STUDIES ON THE NUTRITION OF CLOSTRIDIUM WELCHII IN RELATION TO TOXIN PRODUCTION.

by

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INTRODUCTION

As the builder erects a scaffolding prior to the actual construction of a building, so the scientist, having laid the foundation for his work, erects a "scaffold" built of new ideas and critisms of old ones as a guide for his experimental work. Such is the hypothesis which inspires all scientific research. Gay (1935) has set forth the purpose of an hypothesis so admirably that the writer cannot refrain from quoting his remarks. "Working hypotheses are the soul of experimental science and without them one could scarcely proceed from one experiment to the next. One attempts to explain a curious, unexpected, and chance observation in terms of what is already known, by analogy; a hypothesis is created in proof or disproof of which an experiment is suggested. The new facts that are likely to be elicited are of far greater importance that the corroboration or disproof of the hypothesis in the testing of which the experiment was formulated. In other words, the function of the hypothesis in research is to stimulate the production of new facts rather than to help support what seems to its originator a more permanent explanation or theory of the main subject at issue."

Originally this study was designed to investigate the virulence of Clostridium welchii. It ended far from the original goal, the result being a collection of several new facts and new interpretations of old facts concerning the nutrition of C. welchii.

There is a growing belief that the development of such highly specific nutritive requirements by parasitic bacteria is a result of their continued existence in an environment of prefabricated enzymes and easily assimiable foodstuffs. These organisms allow their own powers of synthesis to deteriorate and become progressively more dependent upon the host cell for their nutritive requirements.

Perhaps then, much of the peculiar disease provoking power of the pathogenic bacteria can be traced to their specific nutritive requirements. What better way to provide clues to the mystery of pathogenicity then, than to study the nutrition of these offenders?

A good deal of experimental evidence must certainly accumulate before an intelligent answer can be given to this question. In the meantime, the profound truth of Robert Bridges' utterance in his "Testament of Beauty" should be pondered by every student of the science---

> "Thy task is first to learn WHAT IS, and
> in pursuant knowledge pure intellect will
> find pure pleasure and the only ground for
> a philosophy conformable to truth."

In the foregoing paragraphs the writer has attempted to present the train of thought which has motivated the experimental work and governed the interpretation of the results of this work. At this point, it seems reasonable to acquaint the reader with the plan that is followed in presenting the

material contained in this paper.

The section which immediately follows, entitled "Historical and General Considerations", is intended to allow the reader to become familiar with the morphology and physiology of C. welchii and with the nature and formation of its toxin. The actual nutritional studies are presented in the sequence in which they would normally occur in the study of the nutritive requirements of any microorganism: namely, the nitrogen requirements, the accessory growth factors or bacterial vitamins needed by the organism, the carbon requirements, and the inorganic requirements. Then, as in any technical paper, follows the discussion of the results and the summary of the accomplishments and the conclusions to be drawn.

HISTORICAL AND GENERAL CONSIDERATIONS

The foundation of all work on anaerobes was laid by Pasteur in 1861 when he reported the isolation of Bacillus butryicus, a nonpathogenic, spore bearing anaerobe.

Clostridium welchii was discovered in 1892 by Welch and Nuttall. They named the newly isolated microbe Bacillus aerogenes capsulatus. In the years that immediately followed, the investigations of Welch and Flexner (1895), and Herter (1907) clearly established C. welchii as a bacteriological entity and demonstrated that this organism is one of the major incitants of gas gangrene.

The organism was also a point of interest elsewhere. In 1893, Fraenkel isolated this same microbe and called it the "gas phlegmon bacillus" or Bazillus phlegmonis emphysematosae. Again in 1898 Veillon and Zuber obtained pure cultures of this bacterium to which they applied the name B. perfringens.

Today in English and American literature the organism is usually referred to as C. welchii. German and French workers, however, still designate this organism as B. phlegmonis emphysematosae and B. perfringens respectively. In the most recent edition (1939) of Bergey's "Manual of Determinative Bacteriology" the organism is referred to as Clostridium perfringens. With all deference to Bergey, the author will, nevertheless, retain the designation of C. welchii because of its widespread usage among bacteriologists and because that name has come to stand for a distinct bacterial entity.

The Nature of Clostridium welchii.

C. welchii is a normal inhabitant of the intestinal tract of practically all higher animals. Because of this it can usually be isolated from any soil specimen. Thus, the spores of C. welchii may be present on anything that comes in contact with dust and dirt. Hence, cultures of C. welchii can be obtained from dairy products, foods, body surfaces of man and animals, and even from normal tissues.

Morphology: C. welchii is a short, plump, rod-shaped organism about 4 to 8 microns in length. The bacilli stain Gram-positive in cultures 15 to 18 hours old. In older cultures the rods appear with ragged indentations of the edges. Many of the bacilli show Gram-positive granules embedded in a Gram-negative matrix. In addition a considerable percentage of the elements are completely Gram-negative. C. welchii produces spores though not readily. Spores are never found in a medium containing fermentable carbohydrate. In order to induce the organism to form spores, it must be cultivated in an alkaline medium rich in proteins. The spores when observed still within the vegetative cell are situated centrally or subterminally and are large enough to cause the sides of the rod to bulge. C. welchii is absolutely non-motile; flagella have never been seen even when dark field illumination methods are used. Although capsules are not evident under all staining conditions, they are readily demonstrated if one subjects the bacilli to the

action of dilute sodium hydroxide solution (Robertson, 1916).

Anaerobiosis: C. welchii is not particularly sensitive to oxygen when compared to the more strict anaerobes, of which C. tetani is an example. Zeissler (1928) reports that welchii is capable of growing in an atmosphere of which the oxygen tension is 40 mm. of mercury.

Growth and Appearance in Various Media:

1. Blood agar: The colonies on an agar plate containing 5 per cent rabbit blood are, at the end of twenty-four hours incubation at 37°C., 1 to 5 mm. in diameter, discrete, circular, and evenly convex. Immediately surrounding the colony is a very distinct zone of complete hemolysis. About this zone is a much wider area in which the hemoglobin exhibits a definite alteration though the majority of the cells appear to be unlaked. The whole colony resembles somewhat that of a target; the colony itself being the bullseye.

2. Plain broth: There is an even clouding with ultimate sedimentation of the organisms.

3. Cooked Meat Medium: Abundant growth takes place even without strict anaerobic conditions prevailing. The meat becomes pinkish in color but digestion is absent. Parenthetically it can be said here that due to difficulties attendant to the sterilization of media of this nature, the writer would hesitate to recommend a medium of this type for routine cultivation of anaerobes, because unkilled

spores lurking in it will most certainly germinate upon inoculation, and the ensuing results are too familiar to bacteriologists to be explained.

4. Milk: In milk one observes the characteristic "stormy fermentation". The clot firmly retracts and is shot through with bubbles of gas produced from the fermentation of lactose. At times the clot is literally blasted out of the whey due to the violence of the gas produced. Zinsser and Bayne-Jones (1939) indicate that "stormy fermentation" of milk is peculiar to C. welchii, but McCoy, F red, Peterson and Hastings (1930) have shown that several species of plectridia anaerobes isolated by them from decaying plant tissues and from mud will produce stormy fermentation of milk.

5. Biochemical characteristics: 1. Gelatin. C. welchii produces a gelatin liquifying enzyme. According to Walbum and Reymann (1933), the production of this enzyme is concurrent with the production of toxin by the organism. This observation may account for the discrepancies appearing in the literature in regard to gelatin liquifaction by C. welchii. 2. Carbohydrate fermentation: Acid and gas are formed from glucose, lactose, sucrose, maltose, galactose, levulose, and sometimes from glycerol and inulin. Mannitol, dulcitol, and salicin are not fermented. Simonds (1915), in his study on the cultural characters of C. welchii has, on the basis of glycerol and inulin fermentation, proposed four types of C. welchii. Kahn (1924), reporting the results

of a study of a large number of strains of C. welchii, accepts Simond's proposal in the main but indicates that there are intermediate types in which the fermentation of these two carbohydrates is not clear-cut and hence bridges over the gaps separating the four types.

THE TOXIN OF CLOSTRIDIUM WELCHII

To the remarkable pathogenic action of C. welchii the older workers ascribed many varied causes. Since the bacillus ferments carbohydrates with the formation of butyric acid, it was regarded by Coleman (1915) as a tissue irritant and debilitant. Stewart and West in 1916 maintained that a true toxin was not responsible for the tissue changes, but that the effect of the acid was sufficient to cause the lesions of gas gangrene. To Bull and Pritchett (1917 a,b,c,) must go the credit of first clearly demonstrating that C. welchii produces a true, antigenic, exotoxin. These workers grew highly pathogenic strains of C. welchii in 0.2 percent glucose broth containing a bit of fresh, sterile, rabbit or pigeon muscle. After 18 to 24 hours incubation the culture was filtered and the filtrate obtained had the power to kill laboratory animals. The animals so injected exhibited the same symptoms that appear in a typical C. welchii infection. Antitoxin which specifically neutralized the toxin was prepared by hyperimmunizing rabbits and horses.

When Bull injected the toxin into the breast muscles of a pigeon he observed edema and extensive necrosis of the muscles. Death followed shortly, if the amount of the

toxin injected was lethal. Injected intravenously the toxin produced death also, but from the result of a speedy and widespread red blood cell destruction. From these results Bull conceived the toxin of C. welchii as consisting of two elements: (1) a hemotoxin, and (2) a substance which caused local tissue injury. This latter element was called a myotoxin by Henry (1922).

The main properties of the toxin were soon determined and a variety of terms were proposed for the various toxic elements of which the toxin was composed. These have been named according to their physiological action. They include the aforementioned hemotoxin or hemolysin, (Bull, 1917 a, b, c; Celerek and Stetkiewicz, 1936; Ford and Williams, 1919; Henry, 1922; Kadisch, 1923; Ouranoff, 1917; Schnayerson and Samuels, 1930; Weinberg and Nasta, 1920; and Wuth, 1923) and myotoxin (Henry, 1922) also called cytolysin (Robertson, 1929). In addition, a neurotoxin has been proposed (Weinberg and Barotte, (1929); a toxin purported to have a specific action on blood vessels (Weinberg and Combiesco, 1930); "the acute lethal toxin" of Shiraishi (1931); "a necrotic and lethal toxin" of Glenny (1936) and a non-specific histamine-like substance found in the growth products of C. welchii (Kendall and Schmitt, 1926; Kendall and Gebauer, 1930; and Kojima, 1922). This substance is found in broth cultures in which the original fermentable sugar was above the optimum for toxin production. It is heat-stable, non-antigenic, and is not

neutralized by antitoxin.

With the exception of the "pseudotoxin", none of the alleged toxin elements have been separated from the "crude" toxin, nor have their individual properties been postulated. Both Robertson (1929) and Gay (1935) infer existence of the hemotoxin and the myotoxin as separate entities.

In the majority of these studies the hemotoxin has been under surveillance. In the discussion which follows the terms toxin and hemotoxin are used interchangeably. C. welchii toxin is destroyed if heated at a temperature of 70°C. for 30 minutes (Bull, 1917 a, b, c, Henry, 1922, and Wuth, 1923). Neil (1926) reveals that the hemotoxin is destroyed if heated at 55°C. for 10 minutes. He reports that toxin inactivated thusly cannot be reactivated by reducing agents.

Neil found that C. welchii hemotoxin loses its activity upon exposure to air. This is a true oxidation reaction in that the hemolytic activity can be restored upon reduction either by bacteria or by sodium hydrosulfite. A further perusal of Neil's paper reveals that the oxidized, inactive hemolysin has the same susceptability to heat as does the reduced active hemotoxin. Neil's claims were substantiated by Reed, Orr, and Campbell (1927). These last-named workers also found that more hemotoxin is required to lyse oxygenated red cell suspensions than reduced red cell suspensions.

Wuth (1923) also studied the hemotoxin of C. welchii. His observations in regard to some of its physical properties are particularly interesting. He found the tosin was absorbed on powdered fibrin, erythrocytes, and minced tissues from various organs. According to Wuth, the hemotoxin is bound by lecithin though not by cholesterol. This lipo-tropic attribute of the toxin is in accord with the exotoxins of certain species of other bacteria (Eaton, 1938).

In order to obtain optimal hemolytic activity of C. welchii toxin correct hydrogen-ion concentration is of utmost importance. Neil's work indicates that the hemotoxin loses its potency if it is exposed to a pH of 7.5 to 8.0.

He (Neil) indicates that a pH of 7.0 is optimal though Walbum (1933) in a more recent study has clearly shown that 6.6 is the optimum pH for the hemolytic activity of C. welchii toxin.

