevisiae*. Since synthetic preparations of this compound were not available as controls, cell-free extracts of *S. faecalis* were incubated with the triglutamate of folic acid to convert it to the formylated, reduced derivative, which could be used to provide a known reference zone when bioautographed. A distinct growth zone at R_F 0.9 (characteristic of the triglutamate of N^5-formylfolate-H_4) appeared on plates seeded with *L. casei*. With the same amount of material, no zone appeared with *S. faecalis*, and only a faint zone was detectable with *P. cerevisiae*. Microbiological assays indicated that the triglutamate was required in at least 50 times the concentration of the other derivatives to produce the same level of growth for the latter organism. This is in agreement with the findings of Silverman and Wright (1956), but not with those of Hakala and Welch (1957). Samples of the synthetic diglutamate derivative of folic acid were not available for use as a known compound. Consequently, we have assumed that R_F values for this compound and the diglutamate of N^5-formylfolate-H_4 are the same (0.55 and 0.8, respectively) as those reported for this solvent system (Hakala and Welch, 1957). Some confirmation is also provided by the fact that the products of hydrolysis by chicken-pancreas conjugase, reported as diglutamate derivatives of both folic acid and N^5-formylfolate-H_4 (Dabrowska, Kazenko, and Laskowski, 1949), have R_F values of 0.55 and 0.8, respectively, in this system. Prior treatment of heat extracts with chicken-pancreas conjugase resulted in a marked increase in zones with these same R_F values. Similar treatment with hog-kidney conjugase, on the other hand, brought about an increase in zones at R_F 0.3 and 0.7, corresponding to folic acid and N^5-formyfolate-H_4. In addition, when digestion with the hog-kidney preparation had been complete (usually requiring at least two successive treatments), all other zones of growth disappeared. This is an indication of the absence in the cell extracts of large amounts of such derivatives as N^{10}-pteroic acid and N^{10}-formylfolate, which have R_F values equal to certain conjugated derivatives of folic acid.

When bioautographs were made with extracts prepared by sonic treatment or from acetone-

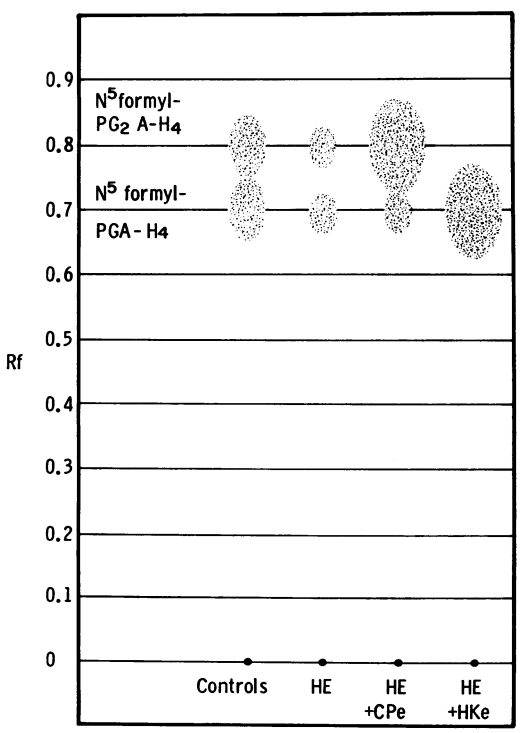

FIG. 2. *Bioautogram with Pediococcus cerevisiae. Controls were spotted in the amount of 2.5 mμg and are the same as in Fig. 1. Samples were spotted in 0.01-ml amounts.* HE = *heat extract of cells,* CPe = *chicken-pancreas conjugase,* HKe = *hog-kidney conjugase.*

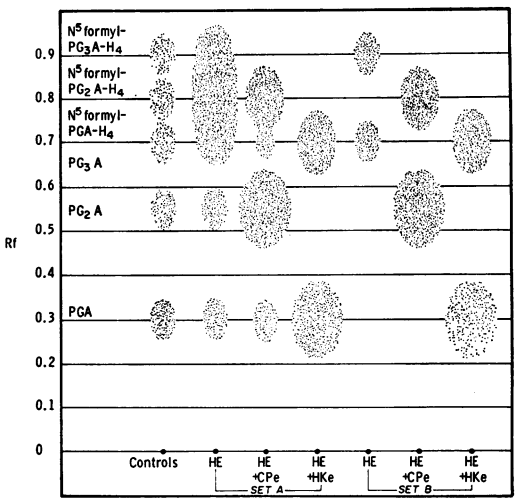

FIG. 3. *Bioautogram with Lactobacillus casei. Controls were spotted in the amount of 2.5 mμg and are the same as in Fig. 1. Samples were spotted in the amount of 0.01 ml (set A) or 0.0025 ml (set B).* HE = *heat extract of cells,* CPe = *chicken-pancreas conjugase,* HKe = *hog-kidney conjugase.*

