When the proteins named are treated with the halogen acids of 0.2 N concentration, in excess, and the mixtures evaporated at a low temperature by standing over sulfuric acid some weeks, followed by similar treatment over solid alkali, and final drying to constant weight at 75°, very constant weights of acid are taken up and held by the protein. These weights of acid are not increased by the excess added, which points to the definite character of the reaction. The amounts are not proportional to the molecular weights of the acids, the combining proportion being relatively greater for HI than for HBr and greater for the latter than for HCl. But the compounds all appear to be salts of the protein molecule and contain many times as much acid as is suggested by the titration combinations. These dry salts undergo dissociation readily when mixed with water.

If the acid-protein mixtures are evaporated on the water bath, in place of being dried at a low temperature, the behavior of HCl and HBr remains essentially the same. No greater amounts of the acid are taken up by a gram of protein and we doubtless reach here a maximum in the combining power of the acid and protein. A salt of a type different from that formed in solution at a low temperature is secured. In the case of the HI, however, there is no such limit to the iodine held, and it is probable that we have here a substitution of the element in the nucleus of the protein molecule, as well as an addition of the acid. As much as 75% of the weight of the original protein may be so held and the combination has a brownish ocher color, with loss of protein reactions.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, CORNELL UNIVERSITY.]

STUDIES ON THE CULTURE MEDIA EMPLOYED FOR THE BACTERIOLOGICAL EXAMINATION OF WATER.

I. THE SCHARDINGER-DUNHAM MEDIUM FOR TESTING FOR THE PRESENCE OF HYDROGEN SULFIDE FORMING BACTERIA.[1]

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Introduction.

Although an enormous amount of time and labor has been spent upon investigations of bacterial culture media with a view of shortening the time required for diagnostic results and producing media yielding more uniform and more constant results, a critical review of the literature fails to show, save in a few instances, that the media which have eventually been proposed have received truly systematic study and that the concentrations suggested are necessarily those which are best fitted for the purposes for which the media have been made.

[1] Papers read at the Rochester Meeting, American Chemical Society, September, 1913.

That slight variations in concentration in most of the media employed in the bacteriological examination of water may lead to relatively great differences in both quantitative and qualitative results has long been recognized and has been the basis of suggested so-called standard media, but the fact has apparently been ignored that in the selection of concentrations, those should be chosen, a slight variation from which will lead to the least possible variation in the diagnostic characters sought; will furnish dependable results in the shortest possible time; and yet will give practically identical results in the hands of different analysts and investigators.

Comparison of the data obtained by different water analysts shows such variations between wide limits, even making due allowances for the personal equation, that it is evident that our standard media need more study and possibly revision.

In the laboratories of the senior author considerable routine work is carried on in the periodic examination of waters and the control of purification plants. In this work he found that the media prepared at different times by himself and coworkers frequently failed to yield identical results.

Although it seemed probable that the only satisfactory solution of these problems lay in abandoning such variable materials as meat broth and peptone and adopting nutritive substances of definite composition—in other words, so-called "synthetic media"—an attempt was made to study the causes of the variation in results obtained in our work and thus to eventually find, if possible, a really satisfactory "synthetic" medium. With these ends in view, investigations of all the different culture media employed in water examinations were begun several years ago and will be continued until all the media have been studied.

The results obtained proved to be so interesting, curious, and at variance with what we expected that it seems worth while to present the facts in a series of articles, of which this is the first.

Part I. Historical.

In 1877 Gayon[1] published what appears to be the first recorded proof of the formation of hydrogen sulfide by bacteria acting upon albuminous material. Two years later, 1879, Miquel[2] succeeded in isolating from sewage, from contaminated waters and also from waters believed to be potable, an organism having the power of changing free and combined sulfur to hydrogen sulfide. Three years later Boehm[3] obtained similar results with an organism isolated from well water. These results paved the way for a large amount of work upon the formation of hydrogen

[1] Gayon, *Compt. rend.*, **85**, 1074 (1877).

[2] Miquel, *Bull. soc. chim.*, [2] **32**, 127 (1879); *Ann. de l'Obser. Mont-Souri*, 1880.

[3] Boehm, *Monats.*, **3**, 224 (1882).

sulfide from organic and inorganic sulfur compounds by the action of bacteria, but no investigator appears to have seriously considered the possibility of making practical use of this property of certain bacterial species in the examination of water until in 1894, Schardinger[1] pointed out the advantages in sanitary water examinations of applying tests which would reveal the presence of hydrogen sulfide producing bacteria. Up to this time even search for the presence of members of the colon group of organisms was but seldom practiced in routine work, the procedure usually followed, namely, that of ascertaining merely the number of micro-organisms in a certain definite volume of the sample, Schardinger did not consider to be a reliable criterion of the quality of the water. In searching for methods which would yield more definite information as to the nature and probable source of the bacteria present in the water, he states that he was guided by the investigations of Nencki, in which it was shown that in the small intestine of healthy men, the bacterial processes are chiefly fermentative, but that in the large intestine they are chiefly putrefactive. Since in the great majority of polluted waters we have to deal with fecal contamination there are two groups of bacteria, those causing fermentations and those causing putrefaction, "concerning which one must inform himself in a suspected water."

The method sugested for testing for the presence of putrefactive bacteria consisted in adding 100 cc. of the sample of water to 10 cc. of a sterile solution containing 1 g. Witte peptone and 1 g. sodium chloride. After incubation for twenty-four hours at 37°, a strip of filter paper impregnated with lead carbonate was hung in the neck of the flask.

"If the investigated water was impure, there appeared in a marked degree (*a*) a pronounced fecal-like odor; (*b*) the turning of the lead carbonate paper yellow-brown to black, immediately or within a very short time, due to the formation of lead sulfide."

Much work upon "putrefactive" organisms followed, but Schardinger's work appears to have escaped the attention of water analysts until 1897, when Dunham[2] investigated the Schardinger method and like his predecessors he appears to have been guided by Nencki's work, for he states that

"All sewage which receives human feces contains the bacillus coli communis, or if it does not, has been subjected to germicidal agencies that would also kill pathogenic bacteria, derived from the cases of disease. It is fair to assume that ordinary sewage would also contain the common bacteria of putrefaction. We must therefore direct our attention to the means of demonstrating the presence or absence of those species in the water under examination."

For the detection of "putrefactive" bacteria Dunham made the following slight modification in the Schardinger medium: To about 90 cc. of water, 10 cc. of a 10% peptone, 5% salt solution, previously sterilized,

[1] Schardinger, *Cent. Bakt.*, 16, 853 (1894).

[2] Dunham, THIS JOURNAL, 19, 591 (1897).

were added. This gave a resulting solution containing 1% of peptone and 0.5% of sodium chloride. The mixture was made in a sterile Erlenmeyer flask, provided with a cotton plug. A strip of paper, impregnated with lead carbonate, was suspended over the mixture and the flask was then placed in the incubator at 37° for twenty-four hours. Under these conditions of temperature and nutrition, Dunham claimed that

"The colon bacillus and the bacteria of putrefaction readily multiply and the latter cause the production of hydrogen sulfide which discolors the lead paper."

Although a few water analysts have made use of the Schardinger-Dunham method in the examination of suspected waters, so far as the authors have been able to ascertain no further work has been published upon the subject of the significance of the presence of hydrogen sulfide producing bacteria in drinking waters nor of methods for their detection.

In the course of some ten or more years of experience with this modified method in the hands of the senior author upon many hundred waters, it was found to be of exceeding great value in forming a definite opinion, especially in waters of the class which would have been graded as "suspicious" were only the usual methods followed. The chief drawback was that the indications were not obtained in a short enough time. With badly contaminated waters strong tests for hydrogen sulfide could be obtained in from twenty-four to forty-eight hours, but with other waters from three to four days of incubation were necessary. Moreover, it appeared probable that the hydrogen sulfide rapidly formed was not due to the colon group but to some other class of bacteria, while the slowly evolved hydrogen sulfide might possibly be ascribed to colon group organisms rather than those classed by Dunham as putrefactive.

