FACTORS INVOLVED IN PRODUCTION OF CLOSTRIDIUM WELCHII ALPHA TOXIN*

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Various simplified media have been described in which *Clostridium welchii* grows well but produces no alpha toxin (1–3). Macfarlane and Knight (4) showed that the addition of autolyzed muscle to a medium which is satisfactory for growth enhanced the production of alpha toxin. Rogers and Knight (5) presented evidence indicating that the effect of autolyzed muscle was in part due to glucosamine. Logan, Tytell, Danielson, and Griner (6) used a pancreatic digest of beef heart as a source of "toxin factor" and furthermore demonstrated the importance of dextrin as a source of carbohydrate in obtaining maximum yields of alpha toxin with certain strains of *Cl. welchii*. Adams and Hendee (7) used a pancreatic digest of casein for "toxin-promoting factor" and confirmed the finding of Logan *et al.* that dextrin was an important factor in obtaining high yields of toxin.

In the course of the present investigation it has been found that for the production of maximum yields of *Cl. welchii* alpha toxin, at least two factors are required in addition to dextrin and the basic growth requirements. One of these factors is present in enzymatic digests of certain proteins such as casein and gelatin. The second factor is present in extracts of pancreas and gastric mucosa. Glycerylphosphorylcholine is responsible, at least in part, for the toxin-promoting activity of pancreas and gastric mucosa.

Methods

Assay of toxin.—The lethal, hemolytic, and necrotizing alpha toxin produced by *Cl. welchii* has been demonstrated to be an enzyme, lecithinase (4). This toxin may be assayed by its lethal effect on intravenous injection into mice, or by the development of turbidity when incubated with human serum (8) or with lecitho-vitellin (9). Amounts of toxin larger than 100 mouse M. L. D. per ml. are more readily assayed by neutralization of the toxin with standard antitoxin, the end-point of the titration being determined by the inhibition of the lecithovitellin reaction (10). The amount of toxin which is just completely neutralized by one international unit of antitoxin has been designated one Lb (antitoxin-binding dose) and is equivalent to from 70 to 100 mouse M.L.D. of fresh toxin (11).

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Culture.—The PB6K strain of *Cl. welchii* obtained from the National Institute of Health has been used throughout this study. Organisms of this strain grow well on media containing an acid hydrolysate of casein or gelatin reinforced with certain amino acids and growth factors (12), but on such media produce almost no alpha toxin. However, when particles of meat, or certain peptones are added to these acid hydrolysate media, toxin is produced.

Certain lots of tryptone (Difco), a commercial pancreatic digest of casein, when added to an acid hydrolysate of casein medium stimulated the production of as much as 400 M.L.D. per ml. of alpha toxin. However, most lots of tryptone are much inferior to this.

EXPERIMENTAL

Preliminary Attempts to Isolate the Toxigenic Factors.—Attempts to isolate the "toxin-promoting factor" from tryptone by butyl alcohol extraction, precipitation with heavy metals, by adsorption to ion exchange resins, and by electrodialysis failed. The activity was distributed over the various fractions and the ratio of activity to nitrogen in the fractions was not greatly increased over the starting material.

Since different lots of tryptone varied greatly in toxin-promoting potentiality it became necessary to develop methods for the preparation of a uniformly effective pancreatic digest of casein. Such digests yielded 600 M.L.D. and 8 Lb of alpha toxin per ml. of culture medium (7). These digests were used in further attempts to isolate the toxin-promoting factor. It was found that treatment of the pancreatic casein digest with calcium phosphate or alumina gel resulted in complete loss of activity, and that charcoal removed some activity. Only very small amounts of toxin-promoting activity could be eluted from these adsorbents. A considerable amount of activity was soluble in 95 per cent ethanol and could be precipitated as an oily layer on the addition of acetone. Little purification was achieved by these methods. It was evident that isolation of the factor from a pancreatic digest of casein would be a difficult problem. It was thought that the problem might be simplified by digesting the casein with purified enzymes, thus avoiding the addition of the autolytic products of pancreas.

