GROWTH REQUIREMENTS OF CLOSTRIDIUM TETANI

III. A "SYNTHETIC" MEDIUM

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Received for publication, August 6, 1943

In the preceding communication (Feeney, Mueller and Miller, 1943) the relationship of our present knowledge of the growth requirements of *Clostridium tetani* was briefly reviewed in connection with the question of toxin production on synthetic media. Experiments were detailed regarding the nature of the components of the medium which limited growth (and presumably toxin formation). In these experiments definite requirements for biotin and for oleic acid were demonstrated, and it was shown that certain other components of the medium, notably Fe, tryptophane and folic acid were rapidly exhausted by growth of the organism. The present paper presents the results of a parallel series of experiments through which it was sought to identify all of the factors essential to growth. Beginning with a basic formula established as the result of earlier work (Mueller and Miller, 1942) and of unpublished experiments, incapable in itself of supporting growth without the use of supplementary mixtures of partially unknown composition, it was sought to identify the components of the supplements which induced growth. The ultimate objective was a satisfactory medium containing only materials of known composition. To a reasonable extent, this has now been accomplished and the results are here presented. A consideration of the application of the findings to the question of toxin production will for the present be reserved.

EXPERIMENTAL

Preparation of the stock basal media. Since the basal media varied as the experimental work progressed, only the formulae of the initial liver-containing medium and the final synthetic medium will be presented. Modifications of the liver medium leading to the identification of the various additional substances will be indicated in the discussion of these materials. Each preparation of basal medium was made up in amounts sufficient for 50–200 10 ml. test lots. The ingredients other than the materials under investigation were dissolved in a small amount of water containing excess hydrochloric acid. The amount of acid added was varied to give an approximate total concentration of 0.6%–0.8% sodium chloride in the completed test medium after neutralizing with sodium hydroxide. Phenol-red indicator was added and the medium diluted to a volume

[1] The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the President and Fellows of Harvard College.

[2] Aided by a grant from the Commonwealth Fund.

one-fourth of that in which the bacteriological tests were conducted. Basal media so prepared were stored in the refrigerator for over a month with no apparent changes.

The amounts of the constituents of the media as listed in the tables or described in the text are given in terms of the media as diluted for a test run (one-fourth the concentration in the stored stock basal media).

Bacteriological techniques, etc. For each set of determinations portions of the basal media were diluted with water, adjusted to pH 7.4–7.6 with sodium hydroxide, and an amount equivalent to 2.5 ml. of the undiluted basal medium added to 150 x 15 mm. Pyrex test tubes which were previously cleaned by standing overnight in strong chromic-sulfuric acid, thoroughly rinsed with water and dried. The solutions of materials to be tested were added to each tube, any changes in acidity rectified, and the contents of each tube diluted to 10 ml. The tubes were covered with glass caps, autoclaved at 10 lbs. steam pressure for five minutes, removed promptly from the autoclave, cooled in water, and inoculated. The glass caps were replaced by previously sterilized cotton plugs and the tubes were immediately placed in an anerobic jar (Mueller and Miller, 1941).

The strain of the organism employed and the procedure of carrying the stock culture by serial transfers in tubes of glucose-peptone-infusion broth were as previously described (Mueller and Miller, 1942).

A large inoculum was employed to facilitate rapid growth. During the introductory stages of the problem the cells of one 10 ml. broth culture were centrifuged, resuspended in sterile saline, and used to inoculate 15–20 test lots of media. This was later slightly refined by washing the cells once with 10 ml. of sterile saline and roughly standardizing the amount of inoculum so that one broth culture served to inoculate 25 test lots of media. The inocula were introduced into the bottom of the tubes by means of sterile Pasteur pipettes.

The extent of growth was usually determined after 24–30 hours of incubation at 37° by reading the turbidity with a Gates suspensiometer and comparing the readings with those of serially diluted glucose-peptone-infusion broth cultures. Longer incubation times and simple visual comparisons of turbidities were occasionally employed.

Protein hydrolysates. The casein and gelatin were hydrolyzed with sulfuric acid and the other proteins with hydrochloric acid. The latter hydrolysates were prepared by refluxing the proteins in 8 N acid for 16 hours and removing the excess of the hydrogen chloride *in vacuo*. The sulfuric acid hydrolysates and the butyl alcohol fractions therefrom (the diamino-dicarboxylic, the proline and the monoamino-monocarboxylic acid fractions) were prepared in the usual way.

