RELATIONSHIP BETWEEN PURINES AND FOLIC ACID-VITAMINS IN THE MICROCOCCUS, *GAFFKYA HOMARI*¹

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Microorganisms that require *p*-aminobenzoic acid or compounds of the folic acid group may have either requirement replaced by a purine and thymine (or thymidine) and several amino acids or vitamin B₁₂ (Lampen *et al.*, 1949; Stokstad, 1941; Baker *et al.*, 1955); the vitamins, *p*-aminobenzoic or folic acids, are believed necessary for the synthesis of those compounds which can bypass them (Shive, 1950). Further support for this concept comes from the observation that the same combination of amino acids, a purine, and thymine annul the inhibition by sulfanilamide of several microorganisms (Winkler and DeHaan, 1948; Shive, 1950). In the micrococcus, *Gaffkya homari*, a new relationship was found: any one of several purines (Aaronson and Rodriguez, 1957) or, alternatively, any of the following vitamins: *p*-aminobenzoic, pteroylglutamic, or folinic acids are required. This suggests that a purine may be necessary for the synthesis of the metabolically active form of folic acid and, conversely, purine synthesis depends on folic acid.

MATERIALS AND METHODS

G. homari strain ATCC 10400 was used. Some of the experiments were performed in a chemically defined medium supplemented with amino acids which stimulate multiplication as described by Aaronson (1956). All chemicals except those mentioned in the acknowledgment were obtained from commercial sources.

Crithidia factor was identified by assay with *Crithidia fasciculata* (Nathan and Cowperthwaite, 1955). Folic acid compounds were identified by chromatography on 1-in paper strips in the following solvent system: isopropanol:glacial acetic acid:water (25:1:74, v/v); the R_f values were determined after bioautography with *Streptococcus faecalis* strain ATCC 8043.

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Bioautographs were prepared as follows: pyrex baking trays (19 by 30 cm, internal dimensions) were sterilized with a plate glass cover; sterile agar (table 1) was poured into the tray to a depth of 1/2 in; the agar was cooled in the refrigerator until firm. The cells from a 24 hr culture of *S. faecalis*, grown in broth (yeast extract, 1 per cent; glucose, 1 per cent; pH 7) were collected by centrifugation, then suspended in 10 ml of sterile water and added to melted, warm (40 to 45 C) agar (table 1) containing Andrade's indicator (1 ml per 100 ml agar). Cells were thoroughly mixed with the melted agar and the agar was then poured over the previous or base layer to form a seed layer of 1/8 to 1/4 in. Trays were returned to the refrigerator until the agar was firm. Paper strips were then placed on the agar surface, the tray and its glass cover were sealed with tape and placed in an incubator at 37 C. Bioautographs were read in 12 to 20 hr. This permitted sufficient streptococcal growth so that the Andrade's indicator would turn red around the area on the paper strip where active compounds such as pteroylglutamic acid, leucovorin (citrovorum factor), etc., were located. These zones were marked on the paper strips and R_f values determined. Each tray contained a paper strip with known compounds in addition to the experimental samples.

RESULTS

The purine requirement of *G. homari* is satisfied by either adenine, hypoxanthine, or guanine (Aaronson and Rodriguez, 1957). This requirement is also satisfied by *p*-aminobenzoic acid or any one of several compounds of the folic acid group (table 2); amino acids or thymine (alone or together) did not satisfy the requirement although amino acids were somewhat stimulatory in the presence of a purine or vitamin adequate for good growth (table 3).

When *G. homari* is grown with a purine or *p*-aminobenzoic acid, small amounts of compounds

TABLE 1

Medium for the bioautography of folic acid compounds with *Streptococcus faecalis* strain 8043

Na Acetate·3H ₂ O	0.2 g
Glucose	1.0 g
L-Cystine	0.01 g
DL-Tryptophan	0.06 g
Casamino acids (Difco)	0.3 g
KH ₂ PO ₄	0.5 g
Biotin	0.04 µg
Thiamin·HCl	0.1 mg
Nicotinic acid	0.3 mg
Pyridoxine·2HCl	0.2 mg
Ca Pantothenate	0.2 mg
Riboflavin	0.1 mg
Thioctic acid	1.0 µg
Guanine·HCl	1.0 mg
Uracil	1.0 mg
Agar	2.0 g
Distilled water to 100 ml, pH 7.0	

TABLE 2

Alternate compounds completely replacing the purines for *Gaffkya homari*

Compounds	Conc Range Tested per 2 ml of Culture
	µg
<i>Replacing</i>	
<i>p</i> -Aminobenzoic acid.....	0.00002-0.2
Pterioic acid.....	0.00002-2.0
N ¹⁰ -Formylpterioic acid.....	0.00002-0.2
<i>p</i> -Aminobenzoylglutamic acid...	0.00002-1.0
Pteroylglutamic acid.....	0.00002-2.0
Ca Leucovorin·5H ₂ O.....	0.00002-2.0
<i>Nonreplacing</i>	
<i>p</i> -Hydroxybenzoic acid.....	0.00002-1.0
Xanthopterin*.....	0.00002-0.2
2 - Amino - 4 - hydroxy - 6 - di-hydroxypropylpteridine.....	0.00002-2.0
	mg
Thymine*.....	0.00002-0.2
Thymidine*.....	0.00004-1.2