Samples of casein were digested with ficin, papain, trypsin, chymotrypsin, and pancreatin. In each case some toxin-promoting factor was released but the maximum yield of toxin was 2 Lb per ml. of culture medium. With pancreatic digests of casein tested under identical conditions the toxin yield was 6 to 8 Lb per ml. These experiments would seem to indicate that the pancreas made some specific contribution to toxin production in addition to furnishing proteolytic enzymes to digest the casein. Consequently, extracts of pancreas were examined for specific toxin-stimulating effect.

The Multiplicity of Toxin-Promoting Factors.—Beef pancreas was homogenized in the Waring blendor with an equal volume of water, allowed to autolyze overnight at 37°C. under toluene, adjusted to pH 4.0, heated to 60°C., and filtered. The filtrate was tested for toxin-promoting factor with acid-hydrolyzed casein and with trypsin-digested casein. The basal medium used and the conditions of the test are described later. Dextrin was the carbohydrate used and the acid production indicated adequate growth of the cultures.

The experiment recorded in Table I indicates that at least two factors are involved in the production of high yields of alpha toxin. One factor is present in enzymatic digests of casein but absent from acid hydrolysates of casein while the second factor is present in autolyzed beef pancreas. The pancreatic factor was found to be present also in lamb and hog pancreas and in smaller amount in yeast extracts.

It has been observed by Taylor (13) that autolyzed hog stomach permits the production of unusually high yields of tetanal toxin with a particular strain of *Cl. tetani*. We have found that autolyzed hog stomach can replace pancreatic

TABLE I

Toxin-Promoting Activity of Autolyzed Pancreas and of Trypsin-Digested Casein

Materials tested	Final pH	Toxin yield
		Lb/ml.
1. Autolyzed pancreas + acid-hydrolyzed casein	5.2	1
2. Trypsin-digested casein	5.3	2
3. Autolyzed pancreas + trypsin-digested casein	5.5	6

TABLE II

The Toxin-Promoting Activity of Digests of Mucosa and Muscle of Hog Stomach

Materials tested	Final pH	Toxin yield
		Lb/ml.
1. Mucosa digest alone	5.2	<1
2. Muscle digest alone	5.4	<1
3. Mucosa digest + muscle digest	5.5	4
4. Trypsin-digested casein alone	6.3	2
5. Casein digest + muscle digest	6.4	3
6. Casein digest + mucosa digest	6.1	6

digests of casein as a source of the toxin-promoting factors for *Cl. welchii*. Furthermore, hog stomach can be mechanically separated to yield two factors analogous to the casein factor and the pancreas factor.

The mucosa was carefully dissected from the muscle of hog stomach and the two portions were separately homogenized in the Waring blendor. Each portion was digested with ficin at pH 6.5 for 40 hours at 40°C. under toluene. The digests were then acidified to pH 4.5, heated to 60°C., filtered, and assayed for toxin-promoting factors, using dextrin as carbohydrate source.

The data of Table II again demonstrate that at least two factors are involved in the production of high yields of alpha toxin. The hog stomach mucosa contains the same factor as does pancreas, while the hog stomach muscle

contains the casein factor, and both factors must be present for maximum yield of toxin.

Preparation and Assay of Toxin-Promoting Factors of Casein and Pancreas.—

Casein Factor: Suspend 50 gm. of vitamin-free casein in 500 ml. of water and adjust to pH 6.0. Add 500 mg. of ficin (Merck) and incubate at 40°C. for 24 hours maintaining pH between 5.6 and 6.0. Toluene is added as preservative. After digestion the pH is adjusted to 4.0, the material is heated to 60°C., and insoluble material is removed by filtration. The toxigenic properties of the filtrate remain unchanged on storage under toluene for several months in the refrigerator.