The liver-extract basal medium. It was found possible to obtain moderate growth in the medium shown in table 1. The evidence for the inclusion of serine, methionine, tyrosine and aspartic acid was based on small increases in growth or toxin production, and in the case of serine and aspartic acid, on the metabolism experiments of Clifton (1942). Nicotinic acid, pimelic acid, beta-alanine, oleic acid and other of the ingredients might well have been omitted in the presence of the liver extract.

dl-isoleucine. The addition of either acid-hydrolyzed casein or gelatin to the liver extract basal medium increased the growth to the level of that obtained in peptone-infusion broth. The amino acid dl-isoleucine largely replaced the protein hydrolysates. Dl-leucine had a small stimulatory effect.

Valine. The basal medium was now modified by the omission of the liver extract and the inclusion of the following materials per 100 ml. of test medium: 30 mg. dl-isoleucine, 30 mg. dl-leucine, 500 gamma adenine, 10 gamma riboflavin, and 10 gamma thiamine. The latter three substances were known to be essential in the absence of the liver extract (Mueller and Miller, 1942). No growth occurred in this medium. The addition of hydrolyzed casein resulted in excellent growth and the addition of hydrolyzed gelatin in poor growth. The Dakin diamino-dicarboxylic acid fraction of the gelatin hydrolysate, however,

TABLE 1

Composition of initial basal medium containing liver extract

SUBSTANCE	AMOUNT PER 100 ML.	SUBSTANCE	AMOUNT PER 100 ML.
l-Glutamic acid	400 mg.	Biotin	0.10 gamma
l-Arginine	60 mg.	Pantothenic acid	25 gamma
l-Histidine	50 mg.	Folic acid concentrate*	0.50 gamma
l-Tyrosine	30 mg.	Na_2HPO_4	100 mg.
dl-Serine	20 mg.	KH_2PO_4	30 mg.
dl-Methionine	40 mg.	Accessories and metals solution†	0.20 ml.
l-Aspartic acid	40 mg.	Liver extract‡	0.10 ml.
l-Cystine	40 mg.	Conc. hydrochloric acid	0.8 ml.
l-Tryptophane	5.0 mg.	$FeSO_4 \cdot 7H_2O$	100 gamma
Glucose (reagent)	1.0 g.		
Oleic acid	100 gamma		

* The concentrate of folic acid was kindly supplied by Dr. R. J. Williams. It was labeled "Potency 38,000."

† A solution with the following composition per 100 ml. $MgSO \cdot 7H_2O$ 22.5 g., $CuSO_4 \cdot 5H_2O$ 50 mg., $ZnSO_4 \cdot 7H_2O$ 40 mg., $MnCl_2 \cdot 4H_2O$ 15 mg., beta-alanine 115 mg., nicotinic acid 115 mg., pimelic acid 7.5 mg., conc. hydrochloric acid 1.5 ml.

‡ A 90% ethanol filtrate fraction of a water extract of liver. This fraction was obtained through the courtesy of the Connaught Laboratories, Toronto, Canada.

was almost devoid of the activity found in the whole hydrolysate. Supplementation of the Dakin fraction with dl-valine largely restored the activity of this fraction to that of the whole hydrolysate.

Nicotinic acid. The basal medium was further modified by the inclusion of 30 mg. of dl-valine per 100 ml. of test medium and the omission of the accessories (pimelic acid, beta-alanine, and nicotinic acid). Likewise, the techniques of washing and standardizing the inoculum were initiated.

Again no growth was obtained in the modified medium. Gelatin hydrolysate was completely inactive while casein hydrolysate supported good growth as before. A hydrolysate of a sample of purified casein was much less effective. Purification of the casein was accomplished by reprecipitation and prolonged extraction with 95% ethanol in a Soxhlet apparatus. The excellent growth pos-

sible with the unpurified casein was again obtained when the hydrolysate of the purified casein was supplemented with nicotinic acid and pyridoxine. The effect of pyridoxine was variable and slight.