When bioautographs were made with extracts prepared by sonic treatment or from acetone-

TABLE 3. *Folic acid content of cells grown in the presence of added folate-H_2 under conditions permitting and not permitting biosynthesis of folic acid*

Sample	Cultural conditions		Folic acid derivatives[c] (mμg/10 mg of dry weight)		
	Drug[a]	Folate-H_2[b]	No addition	+CPe[d]	Nonutilizable[e]
I_1	−	−	59	184	125
I_2	+	+	95	111	16
II_1	−	−	123	374	251
II_2	−	+	610	1,020	410
II_3	+	+	312	327	15

[a] *p*-Aminobenzenesulfonamide (100 μg/ml) was used.

[b] The amount of folate-H_2 was 10 μg/ml.

[c] Measured as equivalents of pteroylglutamic acid by microbiological assay with *S. faecalis*. Average of duplicate determinations done at several sample dilutions.

[d] Assay values obtained after treatment of samples with chicken-pancreas preparation at pH 6.5.

[e] Calculated as that portion of the total not available for growth by the assay organism prior to treatment with chicken-pancreas conjugase.

dried cells, no differences were noted (particularly in the number of zones of growth) when compared with bioautographs made with heated material.

The two genetically distinct amethopterin-resistant strains described above were examined to determine any differences in gross accumulation of folic acid. Microbiological assay and bioautographic data on culture material derived from these strains revealed no significant differences in the amount or type of compounds formed by these mutants and cultures of the wild strain.

In view of the rather large amount of folic acid accumulating in culture material as polyglutamyl derivatives, it was considered of interest to determine, to some extent, the manner in which these compounds are synthesized during growth. An attempt was made to ascertain whether polyglutamate formation occurred at a biosynthetic step preceding, or simultaneously with, the formation of folic acid (i.e., by the addition of preformed homopeptides) or by peptide synthesis after the formation of folic acid. Some information could be obtained by blocking the synthesis of folic acid with sulfanilamide but allowing growth by the addition of a reversing compound to the medium. After a number of unsuccessful attempts with folic acid, it was determined that folate-H_2 was the derivative actually necessary to bring about reversal of inhibition. Cells depleted of folic acid, by culturing them at a subinhibitory level of sulfanilamide, were inoculated into a medium containing (per ml) 100 μg of the same drug, 10 μg of folate-H_2, and 1 mg of ascorbic acid. Growth under these conditions proceeded at the same rate as that which occurred in the absence of the drug, whereas no growth occurred in controls containing only the drug. Cells were harvested at the end of the exponential phase of growth, and heat extracts were prepared in the usual fashion. The results are shown in Table 3 and Fig. 4. Under growth conditions where the total intracellular amount of folic acid accumulated was approximately the same in all cases (values for folic acid obtained with conjugase-treated extracts), practically no polyglutamates were found in cells grown during a block in folic acid biosynthesis. That the relatively large amount of folate-H_2 added did not have a nonspecific physiological effect on polyglutamyl

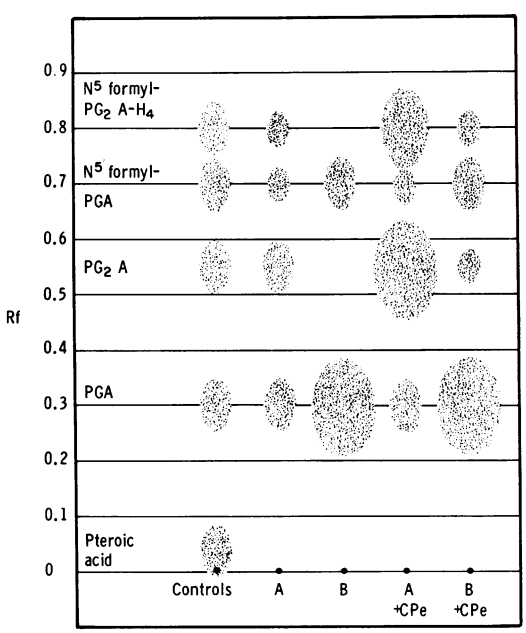

FIG. 4. *Bioautogram with Streptococcus faecalis. Controls were spotted in the amount of 2.5 mμg and are the same as in Fig. 1. Samples were spotted in 0.01-ml amounts.* A = *heat extract of cells,* B = *heat extract of cells grown in the presence of sulfanilamide (100 μg/ml) and folate-H_2 (10 μg/ml),* CPe = *chicken-pancreas conjugase.*

synthesis was shown by data obtained in the second experiment (II_2, Table 3). The amount of polyglutamates was not only equal to, but greater than, that accumulated in the control cells. Possibly an inhibition by sulfanilamide of reactions combining additional glutamic acid residues with folate-H_2 in addition to those leading to the synthesis of this intermediate, although somewhat untenable, might also explain these results. However, under conditions permitting near maximal growth, but where folate synthesis was inhibited more than 95% by sulfanilamide, the proportion of utilizable to nonutilizable conjugated folate accumulating remained unchanged from that normally obtained. When bioautograms were made with these extracts (Fig. 4), a very large zone of growth was seen which corresponded to folic acid (R_F 0.3) in contradistinction to that obtained with control extracts. In addition, after conjugase treatment of extracts from cells grown in the presence of drug, only minute zones of growth appeared at R_F values of 0.55 and 0.8, corresponding to end products of conjugase digestion.