* Not utilized alone or in combination with a mixture of amino acids.

with R_f values similar to that of leucovorin accumulate in the culture supernatant (table 4). When grown with pteroylglutamic acid, compounds with R_f values resembling pteroylglutamic acid, leucovorin, and an unidentified *S. faecalis*-active compound accumulate. Supernatants of *G. homari* grown with either purines or

TABLE 3

Growth response of *Gaffkya homari* to purine, *p*-aminobenzoic acid, pteroylglutamic acid, and leucovorin

Compound	No Ad-dition	Amino Acid Mix-ture*	Thy-mine*	Amino-Acids + Thy-mine
Medium.....	0	0	0	0
+ Hypoxanthine, 0.04 mg/2 ml...	1.02	1.28	1.02	1.38
+ <i>p</i> -Aminobenzoic acid, 0.2 µg/2 ml	0.92	1.06	0.95	1.11
+ Pteroylglutamic acid, 0.7 µg/2 ml	0.74†	0.86	0.78	0.80
+ Leucovorin, 0.7 µg/2 ml.....	0.82†	0.70	0.74	0.71

* Amino acid mixture used at 0.1 ml per 2 ml; thymine used at 0.02 mg per 2 ml.

† Multiplication with pteroylglutamic acid and leucovorin was appreciably slower than with *p*-aminobenzoic acid or purine but it reaches the same densities with longer incubation.

p-aminobenzoic acid accumulate one or more unconjugated pteridines with Crithidia factor activity in addition to these conjugated pteridines. The highest yields of unconjugated pteridines came from cultures grown with *p*-aminobenzoic acid as determined by the optical density of the Crithidia cultures.

DISCUSSION

G. homari is the only known microorganism with an alternate requirement for purine or *p*-aminobenzoic and folic acids. Presumably, all the other functions of these vitamins, i. e., amino acid, and thymine (or thymidine) syntheses proceed with either exogenous purine or *p*-aminobenzoic and folic acids. Other microorganisms requiring *p*-aminobenzoic or folic acids have their requirement satisfied wholly or in part by a mixture of purine, amino acids, and thymine; a purine, amino acids, and thymine replaced *p*-aminobenzoic acid for an *Escherichia coli* mutant (Lampen *et al.*, 1949), a purine and thymine partially replaced the pteroylglutamic acid requirement of *Lactobacillus casei* (Stokstad, 1941), and xanthine, thymine, and vitamin B₁₂ partially replaced the pteroylglutamic acid requirement of *Bacillus stearothermophilus* (Baker *et al.*, 1955).

G. homari can use an exceptionally wide array of exogenous precursors of folic acid and is prob-

TABLE 4
Identification of the Streptococcus faecalis-active materials in culture supernatants of Gaffkya homari

Material	Conc per Spot	R _F of Spot Active for <i>S. faecalis</i>	Ac-tivity
Pteroylglutamic acid	10.0	0.30	+++*
Leucovorin	10.0	0.73	+++
Supernatant of <i>Gaffkya</i> cultures grown on:			
	μg		
Guanine, 0.01 mg/2 ml†	0.12‡	0.76	—
Hypoxanthine, 0.02 mg/2 ml	0.12	0.63	—
<i>p</i> -Aminobenzoic acid, 0.2 μg/2 ml	0.12	0.76	++
Pteroylglutamic acid, 0.64 μg/2 ml	0.12	0.26	+++
		0.50	++
		0.76	+++

* Approximate activity determined from the zone of redness around the spot on the chromatogram.

† Concentration chosen to give good growth.

‡ Total volume of supernatant placed on spot in 0.02 ml aliquots.

ably able to complete the metabolically active form of folic acid if given either *p*-aminobenzoic, *p*-aminobenzoylglutamic, or pteric acids. The active compounds (table 2) contain the *p*-aminobenzoic acid molecule (figure 1) suggesting that this is the site of the metabolic lesion; this idea is negated, however, by the synthesis of this molecule when purines alone are provided (table 4). Two unconjugated pteridines, xanthopterin, and 2-amino-4-hydroxy-6-dihydroxypropylpteridine, were not utilized. Conjugated pteridines of the folic acid series were utilized or else synthesized (tables 2 and 4). Small molecule precursors of folic acid are utilized, and given exogenous purines small amounts of a leucovorin-like compound are synthesized; a leucovorin-like compound also accumulated in the supernatant when the bacteria were grown with *p*-aminobenzoic or pteroylglutamic acid (table 4). We may therefore conclude, not surprisingly, that leucovorin or its enzymatically active relative is the metabolically active form of this vitamin in *G. homari*. This is further borne out if cultures are grown for 5 or more days; leucovorin and pteroylglutamic acid

