THE NUTRITIONAL REQUIREMENTS OF TREPONEMATA

I. Arginine, Acetic Acid, Sulfur-Containing compounds, and Serum Albumin as Essential Growth-promoting Factors for the Reiter Treponeme

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There have been many unsuccessful attempts to cultivate the causative organism of syphilis on both natural and synthetic media since its demonstration by Schaudinn in 1905 (cf. Kast and Kolmer, 1929; Eagle, 1948). Supniewski and Hano (1933) and Scheff (1935) were among the first to approach the problem by studying the metabolism and growth requirements of such treponemata as could be cultivated on artificial media. Their findings, as well as those of Kast and Kolmer (1940), Rosebury and Foley (1942), and Wichelhausen and Wichelhausen (1942), indicated that one or more factors present in body fluids and tissues (e.g., blood serum, ascitic fluid, kidney tissue, liver tissue) were essential for the growth of these organisms. More recently, Little and SubbaRow (1945) and Whiteley and Frazier (1948) reported that the Reiter treponeme, a nonpathogenic organism purporting to have been cultivated from a syphilitic lesion (Reiter, 1926) but serologically related to some of the saprophytic treponemes isolated from the mouth (Robinson and Wichelhausen, 1946; Eagle and Germuth, 1948), could be cultivated in a simplified medium consisting of an acid hydrolyzate of casein (or a mixture of the component amino acids of that protein), supplemented with glucose, several vitamins (ascorbic acid, choline chloride, niacin, calcium pantothenate, pyridoxine, riboflavin, and thiamine), a sulfur-containing reducing substance (sodium formaldehydesulfoxylate or sodium thioglycolate), and serum albumin.

In the present study, also, the nutritional requirements of the cultivable Reiter strain were investigated in the hope that such data might offer clues to the growth requirements of the more fastidious pathogenic *Treponema pallidum*. To date, four substances have been identified as being essential for growth under the conditions of the present experiments: (1) arginine, (2) acetic acid, (3) any one of a series of compounds containing either free sulfhydryl groups or sulfur groups capable of being reduced to the sulfhydryl form and, (4), in confirmation of the data of Little and SubbaRow (1945) and of Whiteley and Frazier (1948), crystalline serum albumin. The latter has been shown to be a substitute for the nondialyzable fraction of whole serum. The phosphate buffer, which was added for its poising action, and ascorbic acid, which was added to maintain anaerobiosis, were apparently not essential nutrients. There remain to be identified

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the growth factors present in the minute amounts of yeast extract and casein digest which must be added to the culture fluid for satisfactory growth. The importance of glucose also remains to be determined.

METHODS AND MATERIALS

The components of the culture fluid which was the starting point of the present investigation were (1) Brewer's thioglycolate medium (Brewer, 1940), with the following composition:

Trypticase (an enzymatic digest of casein)	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
Agar	0.75 g/L

and (2) rabbit or human serum, sterilized by filtration through a glass filter (Corning UF), and heated at 60 to 63 C for 1 to 2 hours. Both were essential, a 9:1 mixture giving luxuriant growth. For the purposes of this study, however, these two components were used in subliminal concentrations which alone did not support the growth of the organisms, presumably because one or more essential ingredients were at less than the threshold concentrations necessary for growth. A variety of substances were then assayed for their ability to permit multiplication of the organisms when added to the otherwise quantitatively inadequate basal medium. These compounds were made up at neutral pH in isotonic concentration, or in solutions adjusted to isotonicity by the addition of sodium chloride. In individual experiments in which they might have complicated the results, cystine, resazurin, and agar were omitted from the basal thioglycolate medium. Similarly, as is indicated in the text and in the tables, washed inocula were used when the presence of the original culture fluid would have complicated the interpretation of the experimental results.

The amino acids, the fatty acids, the phosphate buffer, and the thioglycolate medium were sterilized by autoclaving; ascorbic acid and sulfhydryl compounds were glass-filtered in acid solution and adjusted to pH 6.8 to 7.2 immediately prior to use. The volumes were adjusted with M/7 sodium chloride to a total of 5 ml in 18-by-150-mm pyrex test tubes, and the tubes incubated at 37 C in Brewer anaerobic jars under an atmosphere of hydrogen. Direct microscopic counts (cf. Magnuson, Eagle, and Fleischman, 1948) were made after 4 to 6 days, at the peak of the growth curve.