Part II. Experimental.

Upon studying the data collected, some abnormally short incubation periods were noted and some preliminary work showed that the medium could be sensitized in various ways, and, as the speed with which results can be obtained is a very vital point in the bacteriological examination of water, it was thought that the medium was worthy of a systematic study with a view of obtaining one which would yield positive results in the shortest time and with the greatest possible uniformity.

In the Schardinger medium we have three things which will influence the growth of the bacteria: (1) The concentration of the peptone; (2) that of the inorganic salt present (in this case sodium chloride), and (3) the nature of the reaction of the medium. We may therefore consider that we are dealing with a three-component system and in order to facilitate the investigation the triangular diagram may be resorted to.

The diagram was used in the manner originally proposed by Gibbs and not in the manner later proposed by Roozeboom, which latter is the way in which it is now used by most physical chemists.

By using the diagram according to the Gibbs method, which it will be recalled, gave very excellent results in the hands of Schreiner,[1] in the study of plant nutrients, the concentrations of the three factors, the peptone, inorganic salts, and reaction adjustor could be varied in a systematic manner between wide limits, so as to find the maximum and minimum limits beyond which it would be useless to go in subsequent work, where only one component would be varied at a time. In other words, fields could be found within which the production of hydrogen sulfide would be most rapid and most energetic, and further work confined to those fields.

The rulings of the triangular diagram used gave sixty-six points of intersection and hence in each case sixty-six different combinations of the three components; but in order to obtain combinations in which the salts were high when peptone was low and *vice versa*, and a similar relation of concentrations with respect to acidity recourse must be had to a series of diagrams in which first the order of concentration of one component was reversed, and then another. A series of eight diagrams covers all possible combinations.

It is of course obvious that in each one of the eight diagrams there are a number of points representing possible media which must necessarily be omitted in any given run, as, for example, all those containing no peptone. The actual number of culture media made up in each diagram will therefore never be sixty-six, but will be considerably less than this.

Peptone Used.—The peptone employed was that known as "Witte Peptone" obtained from Kahlbaum. The investigation having extended over a period of three collegiate years, three different lots of peptone were employed. In the case of each shipment, a 10 kilo package was carefully sampled and subjected to chemical and physical tests. The three lots were found to be remarkably uniform and substantially identical in character. But in order to eliminate any possible doubt, simultaneous runs were made on two samples at a time. Identical results were obtained with all three samples in every instance. In addition, it was found desirable to check the compositions by determinations of the acidity, the specific gravity, optical rotation and the coefficient of viscosity of different concentrations of the different samples of peptone at different temperatures. Substantially identical results were obtained.

The chemical analyses of the different samples included moisture, ash, nitrogen, sulfur and phosphates in the ash. The variations in the different samples were no greater than the differences in percentage found in different portions of the same sample.

As is well known, what we ordinarily call a peptone solution is not a solution at all, but is a colloidal suspension, and when peptone is boiled with water there is always formed a heavy sediment which may properly

[1] Schreiner, Bur. Soils, U. S. Dept. Agr., *Bull.* 70.

be considered to be that part of the peptone which is not fine enough to stay in suspension.

In order to ascertain whether this assumption is true and also in order to find out whether any effect would be produced upon hydrogen sulfide production if this sediment were filtered from the medium, the following tests were made:

1. Quantitative determinations were made of the amounts of sulfur, nitrogen, ash, and phosphates in the ash of the sediment and of the peptone. These various constituents were found to be identical within the limits of error of the methods.

2. In each of fifteen of the special culture flasks described below, was placed 10 cc. of unfiltered 30% peptone solution and 0.5 g. of sodium chloride. Another fifteen flasks were similarly prepared, except that filtered peptone solution was used in place of the unfiltered. All of the flasks were plugged and sterilized, and then each was inoculated with 90 cc. of artificial sewage and incubated under similar conditions.

The amounts of hydrogen sulfide produced in the filtered and the unfiltered media were practically identical. It therefore appeared that the precipitate was peptone and that it made no difference whether unfiltered or filtered media were employed. Nevertheless, since it is decidedly advantageous to start with a clear medium so as to detect the growth of bacteria by means of the turbidity and sediment produced in the medium, all peptone solutions were filtered before they were used.

The Witte peptone employed was found to contain 1.006% total sulfur by the Liebig-Koch method,[1] while the peptone in the culture media after filtration was found to contain 0.911% total sulfur, showing that in filtration there is substantially no loss of sulfur.

Koch and Carr[2] have shown that lipoid sulfur compounds are soluble in alcohol, whereas protein sulfur compounds are not. Applying this extraction method to the peptone, two portions were obtained, one soluble in alcohol and one insoluble in this solvent. It was found that the soluble portion contained 0.80% of total sulfur, while the insoluble portion contained 1.08% total sulfur.[3]

Culture media made from alcohol-soluble peptone and from alcohol-insoluble peptone gave in neither case results as good as those made from the original peptone.

Aqueous solutions of peptone are distinctly acid to phenolphthalein, the amount or degree of acidity rapidly rises with the temperature; hence in adjusting media care must be observed that the media shall always be adjusted at the same temperature. Nor must it be forgotten that

[1] Redfield and Huckle, THIS JOURNAL, **38**, 607 (1915).

[2] Koch and Carr, *Ibid.*, **31**, 1341 (1909).

[3] Redfield and Huckle, *Ibid.*, **38**, 612 (1915).

when placed in the incubator the medium becomes more acid than it was at room temperature. After considerable experimentation it was found most convenient to add the reaction adjustor after titrations made at 20°, this being so nearly room temperature as to be readily and quickly obtained. In all the work hereinafter described it will be understood that the stated reactions of the media refers to that at 20°. The acidity of a simple peptone solution or peptone solutions containing simple neutral salts is directly proportional to the concentration.

In the course of some preliminary investigations undertaken some years ago, it was found that the culture flasks employed for testing for the presence of hydrogen sulfide should have wide necks and thus expose a moderate surface of liquid to the air. A 100 cc. modified Pasteur flask was therefore made, having a neck 2 cm. in diameter and of such a size as to bring 100 cc. and 110 cc. levels in the neck of the flask, marks being made for these heights. The small tube of the ground cap was made 4 cm. long and 4 mm. in diameter. This tube serves to hold a cotton plug at its lower end and above the plug a strip of lead acetate paper.

These flasks having answered admirably in routine work were used throughout the present investigation.

After inoculation with a sample, a tin foil cap was placed over the upper end of the tube carrying the lead paper.

As the investigation progressed, evidence was obtained that a better type of flask could probably be devised and further work along this line is being conducted.

The Effect of Peptone Concentration upon Hydrogen Sulfide Formation.—Flasks of media were prepared with the compositions required by the triangular diagram reading clock-wise with concentrations varying between the following limits: 0 to 5% peptone; 0 to 5% acidity in terms of normal hydrochloric acid and 0 to 5% sodium chloride. These runs were made upon reversed diagrams as already described. These flasks were inoculated with equal amounts of sewage[1] and incubated at 38°, and observed at frequent intervals for the first appearance of the blackening of the strips of paper impregnated with lead acetate which had been placed in the tubular top of the caps of the flasks.

It was found in these four runs that the respective fields in which hydrogen sulfide was most quickly developed were as follows:

3.0 to 4.5% peptone;	0.0 to 1.5% NaCl;	0.0 to 2.0% *N* acid.
2.5 to 5.0	1.0 to 3.0	1.0 to 2.5.
4.0 to 5.0	0.0 to 2.5	0.0 to 2.5.
4.5 to 5.0	2.0 to 2.5	2.5 to 3.0.

