THE NUTRITIONAL REQUIREMENTS OF CLOSTRIDIUM PARABOTULINUM, A

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This investigation was undertaken with the purpose of developing a synthetic medium for *Clostridium parabotulinum, type A*, in order to study certain phases of the toxin-producing mechanism.

The results mainly confirmed the findings of Fildes and his coworkers (1933) with respect to the essential nature of certain substances in yeast and pregnancy urines. In addition, certain evidence is presented which indicates that other substances besides the "sporogenes vitamin" are required for growth of *C. parabotulinum* in amino acid mixtures. The nature of these materials will be the subject of subsequent notes.

HISTORICAL INTRODUCTION

Studies on the simplification of media for the cultivation of *Clostridium botulinum* have not often been the primary purpose of researches on this organism. In fact, it is only in recent years that synthetic media have been attained for some members of the obligate anaerobes, notably *Clostridium sporogenes*.

That the complexity of the nutritional requirements of *C. botulinum* and the need for accessory growth factors were realized is stated in a paper by Wagner, Meyer and Dozier (1925). Hosoya and Kishino (1925) demonstrated the importance of the sulfhydryl group in the growth requirements of *C. botulinum* and found that cysteine adequately supplemented deficient tryptic digests of gelatin. Along the same lines, Quastel and Stephenson (1926) found that the lack of reduced sulfur com-

pounds was the limiting factor in the inadequacy of acid and tryptic digests of gelatin, although similar digests of casein did support growth.

Anderson (1928) then attempted unsuccessfully to simplify the routine media for *C. botulinum* by using nucleic acids, ammonium compounds, and mixtures of many amino acids as sources of nitrogen.

In a specific attempt to determine the essential amino acids for growth and toxin production by *C. botulinum*, Burrows (1932) found that acid hydrolysates of casein, if supplemented with tryptophane, would support growth of A and B strains. Such hydrolysates were rendered unfit, by treatment with Norite or by filtration through Seitz pads. Burrows (1933) then reported the successful substitution of an amino acid mixture for the protein hydrolysate and stated that cystine, proline, and leucine were essential acids for *C. botulinum*.

A very intensive study on the nutritional requirements of *C. sporogenes* was summarized in a report by Knight and Fildes (1933) in which was detailed the preparation and proof of the essential nature of a substance in yeast and pregnancy urines. Furthermore tryptophane was found to be an essential amino acid for growth of *C. sporogenes*. These authors also determined that the "sporogenes vitamin" and tryptophane were essential for the growth of *C. botulinum*.

Burrows (1934) reinvestigated the tryptophane requirements of *C. botulinum* and found that this organism differed from *C. sporogenes* both in the tryptophane and "sporogenes vitamin" requirements.

Fildes (1935) returned to the study of the requirements of *C. botulinum*, confirmed his former work and reaffirmed the belief that the difficulty lay in the use by Burrows of impure preparations of amino acids containing tryptophane as an impurity.

Fildes and Richardson (1935) working with *C. sporogenes* finally succeeded in replacing the gelatin hydrolysate by a known mixture of amino acids, most of which had been synthesized, and all of which had been recrystallized several times. They concluded that tryptophane, leucine, phenylalanine, tyrosine and

arginine were indispensable amino acids, while for adequate growth and maintenance of the cultures valine, cystine, methionine, and histidine were required.

Stickland (1934, 1935a, 1935b) cleared up the problem of the source of energy for the organism growing in a medium composed chiefly of amino acids by showing that *C. sporogenes* is able to activate certain pairs of amino acids, one being oxidized, the other reduced.

As to the nature of the "sporogenes vitamin" Pappenheimer (1935) found that it was an acid (pK^1 4.7), whose empirical formula was $C_{11}H_{14}O_4$, that it was most stable as the methyl ester, and that it contained one double bond and one hydroxyl group.

