NUTRITIONAL REQUIREMENTS OF TREPONEMATA

II. Pantothenic Acid, Glutamine, and Phenylalanine as Additional Growth-promoting Factors for the Reiter Treponeme[1]

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It has been shown (Eagle and Steinman, 1948) that the nonpathogenic Reiter strain of *Treponema pallidum* can be cultivated on a medium consisting of arginine, acetic acid, any one of a number of substances potentially capable of supplying sulfhydryl groups, crystallized serum albumin, and a small amount of Brewer's thioglycolate medium[3] (Brewer, 1940), with ascorbic acid and phosphate buffer as poising and buffering agents, respectively.

Three components of the thioglycolate mixture were considered capable of supplying nutritional factors: (1) trypticase, an enzymatic digest of casein, (2) yeast extract, and (3) glucose. Little and SubbaRow (1945) and Whiteley and Frazier (1948) had found a combination of vitamins (ascorbic acid, choline, niacin, pantothenic acid, pyridoxine, riboflavin, and thiamine) as well as glucose to be essential for the Reiter treponeme when used in conjunction with an acid digest of casein. These findings suggested that the thioglycolate medium might be active by virtue of its content of yeast extract and glucose. However, on examination of the components of the thioglycolate medium it was found that the enzymatic casein digest, when used in conjunction with the other factors listed above, completely replaced the thioglycolate medium and that added vitamins and glucose could then be dispensed with.

Three of the growth factors supplied by the enzymatic casein digest are identified in the present communication: pantothenic acid, glutamine, and phenylalanine. As discussed in the text, these three compounds, or substances with similar effects on the growth of the Reiter treponeme, are present in enzymatic digests of crude casein and other proteins. These three factors, with arginine, sodium acetate, serum albumin, a -SH-containing compound, and a small amount of an enzymatic casein digest, constitute a medium in which the Reiter organism can multiply and be carried in serial transfer. There remain to be identified the additional factors present in the minute amounts of the protein digest still necessary.

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[2] With the technical assistance of Elizabeth A. Spence, Vance I. Oyama, and Henry O. Schulze.

[3] Trypticase (15 g/L), L-cystine (0.75 g/L), glucose (5 g/L), yeast extract (5 g/L), sodium chloride (2.5 g/L), sodium thioglycolate (0.5 g/L), resazurin (1 mg/L), and agar (0.75 g/L).

METHODS AND MATERIALS

The methods used in this study were essentially the same as those described in a previous communication (Eagle and Steinman, 1948). In an attempt to define the substances supplied by the enzymatic casein digest, the latter was reduced to a level that just failed to support growth. Any added substance that now permitted growth, and thus significantly decreased the limiting concentration of the casein hydrolyzate, was considered to be a partial replacement for that hydrolyzate.

The various components of Brewer's thioglycolate medium were obtained by courtesy of the Baltimore Biological Laboratory, Inc., as were several of the protein enzymatic digests. Amigen, a commercial enzymatic digest of casein in sterile solution (Mead Johnson and Company), was used in much of the work because of its ready availability in standardized form. The terms "serum albumin" and "plasma albumin" are used interchangeably for crystallized bovine plasma albumin (Armour and Company).

Washed inocula were used in most of the experiments, as indicated in the tables. In later experiments, when the thioglycolate medium was replaced by small amounts of the casein digests, the inocula were sometimes not washed since there was then a negligible carry-over of trace factors. In such cases repeated subcultures were made to ensure the reliability of the results and freedom from trace factors in the inoculum. All solutions were made up at a neutral pH in isotonic concentration or were adjusted to isotonicity by the addition of concentrated sodium chloride.

