ON THE PREPARATION OF SALT-FREE CULTURE MEDIA AND THE GROWTH OF BACTERIA UPON THEM.

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Some time ago I entered upon a study of the relations of different salts and nutrients to the metabolism of bacteria. The first step in the investigation lay in the preparation of a salt-free culture medium. A perusal of the literature indicated that the preparation of such a medium had never been really attempted with natural nutrients; and all the work upon the influence of particular salts upon bacteria is rendered of doubtful value by reason of the fact that the salt in the culture medium was not controlled or removed. For this failure technical reasons have been directly responsible. A culture medium containing natural nutrient materials cannot be controlled in its content of salt. Culture media composed of some amido body, as asparagin, plus a sugar, may be prepared in such a manner as to permit of a control of the salts. But by chemical procedures it is not possible to determine the saline content of a culture medium containing natural nitrogenous substances, nor is it possible to eliminate the salts. Thus far few results of importance have been secured in this investigation beyond the technical preparation of a salt-free medium; and these are herewith reported in the hope that they may prove of service to others engaged in research along similar lines.

PREPARATION OF SALT-FREE CULTURE MEDIA.

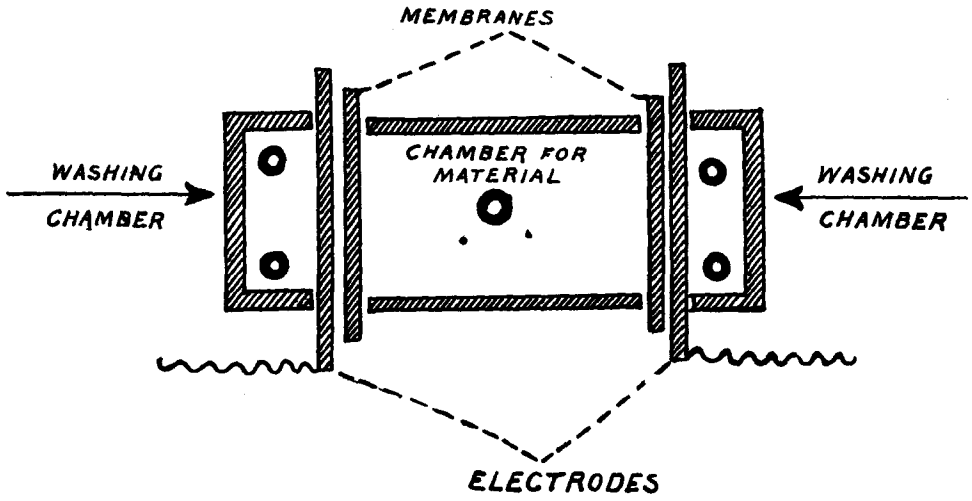
In the beginning a systematic attempt was made to prepare proteins free of salts. Casein may be prepared with an ash content as low as one-tenth of one per cent. Beyond this no chemical method will go. Such a casein is practically insoluble, and

even in the presence of calcium salts it exhibits such alterations in behavior as to suggest denaturation. Insoluble globulin, so-called, may be prepared with an ash content of about the same amount. This globulin, however, while quite soluble in alkaline or acid solution, becomes on manipulation and conservation so denaturated as to be entirely insoluble. Egg-albumin cannot be prepared so poor in salt. Fibrin may be freed of salt to about one-fourth of one per cent. The behavior of the proteoses is no better than that of the coagulable proteins. In fact, proteose is less tractable to purification by dialysis than is simple protein. Peptone may be prepared quite poor in salt by repeated precipitation with alcohol and finally with tannic acid. Dialysis is of little value, as the peptone itself diffuses to some extent, and some of the salts cling to the peptone with the greatest tenacity. The best available method consists in the isolation of the peptone by precipitation with iron and its final separation by alcohol according to the method of Siegfried. This author states that he has secured salt-free peptone by this method; but working with large quantities I have been unable to do so, as colloidal iron, and other salts as well, have always clung to the peptone. In any event this is of little consequence, since these substances (concerning the chemical individuality of which doubts are justified) are poor nutrients for bacteria.

Salt-free protein of the simple type of the protamines may be prepared without great difficulty. I have thus prepared the protamines from the salmon and the striped sea-bass, and also the histone from the star-fish. These all may be prepared so salt-free that the ash is not weighable. But they are not good nutrients for bacteria, and this statement is true for their protones. Moulds grow well on these protamines; bacteria grow poorly.