A stable dried preparation of C. welchii toxin can be prepared by precipitation with ammoniun sulfate, resolution with water and reprecipitation with alcohol (Henry and Lacey, 1920; Kadisch, 1923; and Henry, 1922).

Weinberg (1923) has prepared a toxoid from C. welchii toxin by heating the toxin for 48 hours in the presence of 0.13 to 0.30 per cent formalin.

In a review of this nature it would be inadmissable to omit the mention of several serological studies that have been made with the purpose of establishing the identity of the aforementioned components or at least to exemplify the

complexicity of C. welchii toxin.

There exist several species of bacteria which closely resemble C. welchii both physiologically and in the nature of the toxin produced. These organisms differ from C. welchii in the fact that their pathological effects are confined to enterotoxemias of domestic animals.

In 1923 Dalling isolate an organism which is the etiological factor in lamb dysentery in Great Britian. This organism exhibits minor physiological variations from C. welchii of classical gas gangrene. In addition, the specific antitoxin of Dalling's bacillus neutralizes the toxin of C. welchii, though C. welchii antitoxin does not neutalize its toxin. Another organism of this group, B. paludis, was discovered by McEwen (1929). He obtained it from sheep having a disease called Struck. B. paludis bears a close metabolic resemblance to C. welchii and to Dalling's bacillus yet exhibits a significant difference in toxin-antitoxin relations. The toxin of B. paludis is not neutralized by the antitoxin of C. welchii nor the toxin of C. welchii by the antitoxin of B. paludis. McEwen asserts that his organism differs from Dalling's bacillus in that it does not produce a hemotoxin. Still adding to the complexicity of the picture of the welchii-like organisms is the organism causing enterotoxemia of sheep in West Australia and the "pulpy kidney" disease of sheep in New Zealand. The causative organism, B. ovitoxicus, was discovered by Bennetts in 1932. Bennetts considered his organism a new

and distinct bacterial entity on the basis of the toxin specificity exhibited by cross-protective experiments with C. welchii, B. paludis, and Dalling's bacillus.

Two reports have appeared showing the toxin-antitoxin relationships of this group of organisms. A tabular summary of Wilsdon's (1931) report appears below:

Organism	Toxin type	Toxin factors	Antitoxin neutralize
C. welchii	A	W	A
Dalling's	B	W, X, Z	A, B, C and D
B. paludis	C	W, Z	A, B, and C
B. ovitoxicus	D	W, X	A, D

Wilsdon's report was followed shortly by the publication of a similiar investigation by Glenny, Barr, Llewellyn-Jones, Dalling and Ross (1933). Their paper reveals the existance of toxin fractions α, β, γ and δ. To these Mason (1936) added the factor ε as yet known only in the original strain of Dalling's bacillus. Prigge (1936, 1937) added the factor ζ as yet known only in the true C. welchii. These various factors exert the following physiological effects.

α ---hemolytic, lethal, and necrotic
β ---lethal, necrotic
γ ---lethal
δ ---hemolytic
ε ---lethal, necrotic
ζ ---lethal

Dalling has reported the distribution of these factors in the various toxin types as follows:

Toxin types	Factors					
	α	β	γ	δ	ϵ	ζ
A	+	−	−	−	+	±
B	+	+	+	±	+	
C	+	+	+	+	−	
D	+	−	−	−	+	

It would appear that from the above table that Type B or Dalling's bacillus would protect against the toxins of Types B, C, and D. Montgomerie and Rowland (1936) report what may prove to be a fortunate discrepancy. They find that Type B antitoxin also protects against Type A, or the toxin of the true C. welchii. The antitoxin of true C. welchii should according to the above table, neutralize only C. welchii toxin. It does, however, neutralize Type D toxin which contains the epsilon factor. Such discrepancies among these toxin-antitoxin relationships remain to be explained.

A complete discussion of the enterotoxemias of this group of organisms, and of the possible significance of their toxin-antitoxin relationships to these diseases is beyond the scope of this review. For an admirable digest of the literature on the serological relationships existing in this group, the reader is referred to a review of this subject by McCoy and McClung (1938). Their review is brief, yet adequate, and is accompanied by an extensive bibliography.

In the great majority of papers dealing with either the physiological properties or the serological properties of C. welchii toxin the existence of the so-called toxic fractions, though not established, is, at least, inferred. Perhaps this is not the place for speculation, but viewpoints to the contrary should not, at the present anyhow, be completely outlawed. Landsteiner (1936), in his book, has clearly shown that the specificity of immunologically active substances is dependant upon the atomic configuration of the antigenic molecule. What, then, would prevent one from assuming that the toxin of C. welchii was a singular substance endowed with a variety of pharmocological activities? Following this line of reasoning, the serological differences observed in toxin-antitoxin reactions of the welchii group of organisms might be explained as being due to variations in the atomic arrangement of certain "specificity radicals", or to the addition of one or more of the radicals without the general structure of the toxin molecule being appreciably disturbed.

THE PRODUCTION OF C. WELCHII TOXIN.

In reviewing the literature in regard to the production of C. welchii toxin the writer does not attempt to discuss the various precautions to be observed, the care and maintenance of toxigenic strains of C. welchii, the preparation of mediums best suited for toxin production, etc. Rather the subject will be treated in a chronological manner showing advances in the art of toxin production and significant studies in which workers have endeavored to adjust the metabolism of C. welchii to optimal toxinogenesis.

When Bull and Pritchett established the fact that C. welchii produces a true toxin, the medium they used was a meat infusion broth containing 0.2 per cent glucose. Immediately before inoculation bit of sterile rabbit tissue was added to insure the presence of certain necessary nutritional substances and to maintain supply the means of poising the oxidation-reduction potential of the medium in order that the organism, upon inoculation, would be subjected to conditions optimal for growth. Later DeKruif, Adams and Ireland (1917) demonstrated that hashed autoclaved veal could be substituted for the fresh tissue. Gay (1935b) states that the medium devised by DeKruif is the one in general use in the United States today (1935). This comment being made some years after Walbum and Reymann (1933) showed that the presence of solid meat particles was entirely superfluous to the production of toxin by C. welchii!

Kojima (1922, 1923) actually made the first definite attempts to determine the manner in which C. welchii produces its toxin. His work indicates that the addition of phenanthrenechinon and colloidal sulfur to the culture medium medium are instrumental in stimulating toxin production by C. welchii. Walbum and Reymann (1933), however, state that they were unable to confirm Kojima's results.

The preparation of various enzymatic digests of muscle and other organs and using the resultant product as a base of a liquid medium has been quite popular among various workers in this field. Hartley (1922) advocates the use of tryptic digest broth with meat added to depth of one inch in the bottom of the container. Weinberg and Ginsbourg (1927) have devised a medium whose base is a peptic digest of liver and beef muscle. It is the much heralded 'Vf' medium which has in the past been a prime favorite among French bacteriologists for the purposes of toxin production. Weinberg and Ginsbourg dispense their medium in tall bottles. This eliminates the necessity of a surface seal or other means of maintaining anaerobic conditions. These workers testify that C. welchii growing in 'Vf' medium will produce a toxin of which an intravenous dose of 0.25 ml. to 0.5 ml. will kill a rabbit.

In 1933 Walbum and Reymann published a report of a critical survey of the factors involved in toxin production. Briefly, their results reveal that toxin is most stable at a pH of 6.0 to 7.0, that the concentration of toxin reaches

its maximum in 10-11 hours at 37°, 15-18 hours at 30 C; that the heat-stable poison (histamine) is not formed to any great extent if the substrate contains not more than 0.75 per cent glucose. (The acute poison is formed in appreciable amounts, however, if the medium contains 2.25 per cent glucose). The medium employed by Walbum and Reymann is ordinary veal infusion broth with 1.0 per cent peptone and 0.1 per cent glucose added. This medium is adjusted to a pH of 7.6-7, 8. Calcium carbonate is admixed as an aid in neutralizing the acids formed in the dissimilation of glucose. Anaerobic conditions are maintained by covering the broth with a layer of parafin oil. Using this medium these workers have obtained toxins of which the m.l.d. is 0.015 to 0.013 ml.

Recently Reed, Orr, and Baker (1939) have perfected a medium which they recommend as being excellent for toxinogenesis by members of the gas gangrene group. It is essentially a solution of 5 per cent gelatin and 1 per cent peptone, in which has been incorporated a number of inorganic salts. The experience of the writer is the same as that of Reed, Orr and Baker. The strain of C. welchii used in these experiments has consistently produced large amounts of toxin in this medium.

As has been mentioned above, few projects have been undertaken to explain the mechanism of the production of toxin by C. welchii. The various mediums described have tended toward simplification, but the reasons for introducing them are rather obscure. In the writer's opinion they

represent, nevertheless, definite, though perhaps feeble, attempts to escape the empiricism that shrouds the genuine bacterial toxins.

WHAT IS C. WELCHII TOXIN?

At the present time it is perhaps unwise to ask, "What is C. welchii toxin?" The toxins of the gas gangrene group of organisms have not been the subject of such intensive researches as have the toxins of Corynebacterium diphtheriae and Clostridium tetani. Eaton (1938) in reviewing the recent chemical investigations of the bacterial toxins hesitates to discuss the nature of the toxins of C. welchii and other anaerobic bacteria of the gas gangrene group.

Is the toxin of C. welchii a poisonous by-product of the metabolism of the bacterial cell, or does it play a stellar role as an integral part of some enzyme system of the cell? Eaton notes the possibility that bacterial toxins may be involved in some manner with certain respiratory enzyme systems or oxidation-reduction processes. He cites several investigations which tend to support this supposition on the basis of the close chemical similarity of C. diphtheriae toxin and the compounds of the cytochrome series. Such an implication, of course, precludes similar analogies with the toxins of the anaerobes; cytochrome never having been shown to be present in anaerobic bacteria. Nevertheless, this latter statement would not exclude the possibility of an anaerobe's toxin enlisting as an integral part of a redox system. The fact that C. welchii toxin (see Neil, 1926) is reversibly oxidized might tend to support this supposition. Mueller (1940) indicates that the production of toxin by C. diphtheriae is to a great extent dependent

upon the concentration of iron in the substrate. His review reveals that several investigators have shown that the amount of iron necessary for optimal growth is considerably more than that required for the maximum production of toxin. Though Mueller admits that his premise is not based upon experimental evidence, he is of the opinion that certain iron bearing enzymes or coenzymes are responsible for some function of the usual sequence of metabolic events; and when the iron content of the substrate is depleted below a certain level, the organism in a successful attempt to eliminate a "bottle-neck" in its metabolism relies upon an alternative enzyme system of which the toxin is a member. This "new" system is then forced to carry the whole load, and the toxin is manufactured in increased amounts. The fact that this substance is poisonous for humans is merely an unfortunate accident. Whether this hypothesis can be applied to the toxins of anaerobic bacteria is problematical.

Is the toxin of C. welchii a complex mixture of elements each with its own peculiar physiological effect, or is it a singular substance endowed with the ability to create certain effects on different tissues? The present situation in regard to the actual nature of C. welchii toxin seems a bit chaotic. The tendency of the earlier workers has been to infer the existence of individual elements. Nothing appears in the literature which supports a unitarian concept of C. welchii toxin.

The hemolysin of certain strains of staphylococci

appears to be quite similar to that of C. welchii. As is the case with C. welchii, the hemolytic, necrotic, and lethal toxins of the staphylococci have not been separated nor have their individual properties been established. Glenny and Stevens (1935) have described the properties alpha and beta hemotoxins which, they maintain, exhibit different affinities for rabbit red blood cells and for human red blood cells.

The foregoing questions constituted the motivating influence which resulted in the following experimental studies. It was thought that the development of the simplest medium possible compatible with maximum toxin formation would help answer the question, "What is C. welchii toxin?"

In those studies it will be seen that the mode of attack is along purely nutritional lines. The popular trend in bacterial nutrition is toward the development and perfection of mediums of known chemical composition, so that eventually the fate of essential metabolites and the reason for the formation of various by-products attendant to the economy of the cell may be determined. This trend has been followed in this study.

Before going on to discuss the experimental studies done in connection with this study, the writer should like, at this point, to justify his treatment of this review. An attempt has been made to chart the orderly progress of the science towards the determination of the nature of C. welchii toxin. The great bulk of papers dealing with C.

welchii are in connection with problems of medical importance. Since they primarily deal with the effect rather than the cause, these papers have been ignored. The immensity of the literature pertaining to the anaerobes is almost beyond comprehension. Though the writer feels that the survey of the literature has been adequate, it is quite likely that omissions have occurred. At times the author has completely agreed with the statement of Dr. Heller (1922)," The anaerobist working on bibliography is surely a creature to be pitied, and is not to be blamed for failing to follow the work of others conscientiously and thoroughly."