Pancreatic Factor: Ten pounds of fresh beef pancreas are cut in small pieces and well washed. The pancreas is then homogenized in the Waring blendor with an equal volume of water. Alcohol is added to a final volume of 18 liters and a final concentration of about 50 per cent. The mixture is kept at 37°C. for several hours and in the refrigerator overnight. The mixture is then acidified with HCl to pH 4.5, heated to 60°C., and filtered, yielding 15 liters of clear yellow filtrate.

Dextrin: Suspend 300 gm. of white dextrin (Merck NFV) in 2 liters of water at 60°C. and allow to settle. The supernatant liquid is discarded and the insoluble dextrin filtered off and dried. The yield is about 50 per cent by weight. For use as a carbohydrate source it is suspended in water, 20 gm. per 100 ml., and autoclaved.

Stock Basal Medium:—

$Na_2HPO_4 \cdot 12H_2O$	11.5 gm.
KH_2PO_4	1.0 gm.
$MgSO_4 \cdot 7H_2O$	0.4 gm.
Cystine·HCl	40 mg.
Tryptophane	50 mg.
Growth factor mixture	2 ml.

Water is added to a volume of 1 liter and the pH adjusted to 7.6. The growth factor mixture contains:

Nicotinic acid	20 mg.
Thiamin	10 mg.
Pyridoxine	20 mg.
Calcium pantothenate	30 mg.
Riboflavine	5 mg.
Biotin	5 micrograms

and water to 20 ml.

Assay of Activity of Pancreatic Factor: For each assay the following ingredients are placed in a wide test tube:

Stock basal medium	10 ml.
Casein factor	6 ml.
Pancreatic factor	Various amounts
Dextrin	0.9 ml.
Ferrous sulfate (0.01 per cent solution)	0.4 ml.
Water to a final volume of 20.0 ml.	

The pH is readjusted to 7.6 or if preferred the casein and pancreatic factors can be adjusted to pH 7.6 before addition to the basal medium. The medium may be autoclaved if desired, but in routine testing it was made up as required from the stock solutions and sterilized in a boiling water bath for 15 minutes. After cooling, 0.2 ml. of 1 per cent thioglycollic acid and 0.5 ml. of inoculum are added, and the cultures are incubated aerobically at 32°C. for 16 hours. The inoculum is taken from a 6 hour culture of *Cl. welchii* grown in a medium similar to the test

medium except that casamino acids (Difco) are used in place of the casein digest, glucose in place of dextrin, and the pancreatic factor is omitted.

After incubation of the assay cultures, the organisms are removed by centrifugation, and the amount of toxin in the supernatant fluid is determined by titration against antitoxin using lecitho-vitellin as indicator of the titration end-point.

Under these conditions 2.5 ml. of pancreatic factor per 20 ml. of medium will yield 7 to 8 Lb of *Cl. welchii* alpha toxin per ml. of culture medium, or 140 to 160 Lb of toxin per test. The dry weight of 2.5 ml. of pancreatic factor is 220 mg. and the Kjeldahl nitrogen content is 23.5 mg. or 11 per cent of the dry weight. The yield of alpha toxin is then 730 Lb per gm. dry weight, or 6,800 Lb per gm. nitrogen of the crude pancreatic factor.

Properties and Attempted Purification of the Pancreatic Factor.—The crude pancreatic factor is stable on heating for 1 hour at 100°C. at any pH between 1.2 and 11.3. It is destroyed, however, by autoclaving for 1 hour at 120°C. at pH 1 or at pH 12. The active factor readily dialyzes through a cellophane membrane.

The 15 liters of crude pancreatic factor described above were concentrated *in vacuo* to 2.5 liters. Considerable inactive material came out of solution and was removed by filtration. The filtrate was further concentrated *in vacuo* to 1 liter and placed in the refrigerator overnight. Much more inactive material separated and was removed by centrifugation. The active supernatant was further concentrated *in vacuo* to 500 ml. forming a thick syrup. This syrup was heated to 80°C. and poured with stirring into 2.5 liters of hot ethanol. A heavy gum separated from the alcohol and after cooling the clear supernatant liquid was decanted and concentrated *in vacuo* to 500 ml. On standing in the refrigerator an inactive precipitate separated and was removed by centrifugation. The clear supernatant liquid is called the "alcohol-soluble fraction."