Following this there appeared a paper by Hosoya, Kuwashima, Kayo, Oda, and Kagabe (1936) on a factor essential for "pathogenic bacteria." The source of their active preparation was an "aqueous extract of scomber." In amounts of 5×10^{-6} gamma per 10 ml. gelatin hydrolysate basal medium, growth of *C. botulinum* occurred. The paper deals mainly with the preparative aspects, while information concerning the methods of assay or nature of the material is lacking.

METHODS

Media

(a) Isolation of colonies and purification of strains from stock cultures were effected on blood plates prepared as recommended by Schoenholz (1928).

For carrying stock cultures a beef-heart medium was employed in which Neopeptone was used as the principal nitrogenous ingredient. The inclusion of minced pieces of beef heart at the bottom of the tubes made the use of vaseline seals unnecessary.

For the production of spores a casein-digest medium recommended by Sommer (1930) was used.

A medium which was found to initiate excellent growth, toxin production and enzyme formation contained a filtered infusion of beef heart to which were added 1 per cent glucose, 0.5 per

cent monobasic potassium phosphate and 4 per cent Difco proteose peptone. The final pH was adjusted to 7.4. Sterile petrolatum served as an efficient seal.

Inoculum

Several liters of spores of a carefully purified strain of *C. parabotulinum, type A*, were washed five times in sterile M/15 phosphate buffer, pH 7.0 and eventually suspended in sterile double-distilled water. After the purity and viability of the spores were tested, the suspension was heated at 80°C. for forty-five minutes, cooled to room temperature and aseptically removed in 25 cc. lots to a Flosdorf-Mudd "Lyophile" apparatus (1935). The spores were rapidly desiccated from the frozen state under high vaccum and came out of the apparatus in the form of a white powder, perfectly viable and toxigenic. They were stored in small vials and checked for purity, viability and toxigenicity at monthly intervals.

Preparation of protein hydrolysates

Gelatin and casein, having been shown to support growth of *C. botulinum* under specified conditions, were selected to supply the principal nitrogenous substances in the media to be simplified.

(a) Nelson's photographic gelatin was prepared in the hydrolyzed form as recommended by Fildes (1935).

(b) Washed isoelectric gelatin (Peter Cooper) was prepared according to the method of Northrop and Kunitz (1928) and hydrolyzed by refluxing eight hours with five times its weight of sulfuric acid in 30 per cent solution. The acid was exactly removed with baryta, and the filtrate concentrated *in vacuo* to a thick syrup.

(c) Unwashed Peter Cooper gelatin was hydrolyzed in the same way as (b) and worked up to a syrup.

(d) Casein (Schering-Kahlbaum, according to Hammarsten) was refluxed thirty-six hours with 30 per cent sulfuric and worked up to a syrup as in the previous cases.

"Vitamin" preparation

In the main the original procedure used to prepare the "sporogenes vitamin" followed that of Knight and Fildes (1935a). The active preparation from brewer's yeast was such that 1×10^{-6} gram activated 10 ml. of a deficient medium. The final product was diluted so that 0.1 cc. contained 1×10^{-6} gram of dry material.

EXPERIMENTAL

The medium upon which it was desired to improve was that proposed by Fildes (1935) for *C. botulinum*. It was made up as follows:

Sodium citrate	3.0	grams
Monobasic potassium phosphate	4.5	grams
Water	500	cc.
1N Sodium Hydroxide	26	cc.
Dissolve and add:		
l-cystine	0.05	grams
l-tyrosine	0.05	grams
dl-valine	0.10	grams
Acid hydrolyzed protein (5 per cent solution)	50	cc.
Dissolve; dilute to 600 cc.; pH to 7.4; tube in 6 cc. amounts; autoclave at 121°C. for 20 minutes.		
Before the inoculum was added, all tubes received the following:		
Magnesium sulfate (0.4 per cent solution)	0.1	cc.
Tryptophane (0.2 per cent solution)	0.1	cc.
Sporogenes vitamin	0.1	cc.
1N Sodium Hydroxide	0.25	cc.
Thioglycollic acid (1 per cent in 1N hydrochloric acid)	0.20	cc.
Water to	9.0	cc.
Inoculum (0.01 mgm. dried spores in 100 cc.)	1.0	cc.

