[CONTRIBUTION FROM THE CHEMISTRY DEPARTMENT, CORNELL UNIVERSITY.]

# QUANTITATIVE DETERMINATIONS OF SULFUR IN THE CULTURE MEDIUM FOR THE DETECTION OF THE BACTERIA PRODUCING HYDROGEN SULFIDE.

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By the use of the methods which had been found to give the best results in the determination of sulfur in peptone, as presented in the preceding paper, a study was made of the total amount of sulfur broken down by the so-called putrefactive bacteria in an unadjusted culture medium, containing 3% of Witte peptone and 0.75% of potassium chloride after inoculation; of the forms of sulfur most readily used by them; and of the forms in which the sulfur existed after the action of the bacteria, whether as fixed sulfur, or as loosely bound sulfur, or as easily oxidized sulfur, or as a volatile sulfur compound such as hydrogen sulfide.

**Sulfur in Insoluble Residue.**—Determinations were first made of the total sulfur by the Liebig-Koch method in that part of the peptone which is agglomerated and which appears as an insoluble residue when peptone is boiled with water, in order to ascertain whether this material contained such a large amount of sulfur that its removal by filtration might have an effect upon hydrogen sulfide production.

It was found that so small an amount of sulfur would be removed in this way that peptone solutions may be filtered without detriment to the medium.

**Peptone and Sulfur Used Up by Bacteria in Flasks of Different Size.**—In order to ascertain the amount of peptone used up by the bacteria and also the amount of sulfur used in flasks of different size, when the simple peptone medium was inoculated with artificial sewage and incubated, 23 flasks of 100 cc. capacity were prepared by introducing into each, 10 cc. of 30% peptone solution and 5 cc. of 10% potassium chloride solution, after which the flasks were plugged, sterilized and capped and then 85 cc. of artificial sewage which had been strained through sterile cloth into a sterile beaker was added to each flask. Also one flask of 2000 cc. capacity was prepared by introducing into it 200 cc. of 30% peptone solution and 100 cc. of 10% potassium chloride solution after which it was plugged, sterilized and capped and 1700 cc. of the same strained artificial sewage added to it as was used in the 100 cc. flasks.

Two thousand cubic centimeters of a mixture of 30% peptone solution, 10% potassium chloride solution, and artificial sewage in the same proportions as was used in both the small and large flasks above mentioned, was prepared and sterilized to serve as a blank. The flasks containing the inoculated culture media were incubated for 48 hours at 38°.

**Effect of Potassium Chloride and of Sewage on Sulfur Determinations.**—The grams per 100 cc. of total solids, the grams per 100 cc. of total sulfur by the Liebig-Koch method and the grams per 100 cc. of easily oxidized sulfur by the potassium chlorate-nitric acid method, were immediately determined in the blank.

If the presence of potassium chloride and of artificial sewage has no influence on the sulfur determinations, then the grams of total sulfur per 100 cc. and of easily oxidized sulfur per 100 cc. divided by 3 and divided by 1.0110 (the specific gravity of a 3% peptone solution) and multiplied by 100, should be very close to 1.0061 and 0.5194, respectively, as found in the preceding paper.

Such computations actually give 0.9957 and 0.5176 and, therefore, it is proved that the Liebig-Koch method for total sulfur and the potassium chlorate-nitric acid method for easily oxidized sulfur are as accurate for the culture media employed as they are for peptone itself.

**Sulfur in Cultures.**—The cultures from 20 of the 100 cc. flasks were combined and filtered through a large webbed paper filter and the soluble material all washed through with distilled water; then a hole was punched in the bottom of the filter and the insoluble material was washed with distilled water into a tared beaker. The culture in the 2000 cc. flask was treated in like manner.

The soluble portions of the cultures were then evaporated to dryness to drive off all traces of volatile sulfur compounds, such as hydrogen sulfide; dissolved in hot water and made up to 500 cc., when total solids exclusive of potassium chloride, total sulfur and easily oxidized sulfur were determined in measured portions.