Manganese and various amino acids. With the inclusion of nicotinic acid and pyridoxine at a level of 100 gamma of each per 100 ml. of test medium, the study of the unknown materials supplied by the casein was continued. Testing of the three Dakin fractions, the diamino-dicarboxylic acid, the proline, and the monoamino-monocarboxylic acid, revealed that both the diamino-dicarboxylic acid fraction and the proline fraction were required for growth. This separation was not entirely complete as the diamino-dicarboxylic acid fraction was sufficient by itself at high levels. Since the proline fraction could not be replaced by mixtures of amino acids or other protein hydrolysates, its investigation was temporarily deferred and it was included in the test medium at a level equivalent to 500 mg. of casein per 100 ml. of test medium and the materials in the other fraction studied.

The diamino-dicarboxylic acid fraction was completely replaced by acid hydrolysates of gelatin, edestin, pumpkin seed globulin and largely by an acid hydrolysate of hemoglobin. A partial replacement of this fraction was achieved by adding a mixture of 10 mg. per 100 ml. of medium of each of the following amino acids: lysine, oxyproline, proline, threonine, glycine, alanine, and phenylalanine. The effect of this mixture was extremely inconstant, varying from slight to good growth from test to test and with successive batches of media. This variation was completely removed and good growth was consistently obtained by the further addition of an ash of casein. A fairly large number of metallic salts were tested and, of these, manganese chloride tetrahydrate was found to replace the ash at a level of 100–200 gamma per 100 ml. of medium. The other salts tested were without effect.

Uracil. When the above mixture of amino acids and more manganese were added to the medium and the proline fraction of casein was omitted, no growth occurred. Of a number of materials tested, a 90% ethanol soluble fraction of an aqueous extract of liver appeared the most feasible source for the study of the properties of the unknown active substance.

The activity of the liver extract was not diminished by prolonged acid hydrolysis or by treatment with nitrous acid. It was largely absorbed on Norit from acid solution and eluted from the Norit by boiling 50% ethanol. Extraction experiments with diethyl ether and butanol demonstrated that the active material extracted slowly with ether and rapidly with butanol from strongly acid solution (10% sulphuric acid) but would not extract from strongly alkaline solution. After saturation of the alkaline solution with carbon dioxide, the activity was again extractible.

A concentrate of the active material was easily prepared by utilizing the above described properties. The liver extract was absorbed on and eluted from Norit and the eluate extracted from strongly acid solution with ether in a continuous extraction apparatus. The easily water-soluble solids of the ether extract were dissolved in water and the water solution extracted several times with an equal

volume of ether. The water solution was then made strongly alkaline with sodium hydroxide, extracted 15 times with a double volume of butanol, and finally 15 times with a double volume of butanol after saturation with carbon dioxide. The concentrate obtained in the final butanol extract contained over two-thirds of the total activity of the original liver extract and gave a maximum growth response at a level of 150–200 gamma per 100 ml. of test medium.

At this point it was found that the majority of the activity was precipitated by silver nitrate and barium hydroxide, and several purines and pyrimidines were tested. The pyramidine uracil completely replaced the concentrate while thymine, guanine, and hypoxanthine were without effect. Uracil was included in the medium at a level of 250 gamma per 100 ml. of medium.

TABLE 2

Composition of the synthetic medium

SUBSTANCE	AMOUNT PER 100 ML.	SUBSTANCE	AMOUNT PER 100 ML.
d-Glutamic acid	250 mg.	Biotin	0.10 gamma
l-Arginine	50 mg.	Pantothenic acid	25 gamma
l-Histidine	50 mg.	Folic acid concentrate*	0.50 gamma
l-Tyrosine	30 mg.	Na_2HPO_4	100 mg.
dl-Serine	20 mg.	KH_2PO_4	30 mg.
dl-Methionine	20 mg.	Metals†	0.20 ml.
dl-Aspartic acid	20 mg.	$FeSO_4 \cdot 7H_2O$	100 gamma
l-Cystine	40 mg.	HCl	0.80 ml.
l-Tryptophane	5.0 mg.	Glucose	1.0 gram
dl-Valine	30 mg.	Riboflavin	10 gamma
dl-Isoleucine	30 mg.	Thiamin	10 gamma
dl-Leucine	30 mg.	Nicotinic acid	100 gamma
dl-Threonine	20 mg.	Pyridoxine	100 gamma
dl-Phenylalanine	20 mg.	Uracil	250 gamma
dl-Lysine	20 mg.	Adenine	500 gamma
Oleic acid	250 gamma		

* See table 1.

† As in table 1—without accessories.