The heavy gum that separated from the 2.5 liters of alcohol was dissolved in 1 liter of water. On standing in the refrigerator an inactive precipitate separated and was removed by centrifugation. The active supernatant liquid was concentrated *in vacuo* to 600 ml. and is called the "alcohol-insoluble fraction."

The alcohol-insoluble fraction was further purified by adsorption to charcoal and elution. A 20 ml. sample of this fraction was adjusted to pH 4, and 100 ml. of 40 per cent alcohol and 1 gm. of charcoal were added. The mixture was shaken vigorously for 2 hours. After removal of the charcoal by centrifugation, the supernatant proved to be almost inactive. The charcoal was shaken vigorously with three 100 ml. portions of water adjusted to pH 9.0. The pooled washings were concentrated *in vacuo* to 20 ml. and proved to be quite active. Kjeldahl nitrogen determinations were made on the various active fractions, and titrations of the toxin-promoting activity as described above were also made. The results including the activity in Lb of toxin per milligram of nitrogen as calculated above are recorded in Table III.

The data in Table III indicate that a very considerable degree of purification of the pancreatic factor on the basis of nitrogen content has been achieved. However, with none of the fractions was it possible to achieve as high a concentration of alpha toxin in the culture medium as was obtained with crude pancreatic factor. Purification entailed the loss of some material which contributes to maximum yields of toxin.

Attempts were made to further fractionate the pancreatic factors using various solvents, mercury and lead salts, picric and phosphotungstic acids, and electrodialysis, without achieving a clear cut fractionation into active and inactive fractions. Usually both fractions in any given experiment had some

toxin-promoting activity but the maximum concentration of toxin obtainable with a single fraction was 3 to 4 Lb per ml. of culture medium regardless of how much of the fraction was added to the test medium. If the two fractions of a single experiment were added together to the test medium it was sometimes possible to obtain a maximum toxin yield of 5 to 6 Lb per ml. of culture medium. This would seem to indicate that the pancreatic factor is a mixture of two or more substances each of which contributes something to the maximum toxin production.

TABLE III

Titrations of the Toxin-Promoting Activity of Various Fractions of the Pancreatic Factor

Fraction added in terms of mg. of nitrogen/20 ml. of medium		Final pH of medium	Toxin per ml. of medium	Toxin per mg. N
			Lb	*Lb*
1. Crude pancreatic factor	7	5.1	3	8.5
	14	5.3	5	7.1
	23.5	5.5	8	6.8
2. Alcohol-soluble fraction	3.1	5.0	2	13
	6.2	5.2	4	13
	9.6	5.1	5	10.4
	12.4	5.1	6	10.0
	18.6	5.0	4	4.4
3. Alcohol-insoluble fraction	3.1	5.2	4	26
	6.2	5.1	5	16.1
	9.3	5.3	4	9
4. Charcoal eluate of alcohol-insoluble fraction	0.9	5.2	3	67
	1.8	5.5	5	55
5. No pancreatic factor added	—	5.0	1	—

The alcohol-insoluble fraction of the pancreatic factor was digested under appropriate conditions of temperature and pH with active preparations of pepsin, trypsin, chymotrypsin, papain, ficin, crude pancreatin, and crude intestinal peptidase without alteration of its toxin-promoting activity and without converting it into an alcohol-soluble form.

The alcohol-soluble and alcohol-insoluble fractions of the pancreatic factor had little or no synergistic effect on toxin production when added together to the test medium.

Possible Rôle of Lecithin and Glycerylphosphorylcholine in Toxin Production.—Since the alpha toxin of *Cl. welchii* is an enzyme, lecithinase, it was thought that the production of the toxin might be stimulated in the manner of an adaptive enzyme by including the substrate lecithin, or a related compound in the culture medium. Lecithin was prepared from egg yolk

by precipitating the yolks with 10 volumes of acetone. The precipitate, after thorough washing with acetone, was extracted twice with benzene. To the combined benzene extracts was added 10 volumes of acetone to precipitate the lecithin. The lecithin was redissolved in chloroform and reprecipitated with acetone three times yielding a colorless preparation. A dry weighed sample was dissolved in a small amount of ether and run into a volume of hot water calculated to yield a 1 per cent aqueous suspension of lecithin.