EXPERIMENTAL RESULTS

Arginine as a growth-promoting factor. A typical experiment that demonstrates the effectiveness of arginine as a growth-promoting factor is summarized in table 1. As is there indicated, 0.2 ml of an actively growing culture on thioglycolate medium enriched with 10 per cent serum were used as the inoculum, this inoculum constituting the thioglycolate portion of the basal medium. This

was supplemented by 0.5 ml of heated rabbit serum, 0.5 ml of M/10.5 phosphate buffer (pH 7.4), and cysteine hydrochloride to a final concentration of 1:1,000, all in a total volume of 5 ml. In the absence of arginine the original inoculum of 2 million organisms per ml had disappeared in the course of 6 days. The addition of arginine had a striking effect. The smallest concentration with a significant

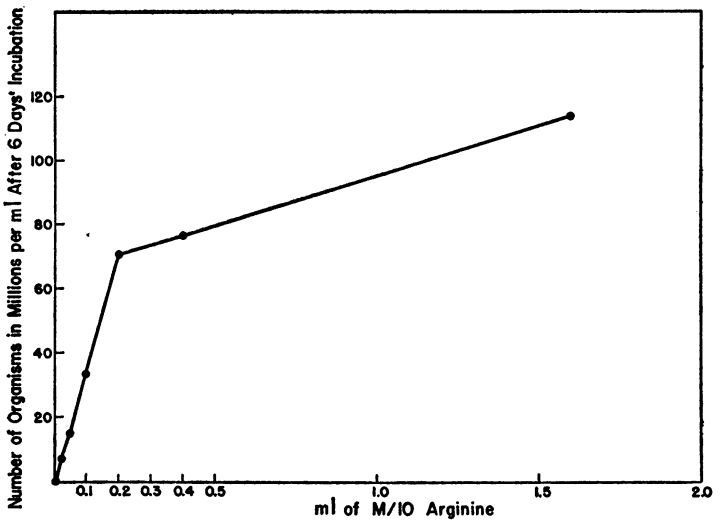

Figure 1. The growth-promoting effect of arginine on the Reiter treponeme as a function of its concentration.

TABLE 1

The growth-promoting effect of arginine on the Reiter treponeme as a function of its concentration

Ml of M/10 arginine in total of 5 ml.....	0	0.025	0.05	0.1	0.2	0.4	1.6
Number of organisms in millions per ml after 6 days' incubation.....	0	7	15	34	71	77	114

Varying amounts of M/10 arginine were added to a basal medium consisting of 0.5 ml heated rabbit serum, 0.5 ml of M/10.5 phosphate buffer (pH 7.4), and 0.33 ml of M/10.5 cysteine hydrochloride, in a total volume of 5 ml (including the inoculum of 0.2 ml of an actively growing culture in the serum: thioglycolate medium). This inoculum served to provide those ingredients essential for growth present in the thioglycolate medium. The initial number of organisms was 2 million per ml; the number present after 6 days' incubation at 37 C was determined by direct count.

effect on growth was 0.0005 M; but beyond that level the number of organisms increased in proportion to the concentration of arginine, 6-day cultures reaching a count of 114 million per ml at an arginine concentration of 0.03 M (0.5 per cent). It is apparent that the arginine did not act as a trace factor; and its activity was quantitatively unaffected by recrystallization.

The dependence of the degree of growth on the amount of arginine used is shown graphically in figure 1, which presents the data of table 1. A number of similar experiments with qualitatively similar results are summarized in table 2. The fact that in some of these experiments there was significant multiplication

