Vol. XXVII [APRIL, 1905.] No. 4

THE JOURNAL

OF THE

American Chemical Society

CONTRIBUTION TO THE BIOCHEMISTRY OF SEWAGE PURIFICATION; THE BACTERIOLYSIS OF PEPTONES AND NITRATES.[1]

BY STEPHEN DEM. GAGE.

Received January 6, 1905.

CONTENTS.

[1] Read at the 31st general meeting of the American Chemical Society, Philadelphia, Pa., December 28, 1904. The studies here reported were made at the Lawrence Experiment Station, under the direction and with the cooperation of Mr. H. W. Clark. In the chemical analyses the writer has been assisted by Mr. Geo. O. Adams, who has also aided in the elaboration of the methods and in the discussion of the significance of the results. In reviewing the literature, free use has been made of the various publications of Doctors Rideal, Chester and Lipman, and in many instances, where the original articles could not be obtained, citations have been made from the reviews of these authors. Published also in the *Tech. Quarterly*, March, 1905.

INTRODUCTION.

IN THE biological treatment of sewage a large number of complex chemical reactions occur, and many types of bacteria are active, some of them playing a good part in the purification, while others exert a hindering action. Hitherto, the analyst has relied on chemical methods in order to judge of the degree of purification accomplished by a given sewage system, and, indeed, as far as they go, the chemical methods have been sufficient for that purpose. In the use of the so-called biological sewage disposal systems, however, with their more scientific control, it has been found that the chemical methods of the present time are inadequate, inasmuch as they do not show what processes are active within the filtration plant further than can be shown by the end products, that is, they do not show in what manner those end products are produced, nor do they indicate in what manner the intermediate processes may be controlled in order to accomplish the greatest amount of purification in the most economical manner. While much knowledge of the methods of filter control has been acquired, this knowledge has been acquired by the cut and try method, rather than by the study of the processes going on in the filters and of the different types of bacteria which are responsible for the various types of reaction.

It has often been noticed that while one method will dispose of a certain sewage in a satisfactory manner, a very different result is obtained when we attempt to treat a different sewage by this process, and this has led to the axiom, that the problem of finding a satisfactory disposal method is an individual one for each sewage. The reason for this is twofold—first, the very great variation in the character and amount of the components of the sewage, and, second, the difference in the active bacterial flora of that sewage.

During the past few years, much study has been given to the bacteriology of sewage at the Lawrence Experiment Station, and recently new methods have been devised for the study of the functions of the bacteria concerned in the purification of sewage, and these methods, with some of the results obtained by their use, have already been published.[1] In the treatment of sewage, the main problem is to accomplish the change of the nitrogenous matter from the organic to the inorganic form, since in the process

[1] Clarke and Gage: *Eng. News*, **53**, 27 (1905).

of changing the form of the nitrogen, the carbonaceous matter, except, perhaps, a very small portion, is usually taken care of. Thus a knowledge of the bacterial flora of sewage and of the changes which different types of bacteria may cause in the form of the nitrogen of that sewage becomes of considerable importance in the proper control of those changes. The greatest difficulty in investigations of this character is the uncertainty of the organisms in the sewage. The least change in the conditions of the experiment, and new species of bacteria develop, with perhaps a total change in the character of the chemical reactions produced, thus assuming that the experiment is conducted under conditions similar to those under which sewage disposal systems are operated. In order to understand the fundamental principles of sewage purification it becomes necessary to study the functions of the various species of bacteria in pure culture, and under conditions which can be controlled, for it is only by such methods that data sufficiently exact to be used in an analysis of the more complex problems where mixed cultures of bacteria are active and the products of their activity react upon one another, may be obtained. The present paper has to deal with the changes caused in the nitrogen content of known solutions by pure cultures of bacteria.

METHODS OF INVESTIGATION.

The method employed in this experiment has been to inoculate two solutions, one containing nitrogen in the form of peptone and the other containing peptone and nitrogen in the form of potassium nitrate, with pure cultures of bacteria, and to determine accurately from day to day the changes taking place in the nitrogen content of the solution as far as could be judged by making quantitative tests of the amount of nitrogen present in the solutions as ammonia, as nitrates, and as nitrites, and by determining at the end of a stated period the total amount of organic nitrogen in the solutions by the Kjeldahl method.

The peptone solutions have been uniformly composed of 0.1 per cent. of Witte's peptone in distilled water, with an organic nitrogen value of about fourteen parts of nitrogen per 100,000 as determined by the Kjeldahl method. It is well known that commercial peptones are not pure and that they contain considerable amounts of albumoses and of other hydrolyzed albumens. This fact, however, is an advantage rather than a detriment in a study

such as the present, since the cultural solutions simulate more closely the character of sewage and are at the same time of sufficiently definite composition to allow of their being readily duplicated in the laboratory.

The nitrated solutions have been made up the same as the peptone solutions, with 0.1 per cent. of Witte's peptone in distilled water and have been of three different strengths at different periods of the investigation, containing nitrogen as potassium nitrate equivalent to 2.7, 6.0 and 22.5 parts per 100,000.

The cultures selected were first purified by plating out on gelatin and were then subjected to a thorough course of revivification by successive cultivations in broth, after which they were transferred to agar streaks. Glass-stoppered bottles, containing about 100 cc. of the peptone solution and of the nitrated peptone solution, were then inoculated with a large loop full of the culture from the surface of the agar streaks, and after thoroughly shaking, the bottle cultures were incubated at 20° C. Portions for analysis were removed from each bottle daily with sterile pipettes, each culture being thoroughly shaken before the sample was removed. At the end of the experiment, determinations were made of the total organic nitrogen in both cultures and uninoculated solutions.

By the use of a peptone solution of known strength both with and without nitrates, we have been able to determine approximately, (1) the amount of ammonia formed from the organic matter, that is, from the peptone; (2) the amount of ammonia formed from organic matter plus that formed by reduction of nitrates to nitrites; (3) by subtracting the amount of ammonia formed from the peptone from that formed in the nitrated peptone solution, we obtain a value for the ammonia formed from the nitrates, although there is a large error in the use of this value as will be shown further on; (4) we also obtain a value for the amount of nitrates destroyed by the culture by subtracting the amount of nitrate in the solution from that at the start; (5) we get the amount of nitrites by direct reading; (6) by subtracting the sum of the nitrites and the estimated amount of ammonia formed from the nitrates from the total amount of nitrate reduced, we obtain a value for nitrogen from nitrates unaccounted for, which may have been liberated as free nitrogen. In obtaining this last value we have the sum of all the errors of the other determinations, so that the

value is itself subject to much doubt, but some of the results have been so characteristic that they are used further on with no further apology; (7) by the use of the Kjeldahl values, in connection with the nitrate, nitrite and ammonia values, we are able to compute, with more or less accuracy, the gain or loss in the total nitrogen content of the test solutions. In addition to the study of the cultures mentioned, examinations have been made of a large number of cultures at the end of a seven-day incubation, and certain of these results are included here.

All of the methods, both chemical and bacteriological, have been subjected to more or less unavoidable error. It should be borne in mind, however, that biological processes are always subject to a large variation, and that all results of biochemical investigation should be interpreted in that light. It has, accordingly, been considered sufficiently accurate to determine the various chemical factors to the nearest 0.1 part in 100,000, and to use the results so obtained, for the purposes of discussion as though the errors had been eliminated.

METHODS OF CHEMICAL ANALYSIS.

The methods for the quantitative determination of ammonia, nitrates and nitrites, in these solutions have varied somewhat from the usual procedure for water analysis. For the ammonia determination one cubic centimeter of the solution was placed in the nessler tube, made up to 50 cc. with ammonia-free water, and nesslerized, direct readings being made against permanent standards.

The direct reading of the ammonia is preferable to the distillation method, as the partially decomposed bacterial products do not show as free ammonia in the direct reading, but many of them are broken down by heat and appear as free ammonia by the distillation process, giving ammonia values which are too high. The main objection to the direct reading is the green color which appears in many cultures upon nesslerizing, probably caused by the presence of amines. The green color interferes very much with the matching of the colors against the usual ammonia standards, and it was necessary to use a special set of standards in order to get comparable readings. These standards are a modification of Jackson's platinum cobalt standards[1] in which the yellow is

[1] Jackson: *Tech. Quarterly*, **13**, 314 (1900).

toned down by the addition of cupric chloride solution. There is considerable variation in the shade of different cultures of bacteria after nesslerization, and many experimental sets of standards were made up before the proportion of copper solution was finally decided upon. Since some of the cultures did not produce the objectionable green shade, it was found advisable to keep on hand two sets of standards, one with and one without the copper. The final composition of the special standards employed is shown in the following table.

TABLE I.—PERMANENT STANDARDS—NITROGEN AS AMMONIA—USED IN DETERMINING AMMONIFYING POWER.

Standard. Cc.	Platinum solution. Cc.	Cobalt solution. Cc.	Copper solution. Cc.
0.1	1.8	0.0	0.6
0.3	3.2	0.0	1.3
0.5	4.5	0.1	1.8
0.7	5.9	0.2	1.8
1.0	7.7	0.5	1.5
1.3	9.4	0.9	1.5
1.7	11.5	1.7	2.0
2.0	12.7	2.2	2.0
2.5	15.0	3.3	0.5
3.0	17.3	4.5	0.0
3.5	19.0	5.7	0.0
4.0	19.7	7.1	0.0
4.5	19.9	8.7	0.0
5.0	20.0	10.4	0.0

Platinum solution=2 grams potassium platinic chloride dissolved in a small amount of water. 100 cc. of strong hydrochloric acid (sp. gr. 1.20) added, and made up to one liter.

Cobalt solution=12 grams cobaltous chloride dissolved in a small amount of water. 100 cc. of strong hydrochloric acid added and made up to one liter.

Copper solution=12 grams dry cupric chloride dissolved in water. 100 cc. strong hydrochloric acid added and made up to one liter.