[1] In most of the runs an artificial sewage was prepared by adding one loopful (0.02 g.) of fresh human feces to one liter of water, shaking thoroughly and pouring the turbid liquid through a sterile cloth. This water was found to contain 2000 to 5000 organisms growing on gelatin at 20°, 100 to 200 colonies on agar at 38°, of which from 20 to 40 were of the *B. coli* group.

From a careful study of all of the data obtained in the four runs, it was found that the very best conditions had been obtained within the following limits:

2.5 to 5.0% peptone; 0.5 to 1.5% NaCl; 0.5 to 1.5% *N* acid.

Consequently a run was now made, in which all of the possible combinations of 2.0%, 2.5%, 3.0%, 3.5% and 4.0% of peptone; 0.5%, 1.0% and 1.5% of NaCl; and 0.5%, 1.0% and 1.5% of normal acid were used, plotted on two component diagrams.

Four checks with 2.0%, 2.5%, 3.0% and 4.0% of peptone, with no added salt and with a reaction of 1.0% normal acid were also prepared.

It was found in the uninoculated media, that of the combinations prepared, the only ones which were clear, with very little sediment, were those containing 3.0% of peptone, 1.0% and 1.5% of NaCl, and with an acidity to phenolphthalein of 1.5% normal acid. It was, moreover, a general rule that the quantity of peptone and of sodium chloride did not seem to have much influence on the amount of turbidity in the solution, but that the reaction did have such an influence; the less the acidity, the greater the turbidity; but there were many exceptions.

Each flask was inoculated with 90 cc. of artificial sewage.

As regards the turbidity of the inoculated media before incubation, none were perfectly clear, with no sediment, but the best combinations were those in which the reaction was equivalent to 0.5% normal acid, and where NaOH had been used to adjust the reaction.

The inoculated flasks were incubated at 38° and frequently observed for evidences of hydrogen sulfide production.

As regards the rapidity of hydrogen sulfide production, it was found to be most rapid in those combinations containing 3.5 and 4.0% of peptone, the concentration of the sodium chloride and the reaction of the media apparently not making a great deal of difference, in the narrow limits used.

The largest amounts of hydrogen sulfide were produced in general in the flasks having from 3.0% to 4.0% of peptone; from 0.5% to 1.5% of NaCl; and with a reaction equivalent to from 1.0% to 1.5% normal acid. These were also the combinations in which the media became the most turbid after incubation and in which the largest amounts of grayish green sediment were produced. It was also noted that in most of these flasks, there were particles of dark green material floating on the surface which sank on tapping the flasks.

In order to obtain more reliable information as to which combinations were really best and produced hydrogen sulfide most rapidly, it was deemed advisable to make up in triplicate those combinations which had given the best results. While none of the combinations used were perfectly clear and free from sediment either before or after inoculation, they were

near enough so for all practical purposes, so far as judging how the growth and development of the bacteria had proceeded on incubation.

The results of all the combinations tried showed that when sodium chloride was the inorganic salt present, there was not much choice between 3.0%, 3.5% and 4.0% of peptone, all of which were much superior to 2.0% and 2.5% of peptone.

In order to throw light upon the question of the effect of the inorganic salt upon the peptone concentration, runs were made, similar in all respects to those recorded above but with the difference that sulfate, nitrate and phosphate of sodium were substituted for sodium chloride; and also in which the chloride, sulfate, nitrate and phosphate of potassium, of ammonium, of calcium and of magnesium were, respectively, substituted for sodium chloride and in which the reaction was adjusted in a variety of ways.

Without giving the details of the results of each run, suffice it to say that in all cases from 3.0% to 4.0% of peptone furnished the best concentrations for most rapid and most energetic production of hydrogen sulfide by the bacterial floras employed. The value of the inorganic salt in the medium will be discussed below.

In most standard culture media it is customary to add an extract of fresh lean beef as one of the components. It was therefore thought essential that the value of such an addition to the Schardinger medium be investigated. To this end fifty flasks were prepared in which varying volumes of meat broth were added. The meat broth used was prepared by digesting 2100 g. of finely chopped beef with 4200 cc. of water. The extract was filtered through cloth and the meat pressed in an iron press until substantially dry. The extract was then boiled, made neutral to phenolphthalein with sodium hydroxide and filtered through paper. The clear meat extract thus obtained contained 2.22% of total solids of which 0.69% was ash. The fifty flasks employed were divided in lots of ten of similar concentration, with respect to meat extract and peptone. Each set of ten was again subdivided, half receiving 0.5% of potassium chloride, the other half no potassium salt. The series after inoculation contained from 0 to 3% peptone and from 0 to 50 cc. meat extract. The 3% peptone contained no meat extract and the 50 cc. meat extract flasks no added peptone, between these two limits the media contained variable quantities of each nitrogenous component. In every case the volume of the media was 65 cc. and to this was added 35 cc. of sewage. At the end of eighteen hours all of the flasks containing meat broth had begun to show hydrogen sulfide, while those containing peptone, but no meat, had not yet developed a test. At the end of twenty-four hours the simple peptone media had begun to show hydrogen sulfide, but the media containing meat broth still showed more. The most powerful evolution

occurred in media containing 2.25 g. of peptone and 50 cc. of meat extract. In all the media in this run the presence of the potassium chloride led to more rapid production of hydrogen sulfide and in larger amounts.

Were conclusions to be based merely upon the time of the appearance of hydrogen sulfide as revealed by the lead acetate paper, it would appear that the medium was greatly improved by the addition of the meat extract. Subsequent investigations and a careful study of data showed that, just as with peptone alone, concentration played an important rôle. Extracts made with meat purchased at different times were found to be variable in composition and therefore could not be relied upon as yielding media of reliable uniformity and concentration with respect to the sulfur containing body or bodies. The media in all of the flasks containing beef extract remained yellow in color even after much hydrogen sulfide had been produced, and what little sediment was present was light yellow in color, while the 3.0% peptone, 0.5% potassium chloride media all turned turbid and green with dark green sediment and dark green floating cakes. This color reaction, joined to an intense fecal odor in the peptone media, constitutes a most valuable indication that the water being tested is seriously contaminated—indications which are usually lost when meat extract is present. It therefore appeared probable that the slight shortening of the period required for the first appearance of hydrogen sulfide was more than outweighed by the greater labor involved and by the loss of valuable diagnostic features.

Further study of the addition of meat broth was abandoned and a study of other possible sensitizing agencies was attempted.

The Influence of the Addition of Inorganic Salts.—Both Schardinger and Dunham recommended the addition of sodium chloride to their culture media. In fact, the addition of this salt to culture media has been quite uniformly practiced by bacteriologists. A careful search of the literature failed to disclose any data upon which a reliable opinion could be based as to the actual influence of this compound, nor evidence that the quantities employed were the best concentrations for the ends in view.

That some inorganic salts in the media were desirable, there was no question, for it had long before been found by one of us that media made up with water from the University water supply gave better results than when made from distilled waters so far as their applicability to the examination of at least the natural waters of the East Central United States. "Tap" water was therefore invariably employed in making up all media.

A study of the data yielded by the runs on the triangular diagrams already tried showed that the presence of sodium chloride was highly desirable.

The problem we next set ourselves was that of ascertaining whether sodium chloride was the best salt to use and the influence of varying con-

centrations of the different salts tried. A series of runs was made in which sodium sulfate, sodium nitrate, potassium chloride, magnesium chloride calcium chloride and ammonium chloride were, respectively, substituted for the sodium chloride.

Advantage was taken of the experience which had been gained in making the runs with sodium chloride, so that with a simple change in the arrangement of the diagram, it was possible to gain as much information from the use of one triangular diagram for each of these salts as had been obtained from running four separate diagrams with sodium chloride. The modified diagram read in a clock-wise direction in the following manner: 1.0 to 6.0% peptone; 5.0 to 0.0% *N* acid; 5.0 to 0.0% salt.

Starting with 1.0% peptone instead of 0.0% had the following advantages: It eliminated the 0.0% and 0.5% peptone solutions which were obviously worthless and it placed the 2.5%, 3.0% and 0.5% peptone solutions in that part of the diagram where the concentrations of salt and acidity had been found to be the best suited to good growth and hydrogen sulfide production.

