# NUTRITION OF THE DIPHTHERIA BACILLUS

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Concerning the earliest reported attempts toward the cultivation of the diphtheria bacillus in media free from protein or its higher cleavage products, little need be said. That they were carried out indicates a recognition of the probable importance of the ability to grow the organism in media of known and simple composition. That they should have resulted in practically complete failure was, in the light of our present knowledge, inevitable. Protein chemistry was still in its infancy. Certain of the amino acids were still unknown. The conception of accessory growth substances, or vitamins, had not yet been introduced, although it is noteworthy that this was to come within a relatively short time through studies with another unicellular organism, yeast (Wildiers' "bios," 1901), more than a decade before it was encountered in the study of animal nutrition.

Consequently the experiments of Guinochet (1892) with "proteid-free" urine, and of Uschinsky (1893) with a medium containing inorganic salts, sodium aspartate, and glycerol were foredoomed to failure. The latter, it is true, claimed to have obtained some growth, but without details of his experiments, the purity of his materials, the size and nature of inocula used, or quantitative data on the amount of growth observed, one may safely assume that it must have been relatively feeble. Attempts to repeat his observations on the part of Fraenkel (1894) and of Hugounenq and Doyon (1896) met with complete failure. To Uschinsky, however, appears to go the credit for being the first to attempt the cultivation of this, and other pathogenic organisms, by means of a reasonably constituted inorganic salt mixture together with chemically defined sources of nitrogen and carbon.

Later, Hadley (1907 a and b) undertook the repetition and extension of Uschinsky's early experiments with the diphtheria bacillus, introducing the amino acid glycine into the medium as a source of nitrogen. A measure of success was reported with some strains, failure with others. One may, perhaps, speculate as to the nature of some of the organisms which grew, described by Hadley as tremendous bacilli of more than 10 micra in length, forming so tough a pellicle that the tube could be inverted without causing its rupture. This modification in morphology and nature of growth he ascribed to alteration accompanying adaptation to the medium. It does not appear to have been observed by later investigators.

Toxin formation, in these early experiments, was demonstrated by the injection of varying amounts, from 0.5 ml. to 1.5 ml. or more of *culture* into a guinea pig, and, as far as one can make out, without the use of control animals protected with antitoxin.

For nearly ten years after the appearance of Hadley's papers very little seems to have been attempted toward an advancement of the general situation. From about 1917, when contributions on the subject began to appear from Rettger's laboratory (Robinson and Rettger, 1917) and continuing to the present time, a considerable volume of material has been accumulating. In order to review this in any detail, certain general considerations regarding objectives and directions of approach are essential, and the implications following from variation in strains must be indicated.

## OBJECTIVES

It is essential to direct attention to this subject because there are at least two different criteria of growth used by the various contributors to the literature. From the following considerations these may be differentiated by the terms "detectable growth" and "normal growth."

If one implants a living bacterial cell in a tube of sterilized medium, and holds the culture at a suitable temperature, one of three things must necessarily occur: either the cell will (*a*) die, (*b*) remain more or less permanently dormant, or (*c*) it will

multiply. If several or many cells are used the situation becomes more complicated, because the death and disintegration of some of the cells may so alter the composition of the medium that some of the survivors begin to multiply. In either case, cell division, if it occurs, may proceed in a variety of ways. The ultimate result will depend on the rate at which division takes place, and on the number of successive generations produced. These facts are all perfectly obvious and have been extensively dealt with by students of cell physiology.

"Growth" may be presumed to have taken place with the division of a single cell into two during an infinite time interval, but its detection at this level would be impracticable. "Detectable growth" begins at some arbitrary point which will depend upon the method used for its determination, but which for practical purposes must imply something quantitatively more definite than delayed fission of a single cell. It will, on the other hand, be far short of the growth which the cell in question is known to be able to attain under optimal conditions. For example, if one represents graphically the degree of growth by a line extending from a zero base to a maximum of 100, detectable growth would fall at any point between perhaps 1 and 10, depending on the method chosen and accuracy of observation.

The significance of these comments, which may appear entirely academic, lies in the fact that the choice of one level of growth, rather than another, is governed by the purpose of the individual investigator. "Detectable growth" has been used by Braun, from whose laboratory have come a number of widely-quoted contributions dealing with the growth of the diphtheria bacillus, to determine the simplest requirements compatible with cell division. He states (Braun, 1931) "Es kommt nicht auf Üppigkeit und Schnelligkeit des Wachstums, sondern nur auf die Beantwortung der Frage der Unentbehrlichkeit und der unmittelbaren Assimilierbarkeit der dargebotenen Nährstoffe an." This, of course, represents a perfectly justifiable objective, and has resulted in valuable observations on minimal nutritive requirements. Yet even Braun in describing his experiments, introduces a kind of quantitative measure of growth together with a time

element when, for example, he states (Braun and Mündel, 1929) that in a particular medium a strain of the diphtheria bacillus grew "rapidly and vigorously," so that five passages could be obtained in 24 days, while a reduction in concentration of one of the components, sodium aspartate, to one tenth, often resulted in much less vigorous growth, 91 days being required for five passages.

The term "normal" as applied to multiplication of bacteria also requires further explanation. The quantitative nature of the phenomenon is inherent in the conception. There must be a standard of comparison, and the experimental results must be stated in some more or less reproducible units, whether they be symbols, (+ to ++++), turbidity measurements, diameter of colonies in millimeters, or weight of bacterial substance in milligrams. The more definite and reproducible the units, and the further removed from individual errors in judgment, the more satisfactory the results are likely to be. As a basis for comparison, it seems reasonable to use the amount and type of growth commonly manifested by any particular microörganism on the usual empirical medium ordinarily used in its cultivation. Such media are the outgrowth of years of observation, and, as a rule, represent at least a fairly satisfactory degree of multiplication. If, in the course of an investigation, it is found possible markedly to increase the amount of growth obtainable in empirical media, by the omission of inert materials, and by suitable increases in concentration of essential factors, such growth is not necessarily abnormally great, and a readjustment of what is held to be normal for that particular organism must be made.

The purpose for which normal growth has been taken as an objective is fundamentally practical. It is an attempt to obtain a reproducible medium of known composition to replace the empirical and complex mixtures now in general use. An adequate appraisal of results must therefore take all these facts into consideration.

Finally, a word must be said regarding differences between growth of an organism in first transplant to a simple medium and its ability to maintain itself through repeated subculture on such

a solution. In this connection one must consider the possibility of transfer of metabolites with the inoculum which may quickly disappear through dilution in subsequent transplants. To offset this, the well-known ability of microörganisms to adapt themselves to an unfavorable environment, depending on the acquirement of new synthetic properties, must be taken into account. An attempt to reduce to a minimum the possibility of transfer of metabolites may add considerably to the complexities of an already difficult situation, whereas the modification of requirements through adaptation may lead to false conclusions. The procedure which is selected and the results obtained must be considered in the light of these facts.

