NUTRITIONAL REQUIREMENTS AND FACTORS AFFECTING THE PRODUCTION OF TOXIN OF CLOSTRIDIUM SEPTICUM*

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Knowledge that immunization with toxoids is effective in the prevention of diphtheria and tetanus has suggested that prophylactic immunization against the toxins of the gas gangrene bacteria may afford a practical method of combatting anaerobic wound infection. Despite the application of modern surgery and chemotherapy to the treatment of wounds, the incidence of gas gangrene among the wounded in the Middle East, according to a recent report (1), appears to be approximately as great as that among the wounded in France in 1918.

The chief obstacle to the preparation of toxoids against the anaerobic spore-forming bacilli has been the poor yield of toxin obtained by the usual methods of cultivation (2). A further objection to the methods which have been employed for toxin production is the use of protein- and peptone-containing media. As noted by Mueller, Schoenbach, Jezukawicz, and Miller (3), tetanus toxoid prepared from media containing extraneous antigenic substances has led to anaphylaxis-like symptoms when used for human immunization.

In the present communication a protein- and peptone-free medium is described which is capable of supporting heavy growth of *Clostridium septicum*. The quantity of toxin produced in the medium is found to be as great or greater than that usually reported to be obtained in media containing serum, tissue, peptone, or other complex nutrients. The medium to be described was developed specifically for strain 44 of *Cl. septicum*, since this strain is regarded as equal or superior to other strains in toxin-producing capacity. Some of the factors influencing toxin production are discussed.

The nutritional requirements of *Cl. septicum* do not appear to have been previously investigated. The growth requirements of certain related bacteria namely *Cl. botulinum* and *Cl. tetani*, have been studied respectively by Burrows (4) and by Mueller (5).

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Methods

Strains.—The strains of *Cl. septicum* employed were as follows: strains 44, 23b, 32, and 59, obtained from the National Institute of Health, Bethesda, Maryland; strain 15192 from Professor Ivan C. Hall, Columbia University; strain BX96 from Dr. Walter L. Koerber of E. R. Squibb and Sons. Unless otherwise stated, strain 44 was used in the experiments to be described.

Preparation of Gelatin Hydrolysate.—100 gm. of Eastman's de-ashed gelatin was refluxed for 20 hours with 250 ml. of 36 per cent HCl of reagent grade and 250 ml. distilled water. After removing excess HCl by distilling *in vacuo* to a thick syrup, the hydrolysate was decolorized with charcoal and made up to 500 ml. with distilled water. 50 ml. was used for 1 liter of medium.

Preparation of Inoculum for Experiments on Nutrition.—Strain 44 was grown for 16 hours at 37°C. in 250 ml. of the medium previously developed for the cultivation of group A hemolytic streptococcus (6). The culture was centrifuged and the sedimented organisms were suspended in 100 ml. distilled water. After standing at room temperature for 24 hours, the organisms were washed four times with distilled water, and made up to 100 ml. with distilled water. The suspension stored in the refrigerator remains viable for several months. Inocula for the test media (10 ml. medium per tube) were prepared by diluting the above suspension ten times and using 0.1 ml. of this dilution for each tube. Cultures were incubated at 37°C. for 16 hours.

Estimation of Growth.—The amount of growth in the test media was determined turbidimetrically by means of a Coleman spectrophotometer at a wavelength of 6500Å. The amount of growth can be expressed in milligrams of bacterial nitrogen since the nitrogen content of washed cultures is a function of the galvanometer deflection of the spectrophotometer. The unit adopted is equivalent to 1 mg. of bacterial nitrogen per 100 ml. of culture.

Culture Technique for Experiments on Production of Toxin and Hemolysin.—(1) Toxin production in cultures of 20 ml.: 20 ml. amounts of the casein hydrolysate medium to be described were distributed in glass-capped, acid-cleaned 6 × ¾ inch pyrex tubes. 0.1 ml. and in some instances 0.01 ml. of frozen cooked-meat broth culture was thawed and transferred to the media. (2) Toxin production in cultures of 500 ml.: 500 ml. of the casein hydrolysate medium to be described, contained in a 500 ml. pyrex flask, was inoculated with 0.1 ml. of stored frozen cooked-meat broth culture, which was thawed just before use. Cultures were incubated at 37°C. until growth was complete.