EXPERIMENTAL WORK

If one entertains an idea to study the nutrition of a particular species of bacteria, two routes of approach to the matter are open to him. One of these ways might be called the "analytical method". Here one would select a medium whose exact composition was unknown and upon which the organism in question grows luxuriantly. Then by painstaking fractionation of the various ingredients of the medium he would ultimately arrive at a point where all of the essential nutrients of the organism had been isolated and identified. Thus, the recombining of these essential nutrients would constitute a medium of known chemical composition. It would then be comparatively easy to ascertain the fate of these substances in the metabolism of the cell.

To the second method the term "synthetic method" might be applied. Following this line of attack one could by trial and error employ various combinations of nutrient substances known to be effective for other bacteria, and ultimately he would arrive at the same destination as one who employed the "analytic method".

In the experimental data that is presented in this section it will be seen that a combination of the two methods has been used.

GENERAL TECHNIC

Cultures: In all of the experiments that are to be described, a single strain of C. welchii has been used. This culture was obtained thru Mr. Edgar H. Beahm of the Creighton University School of Medicine. Upon receipt, it bore the label #SR-12; that designation has been retained throughout. This strain of C. welchii has all the morphological and cultural characteristics as reported for C. welchii by Bergey (1939). The organism is highly virulent, if virulence is to be measured by the ability to produce large amounts of toxin. When 1.0 ml. of an actively growing culture of SR-12 is injected into the thigh of a 300 gram guinea pig, death will occur within 48 hours. Upon post mortem the typical pathological changes are observed at the site of inoculation. The muscles are sodden, friable, pale pink in color, and have a disagreeable sour odor. There is no evidence of putrefactive proteolysis. The organism is easily isolated from the lesion.

Anaerobic methods: Rosenthal's (1937) "chrom-sulfuric acid" method for producing anaerobic conditions is used throughout this study. The chemical reactions which illustrate the manner by which anaerobic conditions are produced by this method follow:

$$(1)\quad Cr + H_2SO_4 \longrightarrow CrSO_4 + H_2$$

$$(2)\quad CrSO_4 + O_2 \rightarrow Cr_2(SO_4)_3$$

The jars in which the anaerobic conditions thus produced were designed and made by the author. Ordinary fruit jars

of a quart of a gallon capacity were equipped with a suitable lid which was fitted with a device that allows the excess hydrogen gas that is generated to "bleed" out and yet maintain a positive pressure of hydrogen sufficient to prevent any air from leaking into the jar. For simplicity's sake the complete set-up, when "charged" and ready for use will be referred to as an "R" jar.

In order to prepare an "R" jar one places 3 grams of chromium metal, 50 ml. of 15 per cent H_2SO_4 and 1 gram of $CaCO_3$ for each liter capacity into the jar. A small amount of iron filings will catalyze the reaction causing it to proceed faster. The tubes or plates that are to be incubated are placed in the jar on a suitable acid-proof stand; the lid is firmly placed on the jar; and it is then ready to be incubated. Of course, the usual precautions in regard to hydrogen and sulfuric acid must be observed. This method has the advantage in that plates may be inverted during incubation since there is no vacuum at any stage in the process.

Very recently Mueller and Miller (1941) have reported on a modification of Rosenthal's chrom-sulfuric acid method. By a remarkable coincidence their modification, including the specifications of their jar, are almost identical with the method and jar devised by the author.

Miles (1940) criticizes the Rosenthal method saying that he found it to be not as efficient as the McIntosh and Fildes (1916) method. Miles bases his assumptions on the

facts that the presence of impurities in the chromium result in the formation of substances toxic for anaerobes, and that the cost of chromium prevents routine use of the Rosenthal method. The writer using the "R" jar has cultivated all of the more fastidious anaerobes and has never observed any evidences of bacteriostasis. As to the cost of the two methods the jars described above can be constructed at a cost of about fifty cents each. Five hundred "R" jars were prepared using a total of four pounds of chromium; the cost of the chromium was $6.80. A complete McIntosh and Fildes jar retails for $20.00.

Measurement of Growth: Several methods for the quantitative estimation of the growth of bacteria in a liquid medium are available to the bacterial nutritionist. Of these the visual inspection of the culture tubes for turbidity is perhaps the most popular, though it is subject to considerable error. More accurate indices of growth can be ascertained by direct count of bacteria and by direct weighing of the mass of bacterial growth. Indirectly the index and bacterial growth can be obtained by determining the nitrogen content of the mass of bacterial growth. These last-named methods are time consuming and where a large number of determinations are being made the time factor is of considerable importance. The use of the photoelectric cell for making turbidity measurements has recently been popularized, and a majority of workers in bacterial nutrition use some form of the photoelectric colorimeter to determine the

effectiveness of various mediums and growth factor preparations. The photoelectric measurement of turbidity eliminates the human factor to a great extent and presents a rapid and simple means of determining bacterial turbidities. The great disadvantage lies in the fact that the cell is exceedingly sensitive and all possible variables must be eliminated before reproducible results can be obtained.

In a majority of the experiments the amount of growth was estimated as turbidity. These turbidities were determined by the Klett-Summerson photoelectric colorimeter. The details of this instrument are described by Summerson (1935). To the writer's knowledge no other worker has used this device so it was necessary to develop a technic that would permit rapid determination of turbidities and at the same time be sufficiently accurate so that duplicate results might be obtained.

It was found that the greatest accuracy is achieved when the liquid phase of a suspension of bacteria is colorless. Hence, the bacteria were removed from the culture medium by centrifugation at 3500 r.p.m. for 30 minutes. The mass of bacteria were resuspended in 10 ml. of water and the centrifugation was repeated. Again the bacteria were resuspended in 10 ml. of water and the turbidity is estimated as follows:

A blue (no. 54) filter is placed on the colorimeter, a special colorimeter tube is filled with 10 ml. of

distilled water and the zero point of the colorimeter is arrived at by adjusting the appropriate controls. The zero point attained is when the scale reading and the pointer both register zero.

Next, to one of the special colorimeter tubes is added the bacterial suspension. The pointer is returned to the zero point by manipulating the scale knob and the reading is made directly from the scale.

A scale reading of 0 to 25 represents poor growth, 26 to 50 moderate growth, 51 to 75 good growth, and above 75 heavy growth.

Estimation of toxin concentration: The concentration of toxin was estimated according to the hemolytic activity of the toxin. As is noted above, the technic of determining turbidities entails collection of the bacteria by centrifugation. This procedure rendering the broth relatively free of bacteria, the supernatant can be tested for toxic activity. The removal of the bacteria by centrifugation also is advantageous in that the toxic activity is not diminished by such treatment. Filtration does result in a loss of toxic activity and is also slow and expensive.

Technic: Serial dilutions of the toxin were made ranging from 1:10 to 1:1280. The dilutent was 0.85 per cent saline solution buffered at a pH of 6.6 (Walbum, 1933, has found that the hemolytic activity of C. welchii toxin is greatest at this pH). The volume of the toxin dilution in each tube was 0.5 ml. After the dilutions of toxin were

made, 0.5 ml. of a 2 per cent suspension of rabbit red blood cells were added. This suspension was prepared from oxalated (1 ml. of 1.1 per cent potassium oxalate for each 5.0 ml. of blood) rabbit blood drawn the day the titration was made. The blood cells were washed twice in buffered saline solution and packed by centrifugation at 3500 r.p.m. for 30 minutes. The 2 per cent suspension is then made from these packed cells. The mixtures of cells and toxin were shaken and incubated for one hour at 37°C. The end point was taken as the highest dilution of toxin causing complete hemolysis of the blood cells in 0.5 ml. of a 2 per cent suspension of rabbit erythrocytes.

In general the bacteriological technics have followed the same general pattern throughout this study. As the experimental work progressed, however, certain variations and innovations occurred from time to time. In order to avoid ambiguity then, the technics used in each individual experiment will be described separately.

EXPERIMENT ONE

THE SOURCE OF ESSENTIAL NUTRIENTS FOR C. WELCHII

In 1939 Reed, Orr, and Baker reported on the preparation of a medium that would consistantly support the production of large amounts of C. welchii toxin. The ingredients of this medium and their respective concentrations are: NaCl, 2.0 grams; $MgSO_4 \cdot 7H_2O$, 0.02 grams; $Na_2HPO_4 \cdot 12\ H_2O$, 5.76 grams KH PO , 0.24 grams; gelatin, Difco, 50 grams; peptone, Difco, 10 grams; glucose, 2 grams; water, 1000 ml. pH is 7.7. The medium is autoclaved at 121°C. for 15 minutes. Reed, et al. state that the phosphate content has little influence on the production of toxin provided that it is present in sufficient amounts to prevent a drop in pH below 6.2-6.0 in 18 to 24 hours. This medium was chosen as a reference medium and all results achieved with test mediums and growth factor preparations are compared with the "ROB" medium. The strain of C, welchii No. SR-12 when grown in "ROB" medium will consistently produce toxin in such concentrations that it will hemolyse a unit of red cells in dilutions of 1:160 or better.

In order to investigate the stimulating effects of substances other than peptone, the above-named experiment was done.

Basic medium: Gelatin, Difco, 50 grams; NaCl, 2.0 grams; $MgSO_4\ 7 \cdot H_2O$, 0.02 grams; KH_2PO_4, 5.45 grams; 5 M NaOH, a sufficient amount to bring the pH to 7.8; water, 700 ml. The medium was tubed 15 X 125 mm. test tubes in 7 ml. amounts and sterilized at 121°C. for 15 minutes. Just before inoculation

0.1 ml. of a 20 per cent solution of glucose, and 100 gammas of thioglycollic acid contained in 0.1 ml. were added to each tube.

Test substances: Solutions of Bacto-peptone, Difco yeast extract, Wheatamin extract, and liver extract, Lilly were made up so that 1.0 ml. contained 1 mgm. of the substance. The solutions were sterilized by filtration through a chromium-plated Seitz filter. Various amounts of the materials were added sterilely to the tubes in the amounts as indicated in Table I. The volume in all tubes was brought to 10 ml. and they were incubated overnight in the 37° thermostat in order to insure sterility.

Inoculum: 0.1 ml. of a twice-washed twenty-four hour culture of SR-12 growth in "ROB" medium.

Incubation: 18 hours at 37°C. in an "R" jar.

Results: The results of this experiment are shown in table I.

Table I

The Effect of Various substances on the growth of C. welchii SR-12

Tube	Assay	Colorimeter reading	Hemolytic activity
1	Basal	20	1:10
2	" , 101 gamma P./ml.	28	1:20
3	" , 100 gammas P./ml.	35	1:20
4	" , 1.0 mgm. P/ml.	43	1:20
5	" , 10 mgm. P/ml.	77	1:40
6	" , 10 gammas W.E./ml.	44	1:10
7	" , 100 gammas W.E./ml.	51	1:10
8	" , 1.0 mgm. W.E./ml.	62	1:40
9	" , 10.0 mgms W.E./ml.	122	1:80
10	" , 10.0 gammas L.E./ml.	44	1:10
11	" , 100.0 gammas L.E./ml	68	1:20
12	" , 1.0 mgm. L.E./ml.	74	1:80
13	" , 10.0 mgm. L.E./ml.	95	1:80
14	" , 10 gammas Y.E./ml.	41	1:20
15	" , 100 gammas Y.E./ml.	54	1:20
16	" , 1.0 mgm. Y.E./ml.	73	1:40
17	" , 10.0 mgm. Y.E./ml.	130	1:80
18	M,R,O,B, medium	100	1:160

P=Bacto-Peptone; W.E.= Wheatamin Extract*; L.E. =Liver extract, Lilly; Y.E. =Yeast extract, Difco.