In the previous runs the peptone had been weighed out for each concentration desired, but from this time on proportional amounts of 30% peptone solutions were used. This had the advantage of using the same identical solution of peptone for all of the inoculations and also of making the uninoculated media of larger volume, thereby better keeping all of the components in solution.

In all of these runs, the same check solutions were used in order to make them as nearly comparable as is possible whenever a fresh lot of sewage must be prepared for each run. These check solutions had the following compositions:

3.0% peptone	0.5% NaCl	1.5% *N* acid
3.5%	1.0%	1.5%
4.0%	1.5%	1.5%
3.0%	0.5%	1.02% (unadjusted)

After inoculation the results obtained with these checks were always similar in character.

The best results obtained with the other media containing the different inorganic salts were as follows:

Sodium chloride from 0.5 to 1.5%, more than 1.5% inhibiting; sodium sulfate from 0.5 to 1.5%, more than 1.5% inhibiting; potassium chloride from 0.5 to 3.0%, required more than 3% to inhibit; magnesium chloride from 0.5 to 1.0%, more than 2% inhibiting; calcium chloride from 0.5 to 2.0%, more than 2% inhibiting; ammonium chloride from 0.0 to 1.5%, more than 2% inhibiting.

The results of these runs seemed to indicate that potassium chloride was the best of the salts tried and that its addition to the media was decidedly advantageous. Nevertheless it was felt that more evidence was

essential before a reliable opinion could be formed and especially did it seem necessary to try as many different combinations as possible of basic and acid ions.

As the net results of some eight hundred runs already made the optimum conditions for the formation of hydrogen sulfide appeared to be in media having a peptone content of 3% and an acidity equivalent to from 1.0 to 1.5% normal acid. It was further found that, when this acidity was obtained from the unneutralized natural acidity of the peptone itself, the results appeared to be a little better than when obtained through the addition of an acid after neutralization with an alkali; in all such cases due allowance being of course made for the increased amount of inorganic salt thus introduced.

Although, as stated above, after a large amount of data had been collected, it was found that just as good and in most cases, better results were obtained when using an unadjusted 3% peptone medium, of which the reaction was always about 1.02% of normal acid; still in the early part of the work many experiments were tried to find the best reaction, and such bases as sodium hydroxide, potassium hydroxide, ammonium hydroxide, calcium oxide and magnesium oxide, and such acids as hydrochloric, sulfuric and nitric were used in adjusting the reaction to neutrality and also to various degrees of alkalinity and of acidity; in combination with various salts and also with no added inorganic salts.

As a result of the data collected in this connection in about five hundred inoculations, sodium hydroxide and potassium hydroxide proved to be the best alkalies to use, with little choice between them, due presumably to the very small amounts of basic elements which were introduced in adjusting the reactions. Hydrochloric acid proved to be the best of the acids. Nitric acid, as would naturally be expected, proved a positive detriment.

When peptone, potassium chloride, hydrochloric acid and potassium hydroxide were used in preparing the media, good results were obtained in some two hundred inoculations over a much wider field than with any other combination, as has already been noted; very good and quick results having been obtained with concentrations of acidity from 0.5% to 3.0%, inclusive, but the very best results were obtained when the acidity was from 1.0% to 1.5% with very little choice between these concentrations, the preference, if any, being for 1.5%. This preference, however, was so slight that it is outweighed by the greater simplicity of leaving the reaction unadjusted at about 1.02%, when 10 cc. of 30.0% peptone is used in 100 cc. of inoculated media.

The limits for the best concentrations of initial acidity for salts other than potassium chloride were found to be as follows, the titrations being made at 20°:

For $NaCl$, from 0.0% to 2.0% *N* acid, best at 1.0%.
For Na_2SO_4, from 0.5% to 1.0% *N* acid, best at 0.5%.
For $MgCl_2$, from 0.5% to 1.5% *N* acid, best at 1.0%.
For $CaCl_2$, from 1.0% to 2.0% *N* acid, best at 1.5%.
For NH_4Cl, from 1.0% to 1.5% *N* acid, best at 1.0%.
For K_2SO_4, from 0.5% to 2.0% *N* acid, best at 1.0%.

Having thus established the limits for acidity, runs were made in which the variable component was the inorganic salt. Concentrations of 0.5, 1.0 and 1.5% of the following salts were tried: $NaCl$, KCl, NH_4Cl, $CaCl_2$, $MgCl_2$, Na_2SO_4, K_2SO_4, $(NH_4)_2SO_4$, $CaSO_4$, $MgSO_4$, $NaNO_3$, KNO_3, NH_4NO_3, $Ca(NO_3)_2$, $Mg(NO_3)_2$. Two check flasks were also prepared containing no added salts.

The quickest and most abundant evolution of hydrogen sulfide was obtained in the media containing 0.5 and 1.0% KCl; 0.5 and 1.0% $NaCl$; 0.5% K_2SO_4; 0.5% $CaSO_4$; 1.0 and 1.5% $MgSO_4$. Again considerably the best results were obtained with KCl. The nitrates all showed a marked inhibiting action.

The five salts which were found to yield the best results were again tried, but in narrow limits of concentration, 0.25, 0.50, 0.75 and 1.0%. Again the best results were obtained with KCl in concentrations of 0.50 and 0.75%, with $NaCl$ next in order. The areas upon the triangular diagram enclosing the best concentrations for these five salts are shown in Fig. 1.

Phosphates were not included in the tables on account of the fact that preliminary trials had already indicated that they were of little, if any, value and probably objectionable. It seemed nevertheless wise to study their effect separately. The following salts were tried, with the results indicated: H_2KPO_4 in concentrations in the finally inoculated media of from 0.0 to 1.5%. Peptone, 3%; acidity, 1.2%. All the solutions precipitated badly. When sterilized, those containing the highest portions were practically turbid jellies before inoculation. None of the series developed hydrogen sulfide at the end of twenty-four hours and only a few at the end of seventy-two hours. Of the checks with KCl and $NaCl$ the former gave the most rapid and abundant evolution of the gas.

The marked inhibiting effect of this salt is probably not necessarily due to the PO_4 ion, since the acidity from a 0.5% solution was found to be as high as 5.0% in terms of normal HCl. It is manifestly impossible to reduce this acidity to an equivalent of between 1 and 1.5% (the optimum acidity previously found), since the addition of an alkali would necessarily change the nature of the phosphate added. HNa_2PO_4, HK_2PO_4, $HMgPO_4$, $HCaPO_4$, $H(NH_4)_2PO_4$, $HKNH_4PO_4$, $HNaNH_4PO_4$, these salts were introduced into peptone solutions, unadjusted in amounts such that the inoculated media would contain 0.05, 0.15 and 0.25% of the basic element present in the salts. The added concentrations equivalent to the best

concentrations obtained with NaCl and KCl. These salts were thus calculated because it had been previously found that it was the potassium or sodium ions which appeared to be beneficial and that the acid ion, so far as Cl and SO_4 are concerned, had little effect. As checks, media containing KCl, K_2SO_4 and NaCl, respectively, were prepared with the K and Na ions present in the proportion 0.05, 0.15 and 0.25%. Other checks contained no added inorganic salt.