Unless otherwise stated, the inoculum was prepared as follows: A smooth toxigenic colony was transplanted to cooked-meat broth. After 16 hours at 37°C., the supernatant fluid was distributed in 0.1 ml. quantities into each of a large number of tubes. The tubes were frozen by immersing them in a mixture of alcohol and solid carbon dioxide for about 3 minutes, after which they were transferred to the freezing compartment of a refrigerator where they were maintained in the frozen state until used.

Estimation of Toxin.—The quantity of toxin present in cultures was estimated by the intravenous injection of mice according to the method described in the preceding paper (7). The unit of toxin employed was the L.D.$_{50}$; *i.e.*, the dose which killed half the mice of a group within 72 hours.

Estimation of Hemolysin.—The hemolytic activity of cultures was estimated by the method described in the preceding communication (7). It has been found that the potency of the lethal toxin in cultures of strain 44 is proportional to the content of hemolysin (7). By reason of this parallelism in lethal and hemolytic activity, considerable reliance has been placed upon the hemolytic test as an indicator of toxin content. Much of the work reported in the present paper has been expedited by measuring hemolysin rather than toxin, and by making use of the observation that the ratio of the L.D.$_{50}$ of toxin to the unit of hemolysin is approximately 13. The validity of this relationship has been verified in many instances in the course of the present investigation.

Experiments on Nutritional Requirements

Preliminary experiments showed that luxuriant growth of *Cl. septicum* could be obtained in a medium consisting of a complete acid hydrolysate of casein supplemented with amino acids, glucose, growth accessory factors, and salts. This medium has been described in detail in connection with the cultivation of *Streptococcus pyogenes* (6). Substitution of 1 per cent acid hydrolysate of Eastman's de-ashed gelatin for 2 per cent acid hydrolysate of casein also yielded excellent growth. The gelatin hydrolysate medium was used as a starting point for further study.

Amino Acids.—An acid hydrolysate of either gelatin or casein supplemented with cystine and tryptophan was found to satisfy the amino acid requirements of the organism under study. In no instance was growth observed to be influenced by the addition of tyrosine or methionine to the medium.

Growth Accessory Substances.—Thiamine (1 mg. per liter), pyridoxine (1 mg. per liter), nicotinic acid (1 mg. per liter), and biotin (1 gamma per liter) were found to be essential for growth. Omitting any one of these substances resulted in negligible growth. Glutamine (20 mg. per liter) although not indispensible for growth consistently shortened the lag period. In its absence, no detectable turbidity appeared until about 24 hours after inoculation, while in its presence, growth was complete after overnight incubation. Growth was not affected by the addition of 1 mg. per liter of pantothenic acid, riboflavin, ascorbic acid, pimelic acid, folic acid, inositol, para-aminobenzoic acid, or *sporogenes* factor.

Metals.—Little or no growth occurred in the absence of iron. When other substances were present in optimal amounts, growth increased with increasing concentration of iron up to an iron concentration of 500 to 1000 gamma iron per liter. Amounts of iron larger than this had either no effect or a slight inhibitory effect upon growth. The iron was added in the form of ferric sulfate dissolved in dilute HCl. Varying the concentration of copper, zinc, and manganese had but little effect on growth. Table I shows the effect on growth of increasing concentrations of iron.

Source of Energy.—No growth occurred in the absence of glucose or other fermentable carbohydrate. In concentrations between zero and 0.7 per cent, growth increased with increasing concentrations of glucose. The substitution of maltose for glucose did not affect the final amount of growth or the final acidity of the cultures. When dextrin was substituted for glucose the cultures failed to grow.

Reducing Substances.—Unless freshly autoclaved media were used, it was found essential to include a reducing substance other than glucose in the medium. Either thioglycollic acid (0.01 per cent) or reduced iron (0.5 mg. per 10 ml.) sufficed. Thioglycollic acid in the concentration stated was found convenient and satisfactory for routine work.