A salt-free medium was finally prepared according to an electro-chemical method suggested by Dr. F. G. Cottrell, Instructor in Physical Chemistry in this University. The method, which combines diffusion with electrolysis, had been previously employed for somewhat similar purposes, and has been used in particular by Dr. Cottrell for the preparation of pure colloids for

physico-chemical studies. The principle consists in the transportation of a powerful galvanic current through the material, whereby the cathions and anions wander respectively to their appropriate electrodes, and, passing through diffusion membranes to reach the electrodes, are removed by lavage of the electrodes with distilled water. Upon the efficiency of this lavage depends the success of the manipulation. The apparatus employed consists of a central glass cell of varying capacity (up to a half-litre), open at both ends and having a perforation through the wall for



the introduction of the material to be electrolysed. The open ends are covered with diffusion membrane of tested quality, back of which are placed sheets of fine platinum mesh, and on the outside of these are clamped tightly the outer glass cells that have openings to permit of the inflow and outflow of the washing fluid. All connections must be water-tight. The lavage chambers behind the platinum electrodes should be shallow, so as to ensure a frequent changing of the water. The connections with the current are made directly to the platinum mesh. The accompanying illustration will render further description superfluous.

When first set in operation the reaction may be rather violent and considerable heat may be evolved. If this be deemed undesirable the current (110 volt circuit) may be reduced by the insertion of resistance, as by the insertion of a few lamps, or the entire apparatus may be immersed in cold water. The greater portions of the salts are soon removed, together with acid or alkali, and as it is these ions that carry the current through the medium, the conductivity decreases as the removal of the salts is accomplished. Finally, the conductivity attains a constant minimum, which will be found to be somewhat above the conductivity of the distilled water employed in the preparation of the medium. This greater conductivity of the medium as contrasted with the distilled water is due to an electrical endosmosis, the particles of colloid being able to transport a small amount of the current. An amperemeter should be inserted into the circuit so that the progress of the reaction may be observed. It not only shows the close of the reaction in favorable instances, but also indicates the occurrence of disintegration of the substrate by the current, since under these circumstances the curve of conductivity in time will not exhibit a progressive descent, but will display wide fluctuations and variations. After every period of prolonged rest, as overnight, the conductivity is for a short time increased, only to drop to the previous level after a few moments. The more nearly complete the process, the less marked is this increase in conductivity after a period of rest. Obviously the method can be applied only to typical non-diffusible colloids.

In this way I have prepared a ten-per-cent gelatine medium. The gelatine was first crudely purified by soaking in weak alkali, and after it had become swollen it was repeatedly washed by decantation. It was then brought into solution by heat and precipitated by the addition of the least effective quantity of alcohol; in this way, while one loses gelatine, one retains less of salt than with precipitation with an excess of alcohol. This process was repeated once, and the final precipitate washed with alcohol and ether and dried, following which it could be weighed for the preparation of the ten-per-cent solution. The

gelatine was dissolved in water that had been twice distilled in a quartz apparatus under the utmost precautions: the conductivity at 16° was 1.1×10^{-6}. The final solution was submitted to electrolysis for five days, by which time the conductivity had reached a constant minimum. The conductivity was determined by the method of Kohlrausch, with the use of a standardized set of resistances. It was 8×10^{-6}—about, therefore, the conductivity of good double-distilled water, from which carbon dioxide has not been excluded during the distillation, and is about x 200 the conductivity of the purest water of Kohlrausch. During the preparation of the gelatine solution and its electrolysis, no attempt was made to exclude entirely carbon dioxide, and the final conductivity of the gelatine may be reasonably attributed in large part to this acid. The material was certainly not salt-free in the absolute sense, but it was very poor in salt.

Such a gelatine contains about two-tenths of one per cent of sulphur. This sulphur is in organic combination; it is a constituent of the gelatine molecule and cannot be detected unless the organic matter is destroyed. The material also contains a trace of phosphorus, likewise in organic combination. If some of the gelatine be digested with hydrochloric acid and potassium chlorate (free from phosphorus), the reactions for phosphoric acid are always to be demonstrated in the digestion fluid. Now the best analyses of gelatine (those of Faust, Paal, and Moerner) have not disclosed the presence of such phosphorus in the molecule of gelatine. It is in all probability best to be explained by the assumption that a trace of other organic matter has accompanied the gelatine, possibly nuclein or nucleo-proteid. In any event, the traces of phosphorus are not to be excluded from the best commercial gelatine. On incinerating a large quantity of the gelatine, after fusing with lye, the presence of the phosphoric and sulphuric acids may be demonstrated in the residue; but no other anions, chlorine in particular, are to be detected. Flame analysis fails to yield any signs of the presence of the ordinary alkali metals. The ash of ten grams of such gelatine, water-free, is not weighable.