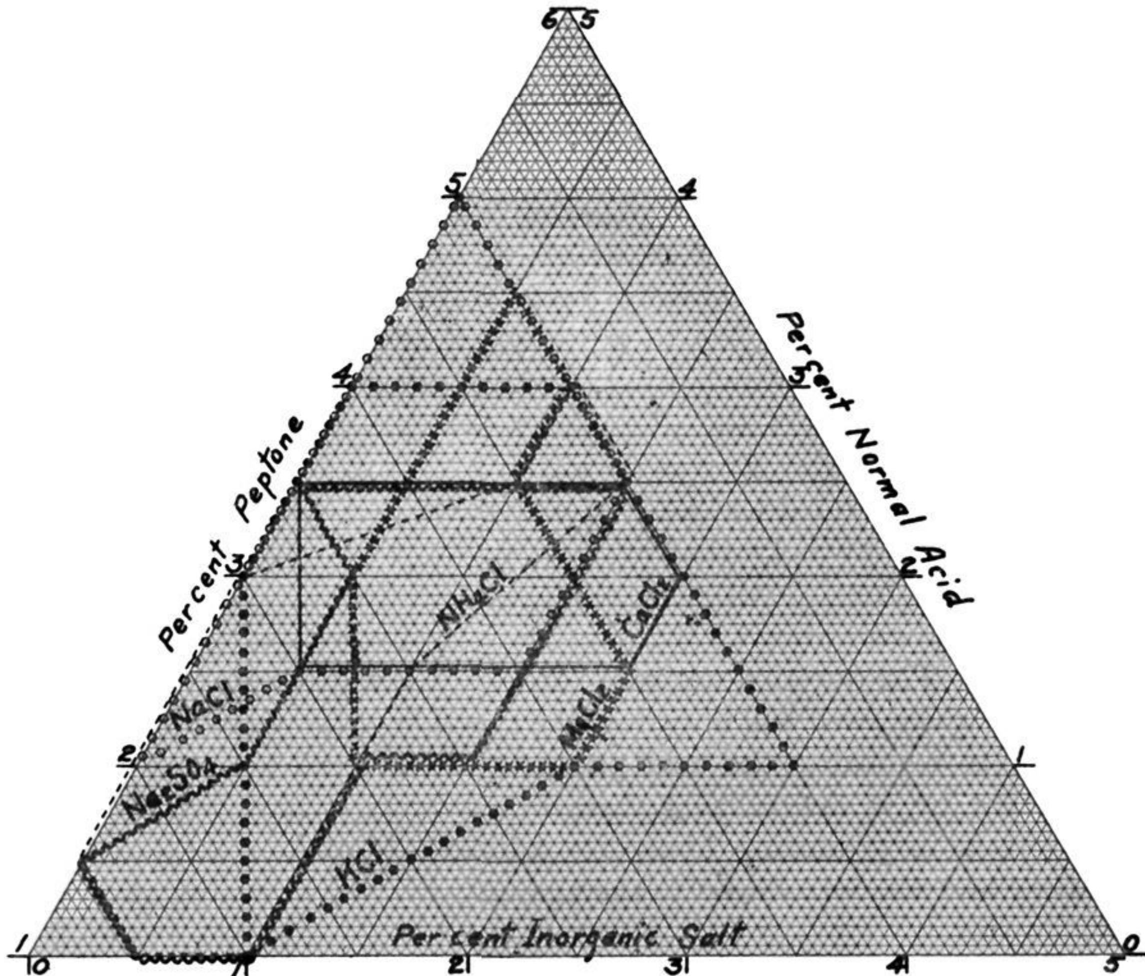

Fig. 1.

Upon sterilizing the phosphate containing media it was found that considerable hydrogen sulfide resulted from a chemical reaction between these salts and the peptone. The largest quantity of this chemically formed hydrogen sulfide appeared in media containing ammonium phosphate, and least in those containing magnesium phosphate.

None of the phosphate containing media gave as good results as the check flasks, while of these the media containing KCl prove to be superior to the others. Of the phosphated media those with $HMgPO_4$ were better than the others, then followed $HNaKPO_4$, HK_2PO_4, HNH_4KPO_4, HNa_2PO_4, $HCaPO_4$, $HNaNH_4PO_4$, $H(NH_4)_2PO_4$.

The influence of tertiary (normal) phosphates was next investigated.

Media were prepared in which were introduced an amount of the basic ion equivalent to that found to have given the best results with KCl. In these runs the following salts were tried: $Mg_3(PO_4)_2$, $Ca_3(PO_4)_2$, NH_4MgPO_4, Na_3PO_4, K_3PO_4, KNa_2PO_4 and NaK_2PO_4. Check flasks with NaCl and KCl were inoculated and incubated at the same time.

In the media containing the phosphates only traces of hydrogen sulfide were formed, while all the checks developed large amounts.

In order that the influence of moderate amounts of phosphates should be studied in all its phases, media were next prepared in which in addition to the phosphates some received KCl, some NaCl and some K_2SO_4. In these media only those containing HK_2PO_4 and KCl yielded appreciable traces of H_2S.

In all of the trials made with phosphates more than mere traces exerted a decidedly deleterious effect so far as the formation of hydrogen sulfide was concerned. As has already been stated part of this deleterious action is unquestionably due to too high an acidity. Although any addition of a compound which would reduce this acidity would necessarily change the character of the phosphates present and probably result in undesirable precipitates, it was decided to try the effect of the addition of a little finely divided $CaCO_3$. Moreover, it had been found that the final acidity to phenolphthalein was about 3.0% in those flasks of media in which much hydrogen sulfide was produced. This is doubtless due to the decomposition of the peptone into simpler amino, or other organic acids. The question naturally arose "Is it not possible that if some carbonate, which itself will not effect the reaction of the medium but which will tend to neutralize the acid products of bacterial activity, is introduced, that by so neutralizing these by-products it may materially aid the bacteria in producing hydrogen sulfide?" Calcium carbonate was first tried and was introduced to the amount of 0.5% in combination with the following:

0.25% K from KCl.
0.25% Na from NaCl.
0.25% K from K_2SO_4.
0.20% K from KCl and 0.05% K from K_2HPO_4.
0.15% K from KCl and 0.10% K from K_2HPO_4.
0.175% K from KCl and 0.075% K from K_3PO_4.
0.10% K from KCl and 0.15% K from K_3PO_4.
0.225% K from KCl and 0.025% K from KH_2PO_4.
0.20% K from KCl and 0.05% Na from Na_2HPO_4.
0.15% K from KCl and 0.10% Na from Na_2HPO_4.

There was a heavy sediment of insoluble calcium carbonate present throughout the run, which is an undesirable feature as it renders difficult, if not impossible, the formation of an opinion as to the character of the growth in the cultures.

As a result of the run, it was found that hydrogen sulfide was developed rapidly and in large amounts in just about as many flasks that contained no carbonate as was the case when the carbonate was present, leading to the conclusion that calcium carbonate has no beneficial influence on the production of hydrogen sulfide by bacteria.

Before abandoning the addition of carbonates it was deemed advisable to try a soluble carbonate and, as it was desired to use one which would least influence the reaction of the medium to phenolphthalein, sodium bicarbonate was chosen. The carbonate, in amount sufficient to give a concentration of 0.25% in the final inoculated medium, was used in combination with potassium chloride, sodium chloride and potassium sulfate, respectively, each of these salts being used in amounts to give 0.25% of the basic element present. As controls, flasks were prepared in triplicate containing 0.25% sodium bicarbonate without other salt; and with 0.25% of basic element furnished by potassium chloride, sodium chloride and by potassium sulfate without any sodium bicarbonate being present; and also three flasks containing no added salt.

As the result of the run, it was found that after sterilization and before inoculation, all of the flasks containing the added carbonate showed an undesirable, slight turbidity, and a marked deposit in the form of an adherent white film on the glass. This deposit, after thorough washing, was tested microchemically and proved to be calcium carbonate in the form of sphero-crystals of minute size. After the flasks had been inoculated and incubated, this film still persisted, being slightly augmented. A quantitative determination of the amount of calcium carbonate was made by titrating hot with 0.1 *N* hydrochloric acid, using ethyl orange as the indicator and it was found to be equivalent to 0.33%.

As far as the production of hydrogen sulfide was concerned, the results were very markedly against the use of sodium bicarbonate, as only one flask containing it showed even a trace of hydrogen sulfide produced. Moreover, when sodium bicarbonate was present, there was no greening of the solution or formation of dark green or grayish green sediment or cakes. The control flasks containing no sodium bicarbonate all produced hydrogen sulfide quickly and in large quantities, those containing potassium chloride giving the best results.