Composition of Medium

It would not be particularly informative to describe the many experiments performed with a view to increasing the growth of the organism under study. In brief, growth equivalent to 3 to 6 mg. bacterial nitrogen per 100 ml. medium was obtained in 1 per cent gelatin hydrolysate supplemented with the compounds listed below.

Since a medium of low cost was desired for purposes of large scale cultivation, acid hydrolysate of technical casein, as previously prepared (6), was substituted for gelatin hydrolysate as a source of amino acids. Somewhat greater growth occurred in the casein hydrolysate medium than in the gelatin hydrolysate medium, and the growth could be further increased by raising the concentration of casein hydrolysate from 1 per cent to 2 per cent. The substitution of bacto casamino acids, a product stated to be a complete acid hydrolysate

TABLE I

Effect of Iron on Growth of Cl. septicum

Fe per 20 ml. medium	Bacterial nitrogen per 100 ml. culture
gamma	*mg.*
0	3.6
0.5	4.4
1.0	5.0
1.5	4.9
2.0	5.7
3.0	6.6
5.0	8.4
10.0	10.9
20.0	12.9

of casein, gave equally good results. The final composition of a simplified medium, capable of supporting growth of *Cl. septicum* strain 44, equivalent to 12 to 20 mg. bacterial nitrogen per 100 ml. is as follows:—

Casamino acids Difco	20 gm.	Phenol red	8 mg.
Cystine hydrochloride	200 mg.	$KH_2PO_4 \cdot 2H_2O$	3 gm.
Tryptophan	20 mg.	$Na_2HPO_4 \cdot 12H_2O$	18 gm.
Glutamine	20 mg.	$KHCO_3$	800 mg.
Glucose	10 gm.	$CaCl_2 \cdot 2H_2O$	100 mg.
Biotin	1 gamma	$Fe_2(SO_4)_3$	1.8 mg.
Thiamine	1 mg.	$CuSO_4 \cdot 5H_2O$	1 mg.
Nicotinic acid	1 mg.	$ZnSO_4 \cdot 7H_2O$	1 mg.
Pyridoxine	1 mg.	$MnCl_2 \cdot 4H_2O$	0.4 mg.
Thioglycollic acid	0.1 cc.	$MgSO_4 \cdot 7H_2O$	0.45 gm.
	H_2O to make final volume 1 liter		

The organisms continued to grow well after 10 serial subcultures in this medium.

Preparation of Medium

Directions are for 1 liter.

Basal Medium.—Dissolve 20 gm. casamino acids Difco, 1 gm. $Na_2HPO_4 \cdot 12H_2O$, 3 gm. $KH_2PO_4 \cdot 2H_2O$, 200 mg. cystine hydrochloride, and 8 mg. phenol red in 300 to 400 ml. distilled water. After adjusting pH to between 7.0 and 7.3 with 20 per cent NaOH, add 100 mg. $CaCl_2$. Boil for 5 minutes and filter through paper. To the filtrate add 20 mg. tryptophan, 1 mg. nicotinic acid, and 1 gamma biotin. Make volume to 500 ml. with distilled water, and autoclave at 15 pounds pressure for 20 to 25 minutes.

To the basal medium, add aseptically:—

Addition mixture (made by dissolving 10 mg. thiamine, 10 mg. pyridoxine, 200 mg. glutamine, and 1 gm. $CaCl_2 \cdot 2H_2O$ in 100 ml. distilled water and sterilizing by filtration)	10 ml.
Salt mixture (made by dissolving 50 mg. $CuSO_4 \cdot 5H_2O$, 50 mg. $ZnSO_4 \cdot 7H_2O$, 20 mg. $MnCl_2 \cdot 4H_2O$, 22.5 gm. $MgSO_4 \cdot 7H_2O$, and 1.0 ml. concentrated HCl in 100 ml. distilled water)	2 ml.
8 per cent $KHCO_3$ (made by autoclaving separately 80 gm. $KHCO_3$ and 1000 ml. distilled water, and mixing aseptically while water is warm)	10 ml.
M/2 $Na_2HPO_4 \cdot 12H_2O$ previously autoclaved	100 ml.
20 per cent glucose sterilized in the autoclave at 10 pound pressure for 10 minutes	50 ml.
$Fe_2(SO_4)_3$ solution (made by dissolving with the aid of a little HCl, 357 mg. $Fe_2(SO_4)_3$ in 1000 ml. distilled water and autoclaving	5 ml.
Thioglycollic acid aseptically neutralized with 20 per cent NaOH. The thioglycollic acid should not be added until just before the medium is inoculated	0.1 ml.
Sterile distilled water	328 ml.