It has long been known that *Corynebacterium diphtheriae* grew well and formed toxin on the usual nutritive bouillon. Blood serum was believed to favor its growth (Loeffler's medium), and a host of complex materials, milk, unheated organ emulsions, and the like had been suggested at one time and another as exerting a favorable influence. In spite of these facts, the work of most investigators on growth requirements of the diphtheria bacillus has been planned along what may be called "synthetic" lines. To the usual salt mixtures were added various known and more or less possible sources of nitrogen and carbon in various combinations and permutations. Incubation was continued for days, or even weeks, apparently in complete disregard of the fact that the organism grows well in 18 to 24 hours on a suitable medium. Had the growth requirements of the organism proved to be simple, such methods would undoubtedly have been the most rapid and direct. Complicated as the situation proved to be, they could have been completely successful only by the most improbable accident. Even so, as will be seen, certain facts of perfectly definite nature did emerge from such work.

Quite the opposite method of approach, which may perhaps be spoken of as "analytical" in character, would begin at the other end. An empirical medium suitable for normal growth, as the bacteriologist knows it, could be broken up into its components, and an attempt made to identify the latter individually, either by direct isolation or by substitution of the known compounds most

likely to be present in a particular fraction. Such a method is almost certain to be slow and laborious, but should be successful eventually, and should result in a medium which equals or exceeds the original empirical one, since each component can be adjusted to the concentration leading to maximal growth.

In effect, the synthetic approach assumes that all the chemical factors essential to the normal development of a microörganism are substances already known and of recognized physiological importance. The analytical method makes no such limiting assumption, and it has become increasingly evident that the compounds of nutritional importance, in bacteriology as in animal physiology, include a considerable number which could not be anticipated and supplied by a method of trial and error. Some of these, in the case of the diphtheria group as in other groups of microörganisms, have now been singled out and identified. Others remain obscure. On the whole, recent progress has been unexpectedly rapid, and one may predict that this will continue in the immediate future.

## DIFFERENCES BETWEEN STRAINS

Before considering in detail the nature of the specific materials which have been shown to be required for the growth of *C. diphtheriae*, it is necessary to discuss the matter of variation between strains. These differences have long been recognized, the partial successes reported by Uschinsky and by Hadley being claimed only for certain strains. Braun and Hofmeier (1927) were probably the first to call attention to this phenomenon when they stated that certain strains of the organism were "anspruchsvoll" or exacting, while others, growing more readily, were non-exacting or "anspruchslos." Successful cultivation on extremely simple media resulted only when representatives of the latter type of strains were used.

The reviewer is unable to write from personal experience with the non-exacting strains, since it has been impossible to obtain cultures of such organisms from Dr. Braun. It is difficult, therefore, to appraise his results on a basis at all comparable with those known at first hand. Significantly, however, in presenting his successful experiments, Braun states that considerable periods

of time were required in order to pass these strains through several generations, (see above). There is no way to judge how well these cultures grew, as compared, for example, to the growth of the same strains on bouillon. Evidently no pellicle-formation occurred, and the extent of growth was estimated by observing the turdidity after shaking. During such long periods one must assume that some adaptation has taken place on the part of the bacterial cell to its environment, and that there need be no direct relationship between the composition of the medium and the original nutritional needs of the microörganism.

There can be no doubt whatever that wide differences in nutritional requirements do exist within the group of organisms classed together as *C. diphtheriae.* It is, therefore, altogether probable that strains of much less exacting nature than any of those which the writer has encountered have been the subject of investigation by others. Similar differences are found among strains of all species of pathogenic bacteria that have been studied, and are probably explicable along lines of functional loss through disuse and re-adaptation. This theory, which attempts to account for differences in growth requirements among bacteria, has been clearly enunciated by Knight (1936). Briefly, it states that the autotrophic bacteria which are capable of growth on the simplest types of inorganic salts, ammonia or nitrate and carbonate, are fully equipped with synthetic mechanisms for the complete production of all of the elaborate chemical groupings of protoplasm. These are held to be the primitive members of the race, since they would have been capable of existence in the absence of complex animal or vegetable materials. In an environment providing an increasingly abundant supply of organic material, certain bacteria, finding many of the components for their own protoplasm already formed, gradually began habitually to depend on such sources for certain groups of substances. Finding it no longer necessary to provide a mechanism for the building up of these particular chemicals, the native synthetic ability for them was gradually lost. Such organisms, placed artificially in a simpler environment, would now have to be supplied with the substances which they had lost the ability to produce.

There are two consequences of such a state of affairs which are

open to experimental verification. In the first place, the adapted cell should still possess the inherent synthetic ability of the primitive one, and it should be possible to train it to regain the lost function. In the second place, failure to require a specific grouping by a given culture should imply that it is able itself to produce the compound in question. Thus, for example, strains of bacteria which do not require tryptophane in their culture medium should be able to synthesize it from simpler compounds. Both of these probabilities have received experimental support which need not be detailed here (see Knight, 1936). For the present, then, this general theory is of considerable service in helping toward an understanding of the descriptive facts which will follow.

We are now in a position to make a general statement of the framework upon which the facts already known in connection with the nutritional requirements of diphtheria bacilli may be assembled. It should then be possible to indicate where further work in collecting new facts is necessary in order to complete the structure. One must balance the possible value to be derived from the filling in of one gap or another in our knowledge, against the time and experimental difficulties likely to be encountered in doing so.

So far as is known, bacterial protoplasm in general, and that of *C. diphtheriae* specifically differs in no fundamental chemical particular from the protoplasm of cells of higher orders of life. Protein, made up of the usual assortment of amino acids is encountered here as in other cells. Nucleic acid, certain carbohydrates and lipoidal materials are characteristic of it as of living material in general. Organic sulfur is present, as well as phosphorus compounds and the ordinary mineral elements. Whichever of these essential building stones the organism cannot produce for itself must be supplied in the medium, and such a compound or element becomes an essential factor for the growth of the organism. If a particular grouping can be built up by the cell only slowly, it may become the limiting factor in growth,

and while not being strictly essential, growth may be greatly hastened or improved by its presence.

We may use, as our criterion of normal growth, that which occurs on some well-known empirical medium in general use. To say that such growth is "normal" is not necessarily correct, but it offers at least a rough standard which will be familiar to every bacteriologist interested in the field. If the growth on a solution of known composition is slower or less in amount than on bouillon, in the case of the diphtheria bacillus, it therefore implies either a deficiency of one or more factors to be found in the bouillon, a badly proportioned formula, or the presence of some inhibiting material.

It is obvious that chemical substances which will supply all the essential elements must be used. Hence nitrogen and carbon compounds, sulfur and phosphorus in some form, and Na, K, Mg, Ca, and Cl, and possibly traces of other elements have to be provided. In addition, certain organic compounds containing specific atom groupings,—the growth accessory substances,—are likely to be required. We shall therefore review the state of our present knowledge of the nutrition of the diphtheria group under the heads of "Nitrogen sources," "Carbon or energy sources," "Sulfur," "Mineral requirements," and "Accessories."

Since so much of the work done with this group of organisms has been inspired by the hope of obtaining a better toxin, or at least one comparatively free from complex substances other than products of bacterial growth, it is desirable to give some consideration to the phenomenon of toxin production, but to attempt a complete résumé of the vast amount of work on this matter would be quite beyond the scope of the present review. The situation in regard to diphtherial toxin will therefore be summarized only to the extent that more precise experiments in the nutritional field have seemed to contribute to the better understanding and control of its production.