All of the above components were sterilized in the autoclave. The amino acids and salts were purified by recrystallization 3–5 times before use. The tubes were plugged with cotton wrapped in washed gauze. The thioglycollic acid was used as purchased. The acid-hydrolyzed protein was diluted so that it was 5 per cent based on the weight of the dry protein used. All incubations were carried out in a phosphorus jar at 37°C.

Experiment 1

The first few experiments on this type of medium were entirely confirmatory in nature, namely, to determine for the strain of *C. parabotulinum* selected the necessity for tryptophane and the "sporogenes vitamin" as previously reported (Burrows, 1932, 1933; Fildes, 1935).

The results indicated that *C. parabotulinum* did require these two components in the hydrolysate medium before any growth would occur. Toxin was produced in ninety-six hours following noticeable autolysis. The occurrence of lysis around the seventy-second hour of incubation was a regular sequel to growth in this type of medium and decreased the usefulness of the medium.

TABLE 1

Growth-supporting ability of protein hydrolysate (medium used as on page 5 but without added cystine, tyrosine and valine)

TUBE	INCUBATION PERIOD		TOXIN 96 HOURS
	24 hours	48 hours	
1 Basal medium + Nelson photographic gelatin hydrolysate	+++	++++	+
2 Basal medium + Peter Cooper gelatin hydrolysate	+++	++++	+
3 Basal medium + Isoelectric gelatin hydrolysate	+++	++++	+
4 Basal medium + Kahlbaum casein hydrolysate	+++	++++	+
5 Basal medium + Egg albumin hydrolysate	++	++++	+
6 Basal medium + Water to volume	0	0	0

Experiment 2

A comparison between the efficacy of acid hydrolysates of gelatin, casein and egg albumen indicated that there was little difference between the abilities of these proteins to support growth in spite of the differences in their amino acid contents.

The slight inferiority of egg albumin was rechecked and confirmed. Efforts to base this on the serine and oxyproline deficiencies of egg albumin by adding these amino acids in amounts equivalent to their respective concentrations in gelatin and casein did not improve the ability of egg albumin to support

growth. Since casein and gelatin supported growth in spite of the many differences in amino acid content (glycine, valine and tyrosine, particularly), it was decided to determine the nature of the substances present in the isoelectric gelatin which was suitable for the growth of the organisms.

Experiment 3. Fractionation of gelatin hydrolysate

The method which gave the best fractionation of the acid hydrolysate of isoelectric gelatin was one involving the formation of the copper salts of the amino acids and the subsequent separation of these salts based on their solubilities in methyl alcohol and water (Towne, 1928, 1936).

Three fractions were separated which were (I) the alcohol-soluble water-soluble group including proline, hydroxyproline, isoleucine, valine and the "hydroxyvaline" of Schryver, (II) the alcohol-insoluble water-soluble group including glycine, serine, alanine, arginine, histidine, lysine and pyrrolidone compounds, (III) the alcohol-insoluble water-insoluble group which includes leucine, phenylalanine, tyrosine and cystine.

The fractions were tested for their growth supporting ability in a deficient medium composed of tryptophane, sporogenes vitamin, thioglycollic acid, magnesium sulfate, and sodium hydroxide at pH 7.4. (The amount of each component is the same as that used in the other tests and is summarized above.)

The results indicate that the alcohol-insoluble water-soluble fraction of amino acids is sufficient to support growth of *C. botulinum*. Apparently, from a mixture containing glycine, serine, alanine, arginine, lysine, and histidine, supplemented with tryptophane and sporogenes vitamin, energy and structural components were minimally satisfied.

The addition of fraction I to II did not materially increase the amount of growth nor in fact did it stimulate an earlier germination of the spores. The increase in the amount and the earlier germination in the presence of fractions II + III and I + II + III was paralleled by the same increase when the amount of II in the medium was doubled or tripled.