DISCUSSION

As reported for a number of other microorganisms, *D. pneumoniae* during growth also synthesizes the majority of its folic acid as polyglutamyl conjugates. The large proportion of conjugated growth factor found intracellularly (as much as 95% conjugated to the extent of three or more glutamic acid residues), as compared with that found in the growth medium, could indicate that the conjugation process is a means for maintaining the necessary intracellular concentration of these important materials. These findings acquire additional significance when one considers the view expressed previously by Rabinowitz and Himes (1960) that folic acid derivatives occur naturally as polyglutamates with a capacity for participation in one-carbon transfer reactions at least equal to, if not greater than, analogues containing one glutamic acid residue. The relatively small accumulation of substituted, reduced derivatives as compared with folic acid precursors could result from the presence in the cell of a less active formylating mechanism, similar to that reported for *S. faecalis* (Albrecht, Johnson, and Hutchison, 1962).

If we assume that the difference among assay determinations performed with each organism on extracts prior to conjugase treatment is due to the inability of *S. faecalis* and *P. cerevisiae* to utilize triglutamates efficiently, then the bulk (about 60%) of the growth factors, both folic acid and substituted folate-H_4, synthesized by *D. pneumoniae* are triglutamate derivatives. In addition to the 10% synthesized as mono- or diglutamate derivatives (both utilized equally as well by assay organisms), the remaining 30% probably occurs as a higher glutamate derivative, viz., hexa- or heptaglutamates described in yeast (Pfiffner et al., 1946) and in *C. cylindrosporum* (Wright, 1955), unless the greater activity obtained with the conjugase-treated extracts is due to a more efficient utilization of the hydrolysis product (diglutamate) by *L. casei*. Indications to the contrary were obtained in reports (Hakala and Welch, 1955, 1957) which show that triglutamates are utilized equally as well as lesser conjugates by *L. casei*. We have also confirmed this by obtaining no increase in *L. casei* activity of solutions of the triglutamate of folic acid and the enzymatically synthesized triglutamate form of N^5-formylfolate-H_4, after chicken-pancreas conjugase digestion. However, in view of the increase in activity shown with conjugase-treated extracts, we have attempted to distinguish by bioautography between triglutamates and higher glutamates using *L. casei*. Extracts, which were diluted to the extent that only the triglutamate derivatives would appear on bioautographic plates, were spotted on paper strips and run in the aqueous phosphate buffer described above and in an ammonia-ethanol-butanol-water solvent (Doctor and Couch, 1953). The strips were placed on agar medium seeded with *L. casei*, some of which contained purified chicken-pancreas conjugase prepared by the method of Laskowski, Mims, and Day (1945). Although the results were not definitely conclusive, areas of growth other than those normally seen at R_F 0.7 and 0.9 (triglutamyl derivatives) were definitely visible on the plates containing the chicken-pancreas conjugase.

The reversal of sulfanilamide inhibition by folate-H_2 and not by folic acid is a most interesting observation. Assuming no permeability factor, this finding could be interpreted to mean that the normal pathway of synthesis, similar to that reported for *Escherichia coli* by Brown et al. (1961), does not proceed via folic acid, but rather via a reduced form of this compound.

The apparent origin in *D. pneumoniae* of folic acid derivatives with additional glutamic acid

residues by the utilization of preformed homopeptides, does not entirely rule out an increase in peptide length at a later stage. The presence of small amounts of polyglutamates in cells grown during a block in folic acid synthesis could be indicative of the existence of such a mechanism in this organism. Furthermore, a similar mechanism must exist in microorganisms, such as *S. faecalis* and *L. casei* (Hendlin et al., 1953), which are unable to synthesize folic acid but form considerable amounts of folic acid polyglutamates from added folic acid.

ACKNOWLEDGMENTS

The authors are indebted to Alberta M. Albrecht for providing the cell-free preparations of *S. faecalis* and for assistance in the preparation of this manuscript.

This work was supported in part by contract no. SA-43-PH-2445 from the Cancer Chemotherapy National Service Center, National Cancer Institute, National Institutes of Health, Bethesda, Md., and by grant T-107C from the American Cancer Society.