## NITROGEN SOURCES

The traditional media are Loeffler's coagulated serum and peptone-meat infusion bouillon. Since the former is prepared by

adding serum to the latter, it is evident that the possible sources of nitrogen occurring in the usual empirical media which are capable of bringing about "normal" growth are extremely numerous. They include in addition to all the amino acids, peptides of varying degrees of complexity, up to fragments of protein just below the stage of being heat-coagulable, and of the most varied composition. Another large group of muscle extractives, many of which may be assumed to be but imperfectly known, have also to be considered. It is our immediate concern to determine which of these nitrogenous materials can serve the diphtheria group as foodstuffs from which its specific protoplasm may be constructed.

It seems to the reviewer to be a great pity that the rather vague chemical concept of "peptone" has for so long a time been held in apparent veneration by many bacteriologists. Initiated, perhaps, by the observations that certain "peptones" were better than others for the growth of this or that organism, or that some particular brand appeared to yield a better grade of toxin, the illusion has been fostered through commercialization. By directing attention to the large mysterious proteoses, the lowly but useful amino acids and accessories have been kept effectively out of sight. It is even possible that the promoters of the products have deluded themselves. If the specificity of a protein depends, however, as all the evidence indicates, on the arrangement of certain amino acids in a particular order, then it is indeed difficult to see how a huge fragment of a molecule of ox fibrin or of casein can be fitted in to the building up of a molecule of diphtheria protein. We do not, of course, know the methods by which new protein molecules are built up, but it is reasonable to suppose that it must occur by single units, or at most by groups of two or three which happen to suit the needs of the particular instant. In support of this view are the experiments of Berman and Rettger (1918) indicating no utilization by the diphtheria bacillus of purified proteose.

It is possible that instances may be encountered in which larger fragments of the protein molecule may serve as metabolites, but so far as our present information is concerned, such a state of

affairs would have to be experimentally established, and cannot be taken for granted.

Reasoning as above, one is justified in a mental simplification of the problem by eliminating all complex materials from consideration, at least provisionally, after which there remain only amino acids and simpler extractives as the most probable sources of nitrogen in the usual media. How far the process of elimination and simplification can extend, becomes now a matter for experiment.

The earliest attempts to substitute ammonia, aspartic acid, or glycine ended in what must be considered as failure. More complex mixtures, including glycine, leucine, tyrosine, aspartic acid, arginine, and lysine, failed in the hands of Galimard and Lacomme (1907) and similar failures have been reported by Koser and Rettger (1919) and by others.

In 1918 Robinson and Rettger reported successful cultivation of *C. diphtheriae* on hydrochloric acid hydrolysates of casein, lactalbumin and edestin. Their materials were so prepared as to make fairly complete breakdown of the proteins to the amino acid stage seem probable. The excess HCl was removed by evaporation on the water-bath. The resulting growth on media prepared with these materials was definite, but not good. It was greatly improved by the addition of Liebig's meat extract, which of itself, gave very poor growth. It is unfortunate that this work, which appears to have provided a definite basis for successful continuation, should not have been followed up.

A year later Davis and Ferry (1919) attempted to build up a synthetic medium from various amino acids and inorganic salts, together with glucose and certain muscle extractives such as creatine, creatinine, xanthine and hypoxanthine. Growth failed unless a very small amount of meat infusion was present. Here again was a clue which could profitably have been followed. It is noteworthy that this work of Davis and Ferry showed definitely that the amino acids cystine and tryptophane were concerned in the nutrition of their strain (a Park No. 8). Curiously, they appeared to act interchangeably on growth, and, moreover, both glutamic and aspartic acids showed similar effects. Sulfuric

acid hydrolysates of gelatine and gliadin were also used, but with no great measure of success.

Parenthetically, the reviewer wishes to admit a certain confusion in his mind over the term "sodium asparaginate." Uschinsky (1893) used "natrium asparaginicum." Fraenkel (1894) used "Asparaginsäures Natrium." Presumably, each of these is sodium aspartate. The amide of aspartic acid, asparagine, is often incorrectly stated to have been used by Uschinsky, and perhaps for this reason has often been used by other bacteriologists. Whether the English use of the term sodium asparaginate has always been employed to denote sodium aspartate, or whether a solution of asparagine in NaOH may have been used, is not clear. Probably the results would differ in no great particular, except that there is evidence of a very specific nutritional effect of glutamine as opposed to glutamic acid (McIlwain et al., 1938),—a perfectly parallel situation.

Braun and Hofmeier (1927) reported that most freshly isolated diphtheria bacilli can be cultivated on a synthetic medium of the following composition:

| | *gram* |
|---|---|
| $Na_2SO_4$ | 0.5 |
| $MgSO_4$ | 0.005 |
| $KH_2PO_4$ | 0.05 |
| $K_2HPO_4$ | 0.15 |
| Na aspartate | 0.5 |
| Cystine | 0.0125 |
| $NaC_2H_3O_2$ | 0.5 |
| $H_2O$ to 100 ml. | |

They state that cystine is indispensable, whereas the aspartic acid can be replaced by glutamic acid. Later Braun and Mündel (1929) reported the possibility of still further simplification of the medium by the omission of salts of K, Mg, Cl and $SO_4$, obtaining successful growth under these conditions. Extending the work, Braun, Hofmeier and Mündel (1929) showed that certain other amino acids could not replace the aspartic acid in the medium. They also showed that strains carried for many passages on the medium retained virulence for the guinea pig and even produced toxin in the medium. A toluol-killed culture was

centrifuged, and 5.0 ml. of the clarified fluid injected subcutaneously into a guinea pig. Considerable swelling and edema appeared, followed by necrosis, but the animal survived. A control animal protected with antitoxin suffered no injury from a similar injection of the material.

Maver (1930, 1931 a, b) has reported confirmation and some extension of Braun's work. She states that strains became adapted to the medium, in which the cystine content was increased somewhat over that of the original formula, and glycine was added. Toxin with an M.L.D. of 0.1 ml. was produced by one strain, while one of Park No. 8 gave a weaker toxin, 0.5 ml. being required to cause death in a guinea pig. Nitsch (1933) has reported a somewhat similar confirmation of Braun's observations.

It must be recalled that Braun has claimed success with his medium only for the non-exacting strains of the diphtheria bacillus. Others failed completely to proliferate. This latter group includes his strain of Park No. 8, and a certain number of freshly isolated organisms. It would be interesting to know something further of the fundamental differences between these two categories in terms of more definite growth requirements.

Mueller and his collaborators (1933) have approached the matter from the point of view of obtaining "normal growth," and proceeded by what has been referred to as the "analytical" method. They also employed a quantitative method (Mueller, 1935 a) of estimating growth by means of nitrogen determinations on the centrifuged and washed bacteria, which was laborious but extremely useful.