To determine the nitrites, one cubic centimeter of the solution was mixed with 49 cc. of nitrate-free water in the nessler tube and read by the usual Greiss method. The nitrates were determined by placing one cubic centimeter of the culture in round-bottomed tubes having a length of about 70 mm., and a diameter of 15 mm. These tubes were placed in wire racks and the contents evaporated to dryness on the water-bath, or better, in the hot-

air-bath at a temperature of 115° to 125° C. Phenoldisulphonic acid was then added directly to the tube and by rolling the tube in the fingers, was thoroughly incorporated with the residue. The contents of the tubes were then washed into nessler tubes, ammonia was added, the contents made up to 50 cc., and the amount of nitrogen as nitrates read against standards as usual. The evaporation in tubes is preferable to the usual evaporation in dishes, both for economy of space and ease in manipulation. Experiments showed that the evaporation to complete dryness at these temperatures did not affect the accuracy of the determinations.

SOURCE OF THE CULTURES.

The cultures selected were obtained from sewage and from the effluents of sewage filters during a study of the functions of the various kinds of bacteria concerned in sewage purification, and were selected as producing as many as possible of the various changes in the nitrogen content of the test solutions. In all, some forty cultures were studied in detail, and from the results obtained, twenty cultures have been selected as giving results sufficiently characteristic to warrant their being included here. The majority of the cultures not included, gave results which showed nothing further than could be shown by the cultures presented, while others of the cultures omitted had breaks in the chain of results due to losses during chemical analysis. The cultures were isolated during February and March, 1904, as follows:

Cultures 1, 16, 17, 23, 40, 41, from Lawrence sewage.

Culture 18, from septic sewage.

Cultures 4, 7, 9, 10, 11, 13, 29, from the effluents of intermittent sand filters receiving Lawrence sewage.

Culture 22, from the effluent of a contact filter receiving Lawrence sewage.

Cultures 31 and 32, from the effluent of a contact filter receiving septic sewage.

Cultures 24, 26, 38, from the effluents of intermittent-continuous filters receiving Lawrence sewage.

It is not essential in a study of this kind that the names of the species be known. Indeed, in sewage disposal, a determination of the presence or absence of definite species of bacteria, other than certain disease-producing organisms, is of small

moment compared with the establishment and determination of the types which play important parts in the conversion of that sewage into a harmless and inoffensive water. While some progress has been made in the study of these cultures, the complete species tests are not complete at the present writing. Enough work has been done, however, in order to be able to say that the cultures are apparently all different species, and indeed, the variation in the nitrogen changes caused by these cultures would alone be almost sufficient to establish this difference.

REVIEW OF THE LITERATURE.

We will now discuss the changes which may occur in nitrogenous matter and the agency of bacteria in causing those changes. As sources of nitrogen in the experimental solutions, we have peptone and atmospheric nitrogen common to both solutions. In the peptone solution the only changes which may be produced by bacterial action are the breaking-down of the peptone into amides and amino-acids, which themselves may then be broken down with the formation of ammonia or free nitrogen, the oxidation of the ammonia to nitrates and nitrites, and the fixation of atmospheric nitrogen. The process going on here is primarily putrefactions or decay, the term putrefaction being used in the sense of breaking down without air or anaerobic action, the term decay being used as indicating an oxidizing or aerobic action.

Owing to the newness of bacteriological methods of sewage disposal we have to turn mainly to the agricultural chemists and bacteriologists for our knowledge of the biochemical processes by which nitrogen is changed from one form to another. As early as 1837, Schultze[1] and Schwann[2] discovered that the cause of putrefaction and decay was always present in unsterilized air, but it was left to Pasteur[3] and his co-laborers to demonstrate that putrefaction was due to the action of micro-organisms. The first step which takes place in the conversion of the albuminous matter in sewage is peptonization or liquefaction of the insoluble proteids into soluble peptones and albumoses. The consideration of this step may be omitted here since we are starting with these latter products. The next step in the process is the conversion of peptones into amino acids and amines.

[1] Schultze: *Pogg. Ann.*, **39**, 487.
[2] Schwann: *Ibid.*, **41**, 184.
[3] Pasteur: *Compt. Rend.*, **48** (1859); **50**, 849 (1860).

The amino acids are next decomposed into organic acids and free nitrogen or ammonia, the organic acids and the ammonia thus formed combining to form ammonium salts, but these salts are rapidly broken up and converted into carbon dioxide, water, ammonia, hydrogen, nitrogen and marsh gas, the last three of which escape with some of the ammonia and carbon dioxide, leaving the rest of the ammonia and carbon dioxide united with the mineral elements in the solution or combined as ammonium carbonate.

Ammonification.—There is much experimental evidence to show that the production of ammonia from organic matter is characteristic of most species of bacteria. Marchal[1] found that out of thirty-one organisms studied, seventeen produced ammonia decidedly, and all of the others gave a slight reaction. He observed that the conversion of organic matter into ammonia is inversely proportional to the amount of organic matter in solution, and varies with the character of the proteid matter, the breaking-down of peptones being more rapid than that of most other albuminous compounds. Sewerin[2] found that the majority of bacteria which he studied produced ammonia, but the rate of ammonification was very variable, some of the species producing ammonia continuously from the start, while others did not show any reaction until after some days. Chester[3] found that the majority of common soil bacteria were able to produce ammonia, and also that the rate of ammonification in a soil which had been manured was directly proportional to the product of the numbers of bacteria in that soil, and of their relative ability to produce ammonia.

The increase in ammonia in sewage on standing, and the conversion of organic nitrogen to ammonia in septic tanks and in contact filters are common knowledge in sewage analysis.

The ammoniacal fermentation of urea, while not directly applicable to the present experiments, has considerable significance in sewage treatment. This subject has been thoroughly studied by Pasteur,[4] Gabler,[5] Felz and Riter,[6] von Jaksch,[7] Miquel,[8] Leube,[9]

[1] Marchal: *Arc. Science*, **8**, 574 (1894).
[2] Sewerin: *Cent. f. Bakt.*, i (2), 167 (1895).
[3] Chester: Bull. 65, Dela. Agr. Expt. Sta., 1904, p. 69.
[4] Pasteur: *Compt. Rend.*, **49**, 1 (1860); *Ann Chem. and Physiol.*, **64** (1862).
[5] Gabler: *Compt. Rend.*, **64** (1874).
[6] Felz and Riter: *Jour. de l'Anat. and Physiol.*, 1874.
[7] von Jaksch: *Zeit. physiol. Chem.*, **5**, 1881.
[8] Miquel: *Ann. de Micrographie*, **1**, 414, 417, 506, 522; **2**, 13, 53, 122, 145, 367, 488; **3**, 275, 305.
[9] Leube: Virchow's *Archiv.*, **100**, 540.

Laureau,[1] Warrington,[2] and a number of others. These investigators find that urea and uric acid are readily and completely fermented by a number of common bacteria, forming ammonia and carbonic acid, which combine and remain in solution unless further acted upon by other bacteria.

Nitrification.—The oxidation of ammonia to nitrites and nitrates, has usually been considered to be the function of a specific class of organisms which will not grow on ordinary culture media, and hence cannot be included in the scope of the present study. The classical work of Winogradsky[3] and Warrington[4] with this class of organisms is too well known to require review here. At the same time there is some experimental evidence, more or less authentic, which would point to the fact that certain common species, not belonging to Winogradsky's group of nitrifying bacteria, are, under certain conditions, able to accomplish the oxidation of ammonia to nitrites and nitrates.

In 1886, Celli and Zucco[5] and Heraeus[6] succeeded in isolating from water by the usual methods a number of species of bacteria which appeared to have feeble nitrifying powers. In 1895, Burri and Stutzer[7] isolated from soil an organism with nitrifying properties, and two years later Stutzer and Hertleb[8] offered a further confirmation of the earlier work of Burri and Stutzer in which they claimed not only to have made the nitrifying organism grow on all ordinary culture media, but also stated their belief that this organism had a pleo-morphic habit, changing its form and character under different conditions. Much doubt has been cast on the purity of the cultures with which these investigators worked, and Fraenkel[9] claims to have isolated from Burri and Stutzer's cultures eleven different species.

In 1896 Woodhead[10] isolated from sewage a number of different

[1] Ladereau: *Compt. Rend.*, **100**, 1252 (1885).
[2] Warrington: *Cent. f. Bakt.*, **6**, 498 (1888).
[3] Winogradski: *Ann. de l'Inst. Pasteur*, **4**, 213, 357 (1890); **5**, 577, 921 (1891).
[4] Warrington: *J. Chem. Soc.* (London), 1891, p. 484.
[5] Celli and Zucco: *Rendiconta della R. Acc. dei. Lincei*, 1886.
[6] Heraeus: *Landw. Jahrb.*, 1887; *Landw. Versuchsst.*, **38** (1888).
[7] Burri and Stutzer: *Cent. f. Bakt.*, **1** (2), 721, 749 (1895).
[8] Stutzer and Hertleb: *Ibid.*, **3** (2), 6, 54, 161 235, 311, 351 (1897).
[9] Fraenkel: *Cent. f. Bakt.*, **4** (2) Nos. 1 and 2 (1898).
[10] Woodhead: Quoted by Rideal, "Bacterial Purification of Sewage," p. 54 (1900).

species which grew well on ordinary culture media, some of them being denitrifying forms and others being able to oxidize nitrites to nitrates.

Richter[1] isolated a micrococcus which reduced nitrates to nitrites and was also able to oxidize urea to nitrites. Furthermore, we must consider that the symbiosis of bacteria, as regards action on nitrogenous bodies, has been little studied. Indeed, it is an open question in the minds of many sewage and soil bacteriologists as to whether the so-called nitrifying group of bacteria are the sole cause of nitrification. That there is some ground for this doubt is instanced by many experiments in sewage treatment in which sterile materials inoculated with pure cultures of these types have failed to nitrify, or at best, have nitrified only very slowly when dosed with ammonia solutions or with sterile sewage, while control filters of the same material have nitrified unsterilized sewage rapidly and completely.

As to the conditions governing nitrification there is some diversity of opinion. The earlier observers uniformly considered that nitrification was essentially an aerobic or oxidizing process. Holdefleiss[2] on the other hand obtained results in his experiments which would warrant the belief that nitrification may often take place under strictly anaerobic conditions. Further evidence of this has not been forthcoming, however, and the results obtained from the operation of sewage filters would indicate that efficient aeration is necessary, if nitrification is to continue.