EXPERIMENTAL RESULTS

Trypticase as the Major Essential Constituent of Thioglycolate Medium

In a medium consisting of (a) arginine, (b) acetic acid, (c) either cysteine or glutathione, (d) whole serum, dialyzed serum, or crystalline serum albumin, and supplemented with (e) Brewer's thioglycolate medium, the thioglycolate medium was effective as a growth-promoting nutrient in final concentrations of 1:6,600 (cf. curve III in figure 1). Growth was arbitrarily defined as satisfactory when an inoculum of 1 to 2 million organisms per ml grew out to at least 30 million organisms per ml after 6 days' incubation at 37 C, an increase of 15- to 30-fold. When the several components of the thioglycolate fluid potentially capable of supplying growth factors were examined individually, the trypticase proved to be a complete replacement for the complex thioglycolate medium (cf. the first portion of table 1). There was good growth in all the trypticase-containing cultures but poor growth in the media to which only yeast extract or glucose had been added.[4] Trypticase was not unique in its capacity to replace the thioglycolate mixture, since enzymatic digests of other proteins, as well as other

[4] This does not mean that yeast extract contains no growth factors for the Reiter treponeme. Actually it supplies at least one of the vitamin factors here shown to be essential for growth (pantothenic acid) and supports growth if glutamine and phenylalanine are also provided (Steinman and Eagle, unpublished).

enzymatic casein hydrolyzates, were also effective (cf. the second portion of table 1).

An Analysis of the Growth Factors Supplied by Trypticase

Pathothenic acid. As indicated in table 2 and illustrated by the curves in figure 1, the minimal concentrations of trypticase and of amigen necessary for growth in the basal medium there used were of the order of 1:1,300. However, when the trypticase was supplied in the form of whole thioglycolate medium, the minimal effective concentration was reduced by a factor of 10 to approximately

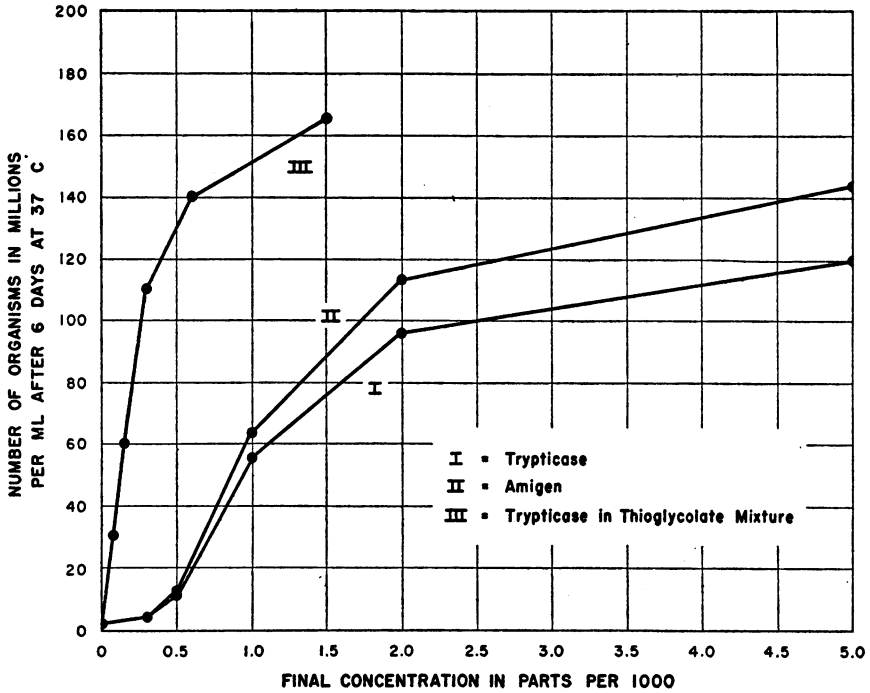

Figure 1. The growth-promoting effect of trypticase, amigen, and thioglycolate mixture (expressed as trypticase) on the Reiter treponeme. Varying amounts of 5 per cent trypticase solution, 5 per cent amigen solution, and 2.95 per cent Brewer's thioglycolate fluid (trypticase content, 1.5 per cent) were added to the basal serum albumin medium described in the first footnote of table 1.

1:13,000. The thioglycolate medium therefore supplied one or more factors that either partially replaced or potentiated the action of trypticase. The yeast extract was shown to be the active factor. Although not alone capable of supporting growth under the conditions of the experiment of table 1, addition of the yeast extract to trypticase resulted in heavier growth; and, as shown in table 2, when the concentration of the protein digest was suitably reduced, the yeast extract became essential.