TABLE 2

The growth-promoting effect of arginine on the Reiter treponeme

DATE	BASAL MEDIA									INITIAL NUMBER ORGANISMS (MILLIONS PER ML)	FINAL NUMBER OF ORGANISMS (MILLIONS PER ML) AFTER 6 DAYS' INCUBATION						
											ml of M/10 arginine						
	Heated rabbit serum, ml	Heated rabbit serum, dialyzed, ml	Heated human serum, ml	Thioglycolate medium, ml	Sodium acetate (M/7), ml	Phosphate buffer (M/10.5), ml	Cysteine hydrochloride (M/10.5), ml	Glutathione (M/7), ml	Ascorbic acid (M/7), ml		0	0.1	0.2	0.5	1.0	1.5	2.0
2-28-44		0.5		0.3			0.33			4.3	0						116
3-11-44	1.0			0.3			0.33			5.4	35						110
		1.0		0.3			0.33			5.4	1						47
3-20-44	0.6			0.3		0.5	0.33			5.0	14			75			
3-30-44	0.4			0.1		0.5	0.33			2.0	2		63		84		58
4-1 -44	0.4			0.1		0.5	0.33			3.4	3						78
4-29-44	1.0			0.1		0.5	0.33			2.5	2			91			
6-22-44	1.0			0.1	1.0	0.5	0.33			1.6	4	58					
	0.5			0.1	1.0	0.5	0.33			1.6	79			390			
		0.5		0.1	1.0	0.5	0.33			1.6	9			119			
11-24-45	0.1			0.05	1.0	0.5	0.33			3.0	14		93	107			
2-19-47	0.5			0.05	1.5	0.5		0.12	0.25	1.0	6					72	
6-24-47			0.5	0.1	1.5	0.5		0.12	0.25	1.4	3					132	

Arginine (M/10) was added as indicated on the right side of the table to the various basal media tabulated on the left side of the table. The experiments were similar to that recorded in table 1.

TABLE 3

Growth-promoting effect of arginine on the Reiter treponeme contrasted with that of a number of other amino acids and related compounds

COMPOUND	NUMBER OF ORGANISMS (MILLIONS PER ML) AFTER 6 DAYS' INCUBATION
Agmatine	0
DL-α-Alanine	0
β-Alanine	1
DL-α-Aminobutyric acid	3
DL-β-Aminobutyric acid	8
ε-Aminocaproic acid	2
DL-α-Aminocaprylic acid	0
DL-α-Amino-α-ethylbutyric acid	6
α-Aminoisobutyric acid	7
DL-δ-Aminovaleric acid	1
L-Arginine	116
L-Arginic acid	9
DL-Aspartic acid	1
L-Benzoylarginamide	0
Betaine	0
DL-Citrulline	6
Creatine	3
Creatinine	8
L-Glutamic acid	4
Glycine	0
Glycocyamine	0
Guanidine	0
Guanidinovaleric acid	0
L-Histidine	3
Homoarginine	9
Homocysteine	6
Hydroxy-L-proline	0
Isoleucine	0
DL-Isovaline	3
L-Leucine	0
DL-Lysine	0
DL-Methionine	0
DL-Norleucine	1
DL-Norvaline	0
Ornithine	2
DL-Phenylalanine	0
L-Proline	8
Sarcosine	4
DL-Serine	0
DL-Threonine	0
DL-Tryptophane	2
L-Tyrosine	0
DL-Valine	3
Control	3

Various amino acids and closely related compounds in isotonic concentration and at a neutral pH were added in a volume of 2 ml to a basal medium consisting of 0.5 ml of heated rabbit serum, 0.3 ml of thioglycolate medium, 0.5 ml of M/10.5 phosphate buffer (pH 7.4), and 0.33 ml of M/10.5 cysteine hydrochloride. The inoculum yielded an initial count of 2 million organisms per ml in a total volume of 5 ml.

in the control tube containing no added arginine is perhaps to be attributed to the presence of sufficient amounts of arginine, or of an adequate substitute for it, in the particular lot of serum used in the medium, or in the growing culture used as an inoculum.

The unique activity of arginine is shown in table 3, in which are given the results obtained with 43 amino acids and related compounds. The initial inoculum throughout was 2 million organisms per ml; with arginine there were 116 million per ml after 6 days, but with the other substances tested the counts varied between 0 and 9 million per ml.

Acetic acid as a growth-promoting factor. When serum was dialyzed, it no longer permitted the growth of the Reiter organism in a thioglycolate-arginine

TABLE 4

The presence in whole serum of at least two distinct growth-promoting factors, one dialyzable and the other associated with the nondialyzable residue

DATE	BASAL MEDIA						INITIAL COUNT (MILLIONS/ML)	MILLIONS OF ORGANISMS/ML AFTER 6 DAYS' INCUBATION					
								Serum fractions added to basal medium					
	Thioglycolate medium, ml	Arginine (M/10), ml	Phosphate buffer (M/10.5), ml	Cysteine hydrochloride (M/10.5), ml	Glutathione (M/7), ml	Ascorbic acid (M/7), ml		Dialyzed serum	Serum ultrafiltrate	Serum dialyzate	Dialyzed serum + ultrafiltrate	Dialyzed serum + dialyzate	Whole heated rabbit serum
6- 5-44	0.15	0.5	0.5	0.33			3.3	2	2		145		140
2-19-44	0.20	1.0	0.5	0.33			1.0	10	1		97		114
4-26-44	0.05	1.0	0.5	0.33			0.5	12	1		71		70
6-19-47	0.20	1.5	0.5		0.12	0.25	2.0	30		3		245	310