Leme,[3] Pickard,[4] and others have shown that an excess of organic matter tends to check or prevent nitrification. Davy[5] observes that the reduction in nitrification is especially marked when the excess of organic nitrogen is of animal origin. The sudden stoppage of nitrification in sewage filters when continually overdosed, and the return of this action when the quantity of organic matter applied has been reduced within the proper limits, are common knowledge to all who have had experience in the operation of sewage disposal works. The conditions affecting nitrification by sewage filters and the amount of nitrogen which these filters will nitrify regularly have been exhaustively studied

[1] Richter: Quoted by Rideal, *Loc. cit.*, p. 91.
[2] Holdefleiss: "Der Stallmist," Breslau, 1889.
[3] Leme: *Naturwis. Rund.*, **5**, 291 (1890).
[4] Pickard: *Compt. Rend.*, **114**, 490 (1892).
[5] Davy: *J. Chem. Soc.* (London), 1879, p. 429.

at the Experiment Station and will be found discussed in the annual reports of the Massachusetts State Board of Health since 1890, under the chapters on filtration of sewage.

Nitrogen Liberation.—There is considerable difference of opinion as to whether or not free nitrogen is formed in the breaking-down of peptones and amino acids. The results of Immendorff,[1] Kellner,[2] Tacke,[3] Ehrenberg,[4] Schlösing,[5] Muntze Girard and Schneidewind,[6] Dietzell,[7] and Pfeiffer,[8] would appear to support the view that no free nitrogen is evolved when the reactions take place under strictly anaerobic conditions. On the other hand, Wollney[9] takes the stand that nitrogen may be given off in putrefaction, and quotes the results of König and Kiesow,[10] Dietzell,[11] Morgan and König[12] to support his arguments. Zoja[13] found that elastin, when fermented under anaerobic conditions, gave off free nitrogen. Wood and Wilcox[14] found that *B. furfuris*, which they believed to be the cause of putrefaction in the tanning industry, liberated nitrogen gas from nitrogenous matter in the presence of starch under anaerobic conditions. Hugonenq and Doyon[15] found that under anaerobic conditions *B. typhosus* and *B. tetanus* evolved considerable amounts of free nitrogen from nitrogenous solutions. The composition of gases given off from putrefying sewage in septic tanks has been carefully studied during the past few years by a number of observers. Although the percentage composition of the gases given off by septic tanks in different places has varied widely, in every case a considerable percentage of nitrogen has been reported, some of the results being as follows: At Lawrence, Mass.,[16] the gases from two different tanks contained 16 per cent.

[1] Immendorff: *Landw. Jahrb.*, **21**, 281 (1892).
[2] Kellner: *Ztschr. physiol. Chem.*, **12**, 95 (1887).
[3] Tacke: *Landw. Jahrb.*, **16**, 917 (1887).
[4] Ehrenberg: *Ztschr. physiol. Chem.*, **12**, 145, 438 (1887).
[5] Schlösing: *Compt. Rend.*, **109**, 835 (1889).
[6] Muntze Girard and Schneidewind: *Journal f. Landw.*, **45**, 173.
[7] Dietzell: *Landw. Versuchsst.*, **48**, 177 (1896).
[8] Pfeiffer: *Ibid.*, p. 243.
[9] Wollney: "Die Zersetzung der Organischen Stoffe," pp. 12, 13.
[10] König and Kiesow: *Landw. Jahrb.*, **2**, 107 (1873).
[11] Dietzell: *Ztschr. des Landw. Vereins* in Bayern, March, 1882.
[12] Morgan and König: *Landw. Versuchsst.*, **30**, 199, 216 (1884).
[13] Zoja: *Ztschr. physiol. Chem.*, **23**, 236 (1897).
[14] Wood and Wilcox: quoted by Rideal, *Loc. cit.*, p. 72.
[15] Hugonenq and Doyon: *Ann. Chem. Phys.*, **7**, 45 (1898).
[16] Thirty-second Annual Report, Mass. Board of Health, 1900, p. 392.

and 19 per cent. of free nitrogen, respectively. At Andover, Mass.,[1] the gas contained 61 per cent. nitrogen. At Worcester, Mass., Kinnicutt[2] found the gas to contain about 17 per cent. of nitrogen. Rideal[3] found that two samples of gases given off from the tank at Exeter, England, contained 39 and 61 per cent. of free nitrogen respectively. Fowler[4] reports the gas from the tank at Manchester, England, to contain 16 per cent. of free nitrogen.

There is also a difference of opinion as to whether nitrogen is liberated in decay, that is, when the decomposition takes place in the presence of air. Ehrenberg,[5] by a long series of experiments in which he made careful measurements of the gases evolved, decided that no gaseous nitrogen was evolved. Hufner[6] also coincides in the opinion that no nitrogen is liberated in decay proper. Kellner and Yoskii[7] found that the loss of nitrogen during decay was very small, even when the experiment was carried over a long period. On the other hand, the results obtained by Tacke[8] led him to believe "that in the oxidation of ammonia, as well as in the reduction of nitric acid to ammonia, conditions may arise by which two nascent nitrogen atoms can combine into molecular nitrogen and escape from the decaying mass." Chabrier[9] found that small amounts of nitrogen are always liberated during nitrification, and his observations have been confirmed by Boussingault,[10] by Pickard,[11] and more recently by Godlewski.[12] Schneidewind,[13] Pfeiffer,[14] and Stutzer and Hertleb[15] all furnish evidence that losses of free nitrogen are likely to occur.

Gerlach and Vogel[16] isolated from soil a number of bacteria

[1] Thirty-second Annual Report, Mass. Board of Health, 1900, p. 392.
[2] Kinnicutt: Quoted by Rafter, "Treatment of Septic Sewage," 1904, p. 80.
[3] Rideal: Interim Rep. Royal Sew. Comm., **2**, 247, Q. 4135 (1902).
[4] Fowler: *Ibid.*, p. 453, Q. 8401.
[5] Ehrenberg: *Ztschr. physiol. Chem.*, **11**, 439 (1887).
[6] Hufner: *J. prakt. Chem.*, 1876, p. 292.
[7] Kellner and Yoskii: *Ztschr. physiol. Chem.*, **11**, 95 (1887).
[8] Tacke: *Landw. Jahrb.*, **16**, 917 (1887).
[9] Chabrier: *Compt. Rend.*, **67**, 1031.
[10] Boussingault: *Ibid.*, **77**, 1480.
[11] Pickard: *Jour. de Agr. Pract.*, **2**, 273 (1884).
[12] Godlewski: *Cent. f. Bakt.*, **2**, 458 (1896).
[13] Schneidewind: *Jour. f. Landw.*, **45**, 176 (1887).
[14] Pfeiffer: *Landw. Versuchsst.*, **48**, 202 (1896).
[15] Stutzer and Hertleb: *Cent. f. Bakt.*, **44** (2), 31 (1898).
[16] Gerlach and Vogel: *Ibid.*, **7** (2), 609 (1901).

which were able to transform soluble inorganic salts into insoluble organic nitrogen, but when organic nitrogen was present this transformation into proteids was always accompanied by a loss of free nitrogen.

Rogoyski[1] arrived at similar conclusions concerning the simultaneous liberation of nitrogen and the fixation of soluble nitrogen as proteids, and also determined that the proteid nitrogen so formed appeared to be non-putrescible and readily nitrifiable without intermediate conversion into ammonia. This latter point is worthy of further study, bearing, as it does, on the storage of nitrogen in sewage filters at times and the ultimate disappearance of this stored nitrogen with the appearance of large amounts of nitrates and nitrites in the filter effluents.[2] Proteids of this class may also form a large percentage of the organic matter in the sediment in the effluents from filters of coarse materials operating at high rates, which, although usually very turbid and containing considerable suspended organic matter, have been repeatedly proved to be non-putrescible.[3] Berthelot,[4] studying the volatility of the ammonia from soil, found that when there was a loss of ammonia extensive loss of free nitrogen occurred at the same time. The same author observes that the activity of bacteria in soils may lead to a very considerable loss of nitrogen either as ammonia or as elementary nitrogen, and the evolution of free nitrogen is independent of the presence or absence of nitrates. Lipman[5] describes an interesting experiment in which he proved that *B. pyocyaneus* caused a greater loss of nitrogen in solution as the surface of the solution exposed to the air was increased. The better aeration thus produced was evidently the cause of the greater loss, although he did not determine definitely whether the loss was due to increased volatilization of the ammonia, or, as was more likely, to the increased oxidation and setting-free of elementary nitrogen. Rideal[6] has calculated the amount of oxygen necessary to completely oxidize certain typical organic compounds either to ammonia or to elementary nitrogen, and it is significant that more oxygen is re-

[1] Rogoyski: *Ann. Agr.*, **26**, 121, 140 (1900).
[2] 26th Annual Report Mass. Board of Health, 1894, p. 486.
[3] 33rd Annual Report Mass. Board of Health, 1901, p. 372.
[4] Berthelot: *Chemie Vegitale et Agricole*, p. 160.
[5] Lipman: Annual Report N. J. Agr. Expt. Sta., 1903, p. 226.
[6] Rideal: "Bacterial Purification of Sewage," 1900, p. 73.

quired for the second reaction than for the first. This would go to prove that nitrogen is more likely to be set free under aerobic than under anaerobic conditions.

Fixation of Atmospheric Nitrogen.—As to the fixation of atmospheric nitrogen in organic solutions there is also considerable controversy and the available data is very meagre. Laurent[1] found that certain algae in symbiosis with bacteria were able to fix nitrogen in sand which was exposed to the air and sunlight. This observation was confirmed by Bouilhac,[2] who decided that the bacteria were the cause of the fixation of the nitrogen, the algae merely furnishing the food supply for the bacteria, and this view has more recently been substantiated by the experiments of Kruger and Schneidewind.[3] Nobbe and Hitner[4] observed that there was a fixation of nitrogen in soils and attributed this action to the agency of micro-organisms. Stoklasa and Vitek[5] believed that many of the denitrifying bacteria under certain conditions have the power to fix atmospheric nitrogen, basing their belief on the fact that in their experiments *B. ellenbachiensis* fixed atmospheric nitrogen in solutions which contained no nitrates. Hiltner[6] found in soil, inoculated with denitrifying bacteria, that the yield was greater than where the soils were sterile. Lipman[7] found in soils containing small amounts of nitrogenous matter, that *B. pyocyaneus*, and a number of other denitrifying bacteria were able to fix atmospheric nitrogen.

