THE NUTRITIONAL REQUIREMENTS OF TREPONEMATA

VI. The Total Vitamin Requirements of the Reiter Treponeme

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A chemically defined medium which suffices for the cultivation of the Reiter treponeme has been described in a previous paper (Steinman *et al.*, 1952). Of the amino acids in that medium, thirteen subsequently proved to be essential and two beneficial. Only one pyrimidine, uracil, and none of the purines proved to be essential.

The vitamin requirements of the Reiter treponeme are considered in the present paper. Three proved to be essential (biotin, nicotinic acid, pantothenic acid), and three were merely stimulatory (choline, riboflavin, thiamin). A number of analogues and derivatives of these vitamins also were found to be active.

MATERIALS AND METHODS

Cultures which had been incubated for five to seven days at 37 C anaerobically under H_2 were centrifuged, washed two times with medium from which the specific vitamins under study had been omitted, and then resuspended in the appropriate medium. The amount of growth was determined by measurement of the optical density at 660 mμ (Steinman *et al.*, 1952). A semisolid medium was used to establish the essentiality of the growth factor under study, as previously described (Steinman *et al.*, 1953).

The vitamin requirements as determined for organisms propagated in the defined medium were found to be the same as for the stock "wild type" organisms continuously propagated in a serum-"thioglycolate" medium.

RESULTS

Vitamins essential for growth of Reiter treponeme. Pantothenic acid previously had been found essential for the growth of the Reiter spirochete (Steinman and Eagle, 1950). By using a completely defined culture fluid, it could now be shown that no detectable growth occurred in the absence of either biotin or nicotinic acid. The growth response to added biotin, nicotinic acid, and pantothenic acid is shown in figure 1. At suboptimal concentrations of these essential vitamins, both the rate and total amount of growth were restricted.

When organisms were inoculated into a semisolid medium deficient in any one of the 3 vitamins in question, no colonies appeared even with an inoculum of 10^6 organisms, while in control cultures essentially all the organisms were viable.

Vitamins beneficial to growth of Reiter spirochete. Figure 2 shows the effect of added thiamin, choline, and riboflavin on the growth of the Reiter organisms. In all three instances, growth occurred in the absence of added vitamin.

(a) The addition of choline served only to accelerate the rate of growth without affecting the maximum population attained. The striking acceleration of the rate of growth effected by choline is shown in figure 2, the time required for 50 per cent growth being reduced from ten to four days.

(b) Riboflavin similarly reduced the time for 50 per cent growth, but only from seven to four days. This suggested a moderate rate of biosynthesis of riboflavin by the organism, which was confirmed by direct microbiologic assay with *Lactobacillus casei* (Snell and Strong, 1939). Organisms grown in a riboflavin-free medium were found to contain approximately 0.7 μg riboflavin per g dry weight.

(c) There is some evidence that thiamin, unlike choline or riboflavin, may actually be essential and not merely stimulatory for growth. In the absence of added thiamin, the organism grew out only to a very limited degree. This limited growth response persisted through several transfers, indicating a lack of any adaptive response. On serial dilution in agar it was found that every organism inoculated grew out, but

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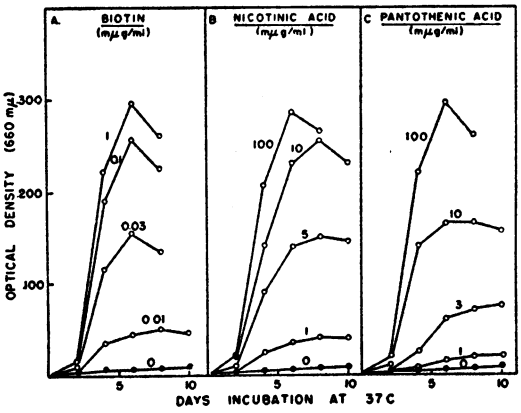

Figure 1. Growth response of the Reiter treponeme to three essential vitamins in the absence of which all the organisms die (●).

formed only pin-point colonies. The dependency of the amount of growth on the amount of added thiamin, as well as the very low concentration required for a maximal growth response, resembled the findings obtained with the 3 essential vitamins and suggested that the medium may have been contaminated with trace amounts of thiamin. On examination of the various ingredients of the defined medium by microbiological assay with *Lactobacillus fermenti* (Sarett and Cheldelin, 1944), it was found that the crystallized serum albumin was in fact so contaminated. However, it has not yet been possible to purify the albumin, and the essentiality of thiamin cannot be considered proved.