Serum fractions obtained by ultrafiltration and by dialysis were tested separately and in combination, in conjunction with various basal media. Each serum component was added in an amount equivalent to 0.5 ml of whole serum, in a total culture volume of 5 ml. The original serum served as a control (last vertical column of table). The inocula were organisms sedimented from an actively growing culture on a serum-thioglycolate medium, and washed with basal medium before addition to the culture in order to remove the serum.

medium. The growth-promoting activity was, however, wholly restored by the addition of a serum ultrafiltrate or dialyzate (cf. table 4). Clearly, serum contributed at least two factors, one dialyzable and present in a serum ultrafiltrate, and one present in the residual nondialyzable fraction. Neither fraction alone supported the growth of the organism.

It was then found that the factor (or factors) present in the dialyzable or ultrafiltrable fraction of serum could be replaced by acetic acid. A typical experiment illustrating this point is summarized in table 5. In this experiment the addition of sodium acetate to dialyzed serum had a growth-promoting effect approaching that of serum dialyzate itself. As in the case of arginine, however, the acetic acid did not act as a trace factor. Although the smallest concentration exerting a significant effect was 0.0036 M, the amount of growth increased with the concentration of sodium acetate to reach a maximum at 0.06 M (0.5 per cent).

This is demonstrated by the data tabulated in table 6 and is graphically shown in figure 2. As in the case of arginine, the amounts of acetic acid necessary for maximum growth were out of all proportion to the amount of growth obtained. As in the case of arginine also, the growth-promoting activity of sodium acetate was quantitatively unaffected by recrystallization.

Unlike arginine, acetic acid was not unique in its growth-promoting activity.

TABLE 5

Acetic acid as a substitute for serum dialyzate in the cultivation of the Reiter treponeme

BASAL MEDIUM					INITIAL COUNT (MILLIONS PER ML)	MILLIONS OF ORGANISMS/ML AFTER 6 DAYS' INCUBATIO				
						Substances added to basal medium				
Thioglycolate medium, ml	Arginine (M/10), ml	Phosphate buffer (M/10.5), ml	Glutathione (M/7), ml	Ascorbic acid (M/7), ml		Sodium acetate (M/7), 1.5 ml	Dialyzed serum, 0.5 ml	Dialyzed serum, 0.5 ml + sodium acetate (M/7), 1.5 ml	Dialyzed serum, 0.5 ml + serum dialyzate 0.5 ml	Whole heated rabbit serum 0.5 ml
0.20	1.5	0.5	0.12	0.25	2.0	3	30	210	245	310

The serum components, sodium acetate (M/7), and inoculum were added to the basal medium under the same conditions as were used in the experiments summarized in table 4.

TABLE 6

The growth-promoting effect of acetic acid on the Reiter treponeme

DATE	BASAL MEDIA						INITIAL NUMBER OF ORGANISMS (MILLIONS PER ML)	FINAL NUMBER OF ORGANISMS (MILLIONS PER ML) AFTER 6 DAYS' INCUBATION				
								ml of M/7 sodium acetate (in total volume of 5 ml)				
	Heated rabbit serum, ml	Heated rabbit serum dialyzed, ml	Thioglycolate medium, ml	Arginine (M/10), ml	Phosphate buffer (M/10.5), ml	Cysteine hydrochloride (M/10.5), ml		0	0.25	0.5	1	2
6–22–44	0.5		0.1	0.5	0.5	0.33	1.6	40			390	
7–18–44	0.5		0.1	0.2	0.5	0.33	6.0	15				114
6–14–44		1.0	0.1	0.5	0.5	0.33	4.4	0		4	29	
6–23–44		0.5	0.1	0.5	0.5	0.33	3.2	2	13		90	
6–30–44		0.5	0.1	0.5	0.5	0.33	1.4	0		12		34
—		0.5	0.1	1.0	0.5	0.33	0.5	0	12	21	36	55

Sodium acetate (M/7) was added as indicated on the right side of the table to the various basal media indicated on the left side of the table, all in a total volume of 5 ml. The inoculum was 0.1 ml of an actively growing serum: thioglycolate culture, representing an initial count of 0.5 million organisms per ml. The last horizontal row represents the experiment of figure 2.