Growth of Other Strains

A number of strains of *Cl. septicum* have been tested on the medium described and the results are summarized in Table II. Four of the six strains tested grew well while two strains grew poorly. The addition of 5 per cent normal horse serum to the medium had little or no effect on growth, except on strain 59, the growth of which was significantly improved by serum. The same six strains were also grown in veal infusion broth containing 5 per cent normal horse serum and 1 per cent glucose. The growth in this medium (Table II, column 4), on the whole was not as satisfactory as in the casein hydrolysate medium.

The nutritional requirements of strain 59 were further investigated. As can be seen in Table II, this strain grew very slightly in the casein hydrolysate medium, although greater growth occurred when horse serum was added to the medium. It was found that the growth-promoting factor present in horse serum can be replaced by pantothenic acid. The addition of 1 gamma of pantothenic acid to 20 ml. of the casein hydrolysate medium previously described supported luxuriant growth of strain 59. There exists the possibility that other strains of *Cl. septicum* require this factor for growth, and it may be desirable, therefore, to include pantothenic acid as a component of the medium which has been described.

Factors Influencing Toxin Production

Dissociation of Strains with Loss of Toxigenicity.—Serial subcultures of *Cl. septicum* in casein hydrolysate medium tended to contain less hemolysin than the primary culture. Table III is a record of the hemolysin content of subcultures of three strains transplanted daily. On direct test in mice, several of the cultures which became devoid of hemolytic activity were found to be completely lacking also in toxic activity.

Transfer of the initial cultures to blood agar plates yielded exclusively or predominately smooth hemolytic colonies, while plates inoculated from subcultures containing little or no toxin contained colonies most or all of which were rough and non-hemolytic. These findings suggested that only smooth colonies are toxigenic and hence a number of smooth and a number of rough

TABLE II

Comparison of Growth of Various Strains in Three Different Media

Strain No.	Bacterial nitrogen per 100 ml. of culture		
	No. 1. Casein hydrolysate medium	No. 2. Casein hydrolysate medium containing 5 per cent normal horse serum	No. 3. Veal infusion serum broth*
	mg.	*mg.*	*mg.*
44	19.0	20.0	9.0
BX96	18.3	17.0	12.0
23b	23.1	26.0	7.8
32	6.2	7.9	10.3
59	3.5	8.0	10.3
15192	16.4	17.3	7.8

* This medium contained 5 per cent normal horse serum and 1 per cent glucose.

colonies of strain 44 were tested for toxigenicity. As colonies inoculated directly into casein hydrolysate medium sometimes failed to grow out, the colonies were inoculated first into cooked-meat broth, and then into casein hydrolysate medium. The cultures from smooth colonies uniformly yielded appreciable hemolysin and toxin, while those from rough colonies yielded either very little or none. There was some evidence also, that all of the smooth colonies were not equally toxigenic.

In later experiments, however, smooth colonies which lacked a zone of hemolysis and which, on subcultivation in fluid media, produced no hemolysin, were frequently observed. The capacity to produce hemolysin is, therefore, not invariably associated with colonies in the smooth phase.

Strain.—In addition to strain 44, the production of hemolysin by five other strains was studied. Although growth of some of the strains exceeded that of strain 44, none of the cultures contained greater hemolytic activity. These strains were not studied with reference to dissociation.