The reaction of those media not containing the carbonate when they showed a trace of hydrogen sulfide production, was about 0.70% normal acid on the average, using phenolphthalein as indicator, while the average reactions in the flasks which contained the sodium bicarbonate was 1.68% normal acid when showing a like amount of hydrogen sulfide. It may be concluded from these figures that it is erroneous to suppose that the presence of a carbonate would be beneficial by reason of the neutralizing effect which it would have upon the acid reacting substances

set free by the bacteria, thus removing from the field the by-products of bacterial life which it was thought might have an inhibiting influence on the development of the bacteria and the consequent production of hydrogen sulfide. Quite to the contrary, they proved that the bacteria must first neutralize this carbonate and then develop an acidity to phenolphthalein of 1.68% normal acid in the solution before they could begin breaking down the sulfur compounds of the peptone to yield hydrogen sulfide.

That hydrogen sulfide had not been produced and then held in solution as sodium sulfide, potassium sulfide, ferrous sulfide or calcium sulfide was demonstrated by the fact that no appreciable amounts of hydrogen sulfide could be detected upon acidifying a number of the cultures with hydrochloric acid.

During the course of the work, and more especially, while making titrations of some of the media after incubation, it was noted that there would be at times a very rapid evolution of hydrogen sulfide. This never occurred during the first twenty-four hours of incubation, and never, as far as observed, before the cultures had reached an acidity of 3.0%. The evolution was so rapid at times that from one-quarter to one-third of the lead acetate papers in the tubes of the culture flasks would be blackened in the half hour, or so, that it took to titrate a group of fifteen or twenty cultures.

It was also noted that the same thing occurred in the incubator in short periods of time.

In order to try out the method with waters of a varying degree of pollution, an artificial sewage was prepared and plated on standard gelatin and on standard agar on lactose-litmus agar and also inoculated into fermentation tubes containing a 2.0% dextrose, 3.0% peptone, 0.5% potassium chloride medium.

After forty-eight hours of incubation, the gelatin plates showed 2800 colonies per cc. with 20 *B. coli communis* per cc., and the agar plates showed 200 colonies per cc. Of the fermentation tubes, those which had been inoculated with 1 cc. and with 0.1 cc. had one-half of the closed arms filled with gas, but those inoculated with 0.01 cc. and 0.001 cc. showed no signs of gas or growth.

For inoculating the media for the detection of hydrogen sulfide, dilutions of the sewage of 5 : 100, 10 : 100, 15 : 100, 20 : 100, 25 : 100, 30 : 100, 35 : 100, 40 : 100, 45 : 100, 50 : 100, 55 : 100, 60 : 100, 65 : 100, 70 : 100, 75 : 100, 80 : 100 and 85 : 100 were made with sterile tap water, and the flasks inoculated with these dilutions were incubated at from 37–38°.

There was, as would be expected, a gradual increase in the amounts of hydrogen sulfide produced and also in the rapidity with which it was produced as the concentration of the sewage was increased, but after eigh-

teen hours of incubation, the sewage which had been reduced to 85 : 100 had given an appreciable amount of hydrogen sulfide with a turning green of the medium and turbidity produced therein; and after thirty hours, much hydrogen sulfide had been produced with a dark green sediment and dark green floating cakes and an accumulation of gas at the top of the solution by even the 10 : 100 dilution; so it may be concluded that a positive test will be given in eighteen hours with a 90 cc. inoculation of a water which would show 2380 colonies per cc. on standard gelatin with 17 *B. coli* per cc.; 170 colonies on standard agar; and gas in dextrose, peptone, potassium chloride medium in fermentation tubes with a 1 cc. inoculation; while a positive test will be given in thirty hours with a water containing only about one-eighth of this amount of pollution, with a proportionate amount of time required by degrees of pollution between these extremes.

These figures are well within the limits of what will be found in sewage polluted waters and hence the method of detecting sewage pollution in a water by means of the hydrogen sulfide produced in the proposed medium by the putrefactive organisms, affords a very valuable method for augmenting and helping to interpret the data obtained by the chemical and bacteriological examinations of water samples.

In order to ascertain whether the feces of domestic animals contributed to contaminated waters bacteria which produce hydrogen sulfide, and in what relative amounts, the following experiments were made:

Into one liter of filtered water taken from the University service pipes there was introduced one loop full (0.02 g.) of the feces of the animal selected for study. Two series of culture flasks were prepared, in each case such that when ready for incubation a final volume of 100 cc. would be obtained containing 3% of Witte peptone, 0.5% potassium chloride, and would have an acidity a trifle over 1.0%. One series contained 90 cc. of culture media, the other 10 cc.; to the first series 10 cc. of the above described artificially contaminated water, and to the second 90 cc. were slowly added.

The results obtained can best be shown by Table I.

It will be seen that in every case hydrogen sulfide was eventually produced, but that in the case of the horses, cows, and sheep, the formation of this compound was slow, only traces being formed under thirty-six hours. Should further investigations substantiate these results their importance in the interpretation of the formation of much hydrogen sulfide in eighteen to twenty-four hours in the case of contaminated waters is not to be ignored.

It was thought essential that the investigation be extended to include a study of the action of pure cultures of the *B. coli* group. We had available thirty-four strains and substrains of organisms believed to be

TABLE I.

Amount inoculated, and kind.	Millimeters blackened of lead acetate papers in				
	18 hrs.	21 hrs.	24 hrs.	36 hrs.	48 hrs.
10 cc. human	0	0	0	4	12
90 cc. human	0	1	5	All	All
10 cc. cow, I	0	1	2	10	All
90 cc. cow, I	0	0	0	8	All
10 cc. cow, II	0	0	0	12	All
90 cc. cow, II	0	1	4	All	All
10 cc. calf, I	0	1	4	All	All
90 cc. calf, I	2	5	8	All	All
10 cc. horse, I	0	0	0	14	All
90 cc. horse, I	0	0	1	6	All
10 cc. horse, II	0	0	0	14	All
90 cc. horse, II	0	0	1	5	20
10 cc. pig, I	0	0	0	17	All
90 cc. pig, I	0	0	1	15	All
10 cc. pig, II	0	1	2	16	All
90 cc. pig, II	0	1	4	All	All
10 cc. sheep, I	0	0	1	8	All
90 cc. sheep, I	0	0	0	6	20

B. coli which had been isolated from contaminated waters and sewage. These thirty-four strains were being employed in the study of carbohydrate media and were taken for use from a series of tubes of Harrison's esculin bile medium and from a series of fermentation tubes of Stokes' neutral red medium. This gave sixty-eight strains or substrains in all. These were inoculated in different bacterial dilutions into media whose final incubated concentrations were 3% peptone, 0.5% potassium chloride, acidity 1.02%. The results obtained were as follows:

18 hours, 1 culture had begun to develop hydrogen sulfide.
24 hours, 5 cultures had begun to develop hydrogen sulfide.
36 hours, 7 cultures developed H_2S, two of them moderate amounts.
48 hours, 9 cultures developed H_2S, two of them moderate amounts.
4 days, 26 cultures developed H_2S, four of them moderate amounts.
5 days, 43 cultures developed H_2S.
6 days, 47 cultures developed H_2S.
7 days, 49 cultures developed H_2S.
8 days, 52 cultures developed H_2S while 16 still showed none.

Although the authors do not consider that these results are conclusive, they do believe that taken in conjunction with the results of the runs made upon animal feces and those obtained from the examinations of contaminated waters, the production of large quantities of hydrogen sulfide in from eighteen to twenty-four hours is to be regarded as indicative of the presence of organisms other than *B. coli*[1] and there appears

[1] This is further substantiated by the work of Kendall, Day and Walker, THIS JOURNAL, 35, 1201 (1913), who find that the proteolytic activity of the *B. coli* group is relatively very low and slow.

to be good grounds for the use of the term "putrefactive bacteria" in water examinations as suggested by Schardinger and by Dunham.