Table 1 lists the half-maximal (ED_{50}) and the fully-maximal (ED_{100}) concentrations of the vitamins required by the Reiter treponeme. The effective concentrations of the merely stimulatory vitamins, choline and riboflavin, were 1,000 to 10,000 mμg per ml in contrast to those of the essential vitamins which ranged from 1 to 100 mμg per ml.

Dispensable vitamins. Growth of the organism after 6 days' incubation in a medium containing the 6 vitamins described above was as good as that obtained with the original thioglycolate-whole serum mixture. The addition of *p*-aminobenzoic acid, folic acid, inositol, pyridoxal, pyridoxamine, pyridoxine, alone or in combination, had no further stimulatory effect.

Because of the metabolic importance of vitamin B_6 the Reiter spirochete was assayed directly for this vitamin. With *Saccharomyces carlbergensis* (Atkin *et al.*, 1943) as test organism, the treponeme was found to contain approximately 1 μg of vitamin B_6 (expressed as pyridoxine) per g dry weight.

As described above, *p*-aminobenzoic acid (PABA) had no demonstrable growth promoting activity. Its probable biosynthesis was indicated indirectly by the fact that sulfanilamide was completely inhibitory to growth at concentrations as low as 10 μg per ml. As shown in table 2, this toxicity could be reversed completely by the addition of *p*-aminobenzoic acid at one-thousandth this concentration (0.01 μg per ml). The antagonism between sulfanilamide and *p*-aminobenzoic acid was competitive, i.e., a 10-fold increase in the concentration of inhibitor required a 10-fold increase in the concentration of the reversing agent.

Three conjugates of *p*-aminobenzoic acid (pteroic acid, folic acid, and folinic acid) were equivalent on a molar basis to *p*-aminobenzoic acid in reversing the toxicity of sulfanilamide. These results are in contrast to those of Sarett (1951), who found that folinic acid (citrovorum factor) was less effective than *p*-aminobenzoic acid as an antisulfanilamide agent for *Lactobacillus arabinosus*, an organism which requires *p*-aminobenzoic acid for growth. 10-Formylpteroylglutamic acid, a close analogue of citrovorum factor, was only a tenth as effective as

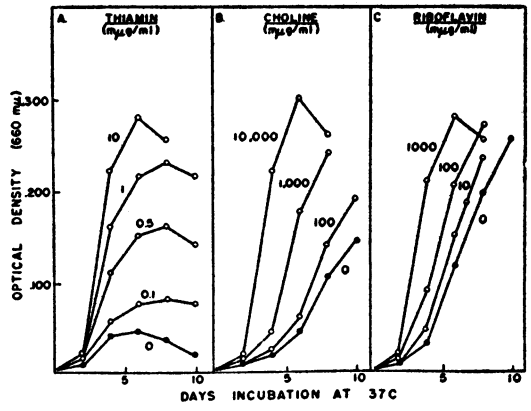

Figure 2. Growth promoting effects of three beneficial vitamins in the absence of which there was growth of all the organisms inoculated into the medium (●). In A (s thiamin) there was limited growth; in B (s choline) there was slow growth which eventually attained the maximum levels obtained by the controls; in C (s riboflavin) there was rapid growth after an initial lag period.