Time, Temperature, and pH.—Study of a large number of cultures whose growth and hemolytic activity were measured at hourly intervals showed that the lethal and hemolytic activities both attain a maximum value, and then decrease, at first rapidly and later more slowly. Except for a slight initial lag, the hemolytic activity paralleled the growth; both hemolytic activity and "growth" as measured by turbidity, reached maxima within 1 or 2 hours of each other. Decline in hemolytic activity was accompanied by decrease in

TABLE III

Loss of Capacity of Cl. septicum to Form Hemolysin When Serially Subcultured in Defined Medium

	Hemolytic activity		
	Strain No.		
	44	23	BX96
	H.U./ml.	*H.U./ml.*	*H.U./ml.*
Initial culture	18	12	13
1st subculture	25	13	11
2nd "	2	0	13
3rd "	0	14	17
4th "	0	16	11
5th "	0	16	11
6th "	0	0	9
7th "	0	2	11
8th "	0	5	10
9th "	0	0	10
10th "	0	0	2
11th "	0	0	2
12th "	0		3
13th "	0		3
14th "	0		
15th "	0		
16th "	0		
17th "	0		

turbidity, probably due to autolysis. When other variables are held constant, the time at which the maximum hemolysin and toxin is reached depends on the size and age of the inoculum. By using a standard frozen inoculum, thawed immediately before use, the time of maximum hemolysin content of a culture could be predicted to within an hour or so. 500 ml. of medium inoculated with 0.1 ml. of standard inoculum contained the maximum amount of hemolysin in about 27 hours.

The maximum hemolysin content of cultures incubated at 30°C. was as great as that of cultures incubated at 37°C.

In spite of the fact that the medium was heavily buffered with phosphate,

the pH of cultures fell from neutrality to 6.0 or less. There is no evidence from the experiments performed, however, that the final pH attained by cultures either hinders the formation of hemolysin or hastens its destruction. The hemolysin content of cultures maintained at or near neutrality by the periodic addition of alkali has not been appreciably greater than that of unneutralized cultures.

Growth.—Provided the inoculum consists of smooth, toxigenic organisms, the production of toxin and hemolysin appears to be directly proportional to growth. Cultures of 6 liters in volume have consistently yielded growth equivalent to 12 mg. bacterial N per 100 ml., hemolysin amounting to 25 to 40 hemolytic units per ml., and toxin whose potency was 400 to 700 L.D.$_{50}$ per ml.

The Absence of Hemolysin in Cooked-Meat Broth Cultures.—Cl. septicum has been cultivated for various purposes during the course of this investigation in tubes of cooked-meat broth. This medium consisted of beef heart infusion

TABLE IV

Partial Inactivation of Hemolysin by Cooked Meat

	After 0 min. at 37°	After 30 min. at 37°	After 60 min. at 37°
	H.U./ml.	*H.U./ml.*	*H.U./ml.*
Hemolysin alone	22	22	22
" plus washed cooked meat	—	18	13
" " ether-extracted, washed cooked meat	—	—	19

broth to which was added before autoclaving particles of previously infused beef heart. Cultures grown in this medium never contained detectable hemolysin or toxin, even when the growth was allowed to become very heavy by the addition of glucose to the medium. An explanation of the absence of hemolysin in these cultures was afforded by the observation that cooked meat inactivated hemolysin at an appreciable rate, while the supernatant fluid of cooked-meat broth did not. Table IV shows that inactivation of hemolysin proceeded at the rate of 9 hemolytic units per ml. per hour in the presence of washed cooked meat. This rate of inactivation probably equals or exceeds the rate of hemolysin formation in growing cultures, and may account for the absence of hemolysin in cooked-meat broth cultures.

As a test of the correctness of this line of reasoning, the hemolysin produced in casein hydrolysate medium to which cooked meat had been added, was estimated and found to be negligible (less than 1 hemolytic unit per ml.) while a similar culture containing no cooked meat contained 25 hemolytic units per ml. The amount of growth in the two cultures was approximately the same.

The hemolysin-destroying factor could be removed from the cooked meat by ether extraction (see Table IV). An aqueous suspension of ether-extractable lipid, however, was not found to be active in promoting the destruction of hemolysin. The demonstration of a hemolysin-destroying factor in cooked meat is of interest in connection with the observations of Wuth (8, 9) and Menk (10) on the inhibitory action for bacterial toxins, of lipids present in emulsions of brain and other organs.