Some evidence has also been obtained by the authors that in a mixed flora the formation of hydrogen sulfide is more rapid than from the isolated pure cultures.

If, therefore, tests for hydrogen sulfide production are to be made in water examinations the analyst must take into account the rate and the amount of this gas formed and the time of its first appearance.

In the course of the investigation a number of purely biochemic questions and problems arose upon which considerable time was spent such as, for example, the actual source of the hydrogen sulfide evolved, whether from firmly or loosely bound sulfur compounds, the actual quantity formed, the rate of formation, the influence of the presence of oxygen, etc. Some of these questions have already been alluded to, but although interesting, appear to have no direct bearing upon the sanitary significance of hydrogen sulfide production. The influence of oxygen, however, needs more than a passing word of comment. It is generally stated in most text-books of microbiology, that hydrogen sulfide is formed by a reducing reaction and therefore the most favorable conditions should be

TABLE II.—SURFACE WATERS.

Source of sample.	Month of year.	Quality from chem. anal.	Colonies per cc. on Agar 38°.	Colonies per cc. on Gelatin 20°.	Gas producers.	Colon group present in 1 cc.	Colon group present in 5 cc.	Colon group present in 10 cc.	Indol production.	Hydrogen sulphide production.
Spring brook	Jan.	Good	50	6	+	—	—	?	+	++
River	Jan.	Bad	25	79,000	+	+	+	+	+	+
Filtered river	Jan.	Good	2	460	—	—	—	—	+	++
Creek	Mar.	Poor	..	460	+	+	+	+	+	++
River	Mar.	Poor	3	2,200	—	—	—	—	+	Trace
Creek	May	Poor	1	300	—	—	—	—	Trace	Trace
Ditch*	July	....	700	120	—	—	—	—	—	—
Ditch	July	....	500	2,500	+	+	+	+	++	+++
Ditch	July	....	3,000	17,000	+	—	—	+	+	+++
River	Aug.	Poor	1,000	2,100	+	—	—	—	++	+++
Lake	Aug.	Good	30	370	+	—	—	—	++	++
Filtered river	Aug.	Poor	12,000	375,000	+	?	+	+	++	+
River	Aug.	Bad	1,000	2,100	+	?	+	+	++	+++
Filtered river	Sept.	Good	3	40	+	—	—	?	+	+++
Spring brook	Oct.	Suspicious	6	70	+	—	?	+	+	+++
River	Oct.	Bad	80	600	+	+	+	+	++	+++
River	Oct.	Bad	200	1,600	+	+	+	+	++	++
Reservoir	Oct.	Suspicious	20	400	+	?	+	+	+	++++
River	Dec.	Poor	160	1,600	+	—	+	+	++	+
River	Dec.	Suspicious	75	4,600	+	—	?	+	++	+++
Filtered river	Dec.	Suspicious	1	8	—	—	—	—	+	++
River	Dec.	Bad	60	50,000	+	+	+	+	+	++
Filtered river	Dec.	Poor	35	5,000	+	—	—	?	+	+

* Hot from exhaust steam from steam pump.

those in which the growth of the organisms producing this reaction takes place in the presence of a minimum amount of oxygen. It is obvious that this has a direct bearing upon the testing for the presence of hydrogen sulfide forming bacteria in water and required investigations. The details of the work done need not be here discussed. In all the experiments made, quantitative analyses showed that more hydrogen sulfide was evolved and less sulfur remained in the peptone culture media if air was present than if it was absent when using a mixed sewage flora. The largest quantities of hydrogen sulfide were obtained when a slow current of sterile air was passed over the surface of the liquids in the culture flasks. Actually 100% more total sulfur was thus converted into hydrogen sulfide.

TABLE III.—SPRING WATERS.

Month of year.	Quality from chem. anal.	Colonies per cc. on		Gas producers.	Colon group present in			Indol production.	Hydrogen sulphide production.
		Agar 38°.	Gelatin 20°.		1 cc.	5 cc.	10 cc.		
Jan.	Good	1	40	—	—	—	—	—	—
Jan.	Good	13	7,200	—	—	—	—	++	+++
Feb.	Good	4	110	—	—	—	—	+	+++
Feb.	Good	4	95	+	—	—	?	Trace	+++
Mar.	Poor	0	15	+	—	—	—	Trace	—
May	Good	1	140	+	—	—	—	—	—
June	Suspicious	30	3,000	+	—	+	+	+	+
June	Good	1	35	—	—	—	—	—	—
June	Poor	12	350	+	+	+	+	?	—
July	Suspicious	3	500	—	—	—	—	—	—
July	Bad	2	170,000	+	—	?	+	+	++
Aug.	Suspicious	65	65	+	—	—	—	Trace	Trace
Aug.	Bad	450	120,000	+	+	+	+	+	+++
Aug.	Good	70	10,000	+	+	+	+	?	Trace
Aug.	Good	22	600	+	?	+	+	Trace	—
Aug.	Good	190	15,000	—	—	—	—	—	—
Aug.	Good	600	3,500	+	—	—	—	+	+
Aug.	Good	30	350	+	+	+	+	Trace	Trace
Sept.	Bad	20	150	—	—	—	—	—	—
Sept.	Bad	0	10	—	—	—	—	—	—
Sept.	Poor	1,900	390	+	—	—	+	+	++
Oct.	Good	15	40	+	—	—	?	—	—
Oct.	Bad	3	210	+	—	—	?	—	—
Oct.	Good	20	800	—	—	—	—	++	+
Oct.	Good	15	730	+	—	?	+	+++	+
Oct.	Poor	0	90	+	—	—	—	—	—
Dec.	Good	70	180	—	—	—	—	Trace	Trace
Dec.	Good	0	9	+	—	—	—	—	—
Dec.	Good	1	3	—	—	—	—	—	Trace
Dec.	Good	0	8	—	—	—	—	Trace	Trace
Dec.	Good	2	150	+	?	?	+	++	+++
Dec.	Good	0	12	—	—	—	—	+	Trace

TABLE IV.—WELL WATERS.

Kind of well.	Depth in feet.	Month of year.	Quality from chem. anal.	Colonies per cc. on		Gas producers.	Colon group present in			Indol production.	Hydrogen sulfide production.
				Agar 38°.	Gelatin 20°.		1 cc.	5 cc.	10 cc.		
Artesian	303	Jan.	Good	0	5	—	—	—	—	—	Trace
Artesian	286	Jan.	Good	1	7	—	—	—	—	—	—
Artesian	280	Jan.	Good	3	3	—	—	—	—	?	+
Artesian	276	Jan.	Good	0	6	—	—	—	—	—	—
Artesian	295	Jan.	Good	0	2	—	—	—	—	—	—
Drilled	342	Jan.	Cl = 312 Solids 4200	400	350	+	—	—	?	Trace	++
Driven	...	Feb.	Bad	95	225	+	+	+	+	+	++
Drilled	90+	Mar.	Cl = 438 Solids 4000	..	720	+	+	+	+	+++	++
Drilled	50+	Mar.	Bad	200	5,000	+	—	+	+	+	++
Driven	85	Apr.	Good	12	100	+	+	+	+	+	—
Driven	50+	May	Poor	95	3,000	+	—	—	—	?	++
Dug	22	May	Bad	290	590	+	—	—	?	Trace	+
Drilled	25	May	Bad	55	700	+	—	—	?	++	+++
Dug	16	June	Poor	650	1,500	+	—	—	?	+	++
Dug	30	June	Poor	8	2,000	+	—	+	+	—	Trace
Dug	30	June	Poor	25	2,000	+	+	+	+	+	++
Dug	..	June	Suspicious	3	1,000	—	—	—	—	—	Trace
Artesian	286+	July	Good	..	35	—	—	—	—	—	—
Dug	40?	July	Good	10	250	+	—	—	?	+	+++
Drilled	..	July	Suspicious	70	145,000	+	—	—	?	?	+++
Dug	17	July	Suspicious	2,000	50,000	+	+	+	+	++	+++
Dug	20	July	Poor	3,000	18,000	+	+	+	+	—	+
Driven	16	July	Good	9	35	+	—	—	—	—	+
Drilled	165	July	Bad	2	170,000	—	—	—	—	—	—

TABLE IV.—WELL WATERS (*Continued*).