LITERATURE CITED

ADAMS, M. H., AND A. S. ROE. 1945. A partially defined medium for cultivation of pneumococcus. J. Bacteriol. **49**:401–409.

ALBRECHT, A. M., A. H. JOHNSON, AND D. J. HUTCHISON. 1962. Studies on mechanisms of resistance to antifolics in *Streptococcus faecalis*. Bacteriol. Proc., p. 105.

BIRD, O. D., B. BRESSLER, R. A. BROWN, C. J. CAMPBELL, AND A. D. EMMETT. 1945. The microbiological assay of vitamin B_c conjugate. J. Biol. Chem. **159**:631–636.

BIRD, O. D., S. B. BINKLEY, E. S. BLOOM, A. D. EMMETT, AND J. J. PFIFFNER. 1946. On the enzymatic formation of vitamin B_c from its conjugate. J. Biol. Chem. **157**:413–414.

BLAKLEY, R. L. 1960. Crystalline dihydropteroylglutamic acid. Nature **188**:231–232.

BRATTON, A. C., AND E. K. MARSHALL, JR. 1939. A new coupling component for sulfanilamide determination. J. Biol. Chem. **128**:537–550.

BROWN, G. M., R. A. WEISMAN, AND D. A. MOLNAR. 1961. The biosynthesis of folic acid. I. Substrate and cofactor requirements for enzymatic synthesis by cell-free extracts of *Escherichia coli*. J. Biol. Chem. **236**:2534–2543.

DABROWSKA, W., A. KAZENKO, AND M. LASKOWSKI. 1949. Concerning the specificity of chicken pancreas conjugate. Science **110**:95.

DOCTOR, V. M., AND J. R. COUCH. 1953. Occurrence and properties of a conjugated form of *Leuconostoc citrovorum* factor. J. Biol. Chem. **200**: 223–231.

FLYNN, L. M., V. B. WILLIAMS, B. L. O'DELL, AND A. G. HOGAN. 1951. Medium for assay of vitamins with lactic acid bacteria. Anal. Chem. **23**:180–185.

FUTTERMAN, S. 1957. Enzymatic reduction of folic acid and dihydrofolic acid to tetrahydrofolic acid. J. Biol. Chem. **228**:1031–1038.

HAKALA, M. T., AND A. D. WELCH. 1955. A conjugated form of citrovorum factor synthesized by *Bacillus subtilis*. Federation Proc. **14**:222.

HAKALA, M. T., AND A. D. WELCH. 1957. A polyglutamate form of citrovorum factor synthesized by *Bacillus subtilis*. J. Bacteriol. **73**:35–41.

HENDLIN, D., L. K. KODITSCHEK, AND M. H. SOARS. 1953. Investigations of the biosynthesis of citrovorum factor by lactic acid bacteria. J. Bacteriol. **65**:466–471.

LASKOWSKI, M., V. MIMS, AND P. L. DAY. 1945. Studies on the enzyme which produces the *Streptococcus lactis* R-stimulating factor from inactive precursor substance in yeast. J. Biol. Chem. **157**:731–739.

PFIFFNER, J. J., D. G. CALKINS, E. S. BLOOM, AND B. L. O'DELL. 1946. On the peptide nature of vitamin B_c conjugate from yeast. J. Am. Chem. Soc. **68**:1392.

RABINOWITZ, J. C., AND R. H. HIMES. 1960. Folic acid coenzymes. Federation Proc. **19**:963–970.

SAUBERLICH, H. E., AND C. A. BAUMANN. 1948. A factor required for the growth of *Leuconostoc citrovorum*. J. Biol. Chem. **176**:165–173.

SILVERMAN, M., AND B. E. WRIGHT. 1956. Microbiological aspects of the diglutamyl derivatives of citrovorum factor and N^{10}-formylfolic acid. J. Bacteriol. **72**:373–377.

SIROTNAK, F. M., R. B. LUNT, AND D. J. HUTCHISON. 1960*a*. Genetic studies on amethopterin resistance in *Diplococcus pneumoniae*. J. Bacteriol. **80**:648–653.

SIROTNAK, F. M., R. B. LUNT, AND D. J. HUTCHISON. 1960*b*. Transformation of resistance to 8-azaguanine in *Diplococcus pneumoniae*. Nature **187**:800–801.

SWENDSEID, M. E., AND J. F. NYC. 1958. Study of folic acid derivatives in *Neurospora crassa* and the recognition of a mutant resistant to aminopterin. J. Bacteriol. **75**:654–659.

WRIGHT, B. E. 1955. Polyglutamyl pteridine coenzymes. J. Am. Chem. Soc. **77**:3930–3931.

ZAKRZEWSKI, S. F., AND C. A. NICHOL. 1955. Derivatives of pteroic and pteroylglutamic acids formed by *Streptococcus faecalis*. Federation Proc. **14**:311.