Beijerinck[8] found that a greater fixation of atmospheric nitrogen occurred in solutions which contained small amounts of organic nitrogen, and this finding was later confirmed by Lipman's[9] experiments. Chester[10] has recently proved without a shadow of doubt that a number of species of bacteria common in sewage are, under certain conditions, able to fix large amounts of atmos-

[1] Laurent: *Ann. de l'Inst. Pasteur*, **6**, 832 (1892).
[2] Bouilhac: *Compt. Rend.*, **123**, 828 (1896).
[3] Kruger and Schneidewind: *Landw. Jahrb.*, **29**, 711, 784 (1901).
[4] Nobbe and Hitner: *Landw. Versuchsst.*, **45**, (1894).
[5] Stoklasa and Vitek: *Cent. f. Bakt.*, **7** (2), 257 (1901).
[6] Hiltner: *Ibid.*, **9** (2), 73 (1902).
[7] Lipman: Annual Report N. J. Agr. Expt. Sta., 1903, p. 230.
[8] Beijerinck: *Cent. f. Bakt.*, **9** (2), 3 (1902).
[9] Lipman: *Loc. cit.*, 1902, p. 231.
[10] Chester: Bull. 66, Del. Agr. Expt. Sta., 1904, pp. 8 to 13.

pheric nitrogen. In addition, there is much recent literature concerning the fixation of nitrogen by bacteria in root tubercles, but the indications at present are that these bacteria are all of one species, and that they do not come within the scope of the sewage analyst.

Denitrification.—In the nitrate solution, besides the peptone and the atmospheric nitrogen as sources of nitrogen, we have present the nitrates, and the changes which may take place are more complex and subject to variation. Here we may have, as in the peptone solution, the fixation of atmospheric nitrogen and the conversion of the peptone into ammonia or free nitrogen or both, and we may also have the reduction of the nitrates to nitrites, to ammonia, to free nitrogen, and we may have the formation of nitrous and nitric oxides. Furthermore, we may have secondary reactions between the nitrites or the lower oxides of nitrogen and the amino acids and amines. The literature on denitrification is particularly rich, owing to its great significance in agriculture.

As early as 1862 Gopplesröder[1] observed that in rich soils denitrification usually took place. In 1867 Froehde[2] observed that a reduction of nitrates frequently occurred when putrefying organic matter was present. In 1868 Schoenbein[3] noticed that many fungi (bacteria?) were able to reduce nitrates to nitrites, and offered the presence of nitrites in drinking water as direct evidence of the presence of micro-organisms. In 1871 Meusel[4] attributed the reduction of nitrates in water to the action of bacteria. Pesci[5] observed that the reduction of nitrates was much more rapid in liquids than in dry or slightly moist material. Gayon and Dupetit,[6] in 1882, observed that in river water containing small amounts of nitrates the nitrates were quickly reduced to ammonia. Deherain and Maquenne[7] and Springer[8] attributed the reduction of nitrates to the agency of anaerobic bacteria which either reduced the nitrates to lower oxides of nitrogen or to free nitrogen. Heraeus,[9] in 1886, isolated from water two species which reduced

[1] Gopplestroder: *Pogg. Ann.*, **115**, 125 (1862).
[2] Froehde: *J. prakt. Chem.*, **102**, 46 (1867).
[3] Schoenbein: *Ibid.*, **105**, 211 (1868).
[4] Meusel: *Ann. Chem. Phys.*, **23**, 161 (1871).
[5] Pesci: *Ber. d. chem. Ges.*, **8**, 259.
[6] Gayon and Dupetit: *Compt. Rend.*, **95**, 544, 365 (1882).
[7] Deherain and Maquenne: *Ibid.*, p. 1365.
[8] Springer: *Ber. d. chem. Ges.*, **16** (1883).
[9] Heraeus: *Ztschr. f. Hyg.*, **1**, 193 (1886).

nitrates to nitrites quickly. Blasi and Fravoli,[1] in 1888, studied twenty-seven species which they had isolated from soil and found that in the majority of instances these species rapidly reduced nitrates to nitrites, and then slowly destroyed the nitrites, although they fail to state what the end-product was. Giltay and Aberson,[2] in 1892, found common in both soil and the atmosphere two organisms, both of which were able to reduce nitrates completely with a liberation of free nitrogen. Eugnow,[3] Burri,[4] Herfeldt and Stutzer, and Schirokokh[5] all isolated and studied species which reduced nitrates. Sewerin[6] studied twenty-nine species, twenty of which were able to reduce nitrates more or less completely. Maasen,[7] in 1902, found that of 109 species of bacteria he examined, eighty-five were able to produce nitrites in solutions containing nitrates. The writer,[8] in a previous paper, reported that of 5,300 cultures of bacteria isolated at the Experiment Station, 85 per cent. were able to reduce nitrates more or less completely, and of 46 different species described at that time thirty-five reduced nitrates to nitrites.

Jensen[9] states that denitrifying bacteria are usually present in the excreta from herbivorous animals, but that they are rare in the effluvia of carnivorous animals. Chester[10] shows that denitrification may take place either under aerobic or anaerobic conditions, confirming the results of Stutzer and Maul,[11] and Boname,[12] who found that denitrification was less rapid under aerobic than under anaerobic conditions. On the other hand, Pfeiffer, Franke, Gotze and Thumann[13] obtained results which led them to believe that denitrification was more rapid when there was free access of air. Frankland[14] concluded from a study of thirty-two well-

[1] Blasi and Fravoli: *Gaz. chim. Ital.*, Palermo, **19** (1888).
[2] Giltay and Aberson: *Abs. in Cent. f. Bakt.*, **12**, 864 (1893).
[3] Eugnow: *Cent. f. Bakt.*, **3**, (2) 504 (1896).
[4] Burri, Herfeldt and Stutzer: *Ibid.*, **1**, 284 (1895).
[5] Schirokokh: *Ibid.*, **2** (2), 204 (1895).
[6] Sewerin: *Ibid.*, **3** (2), 504 (1896).
[7] Maasen: *Ibid.*, **8** (2), 152 (1902).
[8] Gage: 33rd Annual Report Mass. Board of Health, 1901, pp. 401, 419.
[9] Jensen: *Cent. f. Bakt.*, **4** (2), 448 (1898).
[10] Chester: Bull. 98, Penn. Dept. Agr., 1902, p. 64.
[11] Stutzer and Maul: *Cent. f. Bakt.*, **2** (2), 473 (1896).
[12] Boname: Ann. Sta. Agronomic, Mauritius, 1896, p. 74.
[13] Pfeiffer, Franke, Gotze and Thurmann: *Landw. Versuchsst.*, **98**, 189, 245.
[14] Frankland: *J. Chem. Soc.* (London), 1888, p. 372.

known species of water bacteria, more than half of which were able to reduce nitrates to nitrites more or less completely, that the action of these organisms was the same whether they were grown under aerobic or anaerobic conditions.

Stutzer and Jensen[1] showed that a certain amount of organic matter was necessary in solutions for cultures of bacteria to actively reduce nitrates, and the conclusions of these writers have been confirmed by experiments of Kruger,[2] and Wood,[3] Jeannel[4] found that the reduction of nitrates was more rapid when carbohydrates were present.

Liberation of Nitrogen from Nitrated Solutions.—There is some experimental evidence to show that nitrogen is liberated from nitrated solutions, and that in some instances this liberation is due to secondary reaction between the amides and amino acids, and some of the oxides of nitrogen.

As early as 1867 Dr. Angus Smith[5] observed that a reduction of nitrates accompanied by a loss of nitrogen occurred in decomposing sewage. Lipman[6] found that all of the five cultures which he studied, *B. subtilis*, *B. fluorescens*, *B. megatherium*, *B. pyocyaneus* and *B. New Jersey*, liberated nitrogen from organic solutions containing nitrates, although one of the cultures failed to reduce nitrates.

Gerlach and Vogel[7] isolated from soil and manure a number of species of bacteria which, in solutions in which potassium nitrate was the only source of nitrogen, were able to reduce nitrates to nitrites. Later the nitrites disappeared and determinations of total nitrogen showed that there was a loss of nitrogen, although no ammonia had been formed during any portion of the experiment.

Grimbert[8] found that while both *B. coli* and *B. typhosus* are able to liberate nitrogen from nitrated solutions, these species do not liberate free nitrogen unless amido compounds are present, and when nitrogen is liberated by these organisms from

[1] Stutzer and Jensen: *Cent. f. Bakt.*, **2** (2), 2, 7 (1895).
[2] Kruger: *Landw. Jahrb.*, **29**, Nos. 4 and 5 (1900).
[3] Wood: London Rep. Agr. Ed. and Res., 1899, p. 124.
[4] Jeannel: *Compt. Rend.*, **75**, 1244 (1872).
[5] Smith: Quoted by Aikman, "Manures and Manuring," 1867, p. 177.
[6] Lipman: *Loc. cit.*, 1902, p. 222.
[7] Gerlach and Vogel: *Cent. f. Bakt.*, 7 (2), 609 (1901).
[8] Grimbert: *Ann. de l'Inst. Pasteur*, **13**, 67 (1898).

a nitrated medium the volume of nitrogen is always about double the amount of nitrogen lost from the nitrates, thus confirming a previous observation of Hugonenq and Doyon,[1] that *B. coli* reduces nitrates, setting free nitrogen and utilizing the oxygen to oxidize carbonaceous matter.

Ampolla and Gatino[2] studied an organism which reduced nitrates completely, forming a gas which consisted of 85 per cent. of nitrogen and 15 per cent. of carbon dioxide. These authors also found that denitrification is more rapid in alkaline than in acid solutions, and that relatively small amounts of acid were able to prevent losses of nitrogen. That this does not hold true with the organic acids is shown by the experiments of Schlösing,[3] who established the fact that nitrogen is always liberated when the lactic fermentation of sugars occurs in the presence of nitrates.

Deherain and Maquenne[4] studied the gases evolved during denitrification and found that they contained free nitrogen, carbon dioxide and nitrous oxide.