The usual nutrient bouillon was considered to be composed of commercial peptone and meat extract. An acid hydrolysate of casein together with tryptophane, which is for the most part destroyed by acid, adequately replaced the peptone (Mueller, Klise, Porter and Graybiel, 1933). By a process of progressive separation of the amino acids of the hydrolysate into fractions, and substitution of known amino acids, so far as possible of synthetic origin in order to avoid the addition of unexpected physiological substances as impurities, it was possible in the case of the

strain investigated eventually to replace the hydrolysate completely by eight amino acids (Mueller, 1935 b, c). These were glycine, valine, phenylalanine, glutamic acid, methionine, histidine, cystine and tryptophane. A strain of the Park No. 8 organism was then investigated in the same manner by Mueller and Kapnick (1935) and again, with a somewhat different assortment of amino acids, including valine, leucine, methionine, cystine and glutamic acid, as good growth was obtained as in ordinary bouillon. Liebig's extract and inorganic salts by themselves giving minimal amounts of growth were used in the preparation of all such media. The presence of certain amino acids in the meat extract in sufficient quantity to supply the needs of the organism was not excluded, so that conceivably both lists may be longer.

Because of certain striking differences in the apparent amino acid requirements of two strains, other cultures were investigated, including "Park No. 8" organisms from a number of different laboratories. It soon became evident that considerable differences existed, even between the various Park No. 8 strains, and that little profit was likely to result from an indefinite extension of that line of attack.

Considerably later, when it had become possible to substitute known compounds for Liebig's meat extract, the amino acid requirements of a third strain were investigated (Mueller, 1938). Here it was found that valine, proline, aspartic and glutamic acids, cystine, methionine and possibly tyrosine had to be supplied, and even with these, growth was never more than about 85 to 90 per cent as good as in the presence of a complete acid hydrolysate of casein. The question was left open as to whether this may have been due to the presence of some unknown material in the hydrolysate, or simply to the fact that amino acids not supplied had to be synthesized and that as a result there was a certain loss in growth efficiency.

The common factors which developed in regard to amino acid requirements of the various strains, (both published and unpublished results), may be summarized in this way: Either glutamic or aspartic acid seems to be utilizable in greatest absolute amount,

evidently to serve as the source of N and C linkages for the synthesis of amino acids and other compounds not supplied in the medium. With some strains these compounds are quite interchangeable, while with others, one or the other is distinctly better. It is possible in the case of both amino acids to use synthetic materials, thus ruling out the possibility of the presence of active impurities. Only the naturally occurring isomer is utilized in each case. Asparagine appears to be quite as satisfactory as aspartic acid. This supplies corroboration of Braun's observations, and of the value of the quite general use of aspartic acid in much of the earlier work. It also supports the conclusion of Abt (1925), who furnished evidence of quite a different sort that glutamic acid was utilized in considerable quantities.

Of the other amino acids, cystine, or some other unoxidized sulfur compound (see section on sulfur), is essential to all strains. Methionine does not replace cystine, but represents for all strains an additional requirement *for maximal growth.* Tryptophane is definitely required by certain strains, not at all by others. In the case of one or two, there is evidence of improvement of growth by minute traces, and inhibition by larger amounts. One or more of the simple monoamino, monocarboxylic acids (glycine, valine, or leucine) are required for optimal growth by all strains. The growth finally attained on such solutions is several times that given by ordinary bouillon, since it is possible by means of the quantitative method employed to push to the optimum the concentration of each factor used. The organisms grow in the form of a typical pellicle which forms in about 12 hours and thickens progressively up to about 72 hours.

### CARBON SOURCES

Although, carbon is obtained by the diphtheria bacillus from the amino acids which it assimilates, much more abundant growth takes place in the presence of non-nitrogenous organic compounds belonging to the general groups of carbohydrates, acids and alcohols. It will not be our purpose to consider the mechanism by which these substances are utilized, but merely to outline the nature of the materials, and consider briefly the end-products

and the effect on the growing culture brought about in this phase of its metabolism.

From the earliest experiences with the diphtheria bacillus in broth culture, it has been recognized that an initial acidity was likely to develop, which if it did not become too marked, eventually gave place to a slowly developing, alkalinity. In general, the acidity was attributed to products arising from the breakdown of glucose, or some other fermentable material. The later alkalinity was variously held to be due to the formation of ammonia, organic amines, carbonates, etc. The phenomenon has been investigated by numerous workers, particularly in relation to toxin-formation. It has become a part of the tradition attending this matter that an initial acidity must appear, which must be followed by a "reversal" of the reaction, with the development of alkalinity, and that toxin is formed only under these conditions. Among producers of diphtheria toxin have been those who have insisted that sugar-free broth must be used, while others have emphasized the benefit to be derived from the addition of sugars, both glucose and maltose, to the medium.

There is no doubt that glucose is rapidly fermented with acid-formation. With maltose this is not the case, yet the addition of this sugar to the medium greatly improves the growth. Sugg, Fleming and Neill (1927) have shown the presence in the organisms of a heat-labile maltase which breaks the maltose down to the hexose stage. This hydrolysis proceeds slowly (Tasman and Brandwijk 1936), so that the glucose is fermented and its fermentation products are further oxidized as rapidly as they are formed (see below). Maltose, therefore, serves as an ideal source of energy for the growth of the cultures.

The authors just quoted (Tasman and Brandwijk, 1938) later presented evidence that the fermentation of the glucose molecule by the diphtheria bacillus resulted in the appearance of formic, acetic, propionic, lactic, and succinic acids, and ethyl alcohol. Braun and Hofmeier (1927) showed that acetic, lactic, malic and succinic acids, as well as glucose, could be utilized in their very simple medium. Glycerol has been used (Uschinsky, 1893; Hadley, 1907) from the time of the earliest work, and was shown

by Schmidt (1933) to be utilizable, using Braun's method. Formic, propionic, oxalic and tartaric acids are not utilizable according to Braun, Hofmeier and Mündel (1929). The slight discrepancies are easily explained as due either to differences in method, or to variation among strains. That this occurs has been observed by the writer in differences in the effects exerted by glycerol, *d*-lactic acid, and ethyl alcohol on several strains of the Klebs-Loeffler bacillus.

Abt (1925) and Abt and Loiseau (1925) have carried out studies on oxygen-consumption and $CO_2$-production by this organism. They have shown that the gas exchange is considerable, amounting to as much as 4 grams $CO_2$ per 1100 ml. of medium, with a corresponding utilization of $O_2$. From unpublished experiments, the writer is inclined to believe that these figures fall well short of the quantities involved when maltose is present in the medium, since he observed the utilization of approximately 200 ml. of $O_2$ by 30 ml. of culture. Abt believes that many of the amino acids are deaminized, and that the resulting fatty acids are largely burned to $CO_2$ and water. This, he indicates, is particularly true of glutamic acid.

It appears that the above chain of events offers a perfectly adequate explanation of the series of alterations in reaction in the growing diphtheria broth culture. If hexoses are present, an initial acidity develops, due to the rapid (anaerobic) fermentation to organic acids. In their absence, some acidity may still develop due to changes in certain amino acids. If too much glucose is present, probably more than about 0.2 per cent (Tasman and Brandwijk, 1936), the degree of acidity attained is such as to check further growth of the organisms. With small amounts, the organic acids formed are oxidized to $CO_2$ and water with a gradual return of the pH to its original level. A further increase in pH results from changes in the amino acids with the formation of $NH_3$ or other basic substances. Obviously, if sodium acetate or sodium lactate is added to the medium, the oxidation of the organic acid leads to the formation of sodium carbonate or bicarbonate, the initial acid phase is slight or absent, and a more marked alkaline reaction is soon reached. One could

probably control the course of the pH changes throughout the period of growth by the use of suitably chosen quantities of glycerol, ethyl alcohol, sodium lactate, glucose and maltose, together with some regulation of the oxygen supply and $CO_2$ removal.