TABLE I.

| All results are computed to the basis of 3.0 g. of total solids exclusive of KCl per 100 cc. in the blank, 0.0302 g. being taken as the amount of total sulfur. | Grams per 100 cc. of total solids exclusive of KCl. | Grams per 100 cc. of unattacked sulfur. | Grams per 100 cc. of easily oxidized sulfur. | Per cent. which easily oxidized sulfur is of unattacked sulfur. | Per cent. of total solids, exclusive of KCl destroyed. | Per cent. of total sulfur converted into $H_2S$. | Per cent. of easily oxidized sulfur converted into $H_2S$. |
|---|---|---|---|---|---|---|---|
| Peptone, KCl blank | 3.0000 | 0.0302 | 0.0157 | 51.99 | ... | ... | ... |
| 100 cc. flasks. Filtrate after 48 hrs.' incub | 2.5885 | 0.0145 | 0.0103 | 71.03 | 13.72 | 51.99 | 34.39 |
| 2000 cc. flasks. Filtrate after 48 hrs.' incub | 2.8850 | 0.0180 | 0.0106 | 58.89 | 3.83 | 40.40 | 32.48 |
| 100 cc. flasks. Sediment after 48 hrs.' incub | 0.0176 | 0.0001 | .. | ... | ... | ... | ... |
| 2000 cc. flasks. Sediment after 48 hrs.' incub | 0.0121 | 0.0001 | .. | ... | ... | ... | ... |
| 100 cc. flasks. Filtrate and sediment comb | 2.6161 | 0.0146 | .. | ... | 13.13 | 51.65 | ... |
| 2000 cc. flasks. Filtrate and sediment comb | 2.8971 | 0.0181 | .. | ... | 3.43 | 40.07 | ... |

The insoluble portions of the cultures were evaporated to dryness on the water bath and then dried in a vacuum desiccator over calcium chloride at 75° and weighed. As the amount of material was so small, no attempt was made to determine the easily oxidized sulfur, all of the material being used in each case for the determination of total sulfur. The results are given in Table I.

It should be noted that the ratio of total solids, exclusive of potassium chloride, to total sulfur, is very nearly the same in the soluble and insoluble portions of both cultures. Most of the insoluble material is probably peptone and, therefore, it is of no advantage to separate the soluble and insoluble parts of the cultures for analysis and consequently such a separation was not attempted in the subsequent work reported in this article.

**Effect of Amount of Surface Exposed.**—The fact that the total solids, exclusive of potassium chloride, total sulfur and easily oxidized sulfur were all markedly lower in the case of the cultures in the 100 cc. flask than in the case of the culture in the 2000 cc. flask demanded explanation. A possible reason was that in the case of 100 cc. flasks there was a greater amount of surface exposed to a greater amount of air and that, in consequence, the bacteria were more energetic and, therefore, decomposed a larger amount of peptone. This idea is at variance with the belief that the organisms producing hydrogen sulfide are anaerobes. In order to test out the validity of this explanation, two Fernbach culture flasks were prepared by introducing into each 500 cc. from a mixture of 400 cc. of 30% peptone solution, 200 cc. of 10% potassium chloride solution and 3400 cc. of strained artificial sewage; two flasks of 500 cc. capacity were prepared by introducing into each 500 cc. of the same mixture; ten flasks of 100 cc. capacity were prepared by introducing into each 100 cc. of the mixture, while the balance of the mixture was used as a blank.

The blank was immediately sterilized to await analysis, while the inoculated flasks of various size and shape were placed in the 38° incubator. Air was drawn through a wash bottle containing 5 : 1000 mercuric chloride solution to take out any hydrogen sulfide in the air, then through one of the Fernbach flasks. It finally passed through a Fritz Friedrichs wash bottle containing 2.5 g. of cadmium chloride in water solution, in order to catch and hold as cadmium sulfide all of the hydrogen sulfide which might be produced by the bacteria in the culture and swept over by the current of air. The speed of the air current was so regulated that one bubble per second passed through the wash bottles.

By means of a portable gas generator,[1] carbon dioxide gas under constant pressure was generated from calcium carbonate and hydrochloric acid and passed first through a wash bottle containing distilled water, then through the other Fernbach flask and finally through a Fritz Friedrichs

[1] A. W. Browne and M. J. Brown, THIS JOURNAL, 29, 859 (1907).

wash bottle containing 2.5 g. of cadmium chloride in water solution, to absorb any hydrogen sulfide, generated by the bacteria in the culture. The carbon dioxide generator was so regulated that one bubble of gas per second passed through the wash bottle.

The Fernbach flasks through which air and carbon dioxide, respectively, were being passed, and the other flasks through which no gas was being passed but which were being allowed to incubate in the usual manner, were all left in the 38° incubator for 48 hours. They were then sterilized by heat. Without filtering off the insoluble material, this having been proved to be unnecessary in the previous set of inoculations, the blank and the various cultures were analyzed for total solids, for total sulfur, for easily oxidized sulfur, for loosely bound sulfur by the Schultz method, and for sulfur as hydrogen sulfide.