TABLE 1

Vitamin requirements of the Reiter treponeme

COMPOUND	ED_{50}	MAXIMALLY EFFECTIVE CONCENTRATION
	mμg per ml	*mμg per ml*
Essential		
Biotin	0.03	0.5–1.0
Nicotinic acid	6	50–100
Pantothenic acid	10	100–200
Beneficial		
Thiamin chloride	0.8	5–10
Riboflavin	100	1,000
Choline chloride	1,000	10,000

The data are derived from curves similar to those illustrated in figures 1 and 2. The ED_{50} concentrations represent those concentrations at which the growth response was one-half of the maximum observed values. The growth response leveled off at concentrations higher than the listed maximally effective concentrations.

the latter. Similarly, the conversion of folic acid to aminopterin (the 4-amino derivative) destroyed its reversing ability. Aminopterin at 100 μg per ml was itself directly toxic, as has been observed with other non-*p*-aminobenzoic acid requiring organisms (Franklin *et al.*, 1949).

Effect of vitamin analogues. The ability of the Reiter treponeme to utilize certain vitamin precursors and analogues was examined.

Biotin. Biotin, one of the vitamins not synthesized by the treponeme, could not be replaced in any degree by pimelic acid and only very poorly by desthiobiotin (ED_{50} = 3 μg per ml, or 100,000 times that of biotin). Both of these substances are potential precursors of biotin (Snell, 1951). When biotin was present at a concentration of one mμg per ml, avidin at an equivalent ratio to biotin of 4.5 to 1 caused 50 per cent inhibition of growth, and at 8 to 1 inhibited growth completely. This is in accord with the ability of avidin to form a stable complex with free biotin and thus prevent its utilization (Eakin *et al.*, 1941). The fact that the Reiter treponeme requires biotin in addition to aspartic acid and oleic acid (Oyama *et al.*, 1953) indicates a function for biotin over and above the biosynthesis of unsaturated fatty acids and of aspartic acid (Lichstein, 1951; Lardy and Peanasky, 1953).

Nicotinic acid. The nicotinic acid requirement was equally well satisfied by the corresponding amide, niacinamide. The conjugated form, coenzyme I (DPN), was fully equivalent on a molar basis to nicotinic acid. The antimetabolite, pyridine-3-sulfonic acid, inhibited niacin and niacinamide noncompetitively at 100 μg per ml (2,000 times the ED_{100} value of nicotinic acid), but its toxicity was reversed competitively by coenzyme I. Neither tryptophan, which is an essential component of the medium, nor 3-hydroxyanthranilic acid could satisfy the nicotinic acid requirement as they do for certain mutants of *Neurospora* (Mitchell and Nyc, 1948).

Pantothenic acid. As already stated, pantothenic acid was an absolute requirement for the organism. Pantethine, a partially conjugated form of pantothenic acid which is required as such by many strains of *Lactobacillus bulgaricus* and certain other lactobacilli (Snell and Brown, 1953), was one-tenth as active as the free acid. This relative inefficiency of the complex form has been shown to be true for many other microorganisms (Craig and Snell, 1951). Coenzyme A, the fully conjugated form of pantothenic acid (Lipmann, 1953; Novelli, 1953), was as active as pantethine, but again, only one-tenth as active as the free acid.

The corresponding alcohol pantothenol had

TABLE 2

Reversal of sulfanilamide inhibition of the growth of the Reiter treponeme by p-aminobenzoic acid and derivatives

COMPOUND	CONCENTRATION OF PABA OR CONJUGATE IN μG PER ML NECESSARY TO REVERSE SULFANILAMIDE TOXICITY	
	Concentration of sulfanilamide in μg per ml	
	10	100
p-Aminobenzoic acid	0.01	0.1
Pteroic acid	0.1	1.0
Folic acid	0.1	1.0
Citrovorum factor	0.1	1.0
10-Formylpteroylglutamic acid	1.0	10