DISCUSSION

It has been considered desirable to develop a protein- and peptone-free medium suitable for the cultivation of *Cl. septicum* and for the investigation of the factors underlying toxin production. Since hydrolysates of either gelatin or casein, supplemented with tryptophan and cystine, serve as adequate sources of amino acids, and in view of the knowledge that the kind and number of amino acids essential for the growth of different strains of the same species of bacterium are frequently dissimilar, the amino acid requirements were not investigated further.

Not less than four vitamins - thiamine, pyridoxine, nicotinic acid, and biotin - are needed for the growth of *Cl. septicum*. The vitamin requirements, however, are not as numerous as those of *Cl. tetani*, which, in addition to thiamine, include adenine or hypoxanthine, pantothenic acid, riboflavin, folic acid, and probably biotin (5).

Inasmuch as the substitution of an hydrolysate of technical casein for gelatin hydrolysate led to increased growth, and since increasing the concentration of the former from 1 per cent to 2 per cent caused a further increase in growth, it would appear that at least one, and possibly several growth-stimulating factors of unknown identity are present in technical casein.

Although the medium described will support the continued growth of *Cl. septicum*, an inoculum consisting mainly or exclusively of smooth, toxigenic organisms gives rise to non-toxigenic dissociants, which in the course of serial subcultivation dominate the culture population. It would appear, therefore, that the medium as it is now constituted favors the growth of non-toxigenic dissociants over that of toxigenic organisms, and that the addition to this medium of a substance or substances capable of maintaining the organisms in the smooth, toxigenic phase would be a further advance.

Aside from its theoretical interest, the finding that non-toxigenic dissociants may appear in cultures has implications of a practical nature. The inoculum used for toxin production must consist exclusively of toxigenic organisms in order to obtain a maximum yield of toxin. Since non-toxigenic organisms may appear during the growth of a single culture, organisms to be used for toxin production should not be serially subcultured unless it is known that the subcultures remain free from non-toxigenic dissociants.

Parallelism in the production of hemolysin and toxin has been consistently observed throughout the present study. By reason of this parallelism, hemolytic activity has been used in a number of experiments as an indicator of toxic activity. When other variables are controlled, hemolysin production parallels growth so that cultures contain maximum hemolysin at or near the time when growth reaches a maximum. Thereafter, the hemolysin content of cultures diminishes.

The literature contains numerous statements to the effect that certain substances improve toxin production. The evidence in support of these statements, however, is usually absent or incomplete. Substances such as glucose and phosphates unquestionably influence toxin production, but their action is indirect in that suitable concentrations permit greater growth and therefore more toxin than would be obtained otherwise. Although low concentrations of iron have a pronounced effect upon growth, up to the present time there is no clear-cut evidence on the question of whether iron affects toxin production independently of growth, as is the case with the diphtheria bacillus (11).

Animal passage has been employed to raise the level of toxigenicity of strains of *Cl. septicum*. The mechanism which may underlie this effect is suggested by the foregoing observations on the difference in toxigenicity of dissociants, and by the fact that we have not succeeded in attaining greater yields of toxin by passage of smooth toxigenic cultures of strain 44 through mice or guinea pigs.

SUMMARY

A medium consisting of a complete acid hydrolysate of casein supplemented with cystine, tryptophan, growth accessory factors, glucose, and inorganic salts, has been developed for the cultivation of *Clostridium septicum*. Toxin equivalent to 400 to 700 L.D.$_{50}$ per ml. has been obtained regularly in this medium. The principal factors found to affect the yield of toxin are:—

(*a*) Phase of strain employed: Cultures of *Cl. septicum* may contain a number of variants. Some of these may be characterized by their colonial morphology on blood agar as: (1) rough, non-hemolytic colonies; (2) smooth, non-hemolytic colonies; (3) smooth, hemolytic colonies. Of these three variants, only the last produces toxin.

(*b*) Quantity of growth and length of time cultures were incubated: The toxin and hemolysin content of cultures increases as the bacterial population increases, reaching a maximum value when the number of bacteria is at a maximum, or shortly thereafter. Upon further incubation, the toxin and hemolysin content decreases.

(*c*) Presence of a hemolysin-inactivating factor: A substance, possibly lipid, and present in cooked meat, is capable of inactivating hemolysin produced in casein hydrolysate medium and can account for the absence of hemolysin from cultures grown in cooked-meat broth.

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