## SULFUR SOURCES

There is general agreement in the literature that sulfur in the form of cystine is acceptable to all strains of the diphtheria bacillus which have been investigated. Reference has already been made to the work of Davis and Ferry (1919), who showed that the addition of this substance to plain meat infusion, itself just capable of maintaining growth, rendered it capable of producing heavy vegetation.

Hosoya and Kuroya (1923) reported positive results when cystine was used together with other amino acids and an inorganic salt mixture. Braun and Hofmeier (1927) found it to be essential in the growth of their non-exacting strains. Similar findings are reported by Gibbs and Rettger (1927), Maver (1930) (1931 a), Hosoya, Ozarva and Tanaka (1933), Nitsch (1933), Schmidt (1933) and others. Braun and Mündel (1927) have suggested a practical application of the observation. They find that the addition of cystine to ordinary Loeffler's medium greatly improves the growth of *C. diphtheriae* on it, without affecting that of other organisms.

The earliest reported synthetic media, such as Uschinsky's, contained inorganic sulfate, as did some of Braun's media. Braun and Mündel (1929) later obtained evidence indicating that this was not essential, and so far as can be learned, it has never been shown that sulfur in this form is utilized by these bacteria.

In the experiments of Mueller and collaborators (1935 b, 1938) the utilization of cystine was placed on a quantitative basis as a factor in producing normal, heavy growth. Curves were obtained clearly indicating minimal and maximal concentrations. In general, the order of magnitudes found were in good agreement with the quantities used by Braun. The latter appears to have been hampered to a certain extent by the relative

insolubility of cystine, since he found it necessary to add it as a fine suspension. Others have experienced the same difficulty, which in many instances can be avoided by solution of the cystine in dilute HCl. After adding the required amount to the well-diluted medium the pH is suitably adjusted with NaOH, and the presence of the other ingredients of the medium, particularly other amino acids, tends to prevent the cystine from crystallizing.

As to other possible sources of sulfur, cysteine, as would be expected, is effective (Braun, 1938; Locke and Main, 1931; Hosoya, Ozawa and Tanaka, 1933; and others). Locke and Main (1931) find a depression of toxin-formation to occur with cysteine, which they attribute to an alteration of the Cu/Fe ratio in the medium by this amino acid. Scheff and Scheff (1934 a, b) have also observed an irregular diminution of toxigenic action by cysteine, although cystine increased toxin production in their hands. They do not appear to find Locke and Main's explanation involving an effect on Cu to be adequate, but do not offer a better one. Further reference to this matter will be made in the section on toxin.

Braun (1938) has shown that many forms of unoxidized sulfur are capable of replacing cystine in his simple media. These include thio-urea (growth not as good as with cystine), $Na_2S$ and even flowers of sulfur. Braun believes that diphtheria bacilli use sulfur in the form of $H_2S$, and that only compounds capable of being transformed into this material are suitable for its growth.

These observations have been in part confirmed by Compton and Emerson with the writer (unpublished results) using quantitative methods and the criterion of normal growth. Thioglycolic acid and thiolactic acid gave fairly heavy growth, $Na_2S$ somewhat less and flowers of sulfur relatively poor growth. None were as effective as cystine or cysteine. One could perhaps have anticipated that such would be the case.

Further confirmation of the utilization of $H_2S$ by the diphtheria group is found in the report of Beck (1933), who found an increased rate of growth in an atmosphere of $H_2S$, and of Lentze (1930) who made a similar observation. The latter believes, however, that it acts as a stimulant and not as a food-stuff.

The only consistent study of the utilization of methionine by

organisms of the diphtheria group appears to be that of the writer. Hosoya, Ozawa and Tanaka (1933) found that it had no effect in their hands. Scheff and Scheff (1934 a, b) observed what they believed to be some increase in the growth but not in toxin when it was added to a medium already containing peptone. In our own work (1935 b, 1935 c, 1938) it was shown that for each strain fully studied, methionine must be added in order to obtain optimal growth. The effect of its omission, while not leading to complete absence of growth, is considerable. Unpublished and incomplete examinations of several other strains gave indications of the same effect. It was found definitely not to replace cystine, nor of course, was cystine alone adequate. A peculiar effect of this amino acid was observed in the case of the first strain to be studied (1935 b), and has not been again encountered. A low concentration of methionine caused a sharp rise in the growth curve, which stopped abruptly as the concentration was further increased, and growth fell to the original level. When histidine, valine and phenylalanine were added to the basic medium, this drop did not occur, and growth increased regularly with the concentration of methionine to the optimum.

### INORGANIC REQUIREMENTS

One might perhaps anticipate that diphtheria bacilli would manifest a need for the same basic inorganic materials as do other forms of life. Thus, Na, K, Mg, Ca, Fe, and possibly other metals in traces would be expected to be required, and in addition, the inorganic ions Cl and $PO_4$, and perhaps others in minute amounts. The difficulty in connection with obtaining convincing evidence bearing on the matter arises from a consideration of the minute quantities involved, and the practical impossibility of assurance of the absolute purity of materials used. Practically all of the synthetic media which have been tried have contained the usual inorganic salts listed above except Fe; and it is interesting that Uschinsky, in attempting a corroboration of his own earlier observations states (1897) that the addition of a trace of this element improved the growth of the diphtheria bacillus.

Braun and Mündel (1929) seem to have been the first to inves-

tigate the inorganic requirements more closely, and they state that individually neither potassium, magnesium, chloride nor sulfate is required. Mg, Cl and $SO_4$ could be omitted simultaneously and "growth" could still result. Five passages were obtained in 29 days on a medium containing only sodium aspartate, cystine, potassium phosphate and sodium acetate. Whether the protoplasm of the diphtheria bacillus is such that it easily readjusts itself to the absence of such universally distributed ions as K, Mg and Cl, or whether the (presumably) scanty growth was able to eke out some kind of an existence on traces of these elements present as impurities, the reviewer does not feel qualified to suggest. The experiments were carried out in quartz-ware using paraffined stoppers, and presumably with care as to purity of materials. Amino acids are, however, notoriously difficult to purify, and may well have supplied traces of elements so widely distributed as the ones in question. Admittedly the purpose of these experiments has been to reduce protoplasm to its lowest terms, and in this, the workers appear to have been completely successful.

Of other experimental work bearing on the matter one finds very little. Wadsworth and Wheeler (1928) reported that a medium containing peptone, glucose, Na, Ca, Mg, Cl, $SO_4$ and $PO_4$ produced potent toxin only when the Ca and $PO_4$ ions were heated together in the presence of peptone. Their explanation, involving a supposed colloidal peptone-calcium phosphate compound appears to be no longer tenable, but the observation is correct, and supplies the most convenient procedure for removing traces of Fe from culture media. The significance of this fact will appear shortly. Later, Wheeler and Mendez (1937) showed that Na, K, Mg and $PO_4$ ions were necessary for adequate growth, and stated that Ca, while not essential for growth, must be present for toxin-production.