Glycerylphosphorylcholine was isolated from autolyzed beef pancreas by the method of Schmidt (14). From 10 pounds of pancreas, 3 gm. of the $CdCl_2$ complex were obtained with the following analysis:

Found: Cd—30.3 per cent, Cl—18.6 per cent, P—5.08 per cent, N—2.72 per cent, Choline —18.3 per cent

Calculated for $C_8H_{20}O_6NP \cdot Cd_2Cl_4$: Cd—36.0 per cent, Cl—22.7 per cent, P—4.97 per cent, N—2.25 per cent, Choline—19.4 per cent

For testing, a weighed amount of the $CdCl_2$ complex was dissolved in water and treated with excess H_2S to remove the cadmium. After filtration, the phosphorous content was

TABLE IV

Titration of the Toxin-Promoting Activity of Lecithin and of Glycerylphosphorylcholine

Substances added to the test medium	Final pH of medium	Toxin per ml. of medium
		Lb
1. No addition	5.6	1*
2. 2 mg. lecithin	5.5	2
3. 5 mg. lecithin	5.5	2+
4. 10 mg. lecithin	5.5	2++
5. 1.67 mg. glycerylphosphorylcholine	5.5	2
6. 3.3 mg. glycerylphosphorylcholine	5.5	2+
7. 6.6 mg. glycerylphosphorylcholine	5.4	2+
8. 2 ml. of pancreatic factor	5.7	7

* 2+ indicates more than 2 Lb but less than 3 Lb toxin per ml.

checked to determine the actual concentration of glycerylphosphorylcholine in solution.

The lecithin and glycerylphosphorylcholine were tested for toxin-promoting activity in the same way as the pancreatic factor (Table IV).

The data of Table IV indicate that there is some toxin-promoting activity in both lecithin and glycerylphosphorylcholine; however, neither of these substances can account for more than a small part of the toxigenic activity of the pancreatic factor. Neither lecithin nor glycerylphosphorylcholine when added to any of the fractions of the pancreatic factor described above caused any further increase in toxin production.

Bacterial Growth and Toxin Production.—Another property of the pancreatic factors which is probably involved in the production of maximum yields of toxin is stimulation of bacterial growth. Both Logan (6) and Ballentine (15) have reported the existence of an unindentified growth factor which is essential for growth of *Cl. welchii*. We also have been unable to subculture this or-

ganism in a chemically defined medium unless small amounts of pancreas extract or yeast extract are added to the medium. Little is known of the chemical nature of this growth factor except that its properties are not like those of the sporogenes vitamin (16).

The test medium described above, with an excess of sucrose substituted as carbohydrate source, will yield bacterial growth equivalent to 10 to 15 mg. of bacterial nitrogen per 100 ml. of culture without added pancreatic factor. If pancreatic factor is added in an amount optimal for toxin production the bacterial growth is increased to 30 mg. of bacterial nitrogen per 100 ml.

A crude preparation of the growth factor from yeast autolysate was made according to the directions of Ballentine (15). The addition to the test medium of enough of this factor to insure maximum bacterial growth increased the yield of toxin from 1 Lb per ml. to 3 Lb per ml. When in addition lecithin or glycerylphosphorylcholine was added the toxin yield was further increased to 4 lb. per ml. of culture medium.

It would appear from these experiments that the pancreatic factors include some unknown growth factor which improves toxin production by stimulating bacterial growth; a stimulus to adaptive enzyme production which is probably glycerylphosphorylcholine; and other as yet unknown factors, since the two mentioned only partially account for the toxin-promoting effects of the pancreatic extracts.