Sulfanilamide was partially inhibitory at 1 μg per ml and completely inhibitory at 10 μg per ml. The citrovorum factor was obtained in purified form as the barium salt from Dr. M. Silverman. Pteroic acid, folic acid, and 10-formylpteroylglutamic acid were obtained from Lederle Laboratories through the courtesy of Dr. H. Broquist.

only slight activity (less than 1/100,000 that of pantothenic acid). Pantoyltaurine, which might have been expected to be a potent antimetabolite (Woolley, 1952), was only slightly inhibitory of pantothenate and pantethine at a level of 1,000 μg per ml (10,000 times the ED_{100} value of pantothenic acid). Its action was reversed competitively by these metabolites. In contrast, it was much more effective against coenzyme A, but in a noncompetitive manner.

Biosynthetic formation of either the β-alanine or the pantoyl-lactone fragments of pantothenic acid apparently does not occur since neither moiety replaced the condensed molecule even at concentrations as high as one mg per ml. However, there was some activity where the two portions were present together at this level of one mg per ml—a concentration 10,000 times that of the ED_{100} (100 mμg per ml) of pantothenic acid.

Thiamin. Thiamin, as shown above, was highly beneficial, if not essential, to the growth of the organism. The phosphorylated form, cocarboxylase, was one-fifth as active. Although neopyrithiamin is usually toxic for thiamin requiring organisms (Woolley and White, 1943), it did not antagonize either thiamin or cocarboxylase, even at a concentration of 100 μg per ml.

The organism could not condense the two portions of thiamin, 2-dimethyl-4-amino-5-bromomethylpyrimidine dihydrobromide and 4-methyl-5-(β-hydroxyethyl) thiazole, when both were supplied at 1,000 times (10 μg per ml) the ED_{100} concentration (10 mμg per ml) of thiamin. There was some thiamin activity, however, when higher concentrations (1 mg per ml) of the two precursor molecules were used.

Choline. The stimulatory effect on growth by choline also was shown by such closely related compounds as acetylcholine and, to a lesser extent, bromocholine. 2-Dimethylaminoethanol also functioned as a substitute (or precursor) of choline but acetyl-β-methylcholine did not. However, 2-diethylaminoethanol, previously found to be an effective substitute for choline for a strain of Type III pneumococcus (Badger, 1944), actually depressed the growth of the Reiter treponeme in the absence of choline. Betaine was inert.

Riboflavin. As discussed above, riboflavin was stimulatory rather than essential since the spirochete can satisfy its riboflavin requirements by biosynthesis, albeit at a slow rate as evidenced by delayed growth. The 5'-phosphate derivative was equally effective. Galactoflavin, which might have been expected to be an antimetabolite, was in fact a substitute for riboflavin, but at a 10-fold higher concentration level.

Vitamin B_6. No vitamin B_6 requirement could be established, and, as stated above, biosynthesis of this vitamin was demonstrated by direct analysis of the organism. Desoxypyridoxine displayed a partial inhibition of growth at 100 μg per ml which was reversed by pyridoxal at 1 μg per ml. This is in accord with the findings of Rabinowitz and Snell (1953) that 4-desoxypyridoxine is relatively ineffective as an antagonist of vitamin B_6 for organisms that grow in its absence.

p-Aminobenzoic acid. The probable biosynthesis of *p*-aminobenzoic acid by the organism as indicated by the reversible competitive antagonism with sulfanilamide has already been discussed. In addition to the *p*-aminobenzoic acid conjugates (*cf* table 2), *p*-nitrobenzoic acid possessed the ability to reverse the toxicity of sulfanilamide, presumably via reduction by the organism to *p*-aminobenzoic acid. *p*-Hydroxybenzoic acid and shikimic acid were inert.