Although there are several ways in which these results can be

explained, the most attractive approach is by way of the Stickland reaction (1934), in which the source of the energy available to the organisms growing in such a medium is based on reactions between pairs of amino acid molecules.

Fraction II contains alanine which has been shown to be activated as a hydrogen donator by *C. sporogenes*, (Stickland, 1934) plus glycine and serine, the reducible components in the Stickland reaction. This might be one system open to *C. botulinum*. Furthermore, when Fraction III was added to II an initially larger amount of growth appeared which did not

TABLE 2

Effect of gelatin hydrolysate fractions on growth of C. parabotulinum

TUBE	24 HOURS	42 HOURS	96 HOURS
1 Medium + 0.25 cc. Fraction I	0	0	0
2 Medium + 0.25 cc. Fraction II	++	+++	++
3 Medium + 0.25 cc. Fraction III	0	0	0
4 Medium + 0.50 cc. I	0	0	0
5 Medium + 0.50 cc. II	+++	++++	+++
6 Medium + 0.50 cc. III	0	0	0
7 Medium + 0.25 cc. I + 0.25 cc. II	++	+++	++
8 Medium + 0.25 cc. I + 0.25 cc. III	+	+	+
9 Medium + 0.25 cc. II + 0.25 cc. III	+++	++++	+++
10 Medium + 0.25 cc. I + 0.25 cc. II + 0.25 cc. III	+++	++++	+++
11 Medium + 0.75 cc. I	0	0	0
12 Medium + 0.75 cc. II	+++	++++	+++
13 Medium + 0.75 cc. III	0	0	0
14 Medium + 0.50 cc. water	0	0	0

materially increase over that finally obtained with II alone. Fraction III contained leucine and tryosine among other acids from the hydrolysate. Stickland (1934) and Knight and Fildes (1935) reported that, for *C. sporogenes*, leucine is a more readily available oxidizable component than alanine. Fraction III, supplying as it does acids which may be more available in the case of *C. sporogenes*, may act similarly in the case of *C. botulinum*. It remains to test the various amino acids which can be activated by *C. botulinum* to act as donators and acceptors of hydrogen, before carrying over to this organism explanations which may apply only to *C. sporogenes*.

It is also realized that the fractionation of protein hydrolysates by the copper salt method must involve a certain lapping over of fractional components because of faulty technique. If this occurs the magnitude of the concentrations of an amino acid for example may well be within the range of growth-supporting function as demonstrated by Fildes (1935). This would also explain the growth obtained with Fraction II alone.

The amino acids known to be in the various fractions of the protein hydrolysate were substituted for these fractions and tested with a new "sporogenes vitamin" preparation.

The new batch of "sporogenes vitamin" was prepared as previously but with the alteration from the method of Knight and Fildes (1933) that 0.4 per cent hydrochloric acid in methyl alcohol was used as the initial extracting solvent. The extractions were carried out for four days, using fresh changes of solvent, after which the alcohol fractions were evaporated *in vacuo* to dryness, the residue dissolved in hot water, boiled a few minutes and centrifuged. After the supernatant fluid was again concentrated *in vacuo* to a small volume, the steps followed those of Knight and Fildes.

About two hundred milligrams of residual material were obtained from the yeast of which the potency tests in gelatin hydrolysate basal medium revealed that 3.3×10^{-8} gram activated 1 ml. of the deficient medium (detailed on page 433).

Experiment 4

Several different lots of amino acid mixtures were prepared. Lot 1 was composed only of the amino acids known to be contained in the "Towne Fractions II and III." The amounts of each acid in this lot were based on the best available analyses of the amino acids in gelatin. This was further corrected for the amount of hydrolysate, or original protein hydrolysed, which had been shown to support growth.

Lot 2 was more inclusive, containing all the amino acids thought to be in gelatin, and again the amounts of each were based on the reported percentages in gelatin (Bodansky, 1931).