The results of this experiment reveal three significant facts. First, it can be seen (tube 1, table 1) that C. welchii is capable of growing and producing toxin in a medium

*Manufactured by the DePree Company, Holland, Michigan

consisting essentially of 5 per cent gelatin and 0.2 per cent glucose. It might be argued that a sufficient amount of the necessary growth factors to enable C. welchii to grow was carried over with the inoculum. That the technic of washing the organisms prior to inoculation removes all such substances, however, is bourne out in subsequent experiments in which hydrolysates of casein and gelatin were used. The inference gained from this finding is that gelatin itself contains enough of these accessory growth factors to promote the development. of C. welchii. Second, it is seen that Difco yeast extract, Wheatamin Extract, liver extract, and Bacto-peptone are effective in stimulating the growth of C. welchii in the order named. The third and most significant observation to be made from this experiment is that although better growth is obtained with mediums containing yeast extract and Wheatamin Extract, the amount of toxin produced in these substrates in no way approaches the amount produced by C. welchii growing in R.O.B. medium. In addition, one notes (tube 6, table 1) that where the ingredients of the medium are added separately but in such a manner that the ultimate constitution of the medium is identical with that of R.O.B. medium, the growth and the amount of toxin produced are inferior to the R.O.B. medium. The actual reason for these last-mentioned discrepancies is found in the experiments on the "Inorganic requirements".

This reason did not come apparent to the author until much later, however. At the time this experiment was per-

formed, it was thought that the source of nitrogen might influence growth and toxin production considerably. Though the results obtained do not corroborate this hypothesis they do bring out interesting points.

EXPERIMENT TWO

THE EFFECT OF VARIOUS SOURCES OF NITROGEN ON THE GROWTH AND TOXIN PRODUCTION OF C. WELCHII NO. SR-12.

Acid hydrolysate of Gelatin as a source of nitrogen. An acid hydrolysate of Difco gelatin was prepared according to a modification of a method suggested by Mueller and Miller (1941 b). 300 grams of gelatin were dissolved in one liter of 20 per cent sulfuric acid. The resulting black tarry mixture was then heated in the autoclave for four hours at twenty pounds pressure. The hydrolysis of the gelatin was complete, for biuret tests on the solution were negative. The hydrolysate was cooled and the sulfate was quantitaively removed by precipitation as barium sulfate through the addition of 450 grams of anhydrous $Ba(OH)_2$. The resulting precipitate was filtered off by means of a Buchner funnel. The filtrate was then decolorized by the addition of approximately 100 grams of norit. The filtrate obtained from the treatment with activated carbon was a clear, light-yellow liquid. This liquid was concentrated under reduced pressure at a temperature of less than 50 C. This operation took about thirty-six hours with the equipment at the writer's disposal. The concentrate was a thick, sticky mixture containing crystals of various amino acids. Upon the addition of water, the concentrate goes into solution easily.

Basal medium. Acid hydrolysate of gelatin, 8.0 grams, corresponding to 50 grams of gelatin; NaCl, 2.0 grams; $MgSO_4 \cdot 7H_2O$, 0.2 grams; KH_2PO_4, 5.45 grams; 5 M. NaOH, sufficient

to bring the pH to 7.8, usually takes about 7.3 ml. Water. 700 ml. Tube in 15X125 mm. test tubes and autoclave a 15 pounds pressure for 15 minutes. Before inoculating, thioglycollic acid, in a concentration of 100 gammas per ml. of medium, was added to insure good reducing condition of the broth and thereby reduce the lag period of C. welchii. In addition the broth was enriched with 0.2 per cent dextrose.

Test mediums. Each test medium was prepared from a tube of basal medium. Next, varying amounts of substances known to contain accessory growth factors for C. welchii were added as indicated in table II. The volume in each tube was brought up to 10 ml. by adding an appropriate amount of sterile distilled water. All tubes were then placed in the 37°C. incubator overnight in order to insure sterility.

Inoculum. The amount of inoculum consisted of 0.1 ml. of a suspension of a twenty-four hour culture of C. welchii. It was prepared in the following manner: The organism was grown in R.O.B. medium and after the incubationary period, the mass of bacterial growth was thrown to the bottom of the tube by centrifugalizing at 3500 r.p.m. for 30 minutes. The bacteria were resuspended and washed twice in sterile distilled water. This suspension was then diluted until the turbidity was just perceptible.

Incubation. The incubation was carried out in the 37°C. incubator and the incubationary period lasted for 15 hours.

Results. The results are listed in table 2.

TABLE 2

Acid hydrolysed gelatin as a source of nitrogen for Clostridium welchii

Tube	Assay	Colorimeter reading	Hemolytic activity*
1	Basal	8	0
2	" , 10.0 micrograms P/ml.	7	0
3	" , 100 " P/ml.	12	0
4	" , 1.0 mgm. P/ml.	12	0
5	" , 100 mgm. P/ml.	88	1:80
6	" , 10.0 micrograms W.E./ml.	5	0
7	" , 100 " W.E./ml.	5	0
8	" , 1.0 mgm. W.E./ml.	32	0
9	" , 10.0 mgm. W.E./ml.	95	1:40
10	" , 10.0 micrograms L.E./ml.	24	0
11	" , 100.0 L.E./ml.	34	1:20
12	" , 1.0 mgm. L.E./ml.	42	1:80
13	" , 10.0 mgm. L.E./ml.	82	1:80
14	" , R.O.B. medium	106	1:160

*The hemolytic activity of uninoculated controls was zero in all cases.

According to the results as portrayed in table 2, we observe that an acid hydrolysate of gelatin is not as an effective source of nitrogen as gelatin is. However, the added substances, peptone, Wheatamin Extract, and Liver extract exhibit about the same relative stimulative ability as

they did in Experiment 1. The point of interest in the above table, though, is that these substances weren't very effective until their respective concentrations reached 1 per cent (10 mgm. per ml. of the test medium). This points to possible amino acid deficiency in the gelatin acid hydrolysate of gelatin that was allieviated by the presence of the missing substance in the added stimulatory factors. The growth of C. welchii in the test mediums described was in most cases insufficient to warrant an appreciable amount of toxin production. However, when the growth-stimulating substances were present in concentrations of 1 per cent, good growth though poor toxin production occurred. From the data presented one might infer that an acid hydrolysate of gelatin was not as good a source of food as the parent substance. However, in the writer's opinion this is not the case. The reasons for such an assumption will be brought out in subsequent discussions concomitant to certain experiments to be described shortly.

The question again arises as to why the discrepancy between growth and toxin production as compared to the R. O. B. medium (tube 14, table 1). As noted before, the answer will be found in experimental data to be presented later in this paper.

Gelatin is rather unique as a protein because it does not yield any aromatic amino acids upon hydrolysis. Although the literature does not reveal any information as to the amino acid requirements of C. welchii, it was thought probable that the aromatic amino acids such as tryptophane might

be important in the production of toxin or at least in the growth of the organism.

Experiments involving the amino acid requirements of C. botulinum (Burrows, 1934) and C. sporogenes (Knight and Fildes 1933) have been done, and the reports emphasize the need of tryptophane and cystine by these organisms.

It being possible that such may apply to C. welchii, as well, the growth of the organism was observed in a medium containing 1 per cent enzymatic hydrolyzed casein. Such a preparation provides the organism with a source of all "essential" amino acids, including tryptophane and cystine.

2. Enzymatic hydrolysate of casein as a source of nitrogen for C. welchii. A preparation labeled "Amino Acids, Stearns" was used as the source of amino acids in this experiment. It was obtained through the courtesy of Dr. Richard Johnson, of the Fredrick Stearns and Company, Detroit, Michigan. These amino acids are the result of an enzymatic hydrolysate of casein, in which the identity of the amino acids of the casein molecule is preserved intact.

Basal medium. Enzymatic hydrolysate of casein, 10.0 grams; NaCl, 2.0 grams; $MgSO_4 \cdot 7H_2O$, 0.02 grams; KH_2PO_4, 5.45 grams; 5 M. NaOH, a sufficient amount to adjust the pH of the medium to 7.8. Tubed in 7.0 ml. amounts in 15X125 mm. test tubes, and autoclaved at 15 pounds pressure for 15 minutes.

Test Mediums. As in previous experiments, each test medium was prepared from a tube of the basal medium. 0.1 ml. of 20 per cent glucose and 1000 gammas of thioglycollic acid

were added to each tube and the test substances were added as indicated in Table 4.

Inoculum: The inoculum was 0.1 ml. of a suspension of SR-12 prepared in the manner previously described.

Incubation: 15 hours at 37°C. in an "R" jar.

Results:

TABLE 3

Enzyme hydrolysed casein as a source of Nitrogen for Clostridium welchii.

Tube	Assay	Colorimeter reading	Hemolytic activity*
1	Basal	0	0
2	Basal, 10 gammas P/ml.	14	1:20
3	" 100 gammas P/ml.	16	1:40
4	" 1.0 mgm. P/ml.	25	1:40
5	" 10 mgm. P/ml.	40	1:80
6	" 10 gammas W. E./ml.	16	1:20
7	" 100 gammas W. E./ml.	31	1:40
8	" 1.0 mgm. W. E./ml.	68	1:80
9	" 10.0 mgm. W. E./ml.	103	1:80
10	" 10 gammas L. E./ml.	41	1:40
11	" 100 gammas L. E./ml.	53	1:40
12	" 1.0 mgm. L. E./ml.	68	1:80
13	" 10.0 mgm. L. E./ml.	119	1:80
14	R.O.B. medium	134	1:160

The hemolytic activity of the uninoculated controls was zero in all cases.

P=Bacto-peptone; W.E.=Wheatamin Extract; L.E.=Liver extract, Lilly.

The results of this experiment as summarized in table 3 indicate that an enzymatic hydrolysate of casein provides an adequate supply of nitrogen-containing compounds for the development of C. welchii. When compared with gelatin and an acid hydrolysate of gelatin, the enzymatic hydrolysate is of equal value as regards to supplying the cell with sufficient nitrogenous "building blocks" for the synthesis of its protoplasm and for toxin production.

Further, it will be noticed that the hemolytic activity of the toxin produced was not as great in any instance as that which was produced in the "ROB" medium (tube 14, table 3). This question has been raised before; a possible explanation with accompanying experimental evidence will be presented later.

The experimental data presented in tables 1, 2, and 3 agrees with the beliefs of Walbum and Reymann (1933) who worked with C. welchii, and Mueller (1940) working with the diphtheria bacillus. These workers are of the opinion that complex nitrogenous substances in the form of proteins, proteoses, or peptone are unnecessary for the growth of or toxin production by these organisms.

It is true that the added substances (peptone, etc) were all very complex in structure. However, it can be seen that growth occurred when very small amounts of these substances were added. In the writer's opinion the amount of nitrogen supplied by these substances was too limited to be of much value considering that a reasonable amount of

growth occurred.

It is unfortunate that these experiments could not have been followed up with an investigation of the individual amino acid requirements of C. welchii. The writer feels justified in not doing so, however, because all possible variables must be removed before studies of this nature are to be of any significance. The most obvious of these would be, of course, the source of the accessory growth factors of this organism. The experiment that immediately follows deals with this problem. In this connection, it was possible to establish that tryptophane is an effective aid in stimulating the growth of C. welchii. In order to view the effect of tryptophane on the growth of the organism, the reader is referred to the tables accompanying Experiment III in which the experimental data collected is portrayed.

In recent years some evidence has accumulated which may throw some light on the question of the amino acid requirements of the genus Clostridium. Although no specific information is available which applies to C. welchii, Burrows (1933) has made a rather complete study of the amino acid requirements of C. botulinum. He concluded that, of the amino acids, cysteine, proline, and leucine were essential for the growth of C. botulinum, Lysine and glycine, though not essential, were active in promoting the growth of this organism. In a later communication, Burrows (1934) reported that cystine and tryptophane, together or separately, exerted a marked stimulating action on C. botulinum when

they were included in a medium composed of dextrose and acid hydrolysates of casein or gelatin.

Quastel and Stephenson (1926) have offered a possible explanation as to the reason why members of the Clostridia thrive better in the presence of amino acids having a sulfhydryl grouping (-SH-). These workers maintain that this grouping is readily oxidized to the disulfide linkage (-S-S-). Hence, through the agency of the oxidation of substances containing a sulfhydryl grouping (cysteine, methionine, etc), the redox potential of the substrate would be adjusted to a point which would favor the proliferation of the obligate anaerobes.

In 1934, Stickland reported the results of studies of the chemical reactions by which C. sporogenes obtains its energy. He offers experimental evidence to show that two amino acids may combine to form an oxidant-reductant team. Thus, one of them will act as a hydrogen donor, the other as a hydrogen acceptor, and the interreaction between them provides source of energy for the organism.

The role of the amino acids in the metabolism of these anaerobes that are predominately saccharolytic has not, as yet, been the subject of such intensive research. Weizmann and Rosenfeld (1937) have established, however, that asparagin is an essential metabolite for C. acetobutylicum. These workers are of the opinion that this substance plays a dual role in the metabolism of this organism. They suggest that asparagin acts in conjunction with biotin, biotin stim-

ulating the propagation of the bacteria while asparagin promotes the propagation by providing an efficient nitrogen supply. In addition, asparagin may function in a redox system by acting as a hydrogen acceptor in the oxidation of hydroxybutyric acid.