Assay of Activity of the Casein Factor.—The toxigenic activity of the casein factor was assayed in the same test medium as was used for assay of the pancreatic factor except that 1 per cent of casamino acids (Difco) (an acid hydrolysate of casein) was used as a source of amino acids instead of the ficin-digested casein, and an optimal amount of pancreatic factor for toxin production was added. On such a medium excellent growth of *Cl. welchii* was obtained. The assay of the toxin-promoting effect of ficin-digested casein is recorded in Table V.

It is apparent from the data of Table V that the yield of toxin is directly proportional to the amount of casein digest added up to a maximum toxin yield of 8 Lb per ml. The casein digest contained 16.5 mg. N per ml. of digest by Kjeldahl. Since the total culture volume per tube is 20 ml., the toxin yield is 1600 Lb per gm. of casein digest nitrogen added.

The casein factor is stable to heating for 1 hour at 100°C. at all pH values between 1.2 and 11.2. It is destroyed by autoclaving for 1 hour at 120°C. at pH 0.5, but about half the activity survives autoclaving at pH 11.

Attempts were made to replace the casein factor with a mixture of known amino acids in the same proportion as these occur in casein. Included were glycine, alanine, valine, leucine, isoleucine, proline, phenylalanine, glutamic acid, aspartic acid, serine, tyrosine, cystine, histidine, arginine, lysine, tryptophane, methionine, threonine, and asparagine. Added growth factors included adenine, uracil, oleic acid, biotin, pantothenic acid, nicotinic acid, riboflavine, thiamine, pyridoxine, and pimelic acid. Also included were magnesium, phosphate, ammonium chloride, and traces of Fe, Cu, Zn, and Mn. With sucrose as carbohydrate growth was

rather poor, but on addition of pancreas factor or yeast extract excellent growth was obtained. On this medium enriched with pancreatic factor and with dextrin as carbohydrate source, only 1 Lb toxin per ml. of medium was obtained. It would seem that there must be some substance or combination of substances present in enzymatic digests of casein which is essential for production of large yields of toxin by *Cl. welchii*.

In addition certain pure amino acids alone and in various combinations were added to acid hydrolysates of casein to see if it were possible by this means to recreate the casein factor. In particular acid-labile amino acids such as asparagine, glutamine, arginine, and tryptophane were tested with no beneficial effect on toxin yield.

Among other substances tested for possible toxigenic activity were uric acid, xanthine, *p*-aminobenzoic acid, guanine, choline, inositol, phosphocholine, and folic acid. All were inactive as either casein or pancreatic factors.

TABLE V

Titration of the Toxin-Promoting Activity of Ficin-Digested Casein in Presence of Pancreatic Factor, Casamino Acids, and Dextrin

Ficin-digested casein added per 20 ml. total volume of medium	Final pH of medium	Toxin per ml. of medium
ml.		*Lb*
None added	5.1	1
1.0	5.3	3
2.0	5.4	4
3.0	5.6	5
4.0	5.8	6
5.0	5.9	7
6.0	5.8	8
7.0	5.9	8
8.0	5.9	8
6.0 ml. of casein but without added pancreatic factor	5.4	2

A number of crude protein mixtures and several purified proteins were digested with ficin in the same manner as casein and tested for the presence of the casein factor by titration in the presence of adequate amounts of pancreatic factor and dextrin. The egg yolk proteins were obtained by precipitation of egg yolk with acetone followed by thorough extraction with acetone and benzene to remove lipids. The maximum yield of toxin obtained with each protein digest is recorded in Table VI.

From the data in Table VI it is evident that the casein factor is present in a number of proteinaceous materials but appears to be absent from a number of well defined proteins. Further work on the distribution of the casein factor may give some clue as to the nature of the factor, and at the same time yield information about components of proteins.