Our own experiments (1938) in attempting to develop a medium capable of giving "normal" growth have made it possible to show a quantitative need for Mg, K, and $PO_4$, (of the ions so far being considered). It has not been deemed worthwhile to attempt to produce a control-medium free from Na or Cl,

but there have been definite indications in connection with our toxin work that one or both of these would be found essential. The situation as regards Ca appeared to be somewhat different, in that when salts of this metal were omitted, growth took place after a lag of a day or two and became quantitatively as good as when Ca was added, but after a slightly longer time. Without any doubt whatever a certain amount of Ca was present from impurities in other materials. The probabilities are, that with its *complete* elimination, growth would not occur.

We come now to the trace elements, Fe, Cu and Mn. Curiously, these have received the attention of more workers over a period of years than the commoner ones, although most of it has been in connection with studies on toxin-production. We have already noted that Uschinsky observed increased growth with a trace of Fe. Walbum (1921) reported increased yields of toxin after the addition of salts of manganese. Locke and Main (1930, 1931) consider the ratio of copper to iron to be important in toxin-production, and have a theoretical explanation for the phenomenon, which does not appear to be substantiated by the similar experiments of Scheff and Scheff (1934 a, b), nor by certain of our own unpublished experiments. Pope (1932) showed that minute traces of iron increased growth of the diphtheria bacillus and favored toxin-production, whereas the latter process was checked by larger additions. Copper was also found to promote toxin-formation. Strøm (1935) has confirmed both these observations.

The extreme sensitivity of the toxin-producing mechanism of the diphtheria bacillus to traces of Fe, however, seems not to have been appreciated before the work of Pappenheimer (1936) and of Pappenheimer and Johnson (1936). Through an accidental observation that from the same batches of medium, higher titers of toxin were being obtained in certain soft glass Fernbach flasks than in Pyrex glass, the effect was traced to iron, which evidently dissolved out of the soft glass in small quantities. It was found that the medium, prepared according to Wadsworth and Wheeler's (1934) method, was depleted of its Fe by the precipitate of calcium phosphate obtained when heated under

the conditions described above. Without this treatment, the concentration of Fe (present as an impurity in the peptone and other ingredients) is considerably above the optimum, and little toxin is obtained. Its removal by the precipitate is not entirely complete, but usually leaves the concentration somewhat below the optimum. Since the curve of toxin production at varying Fe concentrations is particularly steep on the ascending slope, the traces of Fe dissolving from the soft glass were sufficient to improve the yield noticeably. In general, growth improved with the addition of Fe beyond the optimum concentration for toxin-production, being relatively poor in a medium depleted as thoroughly as possible of its Fe. The concentration of Fe leading to good growth and the best yield of toxin was given by Pappenheimer as 0.000,014 grams per 100 ml.

The writer (1938), investigating the possibility that other metals than Fe might influence growth, fully confirmed the findings of Pappenheimer and Johnson in regard to the growth-promoting effects of Fe in the range described by them. Fairly conclusive experiments were also obtained indicating that Cu, Mn and Zn also somewhat improved growth. These results were in a measure unsatisfactory, because of the difficulty of being certain that the basic medium was sufficiently free from these substances, and must be considered as suggestive rather than conclusive.

In regard to a possible effect of still other substances, it has been reported by Evans, Happold and Handley (1939) that while strains of the diphtheria bacillus belonging to the types *gravis* and *mitis* could be grown with considerable success on a medium whose composition was very similar to that described by the writer, strains classed as *intermediate* grew very sparsely, if at all. Growth of these strains was notably improved by the addition of a solution containing traces of a considerable number of elements, including besides those already mentioned, Al, I, B, Sr, Li, Si, Ti, V and Rb. The effect was not traced to the individual substances involved. The procedure is quite comparable to the use of similar mixtures (so-called A–Z solutions) by plant physiologists.

It therefore appears that for normal heavy growth, the diph-

theria bacillus probably differs in no general way from most other types of living cell.

## GROWTH ACCESSORIES

Under this heading the writer wishes to include organic substances which take part in promoting normal growth, and which do not fall under the heading of nitrogen, sulfur, or carbon sources. It is, therefore, likely to be a heterogeneous group both in chemical composition and function. The boundaries are not particularly well defined, since tryptophane, for example, could well be included here, rather than under the heading of nitrogen sources. Presumably this amino acid acts, not by supplying nitrogen, but by furnishing a specific grouping of carbon and hydrogen which many strains of the diphtheria bacillus are unable themselves to produce.

Very little definite information on the part played by such a group of substances in the nutrition of these organisms was available until quite recently. The probability that such materials existed and were important constituents of the usual empirical media was expressed by the reviewer in his earliest report dealing with bacterial nutrition (1922). The conviction was repeated and elaborated in discussing the first experiments with *C. diphtheriae* (1933), and some direct evidence was presented concerning them. In 1922 it seemed unfortunate to introduce the term "vitamin" into the situation, since both the conception of accessory factors in the growth of bacteria and the significance of the word "vitamin" were still rather nebulous. Now that both matters are becoming better understood, it is evident that the relationship is very close indeed, and perhaps "accessory factors" and "bacterial vitamins" may be considered to be synonymous.

An occasional suggestion of the involvement of vitamins in the growth of the diphtheria bacillus is to be found in the earlier literature. Leichtentritt and Zielaskowski (1922) reported a favorable effect with lemon juice. Hosoya and Kuroya (1923) found that a vitamin B concentrate improved growth on an otherwise relatively simple medium. Weichart (1928) found that

extracts of typhoid bacilli stimulated growth on a modified Uschinsky medium. Dominici (1928) obtained favorable effects with concentrates of vitamins B and D. Clauberg's diagnostic medium (1931) containing heated blood seems to have been based to some extent on the presence of the X and V factors for *Hemophilus influenzae*. Annok and Buchgraber (1933) and Mustafa (1937 a, b) employed yeast extract, and the latter reports a favorable effect on growth and toxin-formation when crystalline vitamin $B_1$ was added to a medium containing fresh beef heart infusion and peptic digest of hog stomach. Since none of the strains so far examined appear to be influenced by $B_1$, and since his basal medium surely contained the substance, it is difficult to understand the significance of this observation.

Using a basic medium composed of an acid hydrolysate of casein, inorganic salts, and lactic acid, the writer and Subbarow (1937) succeeded in showing that certain components of liver extract (or meat extract) were essential for good growth of a strain of *C. diphtheriae*. That there were at least two substances involved which could be separated by ether extraction of the acidified solution was also shown. One of these was isolated and identified as pimelic acid (Mueller 1937 a). The other fraction was subsequently shown to contain two active materials which were in turn identified as nicotinic acid (Mueller, 1937 b) and $\beta$-alanine (Mueller and Cohen, 1937). By the use of suitable amounts of these three substances and an assortment of amino acids, together with salts and lactic acid, extremely heavy growth of this organism was obtained. With Pappenheimer and Cohen (1937) these findings were extended to a strain of Park No. 8, and probably for the first time, toxin of relatively high grade (36 $L_f$) was produced on a medium of known composition, free from peptones or polypeptides.