As shown in table 2 the yeast extract could be replaced by a mixture of the following vitamins: *p*-aminobenzoic acid, biotin, choline, folic acid, inositol, niacin, pantothenic acid, pyridoxine, riboflavin, and thiamine.

TABLE 1

The growth-promoting effect of Brewer's thioglycolate medium, of its individual components, and of several enzymatic protein digests on the Reiter treponeme

	SUPPLEMENTS ADDED TO BASAL MEDIUM*	NUMBER OF ORGANISMS IN MILLIONS PER ML AFTER 6 DAYS' INCUBATION	CONCLUSIONS
I. Whole Brewer's thioglycolate medium and its individual components	Thioglycolate medium (2.95%)	156	
	Glucose (M/7)	3	Glucose and yeast extract are inactive even when added in a higher concentration than in the whole thioglycolate medium
	Yeast extract (2%)	17	
	Trypticase (6%)	117	Trypticase can replace the thioglycolate medium
	Trypticase (6%) + glucose (M/7)	98	
	Trypticase (6%) + yeast extract (2%)	167	Yeast extract accentuates the growth-promoting activity of trypticase
II. Protein hydrolyzates	Amigen (5%)†	119	A number of protein hydrolyzates are equivalent to trypticase as replacements for the thioglycolate mixture
	Protolysate (5%)†	110	
	Gelasate (5%)‡	89	
	Lactalbumin hydrolyzate (5%)§	84	
	Phytone (5%)‖	82	
	Serum albumin hydrolyzate (5%)¶	78	
	Control (no supplement)	1-3	

The various components of Brewer's thioglycolate medium, as well as the whole thioglycolate medium, were added in a volume of 0.1 ml. The relative concentrations of the individual components were 4 times that of the same components in the whole thioglycolate mixture. The various protein hydrolyzates were added in a volume of 0.5 ml. The inocula were organisms sedimented from an actively growing thioglycolate serum culture, washed with the appropriate basal medium, and then taken up in the same medium to an initial count of 2 million organisms per ml in a total volume of 5.0 ml. The number present after 6 days of incubation at 37 C was determined by direct microscopic enumeration.

* The basal medium consisted of M/10.5 phosphate buffer (pH 7.4), 0.5 ml; M/7 ascorbic acid, 0.25 ml; M/7 glutathione, 0.25 ml; M/10 L-arginine hydrochloride, 1.5 ml; M/7 sodium acetate, 1.5 ml. This was supplemented in series I with 0.5 ml of whole rabbit serum previously heated at 60 to 63 C for 1 to 2 hours, and in series II with 0.5 ml of 5 per cent crystallized bovine albumin.

† Enzymatic digest of casein (Mead Johnson and Company).

‡ Enzymatic digest of gelatin (Baltimore Biological Laboratory, Inc.).

§ Enzymatic digest of lactalbumin (General Biochemicals, Inc.).

‖ Papaic digest of soybean (Baltimore Biological Laboratory, Inc.).

¶ Pancreatic digest of crystallized bovine plasma albumin (laboratory preparation).

TABLE 2

The effects of yeast extract and of a vitamin mixture in the cultivation of the Reiter treponeme

ADJUNCTS TO BASAL MEDIUM*	FINAL NUMBER OF ORGANISMS (MILLIONS PER ML) AFTER 6 DAYS AT 37 C			CONCLUSIONS
	No supplement	Yeast extract (1%) added, 0.1 ml	Vitamin mixture† added, 0.3 ml	
I. Enzymatic digests of commercial casein				
Amigen (5%)				
0.02 ml	3	6	5	Yeast extract and the vitamin mixture are both beneficial at threshold concentrations of amigen
0.05 ml	14	46	44	
0.10 ml	58	73	67	
Pancreatic	50		53	Enzymic hydrolyzates of commercial casein are fully active without added vitamins
Papaic	54		50	
Tryptic	58		64	
Peptic	40		44	
II. Acid digests				
Casein (commercial)	4		46	Acid digests of proteins are inactive unless supplemented by vitamins
Serum albumin (crystallized)	1		31	
III. Enzymatic digests of vitamin-free casein				
Pancreatic	2		33	Enzymic hydrolyzates of vitamin-free casein are ineffective without vitamin supplementation
Papaic	2		32	
Tryptic	3		38	
Peptic	1		17	