Rôle of Dextrin in Toxin Production.—Logan (6) has demonstrated the importance of the carbohydrate source in obtaining high yields of alpha toxin with the PB6K strain of *Cl. welchii*. He found that replacing glucose as a carbohydrate source with maltose, glycogen, or dextrin gave increasing yields of toxin

in that order, and that commercial white dextrin extracted with water gave the best results yielding up to eightfold more toxin than glucose. Our results are in complete agreement with those of Logan. The extraction of water-soluble materials from the dextrin is essential for obtaining highest toxin yields. To explain this beneficial effect of water extraction on the toxigenic activity of dextrin, various simple sugars such as glucose and sucrose were added with

TABLE VI

Maximum Toxin Yield from Various Ficin-Digested Proteins Tested with Pancreatic Factor and Dextrin

Protein digest tested	Toxin per ml. of medium
	Lb
Unwashed human red blood cells	5
Human plasma	4
Human pseudoglobulin	<1
Human serum albumin	1
Human fibrin	1
Armour fraction I of beef plasma	3
Beef fibrin	<1
Calf skin gelatin (Eastman)	6
Egg albumin	1
Crystalline hemoglobin	<1
Lipid-free egg yolk protein	5

TABLE VII

Effect of Added Sucrose on Toxin Production in Dextrin-Containing Media

Dextrin	Sucrose	Final pH in medium	Toxin per ml. of medium
mg.	*mg.*		*Lb*
90	—	6.1	7
180	—	5.8	8
90	90	5.6	2
—	90	6.1	1

the extracted dextrin to the test medium with the results shown in Table VII.

From the experiment summarized in Table VII it is evident that the addition of sucrose to a dextrin-containing medium depresses the toxin production from the high level of 8 Lb per ml. to almost the low levels that are usual with sucrose alone. This experiment serves to rule out several possible explanations of the effect of dextrin involving its colloidal properties. For instance Ley and Mueller (17) have explained the stimulating effect of starch on the growth of *Neisseria* in the starch, casein hydrolysate, agar medium as being due to a "detoxification" by the starch of some growth-inhibitory substances present in the agar.

Such an explanation cannot serve in the case of the effect of dextrin on toxin production.

This experiment also renders it very unlikely that the dextrin is serving as a "protective colloid" to minimize surface denaturation of the toxin. This possibility was further checked by adding gelatin to the complete medium with sucrose as the carbohydrate source. The yield of toxin was not increased over that of the control medium which contained sucrose but no added gelatin.

It also would seem unlikely that the stimulating effect of washed dextrin is due to small amounts of some impurity present in the dextrin. To test this possibility the dextrin was digested with salivary diastase with a resultant reduction in toxin yield from 8 Lb per ml. to 2 Lb per ml. of medium. The stimulation of toxin production seems to be peculiar to some property common to glycogen, starch, and dextrin. Lintner soluble starch (Pfanstiehl), which is stated to be free of erythro-dextrin and reducing sugar, is apparently lacking in this property since the toxin yield is only 2 Lb per ml., whereas with gelatinized potato starch the toxin yield is 6 Lb per ml.

It was thought that possibly a phosphorolytic breakdown of dextrin to glucose-*l*-phosphate with a consequent saving of phosphate bond energy might be involved in the toxin-stimulating effect of dextrin. Consequently, glucose-*l*-phosphate and fructose-diphosphate were tested as carbohydrate sources in the absence of other sugars. In both cases growth was very poor and no toxin was produced. When these phosphorylated sugars were added to the test medium with dextrin, toxin production was not depressed. Apparently the phosphorylated sugars cannot be utilized directly as carbohydrate sources by the organism, but this fact does not necessarily rule out the possibility of a phosphorolytic breakdown of the dextrin by a growing culture of *Cl. welchii*.

Among the simple sugars that are utilized for growth by *Cl. welchii* glucose, glucosamine, maltose, lactose, sucrose, trehalose, and inositol were tested and none gave higher yields of toxin than did maltose which gave about twice the yield of glucose. Raffinose and inulin were not utilized by this strain of *Cl. welchii*. Byers, Tytell, and Logan (18) have reported that hyaluronic acid is utilized by *Cl. welchii* as a carbohydrate source, and that in a medium that is otherwise suitable for toxin production, the use of hyaluronic acid results in the production of only 1 Lb per ml. of toxin, but that the production of hyaluronidase is greatly stimulated.