Lot 3 was the mixture developed by Fildes and Richardson (1935) for the growth of *C. sporogenes.*

A brief summary of the mixtures is presented in table 3.

Of those which were not synthetic, the naturally occurring form, purified by recrystallization was selected.

The amino acids were dissolved and added to the following basal mixture:

Monobasic potassium phosphate	4.5	grams
1N Sodium Hydroxide	25	cc.
Water	500	cc.
Adjusted to pH 7.4, diluted to 600 cc., tubed in 6 cc. amounts and autoclaved		

Each tube received the necessary amounts of magnesium sulfate, thioglycollic acid, and the test amount of vitamin preparation previously adjusted to pH 7.4 with 1N sodium hydroxide. Total volume was brought to 9 cc. The inoculum, 1 cc. of a spore suspension, brought the volume to 10 cc.

These media were tested with both available preparations of sporogenes vitamin, the old one, now deteriorating in potency towards gelatin hydrolysate media, and the fresh preparation.

A summary of the comparative activities of the two preparations in the various media is tabulated in table 4.

(1) It will be seen that the old preparation of sporogenes vitamin was completely inactive towards the amino mixtures, whereas the fresh preparation supported a moderate amount of growth in this type of medium. It is also evident in the table above that the old preparation was less effective in the protein hydrolysate medium.

(2) The second point indicated is the loss of activity of the new "vitamin" preparation towards the amino acid mixtures, after ageing about a week.

This loss of potency was by no means an "all or none affair" but was a gradual loss, demonstrable by increasing amounts of preparation required for equivalent turbidities.

(3) The third lot of amino acids was far superior to the other two batches, indicating a striking similarity in growth requirements between *C. sporogenes* and *C. botulinum.*

(4) It was impossible to replace the "Towne Fractions II and

III" by the amino acids known to be contained therein, suggesting the presence of other substances or the overlapping of the fractions.

TABLE 3

AMINO ACID	PER CENT IN GELATIN (BODANSKY)	GRAMS PER LITER		
		Lot 1	Lot 2	Lot 3
Glycine	25.4	0.029	0.029	0.2
s-alanine	8.7	0.085	0.085	0.12
s-leucine	7.1	0.624	0.624	0.17
l-proline	9.5		0.836	0.15
l-oxyproline	14.1		1.24	
s-phenylalanine	1.4	0.123	0.123	0.08
s-glutamic	5.8		0.51	
s-aspartic	3.4		0.308	0.18
s-serine	0.4		0.035	0.14
l-tyrosine	0.01	0.0008	0.0008	0.05
l-histidine	0.9	0.0792	0.0792	0.05
d-arginine	8.2	0.76	0.76	0.05
s-lysine	5.9	0.51	0.51	0.09
l-tryptophane	0.0	0.02	0.02	0.02
l-cystine	0.2			0.06
s-valine				0.15
s-methionine				0.07

The acids marked "s-" were synthetic preparations obtained principally from Eastman Co., and Amino acid Mfgrs., U. C. L. A.

TABLE 4

Effect of sporogenes vitamin preparations

DAILY TEST	GELATIN HYDROLYSATE		AMINO ACID LOTS		
	Growth	Toxin	Number 1	Number 2	Number 3
Vitamin (prepared July, 1936)	++	+	0	0	0
Vitamin (prepared July, 1937)	++++++	+++	0	++	+++
After one week:					
Vitamin (July, 1936)	++	+	0	0	0
Vitamin (July, 1937)	++++++	+++	0	+	+

(5) Toxin was demonstrated in amino acid mixtures supporting growth, but was not particularly potent. One-half cubic centimeter of supernatant fluid from the cultures usually killed

unprotected mice within 36–48 hours, indicating a rather weak toxin. However, this comparison must be qualified because of the fact that growth in the amino acid mixtures, although very apparent, was nevertheless inferior.

