STUDIES IN THE NITROGEN METABOLISM OF BACTERIA. A THESIS

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STUDIES IN THE NITROGEN METABOLISM OF BACTERIA*

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A chemical study of the metabolism of any organism is generally understood to mean a study of the food materials, the changes which take place in these materials within the organism, the agencies which bring about these changes, and the character and composition of the excretory products. It is obvious that the food materials of micro-organisms may be as completely studied as those of higher forms. To determine the changes these undergo within the cell, however, and the exact composition of the excretory products is a problem of a much more difficult nature than the same task would be in the case of the higher plant and animal species.

At present, students of bacterial metabolism must content themselves solely with the study of the beginning and the end of the process. What takes place within the cell can only be surmised from the nature of the enzymes which have been expressed from the bacterial bodies and from the composition of the products of bacterial action. Furthermore, it must be recognized that the substances found in a bacterial culture medium after a period of growth need not all necessarily represent the end products of metabolism. There are numerous possibilities for alteration of the true metabolic-products by means of their mutual action upon one another.

In the knowledge of these difficulties, therefore, we shall have to interpret the title of this paper to mean merely a study of the nitrogenous constituents of the food supply of bacteria and a chemical examination of the products of the action of bacteria upon these food substances.

There has been no attempt on the part of investigators to make a complete study of the metabolism of any micro-organism. In fact, such a study would, in most cases, include so wide a variety of food materials and metabolic end products that the large amount of work involved would hardly be in keeping with the benefit to be gained from

it. Knowledge of the subject has been obtained, therefore, largely by indirect routes, through researches undertaken with some other object in view.

The phenomenon of putrefaction has long been a subject for chemical research, the impetus to its investigation being derived partly from its relation to processes taking place in the intestinal tract of man and animals and partly from its relation to the preservation of food-stuffs. Earlier experiments were all of a general nature, however, being carried out either on spontaneously putrefying masses or on pure proteins inoculated from such masses. The only results were the recognition of a number of compounds as characteristic putrefactive products, and the isolation of many toxic substances. Much benefit was derived from these investigations by the subjects of medicine and biological chemistry, but little was added by them to the knowledge of bacterial metabolism. More productive in this direction has been some of the work of later years, in much of which both pure proteins and pure cultures of bacteria have been used.

The search for new and better culture media, or for media adapted to the growth of certain species of micro-organisms, has been responsible for many valuable contributions, particularly to the knowledge of what sort of nitrogenous compounds can be utilized by bacteria. Likewise, attempts to differentiate species by taking advantage of the dissimilarities in their nitrogen requirements and by noting the different products resulting from the decomposition of the same nitrogenous substance by different species, have led to a more careful investigation of nitrogen sources and of the end products of nitrogen metabolism. It is to these attempts that we owe the extensive data to be obtained on the subject of indol-formation by bacteria, as well as on their reducing and fermenting powers. In recent years the subject of creatinin-formation has been studied with the same object in view.

It is not likely that it is possible for any organism to grow and reproduce without any source of nitrogen in its food supply, tho Fermi[1] asserted that he had cultivated a micro-organism containing no nitrogen in its body substance. In the form, however, in which this nitrogen may be offered there is extremely wide variation. As has been known since the work of Berthelot[2] in 1899, there are many species which thrive with no other source of nitrogen than the uncombined atmospheric nitrogen. On the other hand, there are species which will accept such compounds as the chitin[3] of plant and animal origin, as their only source of this element.

And not only do we notice this variation in the sources of nitrogen among a large number of species, but even with one and the same species it is now well known that compounds of very different degrees of complexity may be utilized. The same organism may grow with a native protein, a peptone, amino-acids, amides such as urea, or even with ammonium salts, as its only source of nitrogen. Even B. tuberculosis, an organism formerly supposed to be exacting in its cultural requirements, has recently been grown successfully on a medium containing nitrogen only in the form of ammonium compounds.[4]

If bacteria show great variations in their choice of food materials, so also do they show wide differences in the ways in which they alter these materials

[1] Schmidt and Weis: Die Bacterien, 1902, p. 102.
[2] Chimie vegetale et agricole, 1899, 1.
[3] Benecke: Botan. Ztg., 1905, 63, p. 227.
[4] Wherry: Jour. Infect. Dis., 1913, 13, p. 144. Kendall, Day, and Walker: Ibid., 1914, 15, p. 417.

in the course of metabolism, and in the kinds of chemical products which they yield by means of such alteration. Two factors must always be considered in studying the chemical products of bacterial action; namely, the species of the organism and the nature of the substance being acted upon. Upon the same substrate different species may yield very different products; likewise, as would be expected from a unicellular organism, the same species may yield quite different products when grown on media of different chemical compositions. The actual chemical processes involved in the decomposition of nitrogenous compounds by bacteria are difficult to study. Equations which have been written to represent such decompositions must, for the most part, be placed in the class of speculations. Such speculations are of great value, however, and, no doubt, frequently arrive very close to the truth.