Although these results were shortly confirmed by Evans, Happold and Handley (1939) for a number of strains of types *mitis* and *gravis*, it soon became apparent from their work that other strains could not be grown satisfactorily without still further additions to the medium. Reference to their observations regarding "trace" inorganic elements has already been

made. Later they showed (Evans, Handley and Happold, 1939) that certain type *gravis* strains required pantothenic acid for growth. Mueller and Klotz (1938) had found that pantothenic acid could replace β-alanine for their test strain, the results on the whole being somewhat more satisfactory than in the case of β-alanine alone. It was suggested that this substance was first built up to pantothenic acid, and from the results quoted above it appears that not all strains are capable of carrying out this synthesis. Here is to be found direct confirmation of the theory of "loss of function" outlined in the introductory pages of this review, and further support appears in the same contribution by Evans, Happold and Handley (1939), who found that sterile filtrates of diphtheria bacilli contained substances having the properties of aneurin, riboflavin and coenzymes I and II.

Some further confirmation of the situation as outlined above has been presented by P. Bordet (1939), who identified as nicotinic acid one of the factors in yeast extract which favors growth and toxin-formation.

More recently, facts have been coming to light which indicate that the picture is even more complicated. Because of the luxuriance of growth obtainable on media which are constituted in the above-described manner, Mueller (1939) investigated the possibility of obtaining a solid medium (by the addition of agar) which could be used for diagnostic purposes. It seemed that the absence of vitamin $B_1$ and of riboflavin, as well as of certain other growth accessories required by the pneumococci and streptococci would make for selectivity and render it particularly suitable. Preliminary experiments showed that in cultures from known cases heavy growth of practically pure *C. diphtheriae* appeared in some instances, whereas not infrequently growth did not occur. A considerable proportion of normal throat cultures also remained without growth, or at most gave only a few scattered colonies. Further investigation revealed that the *size of the inoculum* seemed to be the controlling factor. When plates were streaked, even with strains which grew well on the fluid medium by the usual technic (loopful of pellicle as inoculum) growth was slow in starting, and on the more lightly inoculated

areas of the plate individual colonies did not develop, at least until after two or three days. The indication was clear that a heavy inoculum introduced enough of some still unidentified factor to initiate growth, after which the growing organisms were able to elaborate the material and permit its diffusion into the surrounding medium. It was reported that the addition of whole blood to the medium appeared to supply this deficiency. The substance was in the serum fraction, rather than in the red cells, and remained in solution when the bulk of the serum proteins were removed by heat coagulation.

Snyder, and later Cohen, in the writer's laboratory, have thrown further light on the matter. The former (1940) showed that the effective material was retained by all but the most permeable collodion membranes, and, further, that it could be separated into two components by means of lipoid solvents. They found that not all animal species supplied serum of equal efficacy. Horse and beef serum were particularly suitable, hog and human serum almost without effect. They found that cow's milk offered an abundant and readily available source of material. Cohen and Mueller (1940), and Cohen, Snyder and Mueller (1940) showed that the active substances were precipitated with the casein upon acidification of milk, and could be separated by extraction of this casein, as well as of dry commercial casein, by hot alcohol or cold acetone. The acetone-soluble fraction was extremely abundant in cream and in butter, and has been identified by them as *oleic acid.* This identification appears to be as complete as is possible without the successful substitution of synthetic material. Unfortunately, oleic acid seems not to have been prepared synthetically and our advisors in organic chemistry feel that its attempted synthesis would at this time be attended by great difficulties and considerable uncertainty.

## NUTRITION IN RELATION TO TOXIN-FORMATION

Insufficient data have accumulated since the recognition by Pappenheimer and Johnson (1936) of the critical nature of the iron effect in toxin-formation to warrant dogmatic generalizations. At the moment it is permissible only to state that this

interference on the part of Fe ions with toxigenicity extends to all strains of *C. diphtheriae* which have been studied from this point of view. These comprise several different "strains" of Park No. 8 (Pappenheimer and Johnson 1936) and a number of heterogeneous strains investigated by Happold (1940). The optimum concentration of iron appears to be about the same, at least of the same order of magnitude, in all cases. An extension of the investigation to a considerable number of other strains would be highly desirable. Naturally it is possible that some will be encountered in which the situation is entirely different.

However, in the light of our present knowledge, it seems to the reviewer that diphtheria toxin may possibly be regarded as an abnormal product of the organism, developed as an emergency mechanism when it is forced to grow under conditions of insufficient iron concentration. Perhaps it may be incorrect to consider it as abnormal, for it is still formed in small amounts (as shown by injection of guinea pigs) even in the presence of quantities of iron considerably exceeding the optimal range (Favorite, unpublished observations). A better statement of the hypothesis, then, would be that it is a substance, in some manner connected with a metabolic function which under normal conditions is carried out chiefly by means of iron-containing enzymes. In the absence of sufficient Fe, the alternative system, involving the toxin molecule, is forced to carry the whole load, and the material is formed in greatly increased amount. If one postulates still further the existence of a second emergency mechanism which does not utilize the toxin molecule, it becomes fairly easy to explain differences in "toxigenicity" between strains, since the response in one or the other auxiliary might well vary with the individual culture.

Perhaps such speculation is not suitable to a review. However, the writer hopes that it may serve to furnish in some measure a reasonable basis from which the older work on toxin-formation in connection with growth may be appraised. From what has already been said, at least one of the traditions with which the subject of toxin-formation is replete is now pretty

well shattered. "Peptone" is no longer to be considered an essential for toxin-formation. A second tradition, namely that growth and toxigenicity are not necessarily related, is both supported and explained, since growth improves (Pappenheimer and Johnson 1936) with the addition of iron beyond the optimum for toxin-production.

It is possible that nowhere can a better illustration be found of the complexity and difficulty of biological experimentation than is afforded by this phenomenon. Innumerable investigators have attempted to study the effect of various substances and agencies on toxin-production. Since almost any manipulation or alteration in composition is practically certain to modify the concentration of iron, it is obvious that many of the effects obtained were due to this fact, not to the one supposed to be under experimentation, and that erroneous conclusions have inevitably been drawn.

At this particular stage in the development of our information it appears unwarranted to review in any detailed way the many papers dealing with toxin-formation as a phase of nutrition of the diphtheria bacillus. Many useful facts have been pretty well established, through numerous repetitions in various laboratories, to a point where it would be absurd to suggest that they should not be considered as beyond criticism. An example of this would be the increased yield of toxin through addition of suitable amounts of maltose. Yet it is perfectly possible to purchase maltose in the market which contains either so much iron or so much readily fermentable material (glucose?) that its use would completely ruin an otherwise satisfactory medium.

The review by Strøm (1935) of factors concerned in toxin production has covered the situation very completely up to the time immediately preceding Pappenheimer and Johnson's work. It is therefore superfluous to undertake a further summary at this time. Since 1935 the whole conception of the matter has been altered by the recognition of the extreme sensitivity of the organism to iron, and by the establishment of the fact that powerful toxin can be formed from amino acids and a few other materials of small molecular size and known constitution. Obvi-

ously no further contributions to the subject merit consideration unless these facts are taken adequately into consideration. It will not be sufficient to assume that Fe concentration remains constant when another point is under investigation. The amounts involved are too small to be estimated accurately by chemical methods now available. The opportunities, moreover, for the accidental introduction of significant amounts of iron into solutions used are extremely great.