For making these determinations, except that of sulfur as hydrogen sulfide, the blank and the various cultures were evaporated separately to small volumes, then each was made up to 250 cc. in a measuring flask and measured portions taken for analysis. For the determination of sulfur as hydrogen sulfide, the contents of the cadmium chloride wash bottles were acidified with strong hydrochloric acid, an excess of $N/10$ iodine added and the excess titrated with $N/10$ sodium thiosulfate. The results are given in Table II.

The amounts of total solids, exclusive of potassium chloride, remaining in the media after incubation proved that the bacterial action was most energetic in the Fernbach through which air was passed; next in the cultures having the greatest surface exposed, *i. e.*, in the 100 cc. flasks; next in the cultures in the 500 cc. flasks; and was least energetic in the Fernbach flask through which carbon dioxide was passed; while the amounts of total sulfur remaining, the difference between which and the total sulfur of the blank is a measure of the volatile sulfur compounds produced by the bacteria, proved that the production of volatile sulfur compounds by the bacteria decreased in the same order. Therefore, the orders for general bacterial activity and for hydrogen sulfide production were identical, and in the case of the flora with which this work was done, at least, the organisms producing hydrogen sulfide in largest amounts were aerobes and not anaerobes and for the maximum production of hydrogen sulfide, broad, low flasks giving the maximum surface of culture exposed to air should be used.

**Determination of Hydrogen Sulfide.**—The fact that the amounts of sulfur, as hydrogen sulfide, found by the method employed were far from being the differences between the total sulfur of the blank and the total sulfurs of the cultures in which sulfur as hydrogen sulfide was determined, demanded attention, as it indicated that the method which had been employed was faulty and that the cadmium chloride had failed to stop all of the hydrogen sulfide evolved. It was, therefore, decided to try an iodine solution instead of the cadmium chloride solution in the wash bottle.

For this purpose, air was drawn for 48 hours, first through a wash bottle containing lead acetate solution to remove any gaseous sulfur compounds, then through a Fernbach flask, containing 500 cc. of the culture medium, artificial sewage mixture

TABLE II.

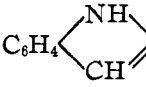
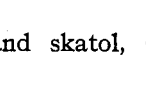
| All results are computed to the basis of 3.0 g. of total solids excl. of KCl per 100 cc. in the blank, 0.0302 g. being taken as the amount of total S. | G. per 100 cc. of total solids excl. of KCl. | G. per 100 cc. of unattacked S. | G. per 100 cc. of easily oxidized S. | G. per 100 cc. of loosely bound S. | G. per 100 cc. of S as $H_2S$. | % which easily oxidized S is of unattacked S. | % which loosely bound S is of unattacked S. | Unattacked S plus S as $H_2S$. | % of S accounted for. | % of total solids, excl. of KCl, destroyed. | % of total S converted into $H_2S$. | % of easily oxidized S converted into $H_2S$. | % of loosely bound S converted into $H_2S$. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Peptone, KCl blank | 3.0000 | 0.0302 | 0.0157 | 0.0078 | .. | 51.99 | 25.83 | 0.0302 | 100.00 | ... | ... | ... | ... |
| Culture in Fernbach. Air passed 48 hrs | 2.6520 | 0.0200 | 0.0090 | 0.0041 | 0.0011 | 45.00 | 20.50 | 0.0211 | 69.87 | 11.60 | 33.77 | 42.68 | 47.45 |
| Cultures in 100 cc. flasks. 48 hrs | 2.7209 | 0.0222 | 0.0105 | 0.0049 | .. | 47.30 | 22.07 | .. | .. | 9.30 | 26.49 | 33.12 | 37.18 |
| Cultures in 500 cc. flasks. 48 hrs | 2.7872 | 0.0244 | 0.0110 | 0.0055 | .. | 45.08 | 22.54 | .. | .. | 7.09 | 19.21 | 29.93 | 29.49 |
| Culture in Fernbach. $CO_2$ passed 48 hrs | 2.8899 | 0.0249 | 0.0096 | 0.0066 | 0.0012 | 38.55 | 26.51 | 0.0261 | 86.42 | 3.67 | 17.55 | 38.85 | 15.38 |

TABLE III.