It is, at present, hardly permissible to speculate on the role of the amino acids in the metabolism of C. welchii. The writer presents the above facts merely to indicate the progress along these lines with other closely related anaerobic bacteria, and to indicate that an analogous situation may prevail with regard to C. welchii.

EXPERIMENT THREE

The Accessory Growth Factors of Clostridium welchii.

Some bacteria, particularly pathogenic organisms, require accessory growth factors or bacterial vitamins in their metabolic processes just as do higher animals. In 1912 Twort, successfully cultivated Johne's bacillus by using extracts of the timothy bacillus which he considered as the wild ancestor of the bacillus of Johne's disease. The train of thought initiated by Twort is best reported in his own words. "The bacillus of pseudo-tuberculous enteritis has lived a pathogenic life from such remote ages that it has lost the original power of synthesis of its wild ancestor-whatever bacillus that may have been and can no longer build up all its necessary foodstuffs outside the animal body." Thus the reason for the need of growth factors by parasitic bacteria. It is interesting to note that though Twort's hypothesis was not pursued for thirty years it might be thought of as the master premise that governs the activity in the study of bacterial nutrition which has gained such impetous in recent years.

The mass of literature on recent developments in bacterial nutrition is so voluminous that it is quite impossible to review it here. Excellent reviews of the subject are available to the reader, and he is therefore referred to Knight (1936), Koser and Saunders (1938), Fildes (1940), and Mueller (1940).

At the time this study was started, nothing, to the

writer's knowledge had been published concerning the growth factor requirements of C. welchii. Since it was found that this organism has nutritional requirements other than sources of carbon, nitrogen, and inorganic salts, an attempt was made to establish the nature of these substances.

A number of substances that had been found to be active as accessory growth factors for other bacteria were at first examined to see if they were also needed by C. welchii. The substances used were Pantothenic acid (Calcium pantothenate, Merck), Thiamin, Riboflavin, Pyridoxine (Vitamin B_6) and Nicotinic acid. With the exception of the last-named, all were obtained in the pure form from Merck and Company.

Fourteen separate experiments were attempted in an effort to establish these substances as possible growth accessory factors for Cl. welchii. In no instance when these compounds were supplied to the organism alone or in various combinations with one another did any stimulation of growth occur of the magnitude that might sanction their being called growth factors for C. welchii. Although a great amount of experimental evidence was accumulated that merely proved a negative hypothesis, the writer feels that, though it did result in inducing the work that is presented immediately following, to present it here would result in confusion rather than enlightenment. Hence, at this point we can categorically remark that pantothenic acid, thiamin, riboflavin, pyridoxine, and nicotinic acid alone or in combination with one another do not stimulate the growth of C. welchii.

In 1937 Wood, Tatum and Peterson described the extraction and concentration of a growth factor which was shown to be active for several species of propionic acid bacteria. Later, Brown, Wood, and Werkman (1938, 1939) showed that this same substance was also active in stimulating the growth of various members of the butyric-acid-butyl-alcohol producing group of bacteria. This growth factor called the "acidic ether-soluble factor" by Wood, et. al. is obtained by extracting Difco yeast extract that has been acidified, with ether. The resulting material when concentrated is able to stimulate the growth of the above-named groups of bacteria in very small amounts. A detailed description of the extraction, and concentration of this factor will be given under the section on "Technic".

Among the butyric acid-butyl alcohol anaerobes that Brown, Wood, and Werkman used in their study was Clostridium acetobutylicum, an organism used in the commercial production of butyl alcohol, and C. butylicum. Both of these organisms were shown to need the acidic ether-soluble factor. When, however, McDaniel, Woolley, and Peterson (1939) effected a partial purification of this factor, the stimulative activity for C. butylicum was increased but was decreased for C. acetobutylicum. At least the writer assumes that this was the case since no mention is made of increased activity of the more active substance for C. acetobutylicum. In 1940 McDaniel, Peterson, and McCoy demonstrated that biotin (Bios II_B, Vitamin H) was the only accessory growth factor required by C.

butylicum, whereas they state that no combination of known growth factors would stimulate the growth of C. acetobutylicum. This last mentioned report is slightly in error, however, because Weizmann and Rosenfeld (1939) reported on the need of biotin by C. acetobutylicum (Weizmann). This fact is confirmed by the work of Oxford, Lampen, and Peterson (1940).

Perhaps the reader is wondering how this discussion is concerned with C. welchii. It is a well known fact that C. welchii produces large amounts of butyric acid during the dissimilation of glucose. Its physiological properties are quite similar to those of the butyric acid-butyl alcohol group of anaerobes, particularly C. acetobutylicum, as witnessed by McCoy, Fred, Peterson, and Hastings (1930).

With these facts in mind, it occurred to the writer that perhaps this acidic ether-soluble yeast factor would stimulate the growth of C. welchii. This proved to be the case, as is demonstrated in Figure 1 and in table 4.

The Extraction and Partial Purification of a Growth Factor for C. welchii. 1. Technic of extraction. 100 grams of Difco yeast extract were mixed with sufficient 10 N.H_2SO_4 to make a thin paste. This was then mixed with plaster of Paris until a dry crumbly powder was obtained that did not set upon standing. The powder was then placed in an extractor with ether for a period of 48 hours. At the end of this time the ether was evaporated off leaving 5.2 grams of a brown resinous material. This was taken up in 100 ml. of

water, neutralized, and sterilized at 10 lbs. pressure for 10 minutes. This treatment does not effect the activity of the substance as was shown by McDaniel, Woolley, and Peterson (1939).

During the first operations with this substance, it was used as the aqueous suspension resulting from the ether evaporation. Although it was active in stimulating the growth of C. welchii in this state, little or no toxin was evident as indicated by the hemolytic activity of the broth cultures. It has been mentioned previously that Wuth (1923) demonstrated that phospholipides had the power to bind the toxin of C. welchii, thus appreciable reducing its activity. In this case, it was reasoned that this might be the cause of the apparent lack of toxin, and accordingly, the aqueous suspension was treated with two volumes of acetone, which resulted in the formation of a white precipitate. The acetone-water mixture was placed in the refrigerator over-night, and on the following day the precipitate was removed by centrifugation. The acetone was removed by evaporation under reduced pressure at a temperature of 50°C., and the remaining liquid was brought up to its original volume with distilled water.

This latter preparation gave indications of being slightly less active towards stimulating the growth of C. welchii but the liquid phase of the culture medium was definitely hemolytic. This was indubitably due to the removal of acetone-insoluble substances which interfer with the activity

of the toxin.

The stimulation of growth of C. welchii by the acidic ether-soluble factor. 1. Technic. The medium used in this experiment had the following composition:

Casein hydrolysate.....1.5%

Tryptophane.....0.025%

NaCl.....0.02%

$MgSO_4$.....0.002%

$FeSO_4 \cdot 7\ H_2O$.....0.005%

The medium was adjusted to a pH of 7.8 by the addition of 5.45 grams of KH_2PO_4 and 7.25 ml of 5 M. NaOH per liter of medium. The medium was distributed in 15X125 Pyrex test tubes in 7 ml. quantities. It was sterilized in the autoclave at 15 pounds pressure for 15 minutes. Before inoculating the medium 0.1 ml. of a 20 per cent sterile solution of dextrose and 1 milligram of thioglycolic acid were added to each tube. The acidic ether-soluble factor was added in the amounts indicated in Fig. 1. In this connection it will be noted that the concentration of the acidic ether soluble factor is expressed in units, a unit being the amount of substance extracted from 1 gram of yeast. This designation was applied by Brown, Wood, and Werkman and is retained here.

TABLE 4

The growth-stimulating effects of the acidic ether-soluble factor on the growth of C. welchii.

Number	Assay	Colorimeter reading	Hemolytic activity
1	Basal medium	0	0
2	" " , ET_2O,0.1unit	76	1:40
3	" " , " , 0.5 "	86	1:80
4	" " , " , 1.0 "	112	1:80

ET_2O = acidic ether-soluble extract.

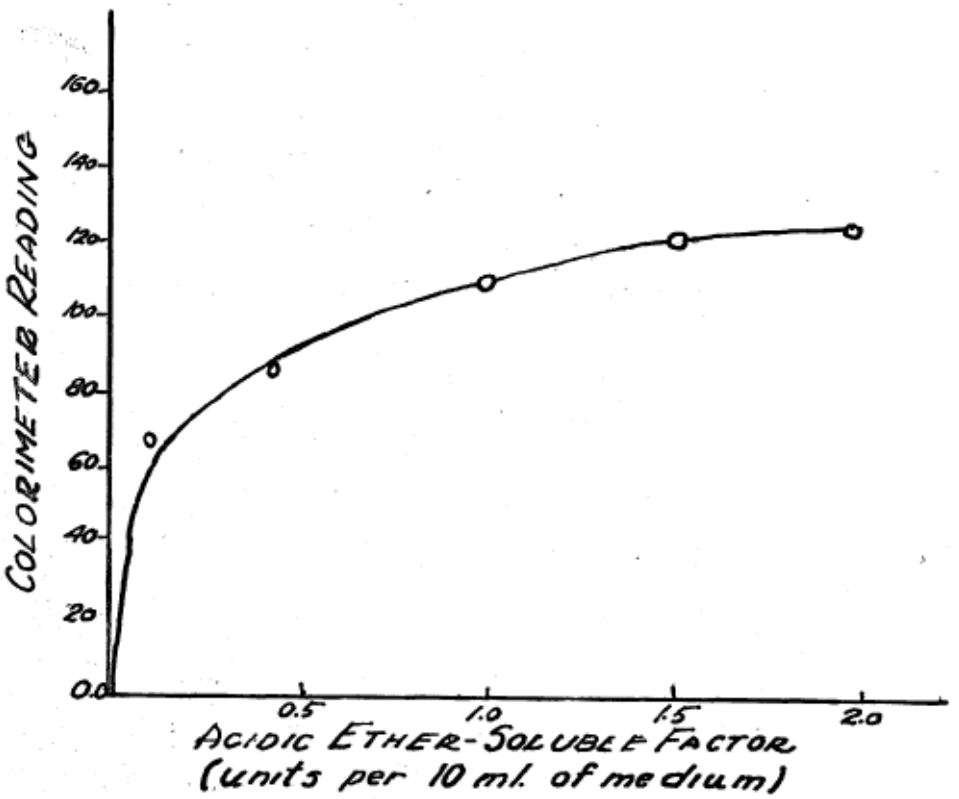

Fig. 1. Effect of increasing concentrations of the acidic ether-soluble factor on the growth of Clostridium welchii (strain SR-12).

The inoculun consisted of 0.1 ml. of a twice-washed suspension of SR-12 prepared in the manner previously described.

The cultures were incubated in an "R" jar for 18 hours at 37°C.

Results: Fig. 1 portrays the effect of increasing concentrations of the acidic ether-soluble factor on the growth of C. welchii. As might be expected, the growth of the organism increases as the factor is supplied in increasing amounts until two units of the substances are present.

The curve (Fig. 1) obtained when photometer readings were plotted against concentrations of the acidic ether-soluble factor is similar to a curve obtained by Porter and Pelczar (1941) when they studied the effects of increasing concentrations of biotin on the growth of certain strains of staphylococci. This fact, gives rise to a possible clue as to the identity of the acidic ether-soluble factor. In addition, the resistance of this factor treatment with acid and alkali; its stability toward autoclaving, and its solubility in water and acetone tend to suggest that this substance might contain biotin inasmuch as biotin exhibits these selfsame properties.

With this fragmentary evidence at hand experiments were set up in an attempt to determine whether or not biotin could replace the acidic ether-soluble factor as an accessory growth factor for C. welchii.

<u>The effect of biotin on the growth of C. welchii</u>.

Technic: The basal medium used in this experiment was the same as that described in section II of Experiment III with the exception that tryptophane was omitted.

The biotin was added in the concentrations indicated in Fig. 2.

The inoculum consisted of a twice-washed suspension of a twenty-four culture of SR-12 prepared in the manner previously described.

The cultures were incubated in "R" jars for 18 hours at the temperature of 37 C. In this experiment the growth-index was determined quantitatively as the bacterial nitrogen content of the mass of bacterial growth. For details of this procedure the reader is referred to the experimental section on "The Inorganic requirements of C. welchii," which immediately follows.