Gayon and Dupetit[5] observed that nitrogen was evolved during denitrification and that the presence of organic nitrogen was necessary for this evolution. They were led to believe that the organic nitrogen is decomposed into ammonia and amines, and that the liberation of free nitrogen is the result of secondary reaction between the amido derivatives of the organic matter and the reduction products of the nitrates. By properly controlling the composition of the fermenting solutions they were able to produce a liberation of nitrogen with or without the admixture of nitrous oxide.

Ampolla and Ulpianni[6] describe two species which changed nitrates in the presence of organic matter into free nitrogen without the intermediate production of nitrites. Frankland[7] found that although *B. aquatilis* was unable to cause a reduction of nitrates to nitrites, yet there was a considerable loss of nitrates from solutions in which it was cultivated.

[1] Hugonenq and Doyon: *Ann. Chem. Phys.*, **7**, 45 (1898).
[2] Ampolla and Gatino: *Cent. f. Bakt.*, **2**, 670 (1896).
[3] Schlösing: *J. Pharm. Chem.*, **8** (4), 213 (1868).
[4] Deherain and Maquenne: *Ann. Agron.*, **9**, 5 (1882).
[5] Gayon and Dupetit: Sta. Agr. de Bordeaux, 1886.
[6] Ampolla and Ulpianni: *Gaz. chim. Ital.*, **28** (1), 410 (1898).
[7] Frankland: *J. Chem. Soc.* (London), 1888, p. 391.

Burri and Stutzer[1] observed that *B. denitrificans II* was able to liberate about 90 per cent. of the nitric nitrogen from certain nitrated solutions, the remaining nitrogen being rendered insoluble. At the start they found that this organism was aerobic, but after denitrification was once started it was able to develop under anaerobic conditions, indicating that the organism was able to use the oxygen set free from the nitrates in its own life cycle.

The foregoing presents a brief and at the same time comprehensive review of the more important literature on bacteriolysis so far as it appears to be directly applicable to the problems confronting the sewage disposal expert, and to the subject-matter of the present paper.

While a number of excellent reviews of different portions of the literature have appeared in the past, nearly all of these, compiled by agricultural bacteriologists, have contained much matter which at present seems to be of no moment in sewage treatment, and none of them have emphasized the points which are of paramount importance to the practical sanitary expert. The viewpoints of the soil bacteriologist and of the man dealing with the disposal of the sewage of large cities are diametrically opposite, the problem of the former being the conservation of nitrogen and the bringing of that nitrogen to the cultivated field in such a form that it shall be stable and readily assimilated by growing plants, while the latter desires to get rid of the nitrogenous matters as quickly and as cheaply as possible. However deplorable the liberation of nitrogen may be in agriculture, and however desirable the assimilation of atmospheric nitrogen, in sewage treatment this same fixation of nitrogen is to be prevented whenever possible, while the liberation of large quantities of organic nitrogen as an inoffensive gas would be of immense advantage.

DISCUSSION OF THE EXPERIMENTAL DATA.

In discussing the results obtained from the study of the change in the nitrogen content of the experimental solutions by the selected cultures, the data have been collected in two tables, Table II showing the progressive change induced by each culture as determined by analyses on successive days, and Table III showing the reduction in the amount of the various nitrogenous constituents

[1] Burri and Stutzer: *Cent. f. Bakt.*, I, (2) 721, 749 (1895).

and the gain or loss of nitrogen during the entire period of the experiment. In considering the progressive change in the nitrogen content of the solutions, as shown by the results in Table II, it will simplify matters somewhat to take up the various reactions by types rather than to discuss the phenomena of each culture separately.

Ammonification.—The amount of ammonia produced in the peptone solution has varied widely with the individual cultures. Of the twenty cultures included in the table, No. 33 showed no ammonification whatever. Five cultures, Nos. 11, 17, 23, 26, and 38, showed only traces of ammonia, *i. e.*, less than 0.5 part per 100,000, and No. 4 showed only 0.7 part. The maximum amount of ammonia produced by any one culture was 18 parts, this being recorded for culture No. 10 on the twentieth day. Four cultures, Nos. 10, 13, 16 and 24, produced over 4 parts of ammonia. As to the rate of ammonification, after two days' growth none of the cultures showed any traces of ammonia. After four days four cultures had begun to ammonify, these being Nos. 10, 24, 31 and 40. On the sixth day three more cultures, Nos. 18, 22 and 41, had begun to produce ammonia. On the tenth day eleven of the twenty cultures showed ammonia production, cultures Nos. 9, 13, 16 and 29 having come into the fold, and on the thirteenth day two more cultures, Nos. 1 and 7, were added to the list.

Denitrification.—In the reduction of nitrates the various cultures have shown many peculiarities, in some cases a steady decrease in the amount of nitrates being noticed, while with other cultures the reduction was spasmodic and erratic. Six of the cultures, Nos. 1, 13, 16, 23, 26 and 38, failed to reduce nitrates at any time in the period during which they were under examination, although culture No. 38 produced small amounts of both nitrites and ammonia in the nitrate solution. Four of the cultures, Nos. 4, 10, 11 and 18, reduced all of the nitrates within the period of incubation, while four more, Nos. 7, 9, 17 and 22, reduced over 50 per cent. of the nitrates. As to the rate of denitrification, two of the cultures, Nos. 10 and 31, show loss of nitrates on the second day. On the fourth day five more cultures showed nitrate reduction, these being Nos. 7, 18, 22, 40 and 41. On the sixth day culture No. 11 showed denitrification, and on the tenth day culture No. 33 began to be

active. Four more cultures, Nos. 4, 9, 24 and 29, showed up on the thirteenth day, and by the seventeenth day culture No. 17 had begun to reduce.

Nitrite Production.—As with the reduction of nitrates, the production of nitrites by the various cultures has been subject to considerable variation. Four of the cultures, Nos. 10, 13, 16, and 23, failed to produce nitrites. Of these, culture No. 10 is of special interest, because, although this culture reduced the nitrates completely, the reduction did not go through the nitrite stage. Three of the cultures, Nos. 1, 26 and 33, produced only traces of nitrites, that is, less than 0.5 part, and culture No. 38 produced less than 1 part. Culture No. 38 is especially interesting, because, although the nitrates were not reduced by the culture, nitrites and ammonia were formed in the nitrate solution in small amounts. The results from cultures Nos. 29, 31 and 41 are interesting, as more nitrites were produced than there was loss of nitrate, and cultures Nos. 29 and 40 showed nitrite production before any reduction of nitrates was noted. These results may be taken as evidence that in the nitrate solution some oxidation or nitrification may have taken place, although in no case were nitrites found in the peptone solution. One of the cultures, No. 11, produced nitrites equivalent to 100 per cent. of the total amount of nitrates reduced, and seven more of the cultures, Nos. 4, 7, 9, 17, 18, 22 and 40, produced nitrites equivalent to 50 per cent. or more of the total amount of nitrates reduced. Of the cultures producing more than 1 part of nitrites, Nos. 31 and 41 gave reactions on the second day, and No. 40 on the fourth day. On the sixth day, cultures Nos. 7, 18 and 22 had begun to produce nitrites actively, and on the tenth day cultures Nos. 9, 11 and 29 were added to the list. Culture No. 4 did not show any great amount of nitrites until the thirteenth day, and cultures Nos. 17 and 24 did not show active nitrite production until the seventeenth day.

Reduction of Nitrates to Ammonia.—The previously discussed functions, the ammonification of peptone, the reduction of nitrates, and the production of nitrites are functions which can be accurately determined and of which we can speak positively in quoting results. As to the production of ammonia from the nitrates, however, we cannot speak so conclusively owing to the indirect method of determination, *i. e.*, by the subtraction of

the ammonia formed in the peptone solution from the total amount of ammonia formed in the nitrated peptone solution, and to the fact that the same culture may not ammonify the peptone in the same ratio in the two solutions, and while we may draw inferences, our results must always be open to criticism and doubt. Nevertheless, some of the results obtained have apparently been of considerable significance. The estimated amounts of ammonia formed from the nitrates, as shown in Table II, are much more erratic and subject to variation than are the figures of the directly determined functions. Eight of the cultures, Nos. 1, 7, 13, 16, 18, 22, 23 and 29, did not show any apparent production of ammonia from the nitrates. Five of the cultures, Nos. 4, 9, 10, 11 and 24, produced ammonia equivalent to more than 50 per cent. of the total amount of nitrates reduced. Cultures Nos. 4, 17 and 24 produced more ammonia during certain periods of the time that they were under examination than was the total amount of nitrates reduced, and one culture, No. 38, produced ammonia, although no reduction of the nitrates occurred. The cause of this overproduction of ammonia is difficult to explain. It may have been due to secondary reactions between the nitrites or other oxides of nitrogen, and the amides or amino acids; it may have been due to the direct fixation of atmospheric nitrogen in the form of ammonia, and it may have been due to an invigorating action of the nitrates on the bacteria themselves, causing them to break down the peptone more completely in the solution containing nitrates than they did in the plain peptone solution. Five of the cultures, Nos. 26, 33, 38, 40 and 41, produced less than 1 part of ammonia; four of the cultures, Nos. 9, 10, 17 and 31, produced between 1 and 3 parts of ammonia, and three of the cultures, Nos. 4, 11 and 24, produced more than 3 parts of ammonia.

Undetermined Nitrogen from Nitrates.—The figures given in this column, as previously explained, are of very doubtful significance, including, as they do, the sum of all the errors in the other determinations. Nevertheless, the results are more or less significant as indicating whether other oxides of nitrogen than nitrites had been formed, which oxides would not be determined by the analytical methods employed, and also as furnishing additional evidence as to the liberation of nitrogen from solutions containing nitrates. Eleven of the cultures did not show losses

of nitrates which could not be accounted for either as nitrites or ammonia, while six of the cultures, Nos. 7, 10, 11, 18, 22 and 23, showed losses, or undetermined nitrogen, amounting to over 50 per cent. of the total amount of nitrates reduced. With cultures Nos. 11, 18, 22, 23 and 40 the largest amounts of undetermined nitrogen occurred during the earlier or intermediate portions of the period of incubation, these amounts decreasing or entirely disappearing towards the end of the incubation period. This would indicate oxides of nitrogen or at least that the nitrogen was in some form which was not determinable, but which was afterwards changed to a form which we were able to determine. With cultures Nos. 11, 33 and 40 the indications are that those losses occurred between the nitrites and ammonia. With cultures Nos. 18 and 22, however, judging from the fact that a very rapid reduction of nitrates occurred, a large percentage of which could not be accounted for either as nitrites or ammonia, but which percentage was later reduced as the nitrites increased, would appear to indicate that some intermediate product between the nitrates and nitrites has been formed. Such a product, as far as we have been able to discover, has never been noted by previous observers, and if the results are not in error, is of much interest. In addition to these cultures three others, Nos. 7, 9 and 31, showed losses or undetermined nitrogen which it would appear was liberated as elementary nitrogen. This is especially marked with culture No. 7, in which the loss of nitrogen increased in direct ratio with the time of incubation.