That proteolysis through the agency of bacteria capable of attacking native proteins pursues the same general course as that brought about by the digestive enzymes of the alimentary tract of animals seems to have been established beyond dispute. Emmerling and Rieser[6] showed that B. fluorescens-liquefaciens digested gelatin with the formation of proteoses and peptones. These were later broken down to lower compounds yielding in the course of a month 25% of their nitrogen in the form of ammonia. Substitution compounds of ammonia were also found in the form of methylamin, trimethylamin, betain, and cholin. That amino-acids were an intermediary product, however, was evidenced by the fact that they were able to identify arginin and leucin. Cultures of the same organism on fibrin solutions contained tyrosin, leucin, arginin, and aspartic acid. Emmerling[7] identified the amino-acids, tyrosin and leucin, in cultures of virulent streptococci on blood fibrin. Mono- and trimethylamins were present here also, as well as pyridin bases. According to Taylor,[8] B. coli digests pure casein mainly to proteoses and peptones, no appreciable quantities of animo-acid being formed. On the egg-meat mixture employed by Rettger[9] this organism produced profound changes, giving rise to the aromatic compounds indol and skatol, the amino-acids tyrosin, leucin, and tryptophan being identified as intermediary products. Proteoses and peptones were formed also.

In the decomposition of proteins the obligate anaerobes play a most important part. In fact, according to Rettger,[9] true putrefactive changes with the production of the foul-smelling mercaptans and hydrogen sulfid are brought about only by this class of organisms, the part played by the aerobes and facultative anaerobes being that of creating an oxygen-free environment and removing the waste products of the strict anaerobes. From his researches it appears that B. putrificus, B. oedematis, and the bacillus of symptomatic anthrax are the most powerful putrefying organisms among the commoner anaerobes. B. tetanus and B. welchii have little or no putrefactive power, the latter being primarily a fermenting organism.

The decomposition of the primary products of protein hydrolysis by bacteria has been studied but little, altho mixtures of peptones and proteoses sold as peptone have long been the favorite basic substance in bacterial culture media. By means of the change in the rotation of polarized light, Abderhalden, Pincussohn, and Walther[10] studied a number of the common pathogens with

[6] Ber. d. deutsch. chem. Gesellsch., 1902, 35, p. 702.
[7] Ibid., 1897, 30, p. 1863.
[8] Ztschr. f. physiol. Chem., 1902, 36, p. 487.
[9] Am. Jour. Physiol., 1903, 8, p. 284.
[9] Rettger and Newall: Jour. Biol. Chem., 1912, 13, p. 341. Rettger: Ibid., 1906, 2, p. 71; 1908, 4, p. 45.
[10] Ztschr. f. physiol. Chem., 1910, 68, p. 471.

respect to the extent to which they break down peptones prepared from pure proteins, and compared the results with the effect on the proteins themselves. Kendall and his co-workers[11-17] recently studied the production of ammonia by a large number of species, using in some cases Witte's peptone and in others a peptone solution containing meat juice. Their aim was mainly to investigate the effect that carbohydrates have on the decomposition of the nitrogenous substances. They took the ammonia-production as a measure of proteolysis. Their data show interesting exceptions to the general rule that carbohydrates have a protein-sparing effect.

The investigation by Glenn[18] of the inhibition of indol-formation by members of the proteus group grown in a peptone-carbohydrate solution also indicates that these compounds materially lessen proteolysis. The author attributes this effect, however, to the inactivation of the tryptic enzymes of the bacteria by the acid products of sugar-fermentation. The decreased gelatin-liquefaction by this group in the presence of sugars fermentable by them he explains in the same way.

Berghaus[19] also published extensive data on the subject of ammonia-formation by bacteria. He furthermore drew curves representing the production of this compound after chemical inhibition of growth.

Kendall and Farmer[11] attempted also to measure the rate of the production of amino-acid, but were unable with the method used (formol titration) to get results of any value.

Kendall and Walker[13] claimed the production of minute quantities of urea from meat-juice peptone solutions, and further stated that the amounts formed day by day were about proportional to the ammonia produced.

Antonoff,[20] using Weyl's test, and Germàn,[21] using Salkowski's method, claimed creatinin-production from Witte's peptone for a large number of species. Both investigators believed the tests to have differentiating value. Fitzgerald and Schmidt[22] repeated these tests, but could find appreciable amounts of creatinin only in cultures of B. proteus. They employed both Weyl's method and Jaffé's picric-acid test.

That polypeptids are produced by bacteria has not been established as far as I know. That they may be utilized, however, as a source of nitrogen is known. Sasaki[23] demonstrated the ability of a variety of species to split some of the simpler peptids into their constituent amino-acids.