Yeast extract and the vitamin mixture were added as indicated to the basal medium supplemented with the adjuncts listed in the first column of the table. The various protein hydrolyzates were added in a volume of 0.5 ml. The enzymatic digests in series I were prepared from a 5 per cent suspension of commercial casein (General Biochemicals, Inc.) treated for 24 hours at 37 C with crude pancreatin, papain activated with cysteine, and trypsin at pH 8 and with pepsin at pH 1.5. The digests were neutralized and then heated at 85 C to precipitate any unhydrolyzed protein. The acid digests of casein and of serum albumin were prepared by refluxing 5 grams of substance in 50 ml of 1 N H_2SO_4 for 24 hours. The hydrolyzates were neutralized and diluted to yield 5 per cent solutions. Since the slight hypertonicity imparted to the final medium by the neutralized solutions had no toxic effects, the solutions were not further treated. The hydrolyzates used in series III were prepared in the same manner as those used in series I from a specially purified vitamin-free casein (General Biochemicals, Inc.). The initial washed inoculum gave a count of 2 million organisms per ml in a total volume of 5 ml.

* M/10.5 phosphate buffer (pH 7.4), 0.5 ml; M/7 ascorbic acid, 0.25 ml; M/7 glutathione, 0.25 ml; M/10 arginine hydrochloride, 1.0 ml; M/7 sodium acetate, 1.0 ml; 5 per cent crystallized serum albumin, 0.5 ml.

† Pooled mixture of 1 ml each of *p*-aminobenzoic acid (1 per cent), biotin (0.001 per cent), choline chloride (1 per cent), folic acid (0.001 per cent), inositol (1 per cent), niacin (1 per cent), calcium pantothenate (1 per cent), pyridoxine (1 per cent), riboflavin (0.01 per cent), and thiamine chloride (1 per cent).

These data indicated (1) that B-complex vitamins were essential for the growth of the Reiter strain, (2) that enzymatic protein hydrolyzates in sufficient concentrations supplied those vitamins, and (3) that the minimum amount of digest necessary for growth could be reduced by the addition of these vitamins. This was further indicated by the fact that protein hydrolyzates produced by

TABLE 3

Pantothenic acid as the active component of a mixture of B vitamins

VITAMIN ADDED	NUMBER OF ORGANISMS (MILLIONS PER ML) AFTER 6 DAYS' INCUBATION	CONCLUSIONS
Control	2	Inactive
p-Aminobenzoic acid	4	
Biotin	4	
Choline chloride	1	
Folic acid	6	
Inositol	1	
Niacin	17	Slightly active
Pyridoxine	12	
Riboflavin	15	
Thiamine chloride	12	
Calcium pantothenate	42	Active
Pantothenate + niacin	42	No potentiation of effect of pantothenic acid by other vitamins
Pantothenate + pyridoxine	44	
Pantothenate + riboflavin	39	
Pantothenate + thiamine	36	
Pantothenate + riboflavin + niacin	41	
Pantothenate + riboflavin + pyridoxine	44	
Pantothenate + riboflavin + thiamine	45	
Pantothenate + riboflavin + niacin + pyridoxine + thiamine	44	
Pool of all 10 B vitamins	52	

To the basal medium described in the first footnote of table 2, supplemented with 0.5 ml of 5 per cent casamino acids (Difco) and 0.1 ml of 1 per cent amigen, solutions of the individual vitamins were added in 0.2-ml volumes. The concentrations of the stock solutions were as follows: biotin and folic acid, 0.001 per cent; riboflavin, 0.01 per cent; *p*-aminobenzoic acid, choline chloride, inositol, niacin, calcium pantothenate, and thiamine chloride, 1 per cent. The pooled mixture was added in 0.5-ml volume. The initial count was 2 million organisms per ml.

acid digestion could not support growth without vitamin supplementation (cf. table 2). Finally, as demonstrated in the last portion of table 2, when enzymatic hydrolyzates were prepared from vitamin-free casein, such digests were also completely inactive unless vitamins were added. One could conclude from these several lines of evidence that, as ordinarily prepared, enzymic hydrolyzates

contain traces of vitamin factors, either present as impurities in the original protein or introduced with the enzyme preparations used for hydrolysis.