TABLE II.—SHOWING THE PROGRESSIVE CHANGE IN THE NITROGEN CONTENT OF PEPTONE SOLUTION AND NITRATED PEPTONE SOLUTION DUE TO THE GROWTH OF PURE CULTURES OF BACTERIA.

		Nitrogen—parts per 100,000.				
				Ammonia from		
Culture number.	Days.	Nitrates, total reduction.	Nitrites from nitrates.	nitrates.	peptone.	Undetermined nitrogen from nitrates.
1.	2.	3.	4.	5.	6.	7.
N-1	2	0.0	0.0	0.0	0.0	0.0
	4	0.0	0.0	0.0	0.2	0.0
	6	0.0	0.0	0.0	0.2	0.0
	8	0.0	0.0	0.1	0.2	0.0
	10	0.0	0.0	0.2	0.3	0.0
	13	0.0	0.0	0.0	1.0	0.0
	17	0.0	0.1	0.0	2.0	0.0
	20	0.0	0.1	1.7	2.1	0.0

Culture number.	Days.	Nitrogen—parts per 100,000. Nitrates, total reduction.	Nitrites from nitrates.	Ammonia from nitrates.	Ammonia from peptone.	Undetermined nitrogen from nitrates.
1.	2.	3.	4.	5.	6.	7.
N-4	2	0.0	0.0	0.0	0.0	0.0
	4	0.0	0.0	0.0	0.1	0.0
	6	0.0	0.0	0.0	0.1	0.0
	8	0.0	0.0	0.0	0.2	0.0
	10	0.0	0.1	0.2	0.3	0.0
	13	3.0	2.3	0.8	0.6	0.0
	17	6.0	5.5	5.5	0.5	0.0
	20	6.0	5.0	6.9	0.6	0.0
N-7	2	0.8	0.8	0.0	0.1	0.0
	4	1.2	0.9	0.0	0.2	0.3
	6	1.7	1.3	0.0	0.3	0.4
	8	2.2	1.6	0.0	0.5	0.6
	10	2.5	1.8	0.0	0.7	0.7
	13	2.5	1.7	0.0	1.1	0.8
	17	3.0	1.6	0.0	2.0	1.4
	20	3.5	1.6	0.0	2.2	1.9
N-9	2	0.3	0.3	0.0	0.1	0.0
	4	0.4	0.4	0.0	0.2	0.0
	6	0.4	0.4	0.0	0.4	0.0
	8	0.4	0.6	0.0	0.7	0.0
	10	0.5	1.2	0.0	1.0	0.0
	13	1.2	0.6	0.6	0.1	0.0
	17	2.5	1.5	0.0	3.2	1.0
	20	5.3	3.0	1.5	2.3	0.8
N-10	2	1.0	0.0	0.6	0.5	0.4
	4	2.7	0.0	1.0	1.5	1.7
	6	2.7	0.0	1.1	2.4	1.6
	8	2.7	0.0	0.0	2.5	2.7
	10	2.7	0.0	2.6	4.0	0.1
	13	2.7	0.0	1.0	10.0	1.7
	17	2.7	0.0	2.4	7.4	0.3
	20	2.7	0.0	0.0	18.0	2.7
N-11	2	0.2	0.2	0.0	0.0	0.0
	4	0.2	0.2	0.0	0.0	0.0
	6	1.5	0.2	0.0	0.0	1.3
	8	1.7	0.7	0.3	0.0	0.7
	10	1.8	1.5	1.1	0.1	0.0
	13	5.0	3.4	1.2	0.2	0.4
	17	6.0	6.0	3.3	0.2	0.0
	20	6.0	2.8	4.5	0.5	0.0

Culture number. 1.	Days. 2.	Nitrogen—parts per 100,000. Nitrates, total reduction. 3.	Nitrites from nitrates. 4.	Ammonia from nitrates. 5.	Ammonia from peptone. 6.	Undetermined nitrogen from nitrates. 7.
N–13	2	0.0	0.0	0.0	0.0	0.0
	4	0.0	0.0	0.0	0.2	0.0
	6	0.0	0.0	0.0	0.2	0.0
	8	0.0	0.0	0.0	0.3	0.0
	10	0.0	0.0	0.0	1.1	0.0
	13	0.0	0.0	0.0	2.9	0.0
	17	0.0	0.0	0.0	4.5	0.0
	20	0.0	0.0	0.0	4.2	0.0
N–16	2	0.0	0.0	0.0	0.0	0.0
	4	0.0	0.0	0.0	0.0	0.0
	6	0.0	0.0	0.0	0.5	0.0
	8	0.0	0.0	0.0	1.2	0.0
	10	0.0	0.0	0.0	3.0	0.0
	13	0.0	0.0	0.0	5.5	0.0
	17	0.0	0.0	0.0	4.9	0.0
	20	0.0	0.0	0.0	7.5	0.0
N–17	2	0.1	0.1	0.0	0.0	0.0
	4	0.4	0.4	0.0	0.2	0.0
	6	0.5	0.5	0.1	0.2	0.0
	8	0.6	0.7	0.2	0.2	0.0
	10	0.5	0.7	0.2	0.2	0.0
	13	0.7	0.9	0.7	0.3	0.0
	17	1.6	1.4	2.0	0.2	0.0
	20	2.3	2.2	2.8	0.2	0.0
N–18	2	0.2	0.3	0.0	0.4	0.0
	4	1.5	0.1	0.0	0.9	1.4
	6	2.7	1.7	0.0	1.1	1.0
	8	2.7	1.9	0.0	1.1	0.8
	10	2.7	2.4	0.0	1.2	0.3
	13	2.7	2.0	0.0	2.4	0.7
	17	2.7	2.2	0.0	2.4	0.5
	20	2.7	2.6	0.0	2.2	0.1
N–22	2	0.5	0.2	0.1	0.4	0.2
	4	1.7	0.1	0.2	0.9	1.4
	6	2.0	1.5	0.0	1.3	0.5
	8	2.0	1.7	0.0	1.5	0.3
	10	2.1	1.6	0.1	1.5	0.4
	13	2.1	1.2	0.0	2.8	0.9
	17	2.1	2.0	0.0	2.8	0.1
	20	2.1	1.8	0.0	3.0	0.3

Culture number. 1.	Days. 2	Nitrogen—parts per 100,000				
		Nitrates, total reduction. 3.	Nitrites from nitrates. 4.	Ammonia from nitrates. 5.	Ammonia from peptone. 6.	Undetermined nitrogen from nitrates. 7.
N-23	2	0.0	0.0	0.0	0.0	0.0
	4	0.0	0.0	0.0	0.2	0.0
	6	0.0	0.0	0.0	0.2	0.0
	8	0.0	0.0	0.0	0.2	0.0
	10	0.0	0.0	0.0	0.3	0.0
	13	0.0	0.0	0.0	0.4	0.0
	17	0.0	0.0	0.0	0.3	0.0
	20	0.0	0.0	0.0	0.7	0.0
N-24	2	0.5	0.0	1.7	0.8	0.0
	4	0.5	0.0	1.3	1.5	0.0
	6	0.5	0.0	1.7	2.2	0.0
	10	0.5	0.0	1.7	3.7	0.0
	13	2.5	0.1	2.6	4.2	0.0
	17	4.5	1.8	3.8	4.8	0.0
N-26	2	0.0	0.0	0.0	0.0	0.0
	4	0.0	0.0	0.0	0.1	0.0
	6	0.0	0.0	0.0	0.2	0.0
	10	0.0	0.0	0.3	0.2	0.0
	13	0.0	0.0	0.3	0.1	0.0
	17	0.0	0.1	0.5	0.2	0.0
N-29	2	0.0	0.0	0.0	0.0	0.0
	4	0.0	0.0	0.1	0.0	0.0
	6	0.0	0.5	0.0	0.8	0.0
	10	0.0	1.8	0.0	1.6	0.0
	13	1.5	4.8	0.0	1.9	0.0
	17	1.5	4.5	0.0	2.7	0.0
N-31	2	2.0	2.6	0.5	0.6	0.0
	4	2.5	4.0	0.5	1.1	0.0
	6	9.0	9.0	1.0	1.5	0.0
	10	9.0	13.0	1.0	1.6	0.0
	13	9.0	7.0	1.4	2.1	0.6
	17	11.0	15.0	1.5	2.4	0.0
N-33	2	0.0	0.0	0.0	0.0	0.0
	4	0.0	0.0	0.0	0.0	0.0
	6	0.0	0.0	0.0	0.0	0.0
	10	1.5	0.0	0.0	0.0	1.5
	13	1.5	0.1	0.1	0.0	1.3
	17	1.5	0.5	0.6	0.0	0.4

Culture number.	Days.	Nitrogen—parts per 100,000. Nitrates, total reduction.	Nitrites from nitrates.	Ammonia from nitrates.	Ammonia from peptone.	Undetermined nitrogen from nitrates.
1.	2.	3.	4.	5.	6.	7.
N-38	2	0.0	0.0	0.0	0.1	0.0
	4	0.0	0.0	0.0	0.1	0.0
	6	0.0	0.0	0.0	0.4	0.0
	10	0.0	0.3	0.2	0.4	0.0
	13	0.5	0.7	0.1	0.4	0.0
	17	0.0	0.9	0.7	0.4	0.0
N-40	2	0.0	0.8	0.0	0.6	0.0
	4	2.5	1.6	0.0	1.0	0.9
	6	2.5	2.0	0.1	1.1	0.4
	10	2.5	2.4	0.2	1.3	0.0
	13	2.5	1.8	0.2	1.4	0.5
	17	2.5	2.2	0.4	1.5	0.0
N-41	2	0.5	1.5	0.0	0.5	0.0
	4	2.5	2.4	0.0	0.8	0.1
	6	2.5	3.2	0.0	1.0	0.0
	10	2.5	3.5	0.5	1.0	0.0
	13	2.5	5.5	0.4	1.1	0.0
	17	2.5	4.0	0.3	1.8	0.0

Gain or Loss of Nitrogen.—On all the cultures which were studied, besides the daily tests for nitrates, nitrites and ammonia, tests were made for total organic nitrogen in the solutions by the Kjeldahl method at the beginning and end of the period of incubation. By the use of these Kjeldahl values, together with the values for ammonia, nitrites and nitrates, assuming that none of the nitrogen is present in forms which would not be determined by these methods, we have been able to compute the gain or loss of nitrogen in the solutions, and to compare the reduction of organic nitrogen with the reduction of nitric nitrogen.