support multiplication almost as well as *p*-aminobenzoic acid on a molar basis (table 5). Other workers have found that leucovorin is a stable form of metabolically active folic acid when cells or cellular homogenates are either grown or incubated with *p*-aminobenzoic or pteroylglutamic acid. Reid and Couch (1955) found that chicken liver homogenates converted pteroylglutamic acid to leucovorin; Nichol (1954) reported that cell-free extracts of *S. faecalis* converted pteroylglutamic acid to leucovorin; Lascelles and Woods (1954) reported that suspensions of *S. faecalis* strain ATCC 8043 incubated with pteroylglutamic acid produce leucovorin; Davidson *et al.* (1956) fed radioactive *p*-aminobenzoic acid to a *p*-aminobenzoic acid-requiring mutant of *E. coli* and isolated radioactive compounds which seemed to resemble leucovorin.

Besides making conjugated pteridines such as leucovorin, *G. homari* cells also make unconjugated pteridines with Crithidia activity in the presence of purines or *p*-aminobenzoic acid. Aside from their role as part of the folic acid molecule pteridines may also supply an unknown precursor of riboflavin (Nathan *et al.*, 1956).

Perhaps in *G. homari* exogenous purine is needed for the synthesis of *p*-aminobenzoic acid, leucovorin, and unconjugated pteridines as well as for direct utilization for the synthesis of nucleic acids and cofactors. Conversely, exogenous *p*-aminobenzoic or folic acids may replace the need of *G. homari* for exogenous purines; they then can synthesize purines for nucleic acids and cofactors, other pteridines as well as amino acids, thymine, etc. There is no present indication of a direct metabolic relationship between *p*-aminobenzoic acid and purine; there is some evidence that purines may serve as pteridine precursors. Albert (1957) has obtained a purely chemical conversion of several purines into pteridines. Ziegler-Günder *et al.* (1956) found that after an injection of 2-C¹⁴-guanine into the larvae of the amphibian, *Xenopus*, a pteridine became radioactive; they were unable to show this in adult amphibia.

Purines have been implicated in riboflavin as well as pteridine synthesis. McNutt (1954) showed that riboflavin was labeled when radioactive adenine was added to growing *Eremothecium ashbyii*. Brown *et al.* (1955) using *E. ashbyii*, found that low levels of aminopterin inhibited growth and stimulated flavogenesis, while azaxanthine, which had no effect on growth, de-

Pteroylglutamic acid

Leucovorin

p-Aminobenzoylglutamic acid

p-Aminobenzoic acid

Pterioic acid

N¹⁰-Formylpterioic acid

Figure 1. Structure of *p*-aminobenzoic acid and compounds of the folic acid group

TABLE 5
Comparison of the utilization of several vitamins by
Gaffkya homari

Compound	Conc <i>μmoles/2 ml</i>	Optical Density
<i>p</i> -Aminobenzoic acid	1.5×10^{-3}	0
	4.5×10^{-3}	0.38
	1.5×10^{-2}	0.45
	4.5×10^{-2}	0.96
	1.5×10^{-1}	1.02
	1.5	1.16
Pteroylglutamic acid	4.6×10^{-4}	0
	4.6×10^{-3}	0.33
	1.4×10^{-2}	0.49
	4.6×10^{-2}	0.52
	1.4×10^{-1}	0.64
	4.6×10^{-1}	0.96
Ca Leucovorin·5H ₂ O	1.4	0.98
	4.6	1.14
	3.3×10^{-3}	0
	1.0×10^{-2}	0.38
	3.3×10^{-2}	0.71
	1.0×10^{-1}	0.82
	3.3×10^{-1}	0.98
	1.0	1.00
	3.3	1.06

pressed flavogenesis; they surmised that aminopterin increased flavogenesis by blocking utilization of the same precursor for folic acid synthesis while azaxanthine interfered with the conversion of this precursor—probably a purine—to riboflavin while not interfering with nucleic acid synthesis as reflected in growth.

Purines, pteridines, and riboflavin may have common precursors (Weygand and Waldschmidt, 1955; Plaut, 1956); we suggest that the pteridine-containing vitamins come from purine precursors which are also the precursors for riboflavin.

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SUMMARY

Gaffkya homari has a requirement for a purine or alternatively for *p*-aminobenzoic acid or several members of the folic acid group of vitamins. This requirement is unusual because the purine

may completely replace the vitamin requirement in the absence of amino acids and thymine.

G. homari accumulates a citrovorum-like factor when grown with either purines or *p*-aminobenzoic or pteroylglutamic acids. Unconjugated pteridines of the Crithidia factor type are also accumulated when the bacteria are grown with either purines or *p*-aminobenzoic acid.

The purines are postulated to be precursors of the pteridines, both of the folic acid and the Crithidia factor series. One of these precursors of pteridine originating from purine is also thought to be a precursor of riboflavin.

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