Pyridoxine. Since pyridoxine had originally been added to the medium with little basis for its use, the effect of its omission was now studied. In primary culture little effect was noted, but upon subculturing the organism in the medium it proved essential. Though the growth with pyridoxine was slower and a little less heavy in subculture, no growth was obtained in its absence. Pyridoxine was therefore retained in the medium.

The final constitution of the synthetic medium and requirements in this medium. Various of the amino acids which had previously been incorporated in the medium were now found to have no influence on growth and were omitted. The final constitution of the synthetic medium is listed in table 2. Though the growth in this medium was consistently good, it was slower and a trifle less heavy than in glucose-peptone-infusion broth. The growth obtained after 24–30 hours of incubation was usually 80–85% of that obtainable in broth.

When the amino acids of the optimum mixture were omitted from the medium one at a time (with the exception of tryptophane and cystine), varying effects on growth were observed (table 3). Tyrosine, leucine, isoleucine, histidine, valine and arginine were essential for growth. Threonine, serine and phenylalanine were strongly stimulatory, while methionine, lysine and aspartic acid appeared only slightly stimulatory. Glutamic acid was not essential and had little effect on the stimulation of more rapid growth but permitted much heavier growth. Synthetic dl-glutamic acid replaced the natural amino acid. Although no attempt was made to study more extensively the action of glutamic acid and the possibility of its replacement by other substances, it was considered as serving as a source of energy for the organism. The minimum requirement of each of the

TABLE 3

*Growth of organism in absence of individual amino acids**

AMINO ACID OMITTED	RELATIVE GROWTH AFTER INCUBATION FOR†		
	24 hours	48 hours	72 hours
None	++++	++++	++++
d-Glutamic acid	++	++	++
l-Tyrosine	−	tr	tr
dl-Leucine	−	−	−
dl-Isoleucine	−	−	−
l-Histidine	−	−	−
dl-Valine	−	−	−
l-Arginine	tr	−	−
dl-Serine	tr	++	++++
dl-Phenylalanine	++	+++	++++
dl-Threonine	tr	++	++++
dl-Lysine	+++	++++	++++
dl-Methionine	+++	++++	++++
l-Aspartic acid	++	++++	++++
All thirteen above	−	−	−

* In synthetic medium.

† Relative growth estimated by visual observation. − = none, tr = barely visible turbidity, + to ++++ gradations to maximum.

essential amino acids (except tryptophane) was between 100 and 300 gamma per 10 ml. of test medium. The optimum amount of glutamic acid was between 15 and 25 mg. per 10 ml. of test medium.

Oleic acid, biotin, nicotinic acid, uracil, and pyridixone were all essential. No growth was obtained after 24–30 hours of incubation when any one of these substances was omitted from the medium. After three or four days of incubation only the cultures from which uracil had been omitted had any visible growth. This growth was 15–20% of the growth obtained in 24–30 hours in the presence of uracil and did not increase with still further incubation. In table 4 are listed the responses of the organism to graded amounts of these substances. The minimum requirements per 10 ml. of medium for each of these materials were as follows: nicotinic acid 0.50 gamma, pyridoxine 0.5 gamma, biotin 0.0075 gamma,

uracil 5.0 gamma and oleic acid 7.5–10.0 gamma. Nicotinamide replaced nicotinic acid microgram for microgram. The above experiments concerning pyridoxine were performed in subculture.

TABLE 4

Response of organism to graded amounts of nicotinic acid, uracil, biotin, oleic acid, and pyridoxine

MATERIAL		RELATIVE GROWTH†
Omitted*	Amount added	
	per 10 ml.	*per cent*
Oleic acid	10.0 gamma	100
	7.5 gamma	100
	5.0 gamma	55
	2.5 gamma	25
	none	0
Uracil	10.0 gamma	100
	5.0 gamma	100
	2.5 gamma	65
	1.0 gamma	45
	0.5 gamma	25
	none	0
Biotin	0.0100 gamma	100
	0.0075 gamma	90
	0.0050 gamma	50
	0.0025 gamma	10
	none	0
Nicotinic acid	0.100 gamma	100
	0.050 gamma	100
	0.025 gamma	70
	0.010 gamma	55
	0.005 gamma	35
	none	0
Pyridoxine‡	10.0 gamma	90
	1.0 gamma	90
	0.2 gamma	45
	none	0

* From synthetic medium.