Although its beneficial action was greater than that of any of the 10 homologous acids tested (formic, acetic, propionic, butyric, valeric, caproic, enanthylic, caprylic, pelargonic, and capric), a qualitatively similar effect was obtained with at least 15 of the 37 related compounds tested (cf. table 7). The results with many of these substances were obscured by their toxicity at the higher concentrations at which acetic acid was most effective.

Crystalline serum albumin as an adequate replacement for the nondialyzable frac-

tion of serum. As previously indicated (table 5) the dialyzable fraction of serum essential for the growth of the Reiter spirochete could be replaced with acetic acid. It was subsequently found that the nondialyzable residue could be replaced with crystalline bovine serum albumin. A typical experiment, in which a washed inoculum was used, is given in table 8. A number of similar experiments that show the growth-promoting effect of serum albumin are summarized in table 9. The growth-promoting activity of serum albumin was usually not apparent until a concentration of about 0.1 per cent was reached, and increased up to the highest concentrations tested (1 per cent).

Sulfur-containing compounds as essential growth factors for the cultivation of the Reiter treponeme. A series of experiments that demonstrate the essential role of cysteine are summarized in table 10. In these experiments also, the organisms

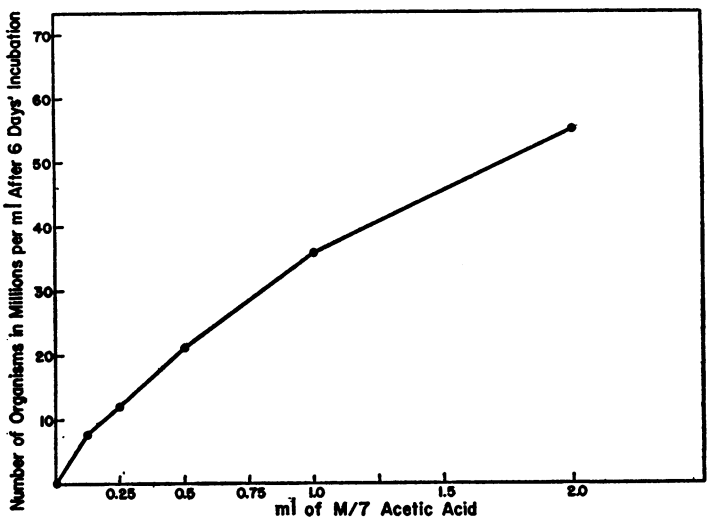

Figure 2. The growth-promoting effect of acetic acid on the Reiter treponeme as a function of its concentration.

were centrifuged and washed free of the culture medium in order to remove reducing substances. That the function of cysteine was not merely the rapid induction of anaerobiosis in the culture medium was shown by the fact that when ascorbic acid was added to a concentration of 1:1,000 (sufficient to induce the rapid reduction of methylene blue or resazurin added as redox indicator in the culture media) no growth was obtained unless cysteine was also added. Under such circumstances, as little as 0.0042 ml of an M/10.5 cysteine solution, equivalent to a final concentration of 0.00008 M, or 0.001 per cent, was able to promote growth in an otherwise —SH-deficient but anaerobic, culture medium. Here also, the efficacy of the cysteine increased with increase in concentration up to the largest amount used (0.1 per cent). A typical experiment illustrating this point is graphically shown in figure 3.

The data of table 11 (which includes the experiment of figure 3) show that

TABLE 7

The growth-promoting effect of acetic acid and related compounds on the Reiter treponeme