Impure gelatine does not give the same result on electrolysis,

and decomposition always occurs. Apparently a certain degree of purity is necessary in order that the reaction shall not disintegrate the gelatine. If the method be employed on a good gelatine at a low temperature, the gelatine will lose nothing of its power of gelatification. At higher temperatures, however, the faculty of gelatification is rapidly lost, more rapidly, indeed, than would seem to be accounted for by the action of the heat alone. A finished gelatine product is absolutely neutral when tested with the Nernst gas chain, using hydrogen electrodes.

I am advised by Dr. Cottrell that, for reasons not yet clear, the method fails with agar-agar. The galactan does not become salt-free, but does lose its power of gelatification. The galactan is either dependent upon some mineral combination for its power of gelatification, or else it suffers during the electrolysis some disintegration attended with the loss of this attribute.

GROWTH OF BACTERIA ON SALT-FREE GELATINE.

This simple salt-free gelatine I have tested upon many bacteria. None of the ordinary saphrophytic or pathogenic bacteria will grow upon the medium. One large bacillus is able to develop upon this gelatine, though very slowly. This bacillus, which in some respects resembles the group of the hay bacillus, is a saphrophyte native to our city water, and since the spores are very resistant to heat it tends to contaminate all media sterilized by fractional sterilization. It forms on ordinary media yellow projecting colonies, is stained by Gram's method, ferments glucose, liquefies gelatine very slowly, produces traces of indol, does not coagulate milk, produces no acid reaction, and is feebly motile. Since the colonies project upward, it is possible to remove the top of a colony, and after suspending it in distilled water the germs may be plated upon the gelatine; from this plating a second suspension in distilled water is prepared, and in this way the danger of carrying over salts is avoided. The bacillus grows on the salt-free gelatine very slowly and months are required to produce a growth, which on ordinary gelatine would be developed in a week. After prolonged cultivation the micro-organism seems to become better inured to

the nutriment, and a more rapid growth is to be noted. Liquefaction, however, is much delayed. When the gelatine has become dried out (this desiccation is much slower than with the same gelatine before electrolysis) the culture dies.

The crucial experiments were carried out in quartz. The same results were secured with well-steamed Jena glass. Control tests with Jena glass showed that, after a proper steaming of the test tubes, water would not in a month dissolve enough of the glass at the temperature employed (20°) to increase measurably the conductivity of the water.

The growth of this bacillus is greatly retarded in an atmosphere free of carbon dioxide or of ammonia. From this it must be inferred that these gases are utilized in the metabolism of the plant. The retardation is most marked in an atmosphere free of carbon dioxide. Apart from the ammonia of the atmosphere the plant has only the nitrogen of the gelatine available for the necessities of its nitrogenous metabolism. Nitrogen is needed for the synthesis of common protein, the purin nucleus, and the pyrimidin ring,—the necessary constituents of the bacterial protoplasm. That ammonia may be utilized to these ends is of considerable interest, and though we are not in a position to speculate, it may be provisionally assumed that the process of building up from ammonia is in general the converse of that by which in higher organisms protein and nuclein are broken down. To what extent the molecule of gelatine must be disintegrated before it can furnish material suitable for these syntheses, can only be conjectured.

Beyond the carbon of the molecule of gelatine the medium contained no available carbon. The utilization of the atmospheric carbon dioxide classes the bacillus with higher plants. I am not acquainted with studies tending to show to what extent bacteria may utilize carbon dioxide for nutritional purposes. We do not know in what form the carbon exists in the molecule of gelatine and cannot even speculate upon what may be the steps in the process of its utilization.

For the synthesis of nuclein, phosphorus is needed. As stated the gelatine contained traces of phosphorus in organic com-

bination, sufficient doubtless for the needs of the nucleinic metabolism. The sulphur needed in the synthesis of the protein of the bacterial protoplasm was also available in the molecule of gelatine.

For practical purposes it may be stated that the atmospheric ammonia, and that derivable from the desintegration of the gelatine, were the only cathions present. To what extent the atmospheric ammonia figured as a cathion, and to what extent it was simply a nutriment cannot be determined. Substances that readily yield ammonia, such as amido bodies, are great promoters of growth of this micro-organism in the gelatine, from which the importance of ammonia as a nutrient substance may be deduced. That ammonia was the sole cathion present cannot be maintained in any absolute sense; hence the experiments ought simply to be interpreted as an illustration of the minimal cathion requirement for bacterial life. In an entirely similar manner the media were not in the absolute sense free of salt, and likewise the experiments should be accepted as an illustration of the minimal saline requirements for bacterial development.

Other peculiarities observed were the darker color of the pigment produced on these media and the absence of indol formation. Old cultures although faintly alkaline are much less alkaline than cultures grown upon ordinary media.