A study of the metabolism of bacteria grown on media containing nitrogen only in the form of amino-acids has been productive of much information that is interesting and valuable. We may deal here with synthetic as well as analytic products. That proteins are synthesized from amino-acids by micro-organisms

[11] Kendall and Farmer: Jour. Biol. Chem., 1912, 12, pp. 13, 19, 21, 465, 469.
[12] Kendall, Farmer, Bagg, and Day: Ibid., p. 219.
[13] Kendall and Farmer: Ibid., 1912, 13, p. 64.
[14] Kendall, Day, and Walker: Jour. Infect. Dis., 1913, 13, p. 425.
[15] Kendall and Walker: Jour. Biol. Chem., 1913, 15, p. 277.
[16] Kendall, Day, and Walker: Jour. Med. Research, 1913, 28, p. 465.
[17] Kendall, Day, and Walker: Jour. Am. Chem. Soc., 1913, 35, pp. 1201, 1208, 1211, 1217, 1225, 1237.
[18] Centralbl. f. Bakteriol., I, O., 1911, 58, p. 481.
[19] Arch. f. Hyg., 1908, 64, p. 1.
[20] Centralbl. f. Bakteriol., I, O., 1906, 43, p. 209.
[21] Ibid., 1912, 63, p. 545.
[22] Proc. Soc. Exper. Biol. and Med., 1912, 10, p. 55.
[23] Biochem. Ztschr., 1912, 41, p. 174; 1913, 47, pp. 462, 472.

follows as a matter of course when we say that growth is supported by them. That proteins other than those of the bacterial bodies are formed seems to be true also.[24] Moreover, it has been shown by Fränkel[25] and others[26] that the characteristic toxins of diphtheria and tetanus are formed in media containing only amino-acids as a source of nitrogen.

The fraction of the nitrogen of the amino-acid that is used in synthesis is always very small. The greater portion is found in the form of simpler compounds. Frouin and Ledebt[27] grew several species on the amino-acids resulting from the hydrolysis of serum proteins and observed that in all cases a primary acidity was produced which was followed later by strong alkalinity. Rivas[28] found that a short digestion of peptone with trypsin made it a much better culture medium than the undigested peptone. On such a medium he obtained indol reactions in from 5 to 6 hours.

Of the amino-acids which have been used alone as a source of nitrogen for micro-organisms, asparagin has been most studied. A very large number of organisms are capable of utilizing this compound. The main manner of decomposition is deaminization with formation of aspartic acid and a subsequent production of ammonia from the latter. That nitrogenous products other than ammonia are usually formed also is probable. Nawiasky[29] made a rather exhaustive study of the action of B. proteus on asparagin when large quantities of the organisms are added to pure asparagin solutions. The most of the nitrogen was recovered in the form of ammonia. About 5% of the asparagin which disappeared was not accounted for by the ammonia recovered.

Tyrosin is broken down by B. coli and yields 78.7% of the theoretical amount of p-oxyphenylethylamin.[30] Traetta Mosca[31] found another organism which decomposed this acid with the formation of p-hydrocoumaric acid and ammonia.

Of the nitrogenous compounds other than amino-acids, special interest attaches to those found in more or less abundance in the urine of man and animals. That urea, uric acid, and hippuric acid are attacked by a number of species of bacteria has long been known. Kossiwicz[32] showed that a number of molds were capable of utilizing these substances also. Liebert[33] found several varieties of bacteria that decompose uric acid to ammonia, and he stated that allantoin and urea were intermediary products. Certain other organisms have been isolated[34] which yield only urea from uric acid, no ammonia being formed.

That a very large number of species exist capable of converting urea to ammonium carbonate is evident from the researches of Miquel.[35] It is probable also that many of the common laboratory forms show this property.

[24] Muller: Pflüger's Arch., 1906, 112, p. 245.
[25] Hyg. Rundschau, 1894, 4, p. 769.
[26] Uschinsky: Centralbl. f. Bakteriol., 1893, 14, p. 316. Arch. de méd. expér. et d'anat. path., 1893, 5, p. 293.
[27] Compt. rend. Soc. de biol., 1911, 70, p. 24.
[28] Centralbl. f. Bakteriol., I, O., 1912, 63, p. 547.
[29] Arch. f. Hyg., 1908, 66, p. 209.
[30] Sasaki: Biochem. Ztschr., 1914, 59, p. 429.
[31] Gazz. chim. Ital., 1910, 40, p. 86.
[32] Ztschr. Gahrungsphysiol., I, 60, and II, 51.
[33] Botan. Centralbl., 1910, 114, p. 361.
[34] Ulpiani: Jahrb. f. Tierchem., 1903, 33, p. 1034. Gerard: Compt. rend., 1896, 122, p. 1019; 123, p. 185.
[35] Lafar's Handbuch der technischen Mykologie, 1904-1906, 3, p. 71.