COMPOUND	NUMBER OF ORGANISMS (MILLIONS/ML) AFTER 5 DAYS' INCUBATION			
	ml of compound added			
	0.025	0.1	0.4	1.6
Acetic acid	7	8	12	36
Acetaldehyde	40	25	16	0
Acetamide	29	15	5	3
Acetone	15	28	—	31
α-Alanine	—	14	26	33
β-Alanine	17	—	16	20
Aspartic acid	7	—	—	6
Betaine	27	23	20	12
β-Bromopropionic acid	—	0	0	0
Chloral hydrate	12	13	0	0
Chloroacetic acid	8	0	0	0
Chloroacetamide	15	0	0	0
Choline	—	5	—	6
Creatine	10	8	—	9
Creatinine	10	6	—	4
Cyanoacetamide	38	15	—	13
Ethanolamine	—	0	0	0
Ethylamine	13	10	12	0
Ethyl acetate	24	30	21	0
Ethyl alcohol	16	16	18	21
Gluconic acid	6	6	—	5
Glucose	18	25	—	23
Glutamic acid	10	15	—	22
Glycerol	0	5	—	3
Glycine	8	9	3	0
Glycinamide	12	18	20	28
Glycocyamine	11	12	8	5
Glycolic acid	—	9	—	14
Lactic acid	—	15	17	20
Malonic acid	9	10	—	10
Oxalic acid	20	15	10	0
Phenylalanine	8	7	—	0
Pyruvic acid	21	—	23	40
Saccharic acid	6	—	3	3
Sorbitol	—	18	34	30
Succinic acid	—	8	9	15
Thioacetamide	16	18	22	10
Thioglycolic acid	20	5	3	0

A series of compounds in isotonic solution and at neutral pH were added to a basal medium consisting of 0.5 ml of dialyzed heated rabbit serum, 0.1 ml of thioglycolate medium, 1.0 ml of M/10 arginine, 0.5 ml of M/10 phosphate buffer (pH 7.4), and 0.33 ml of M/10.5 cysteine hydrochloride. The initial count was 0.5 million organisms per ml in a total volume of 5 ml. The count after 5 days in a control tube containing no added compound was 3 million per ml.

TABLE 8

Serum albumin as an adequate replacement for the nondialyzable fraction of serum in the cultivation of the Reiter treponeme

BASAL MEDIUM						INITIAL COUNT (MILLIONS PER ML)	MILLIONS OF ORGANISMS/ML AFTER 6 DAYS' INCUBATION			
							Serum factors added to basal medium			
Thioglycolate medium, ml	Arginine (M/10), ml	Sodium acetate (M/7) ml	Phosphate buffer (M/10.5), ml	Glutathione (M/7), ml	Ascorbic acid (M/7), ml		Control (no added serum factors)	Crystalline bovine serum albumin	Dialyzed human serum	Whole heated human serum
0.20	1.5	1.5	0.5	0.12	0.25	2.0	3	148	208	310

In an experiment similar to that described in table 5, 0.5 ml of whole serum, 0.5 ml of dialyzed serum, and 0.5 ml of 5 per cent crystalline serum albumin, respectively, were added to the basal medium. The inoculum was a suspension (in the serum-free basal medium) of organisms sedimented from an actively growing culture and washed in the serum-free medium.

TABLE 9

The growth-promoting effect of serum albumin in conjunction with serum ultrafiltrate or sodium acetate in the cultivation of the Reiter treponeme

DATE	BASAL MEDIA							INITIAL COUNT (MILLIONS PER ML)	MILLIONS OF ORGANISMS PER ML AFTER 6 DAYS' INCUBATION					
									ml of 5% crystalline bovine serum albumin (in total of 5 ml)					
	Thioglycolate medium, ml	Arginine (M/10), ml	Sodium acetate (M/7), ml	Phosphate buffer (M/10.5), ml	Cysteine (M/10.5), ml	Rabbit serum ultrafiltrate	Ox serum ultrafiltrate		0	0.05	0.10	0.25	0.5	1.0
6-14-44	0.1	0.5	1.0	0.5	0.33			4.4	1					25
9- 1-44	0.1	0.1	2.0	0.5	0.33	1.0		2.5	8					94
9-18-44	0.1	0.02	1.0	0.5	0.33	1.0		3.0	12					134
11-24-44	0.1	0.2	1.0	0.5	0.33			3.0	2			20	42	
2-19-45	0.2	1.0	2.0	0.5	0.33	0.5		0.1	0					34
2-19-45	0.2	1.0	2.0	0.5	0.33	0.5		1.0	0					89
4- 9-45	0.05	1.0	1.0	0.5	0.33	0.5		0.5	0		9	19	25	35
4-16-45	0.05	1.0	1.0	0.5	0.33		0.5	0.5	0				26	
4-26-45	0.05	1.0	2.0	0.5	0.33		0.5	0.5	1	24	26		28	

Serum albumin (5 per cent) was added to the various basal media as described in the experiments of table 8.