At present, the only practicable method of accomplishing control of the iron supply appears to be the rather cumbersome one of purposefully varying the iron through its optimal range for each experimental modification of a basic medium (Mueller and Miller, 1940). This can be done by removing the iron so thoroughly from all materials used that the completed medium is definitely below the optimum for growth as well as toxin-formation. A series of 5 or 6 flasks of similar quantities of such a medium is then prepared, one left without added iron, and small additions of ferrous sulfate or some other iron compound made to the others. If 30 ml. of medium are used in 125 ml. flasks, the amounts of $FeSO_4 \cdot 7H_2O$ which have been found suitable are 3, 6, 9, 12 and 15 gamma. This is conveniently done by using a freshly prepared 0.01 per cent solution, and adding 0.03 ml., 0.06 ml., etc. before using the medium.

Confirmation of these facts has come through the work of Happold (1940), who has reported that several freshly isolated strains of the type *gravis* and one classified as *intermediate* produced moderate amounts of toxin (10 to 15 $L_f$) under these conditions, and gave best results in the range of Fe concentration mentioned above. Further confirmation has come from the experiments of Taylor in the Connaught Laboratories (personal communication).

By the method outlined above, Mueller and Miller (unpublished results) have investigated the effect on toxin-production of most of the individual factors known to be involved in the growth of their strain of Park No. 8. As might be anticipated it is relatively easy to establish an optimal concentration for each individual component of the medium in the presence of

suitable amounts of all others. To arrive at an absolute simultaneous optimum for all constituents in any convincing experimental way involves the manipulation of far too many variables, and could only be accomplished accidentally. It has been possible, however, to formulate a reproducible medium containing acid hydrolysate of casein (Mueller and Johnson, 1940) as the basic source of nitrogen and suitable amounts of all other materials shown to foster the growth of the toxigenic strain, which has consistently yielded 100 ± 10 $L_f$ of toxin in their hands (Mueller and Miller, 1940). These results have been obtained in both small- and large-scale lots, and the method is entirely suited to practical production.

By way of a summary, it may be of interest to bring together the various facts that have been discussed in the form of specifications for suitable synthetic culture media for the diphtheria bacillus as they have been developed up to the time of writing.

I. A medium giving "detectable growth" with "non-exacting" strains of *C. diphtheriae* (Braun and Hofmeier, 1927).

| | gram |
|---|---|
| $Na_2SO_4$ | 0.5 |
| $MgSO_4$ or $MgCl_2$ | 0.005 |
| $KH_2PO_4$ | 0.05 |
| $K_2HPO_4$ | 0.15 |
| Sodium aspartate | 0.5 |
| Cystine | 0.0125 |
| $NaC_2H_3O_2$ | 0.5 |
| $H_2O$ to 100 ml. | |

II. A medium giving "normal growth" with an "exacting" strain of *C. diphtheriae* (Mueller, 1938).

| | gram |
|---|---|
| *l*-Cystine | 0.07 |
| *dl*-Valine | 0.2 |
| *dl*-Methionine | 0.06 |
| *l*-Tyrosine | 0.05 |
| *l*-Proline | 0.075 |
| *l*-Aspartic acid | 0.5 |
| *d*-Glutamic acid hydrochloride | 0.75 |
| KCl | 0.04 |
| $Na_2HPO_4$ | 0.3 |

| | gram |
|---|---|
| $MgSO_4 \cdot 7H_2O$ | 0.1 |
| Pimelic acid | 0.000,015 |
| Nicotinic acid | 0.000,23 |
| β-Alanine | 0.000,23 |
| Ethyl alcohol | 0.7 ml. |
| *d*-Lactic acid | 1.75 ml. |
| $CaCO_3$ (in HCl) | 0.02 |
| $FeSO_4 \cdot 7H_2O$ | 0.000,5 |
| $MnCl_2 \cdot 4H_2O$ | 0.000,25 |
| $CuSO_4 \cdot 5H_2O$ | 0.000,5 |
| ZnO (in HCl) | 0.000,25 |
| $H_2O$ to 100 ml. | |

III. A medium yielding, with a Park No. 8 strain, toxin having 36 $L_f$ per ml., L+ = 0.05 ml. and M.L.D. = 0.000,75 ml. (Pappenheimer, Mueller and Cohen, 1937).

| | gram |
|---|---|
| Glycine | 0.05 |
| *dl*-Valine | 0.1 |
| *dl*-Leucine | 0.05 |
| *d*-Glutamic acid hydrochloride | 0.5 |
| *l*-Cystine | 0.02 |
| *dl*-Methionine | 0.02 |
| *l*-Tryptophane | 0.01 |
| *l*-Tyrosine | 0.01 |
| Pimelic acid | 0.000,1 |
| β-Alanine | 0.000,1 |
| Nicotinic acid | 0.000,2 |
| $K_2HPO_4$ | 0.2 |
| NaCl | 0.5 |
| $MgSO_4 \cdot 7H_2O$ | 0.03 |
| $CuSO_4 \cdot 5H_2O$ | 0.000,5 |
| Sodium lactate | 0.74 |
| Maltose | 0.3 |
| Glucose | 0.15 |
| $CaCl_2$ | 0.006 |
| $H_2O$ to 100 ml. | |

It is clear that developments in the conception of bacterial nutrition are proceeding at a rapid pace, as are those in the field of animal nutrition. The goal of each is the same,—namely a complete chemical understanding of all factors connected with normal growth. Each field has already contributed significantly to the other and this will doubtless continue to be the case. Within the field of bacterial nutrition an even greater relationship

is found as studies on one or another species of organism are continued and compared. The diphtheria group is evidently a particularly fortunate one with which to work. Within itself there appear to be many shades of adaptation or loss of function, in terms of Knight's theory. While this is recognized in Braun's conception of exacting and non-exacting strains, it goes much farther than such a simple and categorical separation into two groups: It is probable that the state of affairs more closely approaches a spectrum, involving a very considerable number of individual functions. These conceivably cover every phase of cell physiology from oxidative and energy-producing to the most complex synthetic functions concerned in the production of all the organic groups and masses of protoplasm itself.

It seems to the writer that up to this time only a beginning has been made toward exploring the tremendous possibilities for advances in knowledge which this group of bacteria supplies. The individual metabolites have been quite completely worked out for a very few strains. These have manifested striking and doubtless significant differences, as well as many similarities. The necessarily attractive goal of practical application has perhaps unduly influenced the direction of certain of the researches. To replace this by the quest for facts of purely scientific import can be done with complete certainty that as such facts accumulate, unexpected interrelationships and significant applications will become apparent at every stage.

Certainly a number of other strains, representing the various types, *gravis*, *mitis* and *intermediate*, should be completely studied. Strains representing Braun's two classifications should be examined to learn whether his differentiation may be fundamental, and the nature of the facts on which it is based. The matter of variation in toxigenicity is still entirely mysterious and is of the utmost importance, theoretical as well as practical. The mechanism of the effect of iron on this phenomenon offers at least one method of approach, and is itself of great interest. If strains are encountered in which the effect of iron is qualitatively or quantitatively different, extremely careful comparisons of these with other strains should certainly be made.

These are a few of the general lines along which it seems that developments may be anticipated. Others will naturally have suggested themselves to the reader. It is not too much to predict that in the next few years very great advances will have been made in our present conception of the whole matter.

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