† The maximum growth under these conditions was approximately 80% of that obtainable in broth. The relative growths are expressed in percentages of this maximum growth.

‡ The experiments on pyridoxine were performed in first subculture after 36 hours of incubation.

DISCUSSION

Although good growth of *Clostridium tetani* occurred in the synthetic medium, unknown stimulatory substances still exist and it is possible that unknown essential substances also exist. No attempt was made to carry the organism further

than the first subculture and even the growth in the first subculture was not optimum. Serial subcultures with a small inoculum might well reveal a further deficiency of an essential substance. One such substance might be the sporogenes vitamin which has been shown to be required by various pathogenic clostridia (Fildes, 1935). None of the materials found essential for the growth of this strain of the tetanus bacillus appears to have the chemical properties ascribed to the sporogenes vitamin (Knight and Fildes, 1933) (Pappenheimer, 1935). The natural amino acids, glucose or the folic acid concentrate may possibly have contained essential impurities.

Beyond the demonstrated requirements for K, Mg, PO_4, Fe and Mn, nothing is known of the mineral requirements of the organism. The copper and zinc salts included in the medium have been omitted without any effect on growth.

An interesting observation in the course of the problem was the apparent presence of the amino acid valine in the gelatin hydrolysate, as judged from the growth response of the organism. A sample of the same gelatin hydrolysate was found to support the growth of *Lactobacillus arabinosa* when added to a medium containing no valine.[3] Since this organism has been found to require valine for growth (Hegsted, 1943) it would appear that our observation has been substantiated, and that gelatin contains a small amount of valine. This is contrary to accepted analytical data, (Dakin, 1920).

Uracil was not isolated from the concentrate and only a very few materials were tested in its place, but it seemed probable that the active material in the liver extract was uracil. It was well defined as a weakly acidic substance without a basic functional group. The concentrate was approximately one-fourth as active as uracil on a weight basis.

An extensive report of the requirements of the organism for riboflavin, thiamin, folic acid, pantothenic acid, adenine and tryptophane in a well-defined medium has been published (Mueller and Miller, 1942) and protocols concerning these materials have been presented. Both glucose and cystine were included in the medium, though either one of these alone was previously reported as being sufficient. Oleic acid, biotin, histidine, and glutamic acid were reconsidered as the original investigation concerning their requirement was conducted in a medium of unknown composition (Feeney, Mueller and Miller, 1943).

Little information concerning the nutrition of the other pathogenic clostridia has been published to date. Fildes and Richardson (1935) reported that leucine, arginine, tyrosine, phenylalanine, and tryptophane were essential and valine, histidine, cystine, and methionine were stimulatory and possibly essential for the growth of *Clostridium sporogenes*. Following the discovery of oleic acid as an essential factor for *Clostridium tetani*, Dr. A. M. Pappenheimer, Jr., has found *Clostridium welchii* to require oleic acid, pyridoxine, and uracil.[4] It appears possible that the majority of the pathogenic clostridia will be found to have as complex nutritional requirements as is the case with *C. tetani*.

[3] The authors are grateful to Dr. D. M. Hegsted for this determination.

[4] The authors are indebted to Dr. A. M. Pappenheimer, Jr., for suggesting that uracil might be required by *Clostridium tetani*.

Since the fundamental purpose of these investigations has concerned the production of tetanus toxoid for the armed forces, various interesting but academic phases of the problem have been neglected for the present. Further experiments on the nutrition of the organism will be conducted only insofar as studies on toxin production indicate their importance. Toxin studies with a medium similar to that reported here are encouraging at the present time.

SUMMARY

1. Good growth of a strain of *Clostridium tetani* has been obtained in a synthetic medium. Initial subculture was possible but was not entirely satisfactory.

2. In addition to the following previously reported essential substances: riboflavin, pantothenic acid, thiamin, folic acid, adenine, biotin, and oleic acid, the following materials have been found essential: uracil, pyridoxine, and nicotinic acid.

3. The following amino acids were essential: arginine, histidine, tyrosine, valine, isoleucine, leucine, and tryptophane.

4. The amino acids, threonine, phenylalanine, and serine, greatly stimulated the rate of growth, while lysine, aspartic acid and methionine stimulated to a small extent. Glutamic acid supported heavy growth but was not essential.

5. Evidence was obtained that manganese is necessary for optimal growth.

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