One conceivable explanation of the results appeared to involve another essential substance present in gelatin and casein acid hydrolysates additive in effect to the yeast preparation. When the organisms were forced to use purified amino acids as their main food source, they required preformed a larger minimum amount of the "sporogenes vitamin," which could be supplied to them in workable volumes of fresh yeast preparations. Assuming that ageing and subsequent deterioration of the "vitamin" resulted in less than the critical amount being available, the organisms would then find it impossible to grow in such synthetic media. In the gelatin hydrolysate, however, enough "accessory factor" might be present to replace the material lost by ageing of the yeast preparation. A statement by Fildes (1935) that gelatin contains minute amounts of "sporogenes vitamin" seemed to support this hypothesis.

If some accessory factor is present in gelatin hydrolysates, sufficient in the presence of yeast preparation to supplement a deficient medium, the addition of small amounts of gelatin hydrolysate plus the yeast factor to amino acid mixtures should raise the concentration of essential material above the critical level and growth should occur.

When amounts of gelatin hydrolysate, sufficient in themselves to support merely a swirl of growth, were added to the amino acid mixtures, containing yeast preparation formerly sufficient to support growth, no additional growth resulted. When the concentration of hydrolysate was stepped up growth was visible, but the control tubes showed that this growth was due entirely to the hydrolysate and was independent of the amino acid base.

The following fractionations of the gelatin hydrolysate were attempted in order to produce a fraction supplementary only in activity and insufficient in itself to support growth.

1. Alcohol extraction

Ninety-five per cent alcohol was added to a portion of the hydrolysate until the precipitate no longer disappeared on heating. A minimum amount of water was added to dissolve the precipitate and the mixture placed in the ice box. After it had settled the precipitate was filtered and washed with alcohol. It was then dried and taken up in distilled water. The original filtrate containing the alcohol-soluble fraction was concentrated *in vacuo* and taken up in water. Growth tests in a deficient medium (page 433) indicated that the materials for growth were concentrated in the alcohol-soluble fraction since this fraction supported growth. The alcohol-insoluble fraction which did not support growth was also non-supplementary to the deteriorated sporogenes vitamin in the amino acid mixture number three.

2. Alcohol-ether extraction

The procedure adopted here had been used by Tatum and co-workers (1936) in their work on the potato fractions active for *Lactobacillus delbruckii*. Five volumes of 95 per cent alcohol were added to one volume of hydrolysate, then six volumes of ether were added. The mixture was shaken continuously while the ether was added. The precipitate was centrifuged down and taken up in distilled water. The alcohol-ether solution was concentrated, freed of organic solvents, and diluted to volume with distilled water. Only the alcohol-ether precipitate fraction, containing the dark material in the hydrolysate, contained essential growth materials, inasmuch as it supported growth in the absence of added amino acids. The alcohol-ether soluble fraction was negative alone and in the presence of added amino acids (mixture 3).

3. Treatment with lead acetate and ammonia

Although admittedly a remote possibility, it was decided to check the hydrolysates of gelatin and casein for the presence of accessory substances hydrolyzable to glucose. The hydrolysates

were first alkalinized with excess ammonia. A saturated solution of lead acetate was added until no further precipitation occurred. The filtrate was boiled to remove ammonia, saturated with hydrogen sulfide to remove the lead, boiled to remove excess hydrogen sulfide and brought to volume. The lead acetate precipitable fraction was taken up in a little water, saturated with hydrogen sulfide to remove the lead as before and worked up to volume. The growth-essential materials of the hydrolysate were contained in the lead-acetate-soluble fraction which alone supported growth. The insoluble fraction was of no value.

4. Treatment with norite

A portion of the original hydrolysate and also an aliquot of the lead-acetate-soluble fraction were treated with purified animal charcoal, boiled for an hour and filtered twice through Whatman #40 paper. The charcoal precipitates were washed twice with hot water and then dried. The filtrates and washings were combined and concentrated on the water bath to their original volume. Growth tests revealed that the essential materials had been completely removed by the norite.

Boiling the charcoal for a few hours with 95 per cent ethyl alcohol eluted the growth materials with much loss involved. A fraction supplementary only in effect could not be obtained.