TABLE 10

The essential growth-promoting effect of cysteine on the Reiter treponeme

DATE	BASAL MEDIA								INITIAL COUNT (MILLIONS PER ML)	MILLIONS OF ORGANISMS PER ML AFTER 6 DAYS' INCUBATION		
										ml of M/10.5 cysteine		
	Heated rabbit serum ml	Crystalline bovine serum albumin, ml	Rabbit serum ultrafiltrate	Thioglycolate medium, ml	Arginine (M/10), ml	Sodium acetate (M/10), ml	Phosphate buffer (M/10.5), ml	Ascorbic acid (M/7), ml		0	0.033	0.33
11-24-44	0.1			0.05	0.2	1.0	0.5		3.0	2		79
12- 6-44	0.1			0.05	0.1	1.0	0.5		5.6	0		67
2-19-45		1.0	0.5	0.02	1.0	2.0	0.5	0.2	0.1	0	15	
2-19-45		1.0	0.5	0.02	1.0	2.0	0.5	0.2	1.0	0	37	

Cysteine (M/10.5) was added as indicated to the various basal media tabulated below, under the same general conditions as were used in the experiment summarized by table 1.

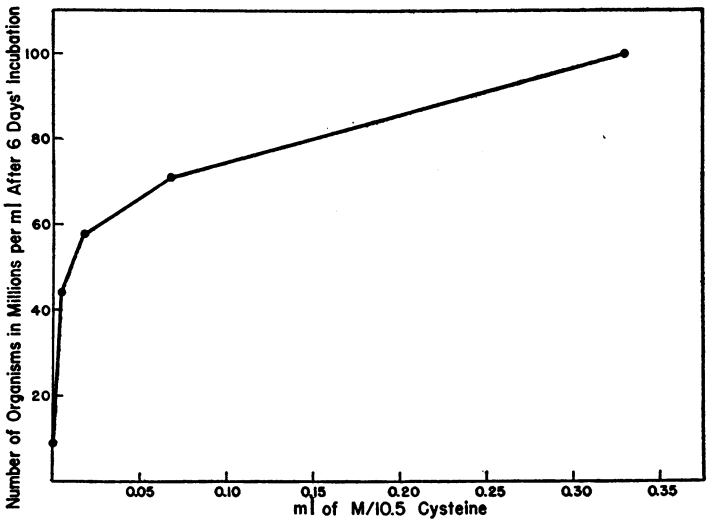

Figure 3. The growth-promoting effect of cysteine on the Reiter treponeme as a function of its concentration.

TABLE 11

The growth-promoting effect of various sulfur-containing substances

SULFUR-CONTAINING COMPOUND	MILLIONS OF ORGANISMS PER ML AFTER 6 DAYS' INCUBATION				REMARKS
	Final concentration of sulfur compounds				
	1:80,000	1:20,000	1:5,000	1:1,000	
Cysteine HCl	44	58	71	100	
Glutathione	32	45	67	71	
Homocysteine	32	—	—	37	Effective
Thioglycolic acid	40	48	41	45	
Thiamine	—	9	50	39	
Cystine	—	47	51	0	Effective,
Sodium sulfide	—	33	0	1	but toxic
Methionine	—	9	0	0	Ineffective

Various sulfur-containing substances at isotonic concentration and neutral pH were added as indicated to a basal medium consisting of 0.5 ml of 5 per cent crystalline bovine serum albumin, 0.05 ml of thioglycolate medium, 1.0 ml of M/10 arginine, 1.0 ml of M/7 sodium acetate, 0.5 ml of M/10.5 phosphate buffer (pH 7.4), and 0.2 ml of M/7 ascorbic acid, all in a total volume of 5 ml. The initial count was 0.5 million organisms per ml. The count after 6 days in a control tube containing no added sulfur-containing compound was 9 million per ml.

cysteine was not unique in its growth-promoting action. Not only were other sulfhydryl-containing compounds (glutathione, homocysteine, and thioglycolic acid) fully active, but several compounds in which the sulfur was present in an

—S— or —S—S— rather than —SH linkage (thiamine, cystine) were also effective. Methionine alone of the substances tested had no growth-promoting effect, perhaps because it is not transformed to an —SH compound under the conditions of the experiment.

Phosphate buffer. The phosphate buffer used in these experiments apparently had no effect over and above its buffering action. In several experiments the same culture medium was used with and without phosphate buffer, with no significant differences in the degree of multiplication. Also, the culture in the phosphate-free medium could be successfully subcultured into the same medium. These data indicate that the phosphate served only to maintain a pH range suitable for growth, and that the buffer otherwise had no beneficial action. This conclusion neglects the possibility that the minute amounts of phosphate probably present in the several unpurified components of the medium (yeast extract, trypticase) may suffice to exercise a growth-promoting effect. The point will require restudy when these as yet unidentified components can be replaced with chemically defined compounds.