As the final step in this series of experiments it was found that pantothenic acid alone could replace the entire pool of B vitamins at threshold concentrations of amigen (cf. table 3). Other vitamins, particularly niacin, pyridoxine, riboflavin, and thiamine, were occasionally able to replace the pooled mixture, but the results were not uniformly reproducible. Furthermore, the vitamins with irregular activity did not, individually or pooled, further enhance the favorable effect of pantothenic acid (cf. second portion of table 3). The inconsistent results obtained with some of these individual vitamins are as yet unexplained.

TABLE 4

The beneficial effect of glutamine on the growth of the Reiter treponeme in the presence of pantothenic acid

ADJUNCT TO BASAL MEDIUM	NUMBER OF ORGANISMS (MILLIONS PER ML) AFTER 6 DAYS' INCUBATION								CONCLUSIONS
	I. No pantothenic acid added				II. Pantothenic acid added				
ml of 1% amigen	ml of M/7 glutamine added				ml of M/7 glutamine added				
	0	0.1	0.2	0.5	0	0.1	0.2	0.5	
0.1	5	6	11	13	8	9	22	34	At threshold concentrations of amigen, glutamine and pantothenic acid promote the growth of the Reiter treponeme
0.2	14	23	30	37	26	47	56	64	
0.5	56	68	76	76	67	79	84	89	
									With excess amigen, neither one is necessary, both factors presumably being supplied by the amigen

Glutamine (M/7) was added as indicated to the basal medium described in the first footnote of table 2, to which had been added varying amounts of amigen. No pantothenic acid was added in series I, and 0.1 ml of 0.1 per cent calcium pantothenate was added in series II. The initial count was 0.5 million organisms per ml.

Glutamine. Supplementation of the basal medium containing arginine, acetic acid, glutathione, and crystallized serum albumin with pantothenic acid, as outlined above, permitted a reduction in the minimum concentration of amigen necessary for growth to 1:2,000. At this point it seemed possible that the factor supplied by the amigen at this limiting concentration might be one or more amino acids or related compounds necessary only in trace amounts. Investigation of a number of such compounds showed that glutamine was capable of substituting in part for amigen. A typical experiment is summarized in table 4. The essential nature of glutamine is demonstrable only at threshold concentrations of amigen, and was particularly striking in the presence of pantothenic acid

(cf. table 4). Conversely, the beneficial action of pantothenic acid was demonstrable only if the vitamin-deficient culture medium contained glutamine, or an equivalent factor as supplied by protein hydrolyzates.

Glutamic acid was a partial substitute for glutamine but would not support growth in serial subculture. Presumably acid digests of protein supply glutamic acid rather than glutamine, and this may contribute to their inactivity as shown in table 2. As illustrated in figure 2, with optimal concentrations of the glutamine and pantothenic acid (1:500 and 1:50,000, respectively), the minimum con-