5. Effect of the Neuberg-Kerb reagent (1911)

The hydrolysate was treated with sufficient 10 per cent sodium carbonate to alkalinize it. To this, a 25 per cent solution of mercuric acetate was added until a permanent brick-red precipitate appeared. Five volumes of 95 per cent alcohol were added. The mixture was then filtered. The filtrate and precipitate were freed of mercury by hydrogen sulfide and concentrated to volume on the water bath. The growth-essential materials were concentrated in the mercury-insoluble fraction. The soluble fraction was negative in the growth tests.

All growth tests with the above fractions were carried out in two basal media, (1) one containing only monobasic potassium phosphate, sodium hydroxide, tryptophane, sporogenes vitamin

and thioglycollic acid in amounts as on page 433, (2) the other being amino acid mixture lot 3. The fractions under test were added in amounts equivalent to that of the original protein hydrolysate which supported growth.

The results are summarized in table 5.

The inability to produce a fraction which was only supplementary in action to the sporogenes vitamin (i.e., which acts only in basal medium number 2) is indicated by the above table.

It was therefore decided to prepare another lot of sporogenes

TABLE 5

Activity of gelatin hydrolysate fractions

FRACTION	INCUBATION PERIOD		TOXIN
	Basal medium 1	Basal medium 2	
Original hydrolysate	++++	++++	+
Alcohol-soluble fraction	+++	+++	+
Alcohol-insoluble fraction	0	0	0
Alcohol-ether soluble fraction	0	0	0
Alcohol-ether insoluble fraction	+++	+++	+
Lead acetate-ammonia filtrate	+++	+++	+
Lead acetate-ammonia precipitate	0	0	0
Norite filtrate, pH 7.0	0	0	0
Neuberg-Kerb filtrate	0	0	0
Neuberg-Kerb precipitate (0.5 cc.)	+	+	0
Neuberg-Kerb precipitate (1.0 cc.)	++	++	0
Neuberg-Kerb precipitate (3.0 cc.)	+++	+++	+
Ethyl alcohol eluate of Norite-adsorbed material	+++	+++	+

vitamin, this time using pregnancy urine as a source of the material and adopting Pappenheimer's published method (1935) for the latter stages of the work. The preliminary treatment of the urine was carried out in accordance with some suggestions kindly sent to me by Dr. Pappenheimer.

One hundred liters of freshly obtained pregnant cow's urine were concentrated to one-tenth of the original volume by distillation *in vacuo*. Equal volumes of normal sulfuric acid and butyl alcohol were added to the concentrate and boiled for twenty-four hours under a reflux condenser. The butyl alcohol layer was

removed and concentrated *in vacuo* to a thick tar. This was partially dissolved in pyridine. An equal volume of peroxide-free ether was added and the insoluble matter removed. The material was then extracted with concentrated acid and the acidified extract alkalinized with sodium carbonate. The rest of the procedure follows Pappenheimer's description (1935).

The yellow material obtained was very active, 1×10^{-7} gram activating 1 ml. of the amino acid basal mixture developed by Fildes for *C. sporogenes*. However, the growth obtained with this amount of vitamin, and with increasing amounts also, did not compare favorably with the growth which the same amount of vitamin supported in gelatin hydrolysate media. One or more essential substances appear to be required for the growth of *C. parabotulinum* in synthetic amino acid mixtures, in addition to the active substance in yeast and pregnancy urines. A further investigation of these findings is being carried on.

SUMMARY

1. Previous reports concerning the essential nature of certain acidic fractions from yeast and pregnancy urines in the growth of *Clostridium parabotulinum* have been confirmed.

2. Growth of *Clostridium parabotulinum* in media composed of amino acids has been obtained.

3. Although the nutritional requirements of *Clostridium parabotulinum* resemble those of *Clostridium sporogenes* in the group of amino acids required for growth, there are qualitative differences in their needs which have not yet been solved to the point of obtaining massive growth in synthetic media.

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