Results: The results of this experiment are presented graphically in Fig. 2. They indicate that biotin does stimulate the growth of C. welchii in amounts as small as 0.01 gamma per ml. of the medium. This would indicate that biotin or at least some substance similiar to biotin, is contained in the acidic ether-soluble factor, and that this substance is responsible for the growth-stimulating properties of this factor. Further, these results indicate that the optimal amount of biotin is in the neighborhood of 0.05 gamma per ml. of medium. The fact that biotin in amounts in excess of this figure do not appreciably increase

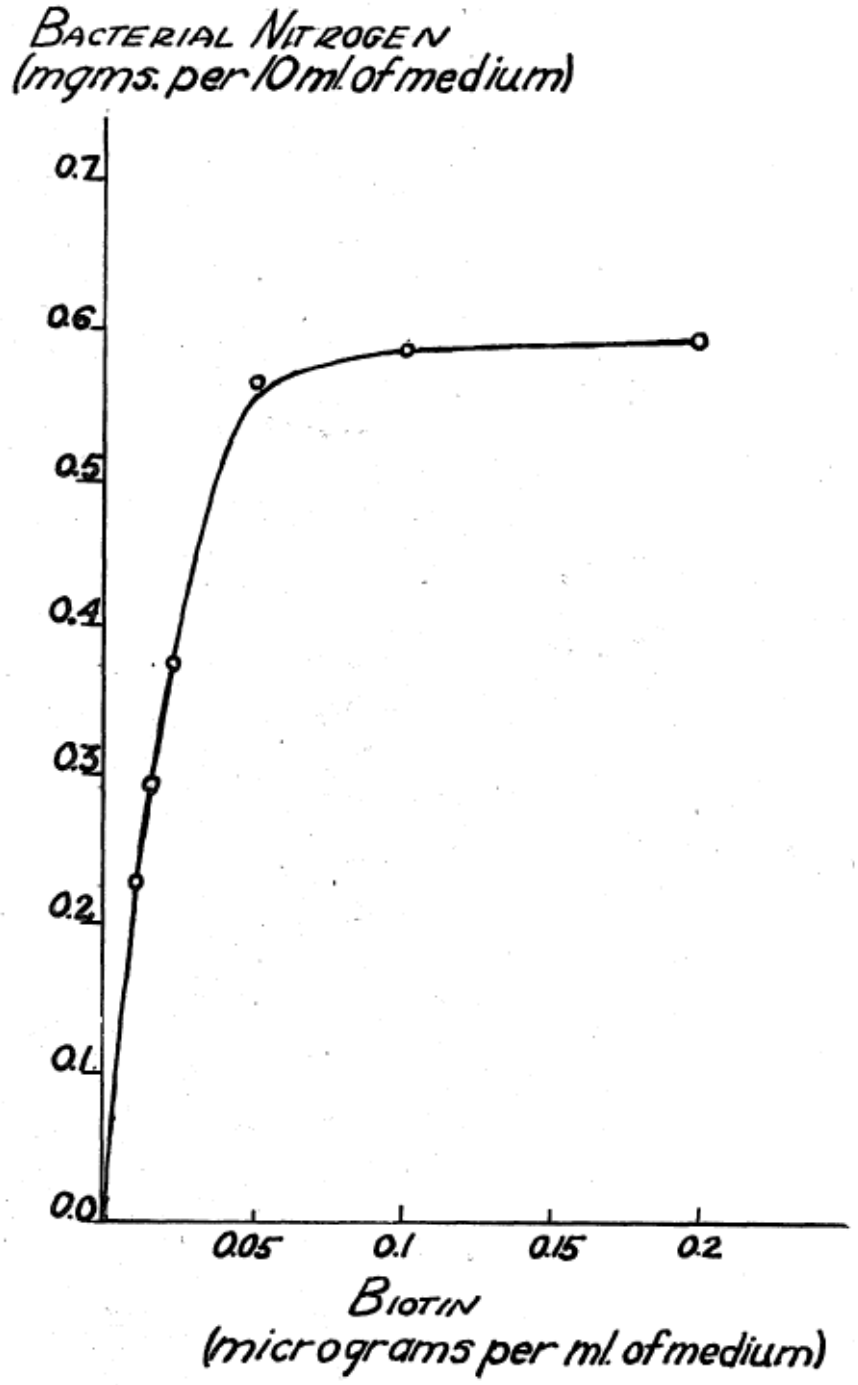

Fig. 2. Effect of increasing amounts of biotin on the growth of Clostridium welchii (strain SR-12).

the growth of this organism might possibly be due to a lack of some other necessary nutrient, the accumulation of harmful by-products of metabolism, or to other limiting factors.

Since several preliminary experiments revealed that certain other growth stimulating factors, known to be active for other bacteria did not effect the growth of C. welchii by themselves, it was thought that these substances might exert some growth-stimulating effect when combined in a medium containing biotin. An experiment fashioned along these lines was accordingly set up. For the results of this experiment, the reader is referred to the one which immediately follows.

The growth-stimulating effects of other substances on the growth of C. welchii in a medium containing biotin.

Technic: The basal medium used in this experiment was the same as that described in section II of Experiment III with the exception that tryptophane was omitted.

The growth-stimulating substances were made up in sterile solutions and added to the basal medium-as indicated in table 5 in the following concentrations: biotin, 0.01 gamma per ml. of medium; thiamin, 0.1 gamma per ml. of medium; riboflavin, 1.0 gamma per ml.; vitamin B_6, 1.0 gamma per ml.; nicotinic acid, 2 gammas per ml.; pantothenic acid, 0.01 gamma per ml. Tryptophane was added to the extent that the final concentration was 50 gammas per 10 ml. of medium.

The inoculum consisted of a twice-washed suspension of a 24 hour culture of SR-12 prepared in the manner previously described.

The cultures were incubated in "R" jars for 18 hours

at a temperature of 37 C.

Results: The results of this experiment reveal two facts concerning the nutrition of C. welchii. First, the need of tryptophane by this organism is clearly established (compare numbers 2 and 3, table 5). In this respect C. welchii resembles other pathogenic anaerobes in that it requires this amino acid, however, it does not correlate with other butyric acid forming anaerobes which have been shown not to require this amino acid (Brown, Wood, and Werkman, 1939). In this connection it may be that C. welchii, living a more parasitic existence, has lost its power to synthesize this amino acid and thus must avail itself of an exogenous source of tryptophane.

TABLE 5

Growth of C. welchii in various test media

Number	Composition of medium	Bacterial N (mgms. per 10 ml.)	Hemolytic activity of toxin
1	Base,	0.00	0
2	" , bi.	0.05	0
3	" , bi.,Tr	0.57	0
4	" , bi,Tr,B_6	0.40	0
5	" , bi,Tr,B_6,B_2	.44	0
6	" , bi,Tr,B_6,B_2,B_1	.44	0
7	" , bi,Tr,B_6,B_2,B_1,NA	0.32	0
8	" , bi,Tr,B_6,B_2,B_1,NA,PA	0.28	0
9	ROB medium	0.75	1:160

bi=biotin. Tr=tryptophane. B_6=vitamin B_6. B_2=riboflavin. B_1=thiamin. NA=nicotinic acid. PA=pantothenic acid.

Secondly, it is shown quite conclusively that vitamin B_6, riboflavin, thiamin, nicotinic acid, and pantothenic acid do not stimulate the growth of C. welchii when they are added to a medium containing biotin.

In this particular experiment the bacteria-free culture mediums (numbers 1-8) exhibited no hemolytic activity. The reason for this is not clear, since the organism was able to produce fairly large amounts of toxin in a medium prepared in exactly the same manner, but containing the acidic ether-soluble factor instead of a biotin concentrate.

An attempt was made, however, to determine wherein the great trouble lay, and a short series of experiments was set up with this view in mind.

The two possibilities as to the seat of this discrepancy were the basal medium and the biotin preparation. Therefore, a concentrate containing biotin was prepared from molasses according to a modification of the procedure of Kögl and Tönnis (1936). In addition, a basal medium was prepared containing 5 per cent of gelatin (rendered iron-free) instead of hydrolysed casein; all other ingredients remained the same.

The inoculum was prepared according to the procedure described elsewhere, and the cultures were incubated under anaerobic conditions for 18 hours of 37°C.

A representative protocol of this series of experiments appears in table 6.

TABLE 6

Comparison of toxin production by C. welchii in various media.

Number	Composition of medium	Hemolytic activity of toxin.
1.	Hyd. cas., 0.1 gamma biotin #1 per ml.	0
2.	Hyd. casl, biotin #2 0.1 ml. 1:10 dil. per ml.	0
3.	Gelatin, 0.1 gamma biotin #1 per ml.	1:80
4.	Gelatin, biotin #2 (as above).	1:160

Hyd. cas.=basal medium containing 1.5 per cent acid hydrolysed casein, Gelatin basal medium containing 5 per cent iron-free gelatin. Biotin #1=biotin concentrate obtained from S. M. A. Corporation assayed to contain 100 gammas per ml. Biotin #2=biotin concentrate prepared from molasses, unassayed.

From the results presented in table 6, it is evident that the basal medium prepared from acid hydrolyzed casein contained some substance which either destroyed the toxin or supressed its formation. Whatever the nature of this substance, it was probably introduced into the medium unwittingly since a similiarly prepared batch of medium afforded the production of a fair amount of hemotoxin.

EXPERIMENT FOUR

THE EFFECT OF VARIOUS CARBOHYDRATES ON THE PRODUCTION OF TOXIN BY C. WELCHII.

Anaerobic bacteria are usually acknowledged as being either proteolyic or saccharolytic according to their predominate physiological activity. In this connection, C. welchii is a saccharolytic anaerobe since it is not capable of attacking proteins in the sense that a proteolytic anaerobe such as C. sporogenes is.

As has been noted before (page4), C. welchii ferments all the common disaccharides and monosaccharides. The organism is able to effect a rapid dissimilation of glucose which results in the formation of lactic and butyric acids and carbon dioxide and hydrogen. Hence, the source of carbon usually supplied to the organism is in the form of glucose.

When toxin production is the goal to be attained, the concentration of glucose in the substrate is a most important factor. When large amounts of dissimiable carbon are available, the organism produces large amounts of butyric acid and the "pseudo-toxin" or histamine with a concomitant decrease in toxin formation. However, it is a well attested fact that the growth of C. welchii is in direct proportion with the amount of assimiable carbon present, providing, of course that nitrogenous compounds, inorganic substances, etc., remain constant. This experiment was designed for the purpose of observing the effects of other fermentable

carbohydrates on toxin production by C. welchii. The substances examined and their effect on growth and toxin production are presented in table 7.

This particular experiment was suggested by a statement of Mueller's (1940). He found that upon substituting maltose for glucose in medium used in the production of diphtheria toxin that the titre of the toxin was increased. According to Mueller this is due to the slower rate of fermentation of maltose by C. diphtheriae.

Technic: The basal medium used in this experiment was that containing the enzymatic hydrolysate of casein as described in the section on "Technic" in Experiment Two. The necessary accessory growth factors were supplied by 0.5 unit of the acidic ether-soluble factor. The carbohydrates were added from sterile solutions in such amounts that the final concentration would be 0.2 per cent.

The test mediums were inoculated with 0.1 ml. of a twice-washed suspension of a twenty-four hour culture of SR-12 grown in ROB medium.

The cultures were incubated in "R" jars at 37°C. for a period of 18 hours.

Results: The results of the effect of various carbohydrates on the production of toxin by C. welchii are presented in table 6.

TABLE 7

Effect of various carbohydrates on the production of toxin by C. welchii

Substance	Colorimeter reading	Hemolytic activity*
Glucose	77	1:160
Levulose	70	1:160
Sucrose	35	1:40
Maltose	79	1:160
Lactose	62	1:80
Inulin	20	0
Glycogen	54	1:40
Mannitol	22	0
Dulcitol	19	0
Salicin	24	0
Calcium lactate	12	0

*All uninoculated controls were not hemolytic.

The data presented in table 7 bears out the well known fact that C. welchii grows best when in the presence of easily fermentable carbohydrates. Thus, it grows best when glucose, levulose, maltose, lactose, and sucrose are supplied in that order. When those compounds which it ferments slowly, or not at all, mannitol, dulcitol, inulin, and salicin, are present; the organism grows poorly and accordingly produces very small amounts of toxin.

From the results as set forth in table 7 it is also evident that the concentration of the toxin formed was greatest in those cases where growth was more abundant.