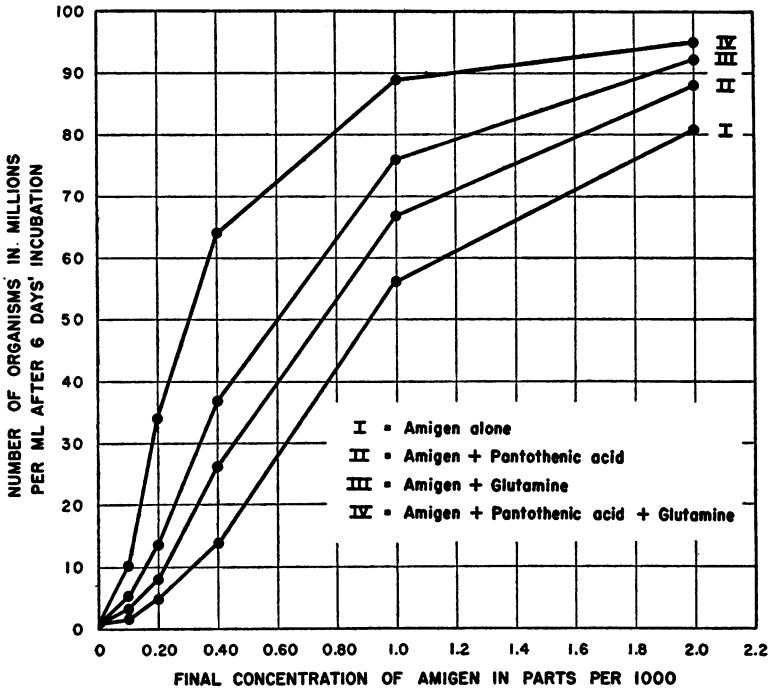

Figure 2. The growth-promoting effect on the Reiter treponeme of pantothenic acid and of glutamine. Under the experimental conditions used in the experiment summarized in table 4 varying amounts of amigen were added to the basal medium described in the first footnote of table 2. The data used to plot curve I were obtained from the control culture fluid, which contained no added factors. Curves II and III resulted from added pantothenic acid (0.1 ml of 0.1 per cent calcium pantothenate) and added glutamine (0.5 ml of M/7 glutamine), respectively, and curve IV was produced by the medium containing both factors together.

centration of amigen necessary for growth in the basal medium described above was reduced to 1:5,000.

Phenylalanine. A number of amino acids were re-examined at the latter threshold concentration of amigen to determine whether one might be the limiting factor. Phenylalanine was the only amino acid with a significant effect (cf. table 5). Under the conditions of that experiment, added phenylalanine, although not essential, produced heavier growth and reduced by one-half (to about 1:10,000) the minimal level of amigen required for satisfactory growth.

The effective concentrations of the phenylalanine were of the order of 1:5,000. Higher concentrations were toxic, D-phenylalanine being more toxic than the L-isomer, with the DL-mixture intermediate. This is consistent with the usual antagonistic effect on bacteria of the unnatural forms of biologically active amino acids.

TABLE 5

The growth-promoting effect of phenylalanine on the Reiter treponeme

ADJUNCT TO BASAL MEDIUM	NUMBERS OF ORGANISMS IN MILLIONS PER ML AFTER 6 DAYS' INCUBATION AT 37 C										CONCLUSIONS
ml of 1% amigen	Control (nothing added)	ml of M/10 L-phenylalanine added					ml of M/10 D-phenylalanine added				
		0.05	0.1	0.2	0.5	1.0	0.05	0.1	0.2	0.5	1.0	
0.02	3	8	14	16	15	8	9	9	7	5	3	Both optical isomers of phenylalanine, although toxic at high concentrations, are beneficial at low concentrations. The L-isomer was somewhat more effective.
0.05	12	32	35	29	18	15	15	20	9	6	3	
0.10	27	43	47	44	38	31	33	31	26	18	8	

Under the experimental conditions used previously, phenylalanine (M/10) was added as indicated to the medium described in the first footnote of table 2, supplemented with 0.1 ml of 0.1 per cent calcium pantothenate and 0.2 ml of M/7 glutamine. One sample of the L-isomer of phenylalanine was obtained from Mann Fine Chemicals, New York. The D-form and another sample of the L-form were obtained by courtesy of Dr. Jesse P. Greenstein. The preparation and properties of these samples are described by Gilbert, Price, and Greenstein (1949).

DISCUSSION

Growth-promoting factors for the Reiter treponeme. The previous identification of arginine, acetic acid, sulfhydryl compounds, and serum albumin as growth-promoting factors for the Reiter treponeme was carried out in the presence of Brewer's thioglycolate mixture (Eagle and Steinman, 1948). In the present paper it has been shown that an enzymatic protein digest is a complete replacement for the thioglycolate medium and that its growth-promoting activity is due largely, but not entirely, to pantothenic acid, glutamine, and phenylalanine (or compounds with similar metabolic effects). When these three were added in optimal amounts, the minimal concentration of casein digest necessary for growth was reduced to 1:10,000.