Creatinin is attacked slowly by bacteria[36] as is creatin also. Nawiasky showed that the latter was decomposed by B. proteus only to the extent of 8.64%. Only 3.69% of the amount attacked was accounted for by the ammonia produced. He assumed that methylguanidin was formed.

THE PRODUCTION BY BACTERIA OF AMINO-ACID AND AMMONIA FROM PEPTONE

That ammonia is the chief end product of the nitrogen metabolism of bacteria seems to have been well established. That the ammonia-production by an organism growing on a protein or peptone medium is always a measure of the organism's proteolytic activity cannot, from this fact, be assumed to be true. It is quite conceivable that, because of the differences in the rate of the decomposition of the primary products of proteolysis, this criterion might lead us astray. We might, for instance, have an accumulation of amino-acids in the medium and a very slight production of ammonia, or, on the other hand, a decomposition of the amino-acids as fast as formed with a consequently high concentration of ammonia. It would give a better idea, therefore, of the rate and extent of protein-decomposition if data were secured on the concentrations of both amino-acid and ammonia. The new method originated by Van Slyke[37] for determining amino-acid nitrogen now makes the procuring of such data possible. The analytical results of the examination of a large number of cultures with respect to their change day by day in amino-acid and ammonia content are given in the following pages by means of tables. Some are shown also in the form of curves.

The free ammonia was determined by Folin's aeration method, in which $Ca(OH)_2$ is used to set the ammonia free from its salts. After the ammonia had been completely removed, the sample was filtered off from the excess calcium hydroxid and a determination of the amino-acid was made by Van Slyke's micro method. The Kjeldahl-Gunning-Arnold method was used for total-nitrogen determinations.

The first organisms investigated were the strongly putrefactive facultative anaerobes, B. proteus-vulgaris and B. pyocyaneus. The medium used was a solution containing 2% peptone and 0.5% NaCl. Five hundred cubic centimeters of this solution were placed in each of two 1000-c.c. flasks. After sterilization in the autoclave at 15 pounds' extra pressure, they were inoculated and placed in an incubator at 37 C. By means of a sterile pipet a sample was withdrawn from each immediately after inoculation, and at intervals of 24 hours thereafter for 11 days. These samples were analysed at once for free ammonia and amino-acids. Creatinin was also determined in the samples from the

[36] Ackermann: Ztschr. Biol., 1913, 62, p. 208; 63, p. 78.
[37] Jour. Biol. Chem., 1913, 16, p. 161.

culture of B. proteus. Tests for this compound in the cultures of B. pyocyaneus were all negative. Folin's[84] method was employed for the determination of creatinin.

Table 1 gives the analytical data for this test. The figures represent the total amount in milligrams of the substance mentioned at the head of the column, that is present in 100 c.c. of the culture fluid on the corresponding day. The third column under each organism gives the ratio between the amounts of amino-acid nitrogen and ammonia nitrogen present on each day of the test. Chart 1 represents the same results in the form of curves.

The data show very marked differences between the two organisms in their action on peptone solutions. In the culture of B. proteus we notice for the first 2 days a decrease in the amino-acid already present in the medium, followed by a rise in concentration on the 3rd, 4th and 5th days. Thereafter the concentration rises and falls without any tendency to get very far from a mean value of about 50 mgm. per 100 c.c. The ammonia nitrogen also decreases for the first 2 days, but thereafter rises rapidly until the end of the experiment, reaching the concentration of nearly 70 mgm. per 100 c.c. The ratio between the two forms of nitrogen decreases rapidly throughout the test.

The culture of B. pyocyaneus likewise shows an initial decrease in its amino-acid nitrogen, followed by fluctuations up and down until the 6th day, after which there occurs a gradual rise in concentration until the end of the experiment on the 10th day. The free ammonia also suffers a decrease in this culture in the first 24 hours. Thereafter there is a general tendency toward a slow increase of this substance, but in two instances, on the 4th and 7th days, a decrease occurs. The ratio, amino-acid nitrogen to ammonia nitrogen, falls in this case also, but the decline is much slower than in the case of B. proteus, and the ratio does not, in the time of the experiment, reach nearly so low a value. These differences are made plainer by the curves. The amino-acid curve of B. pyocyaneus tends to rise rapidly. The free ammonia curve of B. proteus rises very rapidly; that of B. pyocyaneus is much more gradual.

It is interesting to note that both cultures show an initial decrease in both their amino-acid nitrogen and their ammonia nitrogen, indicating that, for purposes of growth and reproduction, these organisms select the simpler forms of nitrogen in preference to the more complex peptone and proteose molecules. This seems to be in accord with the

[84] Jour. Biol. Chem., 1914, 17, p. 469.