DISCUSSION

The pattern of the vitamin requirements of the Reiter treponeme is qualitatively similar to that of other bacteria. The concentrations required for maximal growth ranged from 1 mμg per ml to 100 mμg per ml, increasing in the order biotin, thiamin, nicotinic acid, and pantothenic acid. This concentration pattern parallels the requirements by a wide variety of microorganisms (Snell, 1951). Choline, which is an unusual vitamin requirement for bacteria and is known to be required only by certain strains of pneumococci (Rane and SubbaRow, 1940), was growth stimulatory at relatively high levels (10 μg per ml). Riboflavin at high concentration levels (1 μg per ml) similarly accelerated growth but was not essential. Vitamin B_6 was not required for growth, and its biosynthesis by the spirochete was demonstrated by direct microbiological assay of the organism.

In general, phosphorylated forms of metabolites are utilized poorly, because of cell wall impermeability (Snell, 1951). In this instance,

however, coenzyme I was fully as active as nicotinic acid, and riboflavin-5′-phosphate, as active as riboflavin. Coenzyme A was also equivalent to the dephosphorylated unit, pantethine (but less effective than its basic unit, pantothenic acid). On the other hand, cocarboxylase was significantly less active than the corresponding dephosphorylated form, thiamin.

Desthiobiotin is a potent antagonist towards the utilization of biotin by *Lactobacillus casei* (Lilly and Leonian, 1944). With the Reiter treponeme, however, as with *Saccharomyces cerevisiae* (Dittmer *et al.*, 1944), desthiobiotin functioned as a precursor of biotin rather than as an antagonist. Similarly, galactoflavin, which decreases the rate of growth of rats on a riboflavin-free diet (Emerson *et al.*, 1945), served as a substitute for riboflavin rather than as an antimetabolite. Also, as described above, neopyrithiamin, while highly toxic to a number of thiamin requiring organisms, was inert towards the Reiter spirochete. On the other hand, and in accord with the usual observation that antimetabolites of vitamins function best against organisms requiring external sources of vitamin (Woolley, 1952), pyridine-3-sulfonic acid and, to a much less degree, pantoyltaurine possessed antimetabolite activity towards nicotinic acid and pantothenic acid, respectively, while desoxypyridoxine was relatively ineffective against the Reiter organism which does not require B_6.

p-Aminobenzoic acid was not required for growth and presumably was synthesized by the organism. Sulfanilamide inhibited growth, and that inhibition was reversed by *p*-aminobenzoic acid competitively as described for many organisms. Folic acid and several other *p*-aminobenzoic acid conjugates reversed sulfanilamide toxicity competitively and at molar concentrations equivalent to that of *p*-aminobenzoic acid activity. This is in contrast to the noncompetitive reversal of sulfanilamide toxicity by folic acid observed by Lampen and Jones (1946) which led those workers to conclude that sulfanilamide functions by impeding the conversion of *p*-aminobenzoic acid to folic acid. The substitution of an amino group for the 4-hydroxy radical of the folic acid molecule to yield aminopterin destroyed the ability to reverse sulfanilamide toxicity and rendered the new molecule highly toxic in its own right. The metabolic function of *p*-aminobenzoic acid and its derivatives has been considered to involve the transfer of 1-C groups in the biosynthesis of certain amino acids (e.g., methionine) and the several purines and pyrimidines (Shive, 1953). Since these are all present in the basal medium, additional biochemical functions for *p*-aminobenzoic acid would be implied.

SUMMARY

The vitamin requirements of the Reiter treponeme have been determined. Biotin, nicotinic acid, and pantothenic acid were essential for growth, and the organisms became nonviable on the omission of any one of these from the medium. Thiamin increased both the rate of growth and its amount; while choline and riboflavin were merely acceleratory. *p*-Aminobenzoic acid and the pyridoxine group of vitamins were inert.

The concentration levels for maximal growth ranged from 1 mμg per ml to 100 mμg per ml for the essential vitamins. The highly beneficial vitamin, thiamin, was active in this range. The other growth acceleratory vitamins, riboflavin and choline, were required at much higher concentrations (1,000 to 10,000 mμg per ml).

The organism can utilize certain closely related forms of its vitamin requirements, but otherwise its biosynthetic abilities are quite limited.

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