It was conceivable that the addition of the three new factors to the basal medium, which permitted a reduction in the amount of casein hydrolyzate necessary for growth, might also have altered the growth requirements of the organism with respect to the factors previously determined. On re-examination, however, it was found that the presence of pantothenic acid, glutamine, and phenylalanine, whether supplied as such or in an enzymatic casein digest, did not materially change the need for arginine, acetic acid, a sulfhydryl-supplying substance, or serum albumin.

Acetic acid had previously been shown to replace the ultrafiltrate components of whole serum, and a number of other compounds, including glucose, were found to have a qualitatively similar effect (cf. Eagle and Steinman, 1948). In the refined medium described here, in which the thioglycolate medium has been replaced by chemically defined compounds and a trace of protein digest, it was found that glucose and acetic acid were mutually replaceable in their effects on the growth of the Reiter treponeme.[5] This agrees with the beneficial effect of glucose as reported by Little and SubbaRow (1945) and Whiteley and Frazier (1948). However, unlike acetic acid, glucose at concentrations greater

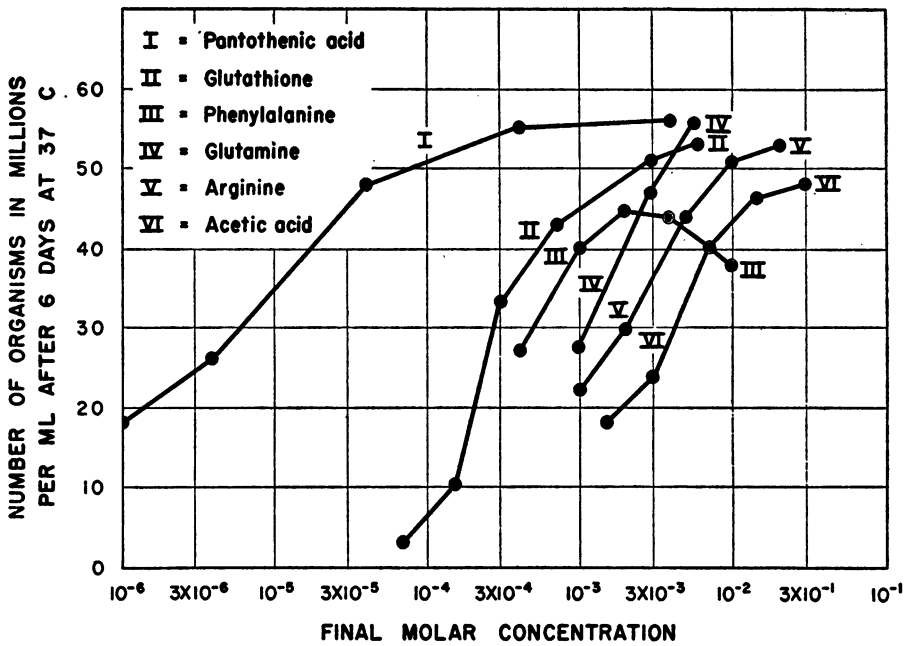

Figure 3. The growth-promoting effect on the Reiter treponeme of pantothenic acid, glutathione, phenylalanine, glutamine, arginine, and acetic acid as a function of concentration. A basal medium was prepared containing 0.1 per cent calcium pantothenate (0.1 ml); M/7 glutathione (0.25 ml); M/10 L-phenylalanine (0.1 ml); M/7 glutamine (0.2 ml); M/10 L-arginine (1.5 ml); M/7 sodium acetate (1.5 ml); supplemented with 0.5 ml of 5 per cent crystallized bovine albumin, 0.5 ml of M/10.5 phosphate buffer (pH 7.4), and 0.25 ml of M/7 ascorbic acid, in a total volume of 5.0 ml. Each of the first 6 factors was then separately varied in order to determine the effect of its concentration on the amount of growth.

than 1:1,000 caused an initial rapid growth followed by a sharp decrease in the total number of organisms. This may be related to acid formation.