Hence, one might suppose that toxin production is a direct function of the metabolic activities of the cell. That is to say, perhaps toxin enjoys a stellar role as an integral part of some enzyme system of the organism, and thus as the bacteria increase in number, the amount of toxin will also increase. Whatever the case may be, one can, according to the data presented, say the heaviest growth occurred in those media containing fermentable carbohydrate; and when the bacteria-free culture medium was examined for the presence of toxin, the hemolytic activity was greatest in those self-same tubes.

Time did not permit a more detailed study of the relation of carbohydrates to the production of toxin by C. welchii. It may be that further studies directed along these lines would reveal the nature and optimal concentration of the source of carbon best suited for the production of C. welchii toxin.

EXPERIMENT FIVE

The Inorganic Requirements of C. welchii.

Bacteria, like other forms of living matter, have certain inorganic requirements that must be supplied to the cell before growth will take place. Until recently, not a great deal of attention has been paid to these requirements except in special, isolated instances. The reason for this apparent neglect is possibly due to the fact that because bacteria require only very small amounts of these substances they are supplied unwittingly as contaminations in the ingredients of the common laboratory media.

However, in connection with those bacteria which form genuine toxins, the inorganic constituents of the substrate have been shown to play a most important part in the nutrition of the organism. Most of the work of this nature has been done on C. diphtheriae (Walbum, 1921; Locke and Main, 1931; Sheff and Scheff, 1935; and Pappenheimer and Johnson, 1937). For a review of the literature on the inorganic requirements of the diphtheria bacillus the reader should consult these papers. As for the inorganic requirements of C. welchii as they are related to its formation of toxin, but a single paper has come to the author's attention. This is the paper of Locke and Main (1931) mentioned above. These workers studied the effects of iron, in various forms, copper, cystine, pyrophosphate, blood and liver extract on the formation of toxin by C. welchii. Their results indicate that ferrous

ions stimulate the formation of toxin to the greatest degree, while copper ions have relatively no effect. Ferric iron was shown to have little or no effect on toxin formation as was ferrocyanide ion. Ferricyanide ion and blood were shown to be definitely antagonistic to toxin formation. In the writer's opinion the results with liver extract must be ruled out since this substance undoubtedly supplied substances other than iron-containing compounds which stimulated the growth of the organism.

This experiment was undertaken for two reasons. First, it was thought that a more detailed study of the quantitative relationships of iron and copper in connection with the nutrition of C. welchii would be desirable. The second reason evolved from an observation made from the experimental data of Experiment One. It was found that when the ingredients of "ROB" medium were added separately instead of being combined and sterilized as is done in the original recipe, that the amount of growth was equal to that of the ROB medium, while toxin production was considerably less. Upon investigation, the iron content of the latter medium was found to be considerably less than that of the former. This reasoning was prompted by a similiar situation which confronted Pappenheimer and Johnson (1936).

Technic: In order to study the effects of iron on the nutrition of a particular organism it is first necessary to go through the tedious process of freeing the ingredients of the medium of iron. This was done according to

the method described by Mueller (1941). This procedure consists essentially of adsorbing the iron on a floc of calcium phosphate prepared by mixing the substance to be freed of iron with calcium chloride and phosphate ions. The amount of iron present in any substance is tested colorimetrically, following the method described by Mueller (loc. cit.) The medium used in this experiment had the following constitution:

1. Acid hydrolysed casein...1.5%
2. Tryptophane.....0.025%
3. $MgSO_4 \cdot 7H_2O$.....0.002%
4. NaCl.....0.2%
5. M/25 phosphate buffer (5.45 grams of KH_2PO_4 per liter with sufficient 5M NaOH added to bring the pH to 7.8).

The medium was tubed in 7.0 ml. amounts and sterilized at 15 pounds pressure for 15 minutes. After sterilization and just before inoculation the following substances were added to each tube: glucose, 0.1 ml. of a 20 per cent solution, thioglycolic acid, 100 gammas and 0.5 ml. of a crude concentrate of biotin (acidic ether-soluble extract). The various test substances in amounts indicated in the tables were added from sterile solutions. Before inoculation 0.1 ml. of 20 per cent sterile dextrose was added to each tube; thioglycolic acid was added to the extent of 100 gammas per ml. of medium, and the amounts of the various substances indicated in the tables were added from

sterile solutions. The final volume of each tube was brought up to 10 ml. by adding the necessary amount of sterile, distilled water. All tubes were incubated for 24 hours to insure sterility.

The inoculum consisted of 0.1 ml. of a twice-washed suspension of a 24 hour culture of SR-12 grown in "ROB" medium.

The cultures were incubated in "R" jars for 18 hours at 37 C.

In this particular case it was found necessary to abandon the use of turbidity readings as an index of bacterial growth. This was because on certain occasions precipitates occurred in the test mediums which caused the introduction of considerable error in the turbidity reading. Instead, the nitrogen content of the mass of bacterial growth was used as an index and a micro-Kjdehal method was employed to measure the nitrogen.

The procedure of washing the cells was essentially that described by Mueller (1935). The cells were "spun down" in an angle centrifuge at 3500 r.p.m. for 15 minutes. This is a shorter length of time than recommended by Mueller, but sedimentation is more rapid in this type of centrifuge than in the common type of machine. The cells were washed in 0.05 per cent acetic acid, recentrifuged, and the supernatant fluid was removed with a capillary pipette.

The micro-Kjeldahl technic for the determination of nitrogen was that of Sobel, Yuskin and Cohen (1937) as adapted

for the determination of bacterial nitrogen by Porter and Pelczar (1941). For details of the technic these original articles should be consulted.

The effect of iron on the production of toxin by C. welchii. Locke and Main showed that the hemolysin content of a medium to which had been added 0.0023 per cent ferrous ion was of 1.7 times that of the control medium. The addition of ferric ion was shown by these workers to exert no effect on the production of toxin by C. welchii. Since these workers did not make quantitative estimations the iron content of their medium or attempt to determine the optimal concentration of iron compatible with maximum toxin production it was deemed worthwhile to direct this study towards these ends.

TABLE 8

Effect of ferrous and ferric iron on the production of toxin by C. welchii.

Number	Micrograms of iron added to basal medium.	Bacterial N (micro-Kjeldahl), mgm. per 10 ml. medium	Hemolytic activity of toxin*
1	0.1 Fe	0.16	1:40
2	1.0 Fe	0.20	1:160
3	10.0 Fe	0.23	1:160
4	50.0 Fe	0.32	1:40
5	100.0 Fe	0.39	1:20
6	0.1 Fe	0.14	1:40
7	1.0 Fe	0.19	1:160
8	10.0 Fe	0.22	1:80
9	50.0 Fe	0.27	1:20
10	100.0 Fe	0.34	1:20
11	ROB medium	0.75	1:160

*All uninoculated controls were negative.

According to the results presented in table 8, the optimum amount of iron for maximum toxin production is between one and ten micrograms per ml. of medium. As the amount of iron added increased up to 100 gammas of iron per ml. of medium the growth of the organism definitely increased as indicated in the table. However, in this case, increased growth was accompanied by a decrease in toxin production.

The results obtained when ferric iron was added to the basal medium are similiar to those when ferrous iron

was present. This does not substantiate Locke and Main's results, but it may be that the strong reducing conditions under which the cultures were grown may have reduced the ferric ions to the ferrous state.

The Effect of Copper on the Production of Toxin by C. welchii. Small amounts of copper seem to be essential in the metabolism of all cells. The function this element performs is unknown. In regard to the effect of copper on the formation of toxins by bacteria, a considerable amount of work has been done in connection with the diphtheria bacillus. With specific reference to C. welchii, however, little has been done on the effect of copper on the growth and toxin production.

Locke and Main carried on several experiments along these lines, and their results indicate that copper, added to the extent of 0.0008 per cent of the medium employed did not influence growth or toxin production. Preliminary experiments along these lines gave indications of the contrary, however, and several experiments were set up in an attempt to establish the optimum amount of copper compatible with maximum growth and toxin production by C. welchii.

Technic: The medium, and test solutions used were prepared in the same manner as described in the section dealing with the effects of iron in relation to growth by C. welchii.

The growth of the bacteria was estimated according to the nitrogen content of the mass of bacterial growth, and

the concentration of the toxin was estimated as its hemolytic activity.

Results: The data which indicates the manner in which the growth of and toxin production by C. welchii is influenced by copper and copper in the presence of iron is presented in table 8. In general these results indicate that copper alone does not particularly stimulate the growth of the organism though it does influence toxin production to a considerable degree (compare tubes 2 and 13, table 10.

TABLE 9

Effect of Copper on Growth and Toxin Production by C. welchii.

Number	Micrograms of copper per ml. of medium	Bacterial N (micro-Kjeldahl) in mgms. per 10 ml. medium	Hemolytic activity of toxin*
1	0.1	0.05	1:20
2	1.0	0.12	1:80
3	10.0	0.00	0
4	50.0	0.00	0
5	100.0	0.00	0

*All uninoculated controls were non-hemolytic.

When iron and copper were both present in the medium, more vigorous growth took place, as well as an increase in the production of toxin. Again the results (table 10) indicate that the presence of 10 micrograms or more of copper pre ml. of medium is enough to inhibit the growth of C. welchii. Parenthetically, it might be said that perhaps 10 micrograms of cupric ion represents the critical concentration, since Locke and Main's results indicate that the addition of approximately 8 micrograms of cupric ion occasioned a very slight increase in the hemolytic activity of the

treated cultures over the controls. Varying the amount of iron in the presence of copper apparently has no effect on either growth or the amount of toxin formed, within, of course, the limits indicated. It is interesting to compare growth and concentration of toxin obtained in the medium devised by Reed Orr, and Baker (table 10) with medium containing the acid-hydrolyzed casein, for example, tube 6, table 10.

TABLE 10

Effect of copper and copper and iron on the growth of and toxin production by C. welchii.

Number	Composition of medium		Bacterial N (micro-Kjeldahl) mgm. per 10 ml. medium	Hemolytic activity of toxin*
	Fe	Cu		
1	0.1 gamma per ml.	0.1 gamma per ml.	0.21	1:160
2	0.1 "	1.0	0.26	1:160
3	0.1 "	10.0	0.00	0
4	0.1 "	50.0	0.00	0
5	1.0 "	0.1	0.18	1:160
6	1.0 "	1.0	0.24	1:160
7	1.0 "	10.0	0.00	0
8	1.0 "	50.0	0.00	0
9	10.0 "	0.1	0.05(?)	(0)
10	10.0 "	1.0	0.24	1:160
11	10.0 "	10.0	0.00	0
12	10.0 "	50.0	0.00	0
13	ROB medium		0.87	1:320

*All uninoculated controls were non-hemolytic

In the first instance, the "ROB" medium supported almost four times as much growth, but the concentration of the toxin was only twice as high. If the ingredients of the semi-synthetic medium could be adjusted to support a more vigorous growth undoubtedly an extremely high concentration of toxin would be found.

The effect of bound iron on the growth and toxin production by C. welchii. It having been shown that iron profoundly affects the production of toxin by C.welchii, it was reasoned that an investigation of the effects of iron-containing compounds-such as the organism might come in contact with in nature-on the formation of toxin. A sample of recrystallized hemin was purchased from the Eastman Kodak Company, and the effects of this substance on the metabolism of C. welchii were accordingly investigated. (Hemin is the chloride of heme, the ferrous-porphyrin compound that is the prosthetic group of hemoglobin. It is similiar in structure to the cytrochrome series of compounds.)

Table 11 shows the effect produced on C. welchii in regard to growth and toxin production. The growth, measured as the nitrogen content of the bacterial mass of growth, was in no instance of the same magnitude as that occurring when inorganic iron was added to the medium (see table 10). Neither did the amounts of hemin added appear to be compatible with efficient toxin production. It is interesting to note that 50 micrograms, or more, of hemin per ml. of the

medium were sufficient to inhibit the growth of C. welchii. This particular part of the experiment was repeatedly done, but the results were always the same. No explanation can be given for this phenomenon inasmuch as neutral solutions sterilized by filtration through a chromium-plated Seitz filter, were added to the base medium.

TABLE 11

The effect of "bound iron" on the growth of and toxin production by C. welchii.

Number	Micrograms of hemin per ml. of medium	Bacterial N (micro-Kjeldahl), mgms. per 10 ml. medium	Hemolytic activity of toxin*
1	0.1	0.15	1:40
2	1.0	0.16	1:40
3	10.0	0.19	1:40
4	50.0	0.00	0
5	100.0	0.00	0

*All uninoculated controls were non-hemolytic

Comparison of the hemolytic activity and the lethal power of C. welchii toxin. In all of the experiments which have been described, the concentration of the toxin has been estimated according to its hemolytic activity. However, it is the fact that C. welchii toxin is lethal which causes it to be of medical importance, and for this reason it seemed advisable to compare the hemolytic and lethal qualities of several preparations of C. welchii toxin.