Of the results obtained with thirty cultures which are included in Table III, eighteen of the cultures showed a loss of nitrogen in the peptone solution and twelve of the cultures showed a gain. In the solution containing nitrates sixteen of the cultures showed a loss of nitrogen and thirteen showed a gain. Nineteen of the cultures agreed as to gain or loss of nitrogen as determined in the two solutions, one of the cultures showed no change in the nitrate solution, and ten of the cultures showed a gain or loss in the peptone solution when the reverse was true in the nitrate solution. With seven of these ten cultures, the gain or loss of nitrogen in one

solution or the other was less than 0.5 part per 100,000 of nitrogen, this being the limit of error of the Kjeldahl method as determined by duplicate analyses of all the cultures. One culture showed a gain or loss of exactly 0.5 per cent. and this may be included with the other seven, leaving only two cultures in which there was an appreciable discrepancy in the two solutions, these being culture No. 9, which showed a gain of 1.2 parts in the peptone solution and a loss of 1.8 parts in the nitrate solution, and culture No. 34, which showed a loss of 1.7 parts in the peptone solution and a gain of 12.1 parts in the nitrate solution. The maximum gain in nitrogen in the peptone solution was made by culture No. 10, which gained 12 parts nitrogen, and the maximum gain in the nitrate solution was by culture No. 34, which gained 12.1 parts nitrogen. The greatest losses were made by culture No. 16, which lost 5 parts of nitrogen in the peptone solution, and by culture No. 32, which lost 6.6 parts of nitrogen in the nitrate solution. Assuming that the error of the methods is 0.5 part per 100,000, twenty-two of the thirty cultures gained or lost more than this amount in the peptone solution, and twenty-three gained or lost more than this amount in the nitrate solution. Comparing the reduction of organic nitrogen by the same cultures in the two solutions, with twenty cultures, a greater reduction occurred in the nitrate solution than in the peptone solution, either through increased activity of the bacteria in the presence of nitrates, or through secondary reactions between the decomposition products of the nitrates and organic matter. With seven of the cultures the reduction of organic nitrogen in the nitrate solution was less than in the peptone solution, and this may possibly be ascribed to the slight deterrent action of nitrates on certain cultures which has been mentioned before. Of the cultures which gained nitrogen in the nitrate solution nine were able to reduce nitrates and four did not reduce nitrates, while of the cultures which showed a loss of total nitrogen in the nitrate solution eleven reduced nitrates and five did not.

Comparing the reduction of organic nitrogen in the nitrate solution with the reduction of nitric nitrogen we find that of the twenty-four cultures which showed a reduction of organic nitrogen of 0.5 part or more, seven failed to reduce nitrates, while of the six cultures which did not cause any material reduction in the organic nitrogen three were able to produce a decided re-

duction of the nitric nitrogen. The figures showing the reduction of organic nitrogen and the reduction of nitric nitrogen, together with the amount of nitrogen determined by the various methods and the probable gain or loss in the nitrogen content of the solutions, are shown in the following table:

TABLE III.—SHOWING THE GAIN OR LOSS OF NITROGEN AS COMPUTED FROM KJELDAHL VALUES AND DETERMINATIONS OF NITRATES, NITRITES AND AMMONIA.

	Peptone solution.			Nitrate solution.				
Culture.	Reduction of organic nitrogen.	Determined as ammonia.	+ = Gain. − = Loss.	Reduction of organic nitrogen.	Reduction of nitric nitrogen.	Total reduction of *initial* nitrogen.	Determined as nitrites and ammonia.	+ = Gain. − = Loss.
	Parts per 100,000.							
1	1.6	2.1	+0.5	2.2	0.0	2.2	3.9	+1.7
2	2.4	1.1	−1.3	0.0	0.0	0.0	1.0	+1.0
4	0.0	0.6	+0.6	4.5	6.0	10.5	12.5	+2.0
6	6.8	1.8	−5.0	0.0	1.0	1.0	0.9	−0.1
7	4.5	2.2	−2.3	3.2	3.5	6.7	3.4	−3.3
8	0.0	2.3	+2.3	0.0	2.6	2.6	3.1	+0.5
9	1.1	2.3	+1.2	2.8	5.3	8.1	6.8	−1.3
10	6.0	18.0	+12.0	8.3	2.7	11.0	12.0	+1.0
11	0.1	0.5	+0.4	4.5	6.0	10.5	7.8	−2.7
12	2.1	3.8	+1.7	2.3	0.0	2.3	1.9	−0.4
13	3.3	4.2	+0.9	1.4	0.0	1.4	1.4	0.0
16	3.5	7.5	+4.0	0.4	0.0	0.4	1.0	+0.6
17	3.0	0.2	−2.8	3.0	2.3	5.3	5.2	−0.1
18	1.8	2.2	+0.4	2.4	2.7	5.1	4.4	−0.7
22	3.1	3.0	−0.1	2.3	2.1	4.4	4.4	−0.0
23	0.0	0.7	+0.7	0.1	0.0	0.1	0.5	+0.4
24	6.2	4.8	−1.4	8.4	4.5	12.9	10.4	−2.5
25	0.5	0.3	−0.2	4.9	9.0	13.9	17.7	+3.8
26	0.4	0.2	−0.2	1.6	0.0	1.6	0.8	−0.8
27	1.5	0.9	−0.6	2.5	0.0	2.5	0.6	−1.9
29	2.3	2.7	+0.4	3.2	1.5	4.7	6.4	+1.7
30	0.5	0.3	−0.2	0.0	2.5	2.5	4.7	+2.2
31	3.2	2.4	−0.8	5.5	11.0	16.5	18.9	+2.4
32	5.3	4.8	−0.5	9.0	1.5	10.5	3.9	−6.6
33	2.0	0.0	−2.0	2.2	1.5	3.7	1.1	−2.6
34	1.7	0.0	−1.7	4.1	2.5	6.6	18.7	+12.1
36	1.1	0.4	−0.7	1.5	0.0	1.5	0.6	−0.9
38	0.6	0.4	−0.2	2.1	0.0	2.1	2.0	−0.1
40	2.9	1.5	−1.4	2.8	2.5	5.3	4.1	−1.2
41	2.3	1.8	−0.5	3.1	2.5	5.6	6.1	+0.5

The Distribution of Ammonifying and Denitrifying Bacteria in Sewage and Effluents from Sewage Filters, and the Relation of Peptonization to Other Biochemical Functions.—Of the biochemical functions of bacteria concerned in sewage disposal the most important and the ones which can be most accurately determined are the power to liquefy insoluble organic matter, and the power to break down the soluble proteids, peptones, albumoses, etc., into ammonia. These two reactions are the first essentials, and are closely seconded in importance by those reactions by which this ammonia is converted into some stable product such as nitrates, in which form it is at least inoffensive. Denitrification, while in a measure a retrograde process, undoubtedly does occur to a greater or less extent in all biological methods of sewage treatment, and it is an open question whether the secondary reactions between the reduction products of the nitrates and the intermediate products of peptonization are not of more importance and more worthy of careful study and control than has generally been thought to be the case. While these changes may be purely chemical in their nature, or may be due to the action of enzymes, they are brought about directly or indirectly by the action of bacteria, and for this reason some figures as to the relative numbers of bacteria in sewage and in the effluents from some of the various types of sewage filters, may properly be inserted in a paper of this kind.

During a study of the biochemical functions of the bacteria concerned in sewage purification, previously reported, determinations of the ammonifying and denitrifying powers of over 300 cultures of bacteria were made, and in addition, tests were made for the amount of liquefaction which many of these cultures were able to produce in gelatin. The solutions and methods of procedure for the nitrogen tests were the same as previously described in this paper, a standard period of incubation of seven days at 20° C. being employed. The method of making the quantitative gelatin tests was as follows: Test-tubes of a uniform bore of ten millimeters were filled to a depth of 100 millimeters with standard beef peptone gelatin, and the entire surface of the gelatin was inoculated with the culture. Any liquefaction which occurs must proceed straight down from the surface of the gelatin, and the depth of this liquefaction in millimeters, in a given time is a numerical measure of the rate or amount of liquefaction.

The cultures were selected from the colonies on the gelatin plates in such a way as to represent as closely as possible all of the kinds of bacteria in the samples, that is, all of the kinds of bacteria which would be shown by the ordinary plate method. From the data thus accumulated, it has been possible to compute the percentage of the total number of bacteria in the individual samples which possessed these functions, and by averaging the percentages in the various samples to determine approximately the percentage of the total bacteria in samples of different kinds which were able to produce ammonia in peptone solution and to reduce nitrates. From these figures it appears that a considerable majority of the bacteria in sewage and in the effluents and material from sewage filters are possessors of both of these functions. The results are shown in the following table:

TABLE IV.—SHOWING PERCENTAGE OF AMMONIFYING AND DENITRIFYING BACTERIA IN SEWAGE AND THE EFFLUENTS FROM SEWAGE FILTERS.