Some indication of the metabolic functions of the various growth factors is afforded by a consideration of their effective concentrations. As shown in figure 3, relatively high concentrations of arginine and of acetic acid (0.005 M and 0.01 M, respectively) were necessary for good growth. In the case of glu-

[5] The quantitative aspects of this finding must await the resolution of the factors remaining in the casein digest.

tamine and of phenylalanine slightly lower concentrations (0.002 M and 0.001 M, respectively) sufficed. Glutamine has been shown to be a growth factor for a wide variety of organisms (cf. Archibald, 1945, 1947). Glutathione was effective at fairly low concentrations (0.0003 M), and pantothenic acid was similarly effective at very low concentrations (0.000001 M), consistent with a vitamin activity for this universally essential substance (cf. Novelli and Lipmann, 1947). Somewhat smaller concentrations of these factors had a demonstrable if suboptimal effect.

The role of the crystallized bovine albumin is not entirely clear. Its activity apparently depends on the intact protein. Serum albumin completely hydrolyzed, either enzymatically or by acid, was inactive. Although partially hydrolyzed bovine albumin could replace whole crystallized plasma albumin, the activity was associated with the nondialyzable fraction. The fairly high level of crystallized serum albumin (approximately 0.1 per cent) required for minimal growth (cf. Eagle and Steinman, 1948) is consistent with either a detoxifying action (Davis and Dubos, 1947) or the fact that it supplies one or more compounds not present in a casein digest.

Commercial enzymatic digests of casein such as trypticase and amigen, although sometimes stated to be vitamin-free, are known to contain traces of vitamins. Since pantothenic acid as such is effective in concentrations as low as 1:10,000,000, it is evident that a 1:1,300 concentration of the protein digest would contain an effective concentration of pantothenic acid if the latter constituted 0.001 per cent of the digest. On direct analysis by microbiological assay, using *Lactobacillus casei* in a chemically defined basal medium (Rickes, Koch, and Wood, 1949), the pantothenic acid content of amigen was actually found to be 15.6 μg per gram, or 0.00156 per cent.

Similarly, the glutamine content of casein digest need be only 1 per cent to account for the glutaminelike activity of amigen at a concentration of 1:1,300. By determination of the ammonia liberated by acid hydrolysis at 100 C for 5 minutes (cf. Vickery *et al.*, 1935) the glutamine content of amigen was estimated to be of the order of 1.5 per cent.

Finally, since amigen contains approximately 4.41 per cent of phenylalanine (Cox, 1950), a 1:2,000 dilution of this casein digest could potentially supply a 1:50,000 concentration of phenylalanine. Again, this value is compatible with the threshold requirements of the Reiter treponeme. Only at subliminal concentrations of the casein hydrolyzate, determined by the specific requirements for each factor, does supplementation of pantothenic acid, glutamine, and phenylalanine become necessary.

Total growth response. In the original complex culture medium consisting of whole serum and thioglycolate fluid medium the Reiter strain gave an average growth of 150 to 300 million organisms per ml for 6 days at 37 C. As these complex ingredients were progressively fractionated and eliminated by the substitution of identified growth factors, the average growth decreased significantly. This suggests that, when the simple growth factors were substituted for the complex constituents of the medium, some factors were lost that were not essential for growth but that promoted the multiplication of the organism.

SUMMARY

The Reiter treponeme had previously been shown to grow in a medium containing arginine, acetic acid, a sulfhydryl-containing compound, crystallized serum albumin, and small amounts of Brewer's thioglycolate medium. The essential component in the latter is here shown to be trypticase, an enzymatic digest of casein. Other enzymatic digests of casein as well as similar digests of gelatin, lactalbumin, soybean, and plasma albumin have been found to be equally effective; acid digests are inactive.

Pantothenic acid, glutamine, and L-phenylalanine have here been shown to be growth factors for the Reiter treponeme. They, or compounds with similar metabolic activities, are supplied by the enzymatic casein digests. Their effective concentrations are 10^{-6} M, 2×10^{-3} M, and 1×10^{-3} M, respectively. The Reiter strain of *Treponema pallidum* can be grownin a medium that, except for necessary supplementation with a 0.01 per cent casein hydrolyzate, is chemically defined. The nature of the substances supplied by this low concentration of casein digest are under present study.

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