The technic of determining the minimum lethal dose (m.l.d.) was as follows: mice weighing from 17 to 20 grams

were selected for the test and were injected intramuscularly with varying amounts of the bacteria-free culture medium to be tested. The least amount of toxin killing the mouse in 24 hours was recorded as the m.l.d. The mice selected as controls were injected with 1.0 ml. of a culture filtrate that had been heated at 70°C. for 30 minutes.

A representative protocol of these determinations is presented in table 12.

TABLE 12

Comparison of hemolytic activity and killing power of the toxin of C. welchii produced in various media.

Number	Type of Medium	Hemolytic activity	Killing power (m.l.d.)
1	Non-synthetic*	1:160	0.5 ml.
2	Semi-synthetic**	1:80	0.1 "
3	Semi-synthetic***	0	>2.0 "
4	Control	0	0.0 "

* Medium of Reed, Orr, and Baker
** see Experiment 5, table 8, no.3
*** see Experiment 3,

From the results presented in table 12 one might suppose that since the hemolytic activity and the lethal effects of C. welchii toxin do not parallel, the use of the hemolytic activity as an index of toxic strength does not give a true picture of the actual toxicity of the culture medium. However, in cases where a large number of test media are being examined to note the effect of a single ingredient of the

medium on the growth and toxin production of C. welchii, one is justified in employing such an index for two reasons: first, the determination of the hemolytic activity of the toxin does give an accurate index of the relative toxic qualities of the test medium; second, the titration of the hemolytic activity is cheaper and less time-consuming than determination of the m.l.d. or the L_f dose of toxin as determined by the Ramon flocculation technic.

It is interesting to note that the killing power of the toxin produced in the semi-synthetic medium was five times as great as that produced in the non-synthetic medium (compare numbers 1 and 2, table 12). Superficially, these results might lead one to concur in the belief that C. welchii toxin represents a mixture toxic elements. On the other hand, the evidence submitted by Glenny, et al. (1931), and by MacFarland, Oakley, and Anderson (1941) which infers that C. welchii toxin is a singular substance is quite convincing These last-named workers have shown that the use of phosphate buffers (which were used in these experiments) in the toxin and cell suspension diluent seriously interfers with the hemolytic activity of the toxin. A further hint which would tend to support the "singular" theory is obtained by inspecting the results presented in number 3, table 12. Here, in a medium in which C. welchii grows readily, neither hemolytic activity nor killing power of the toxin is detectable. In other words, when the "hemotoxin" is not formed neither is the "lethal" toxin.

DISCUSSION

In reviewing the literature on the toxinogenesis of C. welchii, one is struck by the fact that a majority of workers are hesitant to use a medium of relatively simple constitution. However, Walbum and Reymann (1933) and Reed, Orr, and Baker (1939) have shown that C. welchii will produce potent toxin in a medium devoid of solid meat particles. When the toxin thus formed is to be employed as an antigen for the production of antitoxin, such media are indeed satisfactory. But it seems apparent to the writer that in experimental procedures designed for the purpose of establishing the identity of the toxin and its role in the metabolism of the organism, a simple medium of definite chemical composition ought to be used.

In the experimental work described in the preceding section, evidence that C. welchii will produce its toxin in such a medium is afforded by the fact that an ample amount of toxin was produced by the organism when grown in a medium consisting essentially of hydrolysed casein, glucose, inorganic salts, and the necessary accessory growth factors. As a matter of fact, the amount of toxin formed when estimated as the m.l.d., was found to be considerably greater than the amount formed in a non-synthetic medium. This being the case, the writer feels that the belief that C. welchii requires peptone, proteose, or other complex nitrogenous compounds in order to produce its toxin is no longer tenable.

With regard to the specific nutritive requirements of

C. welchii, it is regrettable to admit that studies on the exact amino acid requirements of this organism could not be attempted. Although it was established that the growth of C. welchii is considerably enhanced by the addition of tryptophane, further experiments directed toward establishing the identity of the amino acids essential for the growth of C. welchii should be attempted.

According to the experimental evidence available at present, glucose is undoubtedly the best and most logical source of carbon for the organism when toxin production is the desired end. C. welchii owes its pathogenicity to the fact that it is able to produce its toxin when it is residing in the tissues of its host. Glucose is present in blood and the tissue fluids in amounts in the neighborhood of 100 mgms. per cent, this amount being in accord with the much-observed fact that the maximum amount of glucose for optimal toxin production by C. welchii is 0.2 per cent.

The question of the accessory growth factors needed by C. welchii has been dealt with to the extent that it can categorically be said that this organism must have available an exogenous source of biotin in order that growth may take place. In addition, it is quite possible that C. welchii has need of at least one other accessory growth factor, inasmuch as the organism always grew more luxuriantly on the medium of Reed, Orr, and Baker, which, as pointed out before, consists mainly of gelatin, peptone, glucose, and various inorganic salts. Needless to say, the nature of this substance,

if it does exist, is entirely unknown.

One can, at this time, only speculate as to the part played by biotin in the nutrition of C. welchii. Because it is active in such small amounts, biotin undoubtedly forms a part of some enzyme or coenzyme. Our present knowledge of the function of the accessory growth factors as a whole is quite empirical, and until the biochemists are able to formulate the nature and action of the basic enzyme systems which are concerned in the anabolic activities of the cell, the bacteriologists can scarcely do better than to speculate as to the nutritional role of the bacterial accessory growth factors.

In the section on the experimental work, it was pointed out that very little appears in the literature concerning the inorganic requirements of C. welchii and their effect upon toxin production. Repeated references were made however to the very impressive paper of Locke and Main (1931). To the writer's knowledge, this paper is the only one in which the effects of various inorganic substances on toxin production by C. welchii were studied in detail. The observation by these workers that ferrous iron stimulates the growth of and toxin formation by C. welchii has been confirmed. In addition, it was shown that, within certain limits, the amount of toxin produced is inversely proportional to the amount of ionized iron present in the substrate. In other words, the organism grows poorly in an iron-deficient medium,

but when the iron content of the medium exceeds 100 gammas per ml. of the medium, the C. welchii grows readily; but little or no toxin is formed. The presence of cupric ions in very limited amounts appears to stimulate the growth of C. welchii as well as toxin production.

The effects "bound iron" on the growth and toxin production were studied. In this connection, it is interesting to note that hemin has a definite antagonistic effect upon C. welchii. The culture medium containing 50 gammas of hemin per ml. of medium would not support the growth of the organism. Although it was not determined whether the organisms were destroyed or merely prevented from proliferating, it would be interesting to view results of additional pursuits along these lines. Such studies might reveal the possibility of hemin as a therapeutic agent for gas gangrene.

If one further considers the role of ionized iron in the mechanism which results in the formation of toxin by C. welchii, it might be of value to compare the iron content of the contents of the intestinal tract with that of the tissue fluids. The normal habitat of C. welchii for all practical purposes can be considered to be the intestinal tract. Here, the organism does not produce its toxin, but cultures of C. welchii isolated from feces and introduced into muscular tissue are capable of inciting gas gangrene. Can it be that the amount of iron in the intestinal contents (shown to be about 550 gammas per gram of dry feces by Shohl, 1939) is sufficiently large to supress toxin formation, while the iron present in the

tissue fluids represents the optimal amount for toxin production? This latter figure has been shown to be about 2 gammas per ml. of tissue fluid (Moore, et. al., 1937). Of course, such reasoning should not be considered to be the ultimate answer to the question of toxin formation in the tissues since numerous discrepancies are known to exist.

Two theories of the formation and function of C. welchii toxin have been proposed; one by Dernby and Walbum (1923) concerning the formation of diphtheria toxin which Walbum, in a later communication (1933), suggests equally applies to the formation of C. welchii toxin; the other that of Locke and Main (1931).

In brief, Dernby and Walbum are of the opinion that toxin is not a secretory product of the cell, but rather is formed extracellularly as a result of the degradative activity on proteins in the substrate by certain proteolytic enzymes. With specific mention to C. welchii, Walbum bases his argument on the fact that the activity of the gelatinase produced by C. welchii increases with the potency of the toxin.

Locke and Main formulate an enzymic concept of C. welchii toxin. To them the toxin of C. welchii appears as a sort of respiratory substance having ferrous iron as the predominant catalyst. This type of substance, they say, resembles the respiratory enzymes of the dehydrogenase type and the proteoases of the pH 4 papain type, which are not inactivated by sodium cyanide or cysteine, but are (with the possible exception of dehydrogenase) inactivated by the cupric ion.

The writer neither subscribes to nor denounces these proposals. They are presented merely to indicate the present status of the toxin of C. welchii.

Very recently there has appeared in the literature a paper concerning a new property of C. welchii. MacFarland, et al., (1941) have reported the results of investigations concerning the lipoprotein-splitting property of the α toxin of C. welchii. Their results reveal that this toxin is capable of hydrolyzing the lipoproteins of certain human serums and also lecitho-vitellin obtained from egg yolk. This property could be quantitatively supressed by the addition of antiserum, and, in addition, these workers present evidence to show that this toxin can not hemolyze red blood cells in the absence of ionized calcium. It is pointed out in this paper that the hemolytic activity of the toxin and the lipoprotein-splitting property are both manifestations of the same enzyme reaction. Thus, the stroma of the red cell being weakened, or destroyed, through the agency of the agency of the action of this enzyme on its constituent lipoproteins allows the hemoglobin contained within to be released into the surrounding menstrum.

Thus, with this evidence at hand, and if a bit of ideology will be adjudged permissible, the writer should like to present his concept of the formation and function of C. welchii toxin.

It is assumed that the toxin of C. welchii is a singular substance, secreted by the cell for the purpose of reducing substances, other than carbohydrates, into less complex molecules, and thus obtaining from these last-named substances,

a source of energy. Following this line of reasoning, one might suppose that the liquefaction of gelatin and the splitting of lipoproteins are due to the action of the same hydrolytic enzyme. The variety of physiological effects ascribed to the toxin, could easily be produced by its direct degradative action on the various tissues, and the products produced by such action.

In support of this hypothesis, it can be said that when an ample source of energy is available in the form of an easy fermented substance such as glucose, no toxin is formed. To the contrary, it may be argued that C. welchii is able to produce its toxin in a synthetic medium containing no complex substances such as proteins. However, though such a medium supplies all the essential nutrients necessary for the organism to multiply and to maintain its normal metabolic activities, it must be remembered that these conditions represent an unnatural environment for virulent, toxin-producing strains of C. welchii. In all probability, prolonged cultivation of the organism in this type of medium would result in a "loss of virulence", because the organism, having no further use for its hydrolytic enzyme, would cease to manufacture it.

Though nutritional studies of the toxigenic bacteria, as have been described, may aid in the eventual establishment of the identity of a particular bacterial toxin, their real value lies in the fact that they provide a rational basis for investigations directed toward the development of specific chemotherapeutic agents. A paper by Fildes (1940) provides

the inspiration for the above statement. In it he states that a knowledge of the "essential metabolites of bacteria" (an essential metabolite being described as a substance or chemical group which takes an essential part in a chain of syntheses necessary for bacterial growth) is a distinct advantage in research in chemotherapy inasmuch as these anti-bacterial substances function by interfering with an essential metabolite and thus inhibit growth.

SUMMARY

An investigation of the nutritive requirements of C. welchii in relation to toxin formation by this organism showed that:

1. C. welchii is able to produce its toxin in a semi-synthetic medium composed of hydrolyzed casein, glucose, inorganic salts and the necessary accessory growth factors.

2. When solutions of amino acids, i.e., hydrolysates of casein or gelatin, form the source of nitrogenous "building materials" in a medium, C. welchii is able to proliferate and produce its toxin. This information would indicate that more complex nitrogenous materials are unnecessary for toxin production.

3. Biotin is an accessory growth factor of C. welchii.

4. Certain substances which are growth factors for other species of bacteria do not stimulate the growth of C. welchii. These include riboflavin, thiamin, pyridoxin (vitamin B6), nicotinic acid, pantothenic acid.

5. The production of toxin by C. welchii is influenced to a considerable degree by the concentration of ionized-iron and copper in the substrate, and that under the experimental conditions set forth, hemin exerts an antagonistic effect upon the organism.

6. C. welchii may require additional accessory growth factors of other nutrients necessary for maximum growth to occur, as is evidenced by the fact that a non-synthetic medium supported a more luxuriant growth than did the semi-synthetic medium.

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