Kind of well.	Depth in test.	Month of year.	Quality from chem. anal.	Colonies per cc. on		Gas producers.	Color group present in			Indol production.	Hydrogen sulfide production.
				Agar 38°.	Gelatin 20°.		1 cc.	5 cc.	10 cc.		
Driven	75	Aug.	Good	750	2,000	+	—	—	—	—	—
Artesian	428	Aug.	Good	250	500	+	—	—	—	—	—
Dug	17	Aug.	Suspicious	2,500	15,000	+	+	+	+	++	+++
Drilled	165	Aug.	Bad	1,800	18,500	—	—	—	—	—	Trace
Drilled	115	Aug.	Good	200	75,000	+	—	—	?	?	+++
Drilled	25	Aug.	Bad	60,000	50,000	—	—	—	—	Trace	Trace
Driven	41	Sept.	Good	..	330	+	—	?	?	+	+
Dug	..	Sept.	Poor	4	70	—	—	—	—	?	Trace
Dug	16	Sept.	Good	25	750	+	—	—	—	—	—
Dug	20	Sept.	Poor	1,100	3,800	+	+	+	+	++	+++
Driven	50	Oct.	Suspicious	60	130	+	—	?	+	—	+
Driven	46	Oct.	Poor	5	10	+	—	—	?	+	++
Driven	80+	Oct.	Good	0	6	—	—	—	—	—	—
Dug	..	Oct.	Poor	35	300	+	—	—	—	—	—
Dug	..	Oct.	Good	3,900	39,000	+	—	—	?	++	+++
Dug	..	Oct.	Poor	5	100	+	—	?	+	++	+++
Artesian	303	Oct.	Good	15	3	—	—	—	—	—	—
Artesian	286	Oct.	Good	2	3	—	—	—	—	—	—
Artesian	280	Oct.	Good	4	3	—	—	—	—	—	—
Artesian	276	Oct.	Good	1	2	—	—	—	—	—	—
Dug	12	Oct.	Good	100	1,400	+	—	—	—	+	+
Dug	20±	Oct.	Bad	30	120	—	—	—	—	+	+++
Dug	15	Oct.	Bad	60	550	+	?	+	+	++	+++
Driven	..	Oct.	Bad	30	130	+	+	+	+	+	+++
Dug	..	Dec.	Suspicious	500	1,500	+	—	?	+	++	+++
Dug	40	Dec.	Suspicious	20	500	+	—	—	?	+	++

In routine testing, therefore, it is advantageous to use flasks with wide neck tubes of large diameter in order that a considerable surface shall be exposed to the air.

In the accompanying tables are given the results obtained with the tests for hydrogen sulfide production upon a number of different water samples. Although the data have been greatly abridged it is hoped that sufficient information has been given to enable the reader to properly grade the water samples. Since most natural waters usually exhibit a very marked seasonal change in their bacterial flora, the samples have been tabulated by months.

TABLE V.—MISCELLANEOUS WATERS.

Source of sample. Water.	Month of year.	Quality from chem. anal.	Colonies per cc. on		Gas production.	Colon group present in			Indol production.	Hydrogen sulphide production.
			Agar 38°.	Gelatin 20°.		1 cc.	5 cc.	10 cc.		
Stored, ground	Jan.	Good	0	50	—	—	—	—	+	—
Ice	Jan.	Poor	2	3,000	—	—	—	—	—	Trace
Ice	Feb.	Good	1	13	—	—	—	—	—	Trace
Ice	Feb.	Good	2	10	—	—	—	—	—	—
Ice	Feb.	Poor	10	110	+	—	—	+	+	+++
Ice	Mar.	Poor	25	900	—	—	—	—	—	—
Tank wagon	July	Good	30	450	—	—	—	—	—	—
Tank wagon	Aug.	Good	70	1,400	+	+	+	+	+	++
Cistern, rain	Oct.	Poor	3,300	45,000	+	—	?	?	++	+++
"Sterilized"*	Oct.	Poor	10,000	15,000	+	+	+	+	Trace	Trace?
"Sterilized"	Oct.	Good	325	1,200	—	—	—	—	Trace	+
Tank wagon	Nov.	Good	350	10,000	+	+	+	+	Trace	+++
Bottled spring	Nov.	Good	750	1,500	+	?	+	+	—	—
"Sterilized"	Nov.	Poor	1	270	—	—	—	—	—	Trace
Stored, ground	Dec.	Good	0	10	—	—	—	—	—	—
Stored, ground	Dec.	Bad	1,000	6,300	+	+	+	+	+	++

*From small plant using a patented "water sterilizer."

Summary of Results Obtained.

1. Irrespective of the inorganic salts present and of the acidity of the medium, a concentration of between 3 and 4% peptone in the final inoculated and incubated medium appears to be best for the most rapid and energetic production of hydrogen sulfide.

2. The addition of beef broth to simple peptone media slightly increases its sensitiveness, but not in proportion to the increased trouble and labor involved.

3. If sodium chloride is used, the quantity added must not be over 1.5%. Cultures to which this salt was added showed greater hydrogen sulfide production than those which contained none.

4. In 3% peptone media, the presence of from 0.5% to 1% of potassium chloride had a decidedly beneficial influence and led to quicker, better and far more uniform results than any other inorganic salt tried.

5. Positive results of hydrogen sulfide formation may be obtained in eighteen hours.

6. No hydrogen sulfide formation is obtainable in as long a period as seventy-two hours from natural waters which are truly "clean," while much is formed in from twelve to twenty-four hours with contaminated waters.

7. The feces of domestic animals contain bacteria which are capable of producing hydrogen sulfide from a simple peptone medium in as large amounts as is the case of the bacteria from human feces.

8. The large amounts of hydrogen sulfide *rapidly* produced by organisms of sewage appears to be not due primarily to members of the *B. coli* group.

9. This group of hydrogen sulfide producing bacteria do not actively ferment carbohydrates. Hence testing for their presence is a valuable aid supplementing tests for gas producers and is of especial value in polluted waters in which the *B. coli* group is absent.

10. Some evidence has been obtained which apparently indicates that hydrogen sulfide is more rapidly produced in waters containing a mixed bacterial flora than by the isolated pure cultures alone.

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[CONTRIBUTION FROM THE COLLEGE OF AGRICULTURE, THE UNIVERSITY OF MINNESOTA.]

ON THE ORIGIN OF THE HUMIN FORMED BY THE ACID HYDROLYSIS OF PROTEINS.

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Introduction.

It is well known that, when proteins are subjected to hydrolysis by boiling acids, a blackening of the solution occurs, and that, when the boiling is continued for some time, black insoluble particles separate from solution. These compounds may be purified to a greater or a less degree by solution in alkali and reprecipitation by the addition of acid. They are, however, insoluble in all of the usual organic solvents and have never been obtained in crystalline form.

A very considerable amount of work has been done on these humins or "melanoidins" as Schmiedeberg[1] calls them. Schmiedeberg found that indol and skatol were formed in an alkali fusion and Samuley[2] by reduction obtained evidences of pyridine formation. Inasmuch as humin was not formed from proteins when the hydrolysis was carried out in the presence of stannous chloride, Samuley concludes that the formation of this dark-colored product is due to an oxidative process.

[1] *Arch. Exper. Path. u. Pharm.*, **39**, 1–84 (1897).

[2] *Beitr. chem. Physiol. u. Path.*, **2**, 355–88 (1902).