Source.	Number of samples.	Percentage of total bacteria which ammonify peptone.	Percentage of total bacteria which reduce nitrates.
Sewage	3	90	70
Septic sewage	6	56	59
Effluent contact filters	3	60	61
Effluent trickling filters	4	59	71
Effluent sand filters	15	70	70
Sand from sewage filters	3	90	70

The tests for gelatin liquefaction were made on 157 of the before-mentioned cultures and a comparison of the results of these tests with the various changes in the nitrogen content of the test solutions enables us to trace, in a general way, the relation between the peptonizing power as represented by the amount of liquefaction of gelatin and the denitrifying and ammonifying powers. These results have been grouped in Table V, according to their liquefying power. Comparing the results obtained with cultures which liquefied with those which did not liquefy we find that the liquefiers have an average ammonifying power nearly twice as great as the non-liquefiers and that they have a denitrifying power about three times as great. In each group of cultures, both liquefying and non-liquefying, we find some which cause large changes in the nitrogen content and others which cause no change whatever. A study of the individual analyses reveals, however, that

while 30 per cent. of the non-liquefiers were unable to reduce nitrates, only 8 per cent. of the liquefying cultures failed in that function. Again, 32 per cent. of the non-liquefying cultures did not produce nitrites in the nitrate solution and the same percentage of cultures failed to show any ammonia production in the peptone solution. On the other hand, the number of cultures which failed to produce nitrites was 15 per cent. or less than one-half as many, and the percentage of cultures which failed to break down peptone into ammonia was only 2 per cent., or one-eighth as many as in the case of the non-liquefying cultures. In other words, as shown by the amount of change and by the percentage of cultures reacting, the liquefying cultures are much more active in causing changes in the form of the nitrogenous ingredients of the solution than are the non-liquefying cultures. The figures showing the average amount of nitrogen lost from nitrates which was not determined as nitrites or ammonia, also appear to follow the same rule as above stated, although the results are of doubtful significance for reasons which have been previously discussed. Comparing the relative liquefying power with the other functions, we find that as this power increases the power to reduce nitrates also steadily increases. The relation between the increase in the liquefying power and the nitrite production and the ammonification of peptone, however, is less marked. It is probable however, that with a more extended study covering a larger number of cultures the ratio between these functions would be more uniform.

CONCLUSIONS.

With the advent of the various biological methods of sewage disposal in which the activity of various classes of bacteria have to be carefully controlled in order to accomplish the desired end, a knowledge of the various biochemical reactions taking place and the bacteria which produce these changes becomes necessary. Of the various changes occurring in the sewage during treatment, the main problem is to change the nitrogenous matter from a highly objectionable form in which it is present into a non-putrescible and inoffensive form, since in the process of changing the form of the nitrogen content the carbonaceous matter is also converted into simple elementary compounds. Consequently, the problem narrows down to a study of the bacteria causing changes in the nitrogenous matter, and since the study

TABLE V.—SHOWING RELATION BETWEEN LIQUEFYING POWER AND DENITRIFYING AND AMMONIFYING POWER.

	Cultures which did not liquefy gelatin.	Cultures which liquefy gelatin.	Liquefying power between 1 and 10.	Liquefying power between 11 and 20.	Liquefying power between 21 and 30.	Liquefying power between 31 and 40.	Liquefying power between 41 and 50.
Average liquefaction.	. .	. .	6.	15.	24.	35.	49.
Nitrate reduction, average	1.8	5.3	3.1	5.2	6.2	6.8	6.9
" " maximum	7.0	9.6	8.7	9.3	9.3	..	9.6
" " minimum	0.0	0.0	0.0	0.0	0.0	..	2.0
Per cent. of cultures did not reduce nitrates	30.0	8.0	29.0	3.0	20.0	..	0.0
Nitrite production, average	1.6	6.1	3.5	6.5	7.7	0.0	6.5
" " maximum	11.0	12.0	11.0	12.0	11.0	..	11.0
" " minimum	0.0	0.0	0.0	0.0	0.0	..	0.0
Per cent. of cultures did not produce nitrites	32.0	15.0	35.0	8.0	20.0	..	14.0
Ammonification of peptone, average	0.4	0.7	1.1	1.8	1.4	0.6	1.4
" " " maximum	1.9	5.2	3.2	5.2	2.1	..	4.5
" " " minimum	0.0	0.0	0.0	0.0	0.0	..	0.1
Per cent. of cultures did not produce ammonia from peptone	32.0	2.0	6.0	0.0	10.0	..	0.0
Reduced nitric nitrogen not determined as nitrites and ammonia, average	0.7	1.2	0.3	0.2	0.1	5.3	1.1
maximum	4.6	5.6	2.4	1.8	0.8	..	5.6
minimum	0.0	0.0	0.0	0.0	0.0	..	0.0
Per cent. of cultures which did not show undeterminable nitrogen	59.0	45.0	76.0	27.0	80.0	..	62.0
Number of cultures	44.	113.	17.	64.	10.	1.	21.

of these bacteria under natural conditions, *i. e.*, in the sewage and in the filters, surrounded, as they are, by other types of bacteria and under conditions which are constantly changing, is beset with difficulties, it becomes necessary to study these bacteria in pure culture and under constant conditions, in order to acquire data sufficiently exact to be used in an analysis of the more complex problems which occur in actual practice. The present paper is a portion of a more extended investigation into the types of bacteria normally present in sewage and the effluents of sewage disposal systems, and their rôle in the causation of the biochemical reactions occurring during the processes of treatment, and has to deal with the specific changes which representative types of bacteria in pure culture may produce in solutions containing nitrogen in forms more or less common in sewage during its purification. Thirty cultures of bacteria, common in sewage and the effluents from sewage filters, have been studied carefully to determine the character and amount of change brought about by their action on peptone and nitrates.

From the results obtained in this study it becomes possible to state definitely that bacteria, common in sewage purification, are able to produce ammonia from organic matter, to reduce nitrates to nitrites, to ammonia and probably to elementary nitrogen, to liberate nitrogen from solutions of organic matter either with or without the presence of nitrates or its reduction products, and also to fix atmospheric nitrogen under the same conditions, all of which reactions have been noted by other observers, but about which there has been more or less controversy. Many sewage bacteria probably also produce the lower oxides of nitrogen as reduction products of nitrates, which oxides may play an important part in the further decomposition of the organic matter in solution, either through catalytic action or by direct chemical reaction. Furthermore, certain of these bacteria may produce an oxide or at least a compound of nitrogen intermediate between nitrates and nitrites, which has apparently not been noted hitherto. The amount of ammonia produced by the different cultures has ranged from 0 to 18 parts per 100,000, and the rate of ammonification has varied considerably, some of the cultures reacting as early as the fourth day, while other cultures, which eventually reacted strongly, did not begin to ammonify until after periods of ten to fourteen days. Similar phenomena have been

noted with regard to the reaction of the cultures on nitrates. Some have reduced the nitrates rapidly and completely, some have denitrified slowly, some have reacted only after a considerable time of incubation, and others have caused no change. The character of the reduction products, both as regards amount and rate of formation has also been interesting. Some cultures have been able to reduce the nitrates to nitrites, ammonia and elementary nitrogen continuously from the start, while with other cultures the reduction to these various bodies has occurred consecutively, and with still others one or another of the reduction products has not occurred during the period over which the examination of the cultures extended. In a few instances nitrites have been produced in quantities far greater than the amount of nitrates, and if the results are not in error, this can be explained only on the supposition that a direct oxidation or nitrification has occurred, caused either by direct bacterial action or by some undetermined secondary reaction.

Regarding the distribution of ammonifying and denitrifying bacteria in sewage and the effluents from sewage filters, based on a study of the biochemical functions of over 300 representative cultures from a variety of samples, it may be said that a majority of the bacteria from these sources, which are determinable by the ordinary plate methods, using gelatin as a culture medium, reduce nitrates and decompose peptone into ammonia, although these two functions are not always correlated with the same species. By the use of quantitative tests for the amount of liquefaction produced in gelatin by over 150 of these cultures, we have been able to determine approximately the relation between the peptonizing power or power to liquefy insoluble organic matter, and the ammonifying and denitrifying powers of sewage bacteria. We find that while many non-liquefying bacteria are able to reduce nitrates, or to ammonify peptone or to do both, and many liquefying bacteria do not possess these functions, nevertheless, in a large majority of cases the possession of the first function means possession of one, and usually both of of the last two functions, and the average amount of change produced in the nitrogenous matter in solution is much greater with peptonizing bacteria than with those which are unable to liquefy gelatin. Furthermore, an increase in the liquefying power ap-

pears as a rule to be synonymous with increased ability to reduce nitrates and to ammonify peptone.

In the present paper we have dealt with the reactions produced by pure cultures of bacteria, it being necessary that these elementary reactions should be thoroughly understood before we attempt the study of mixed cultures, with the complicated series of secondary reactions which may occur between the reduction products of the different species. A practical application of the quantitative tests for the biochemical functions of various species combined with the numbers of bacteria of those species present in water and sewage has already been made, and the field is one which promises to bear fruit in the near future in a better understanding of the biological processes occurring in sewage treatment and in the more scientific control of the conditions which govern those processes.

LAWRENCE, MASS.

ON THE SPECIFIC ROTATION OF SALTS OF CASEIN.

BY J. H. LONG.

Received March 7, 1905.

IN THE literature there are a few references to the optical rotation of "casein" in solutions of weak alkalies, acids or neutral salts, but most of them refer to the old work of Hoppe-Seyler on products of doubtful purity,[1] or to later work by Béchamp with mixtures of somewhat uncertain composition.

Casein, as the term is now understood, is a nucleoalbumin of marked acid character, which exists in milk in the form of a calcium salt mixed or combined with phosphate. This combination may be easily broken up by addition of weak acids, especially acetic, yielding the pure substance, which seems to be quite insoluble in water, but readily soluble in alkali solutions with formation of acid or neutral salts. Like many of the proteins, this casein holds certain groups which impart to it a basic character by reason of which it combines, to a certain extent, with weak acids, forming another class of salts, but in this short paper the alkali combinations only are considered. Some of these alkali combinations have some commercial importance; nutrose and plasmon are essentially impure sodium salts.

[1] For literature see "Optical Rotation of Organic Substances," Landolt, Long's translation, p. 728, and Hoppe-Seyler's Handbuch der Phys. und Path. Chem. Anal., 6th ed., p. 259.