Apparently this great stimulating effect of carbohydrate source on toxin production is restricted to glycogen, starch, and dextrin, and the mechanism of the effect is not known.

Monod (19) has noted that the fermentation of dextrin by various species of bacteria may be completely suppressed by the presence in the medium of other fermentable carbohydrates such as glucose. None of the dextrin was fermented until all of the glucose present had been glycolyzed. If one assumes

that the production of high yields of *Cl. welchii* lecithinase is in some way associated with the fermentation of starch or dextrin, the depressing effect on toxin production of other fermentable sugars may be readily explained.

In many of the tables in this paper an increase in final pH of the medium appears to be correlated with an increase in yield of toxin. Experiments in which the final pH of the medium has been shifted between 5.0 and 6.2 by changing the relative concentrations of fermentable carbohydrate and buffer have demonstrated that toxin yield is independent of final pH over this range. However, the maintenance of a pH of 7.0 during the entire growth period by frequent additions of alkali resulted in almost complete suppression of toxin production and early autolysis of the culture. The increase in final pH occurring on addition of supplements of casein digest or pancreatic extract is due to the buffering capacity of these supplements and is not itself responsible for increased toxin production.

DISCUSSION

It has been demonstrated that for maximum production of *Cl. welchii* alpha toxin a number of factors must be present together in the medium in addition to the ingredients needed for growth.

One or more such factors are present in enzymatic digests of certain proteins such as casein and gelatin, but absent from digests of many other proteins. This factor is destroyed on acid hydrolysis of the proteins. It is readily dialyzable through membranes and is stable to autoclaving at neutral pH. It was not possible to replace this factor with any combination of the many known amino acids and growth factors tested.

A group of factors involved in toxin production is present in extracts of pancreas, gastric mucosa, and yeast. Part of the toxin-stimulating effect of these extracts is due to an unidentified growth factor which appears to be essential for maximum growth of some strains of *Cl. welchii*. Evidence is presented which suggests that part of the toxin-stimulating effect of pancreatic extracts may be due to glycerylphosphorylcholine. This degradation product of lecithin appears to act like lecithin as a stimulus to the adaptive production of the enzyme lecithinase, which has been shown by Macfarlane and Knight (4) to be identical with the alpha toxin of *Cl. welchii*. Since growth stimulation and adaptive enzyme stimulation together account for only part of the toxin-promoting effect of pancreatic extracts, it must be assumed that one or more additional unidentified factors are present which in combination with the growth factor and glycerylphosphorylcholine contribute to toxin production.

The fermentable carbohydrate used as energy source seems to be of the utmost importance for maximum yields of toxin. Of all the carbohydrates tested only glycogen, starch, and water-insoluble dextrin gave toxin yields more than double that obtainable with glucose. These three polysaccharides increased the toxin yield from 4 to 8 times the yield obtainable with glucose. The ad-

dition of mono- or disaccharides to the polysaccharide-containing media depressed toxin production to a low level. This effect rules out any explanation of the rôle of the polysaccharides involving their possible rôle as protective colloids, as bearers of toxin-promoting impurities, or in the neutralization of growth inhibitors. The toxin-stimulating effect of these polysaccharides seems to be due to some property common to glycogen, starch, and dextrin which is capable of acting only in the absence of other fermentable sugars.

CONCLUSIONS

Maximum production of the alpha toxin by *Cl. welchii* is dependent on the inclusion in the medium of several substances in addition to those required for growth. These factors include:

1. Some substance present in enzymatic digests of certain proteins such as casein and gelatine.
2. Glycerylphosphorylcholine and other substances that are present in extracts of pancreas.
3. The use of starch or dextrin as a carbohydrate source in the absence of other fermentable carbohydrates.

The omission of any one of these factors from the medium results in a very low yield of alpha toxin.

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