DISCUSSION

It is clear from the data of table 3 that arginine alone of the amino acids here studied was essential for the growth of the Reiter strain of treponeme under the conditions of that experiment. The fact that such closely related substances as arginic acid, citrulline, and ornithine did not replace arginine suggests that arginine as such may enter the metabolic cycle of the organisms. However, the large amounts necessary in comparison with the amount of growth obtained suggest also that arginine may be merely substituting for another more active compound, as yet unidentified; and the very necessity for such large amounts may make it difficult to follow the metabolic pathways involved in its utilization.

Acetic acid was found to promote the growth of the Reiter organism under the experimental conditions. Although it was the only one of the low-molecular-weight saturated aliphatic acids tested which was active in this respect, acetic acid could be replaced by a number of closely related compounds such as acetaldehyde, ethanol, and pyruvic acid (see table 7). This may prove useful in tracing the metabolic pathways involved in the utilization of these compounds. Acetic acid (but not arginine) functioned as a substitute for the dialyzable components of serum; i.e., dialyzed serum or serum albumin gave good growth when supplemented with acetic acid on an otherwise inadequate basal medium (cf. tables 2, 5, 6, 7, 8, and 9).

The growth-promoting properties of both acetate and arginine were not manifest unless there were present adequate amounts of at least three other factors: (a) the unidentified factor (or factors) in the thioglycolate medium, (b) serum or serum albumin, and (c) cysteine or some other S-containing compound.

In agreement with previous reports (Little and SubbaRow, 1945; Whiteley and Frazier, 1948), crystalline bovine serum albumin (Armour) was found to be a

growth-promoting factor, and was shown to substitute for the nondialyzable fraction of whole serum (table 9). Its function is, however, not yet clear. Serum albumin plays an essential role in media developed for the cultivation of *Mycobacterium tuberculosis* (Dubos and Davis, 1946) and of *Trichomonas vaginalis* (Sprince and Kupferberg, 1947); and, in the case of the former medium, its favorable effect has been attributed primarily to a detoxifying action (Davis and Dubos, 1947). Studies are now in progress to determine its function in the cultivation of the Reiter organism.

Five components have therefore been identified as essential and together to be adequate for the multiplication of the Reiter treponeme in an anaerobic environment: (1) arginine, (2) acetic acid, (3) any one of a number of sulfur-containing substances, (4) crystalline serum albumin, and (5) an as yet unidentified component (or components) in the thioglycolate medium. Of the four chemically defined compounds only the —SH—containing substances were effective in such small concentrations (0.00008 M) as to suggest that they might act as growth factors rather than metabolites. The present experiments furnish no evidence as to whether the other substances serve merely as sources of energy or are used in building up cell protoplasm.

The "thioglycolate medium" which must still be added apparently contains only three substances that could be contributing to growth: a yeast extract, an enzymatic casein digest (trypticase), and glucose. (The phosphate, resazurin, and agar in the thioglycolate medium have been shown to be probably noncontributory under the conditions of the present experiments; and the cystine and thioglycolic acid have been shown to be replaceable by any sulfhydryl-yielding compound.) Since the yeast extract and casein digest permit growth of the organism when they are added to the other four ingredients in final concentrations of 1:10,000 and 1:3,300, respectively, the active component (or components) must be effective in minute concentration. Studies on the identification of those active components are now in progress.

SUMMARY

A mixture of acetic acid, arginine, any one of a number of sulfur-containing compounds, and crystalline serum albumin, supplemented with minute amounts of yeast extract, an enzymatic casein digest, and glucose, has been shown to permit the multiplication of the Reiter treponeme.

The acetic acid has been shown to replace the dialyzable components of whole serum, and crystalline albumin has been shown to substitute for the nondialyzable fraction.

The minimal effective concentrations of acetic acid and arginine were 0.0036 M and 0.0005 M, respectively, but the rate of growth increased progressively with increasing concentrations up to the highest concentrations studied. The minimal effective concentrations of the sulfhydryl compounds studied were of the order of 0.0001 M, and serum albumin was effective at a concentration greater than 0.1 per cent.

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