| All results are computed to the basis of 3.0 g. of total solids exclusive of KCl per 100 cc. in the blank, 0.0302 g. being taken as the amount of total S. | Absorbent. | G. per 100 cc. of total solids excl. of KCl. | G. per 100 cc. of unattacked S. | G. per 100 cc. of S as non-vol. sulfide. | G. per 100 cc. of S as $H_2S$. | Unattacked S plus S as $H_2S$. | % of S accounted for. | % of total solids, excl. of KCl, destroyed. | % of total S converted into $H_2S$. | % of total S converted into non-vol. sulfide. |
|---|---|---|---|---|---|---|---|---|---|---|
| Peptone, KCl blank | ..... | 3.0000 | 0.0300 | 0.0003 | .. | 0.0300 | 99.34 | ... | ... | .. |
| Culture in Fernbach. Air passed 72 hrs | KOH | 2.4915 | 0.0148 | 0.0003 | 0.0154 | 0.0302 | 100.00 | 16.95 | 51.00 | 0.00 |
| Culture in Fernbach. Air passed 72 hrs | $CdCl_2$ | 2.5535 | 0.0154 | 0.0009 | 0.0140 | 0.0294 | 97.35 | 14.88 | 49.01 | 1.99 |
| Culture in Fernbach. Air passed 72 hrs | $Na_2O_2$ | 2.5118 | 0.0133 | 0.0009 | 0.0144 | 0.0277 | 91.72 | 16.27 | 55.96 | 1.99 |
| Culture in Fernbach. Air passed 72 hrs | $PbAc_2$ | 2.4023 | 0.0133 | 0.0010 | 0.0112 | 0.0245 | 81.13 | 19.92 | 55.96 | 2.32 |

already described, in the 38° incubator, then through what had been calculated to be an excess of $N/10$ iodine solution in a Fritz Friedrichs wash bottle and finally through a $N/10$ sodium thiosulfate solution in a wash bottle.

During the period of incubation, the iodine solution was found to fade rapidly, indicating that larger amounts of hydrogen sulfide than had been expected were being produced, and consequently, small measured amounts of normal iodine solution were, from time to time, added to the iodine solution in the wash bottle. After 48 hours of incubation, the iodine solution and the sodium thiosulfate solution were mixed together and the amount of iodine which had been used up, determined by titration.

It was found that an amount of iodine equivalent to the hydrogen sulfide which would be produced if nearly all of the sulfur of the medium were transformed to hydrogen sulfide, had been reduced. As determinations of total sulfur showed that only about 25% of the total sulfur had disappeared from the medium the results were evidently impossible. The explanation which is offered is that volatile, unsaturated organic compounds like indol,  and skatol,  , were liberated from the medium by the action of the bacteria and that these reduced the iodine solution.

The use of iodine solution having been proved to be of no value, it was decided to try solutions of cadmium chloride containing potassium chloride to prevent colloidal suspension of cadmium sulfide, of lead acetate containing potassium nitrate to prevent colloidal suspension of lead sulfide, of sodium peroxide and of potassium hydroxide, determining the total sulfur retained as sulfide by the Liebig-Koch method and not by liberating with hydrochloric acid and titrating with iodine and sodium thiosulfate solutions as had been done when cadmium chloride was before used.

The Fernbach flasks containing the usual mixture of culture medium and artificial sewage and the Fritz Friedrichs wash bottles containing the various solutions were connected in parallel to the same suction. The blank was immediately sterilized to await analysis and the Fernbachs were incubated at 38° for 72 hours (allowed to incubate 72 hours because the end of 48-hour period came on Sunday), air being sucked through the Fernbach flasks and their wash bottles at as uniform a rate as possible.

After 72 hours, each Fernbach was heated to boiling on a water bath for 30 minutes while air was sucked through it and its respective wash bottles. This was to drive out all hydrogen sulfide held in solution in the cultures. The solution from each Fernbach was then made up to 500 cc. (only a few cc. had been lost by evaporation in each case), and total solids, total sulfur and sulfur as nonvolatile sulfides determined in measured portions of these cultures and of the blank. The results are given in Table III.

The determination of sulfur as nonvolatile sulfides was accompanied by placing 300 cc. of each culture in a distilling flask and adding 25 cc. of concentrated hydrochloric acid. A Vigreux tube was attached and the solution was then distilled for 30 minutes under reduced pressure into sodium peroxide solution (5 : 60) contained in a wash bottle, the purpose being to decompose the sulfides and to distil the hydrogen sulfide formed into the sodium peroxide where it would be held as sodium sulfide, and possibly sulfate, in which form the sulfur was determined by the Liebig-Koch method.

The sum of the total sulfur remaining in the medium plus the sulfur found as hydrogen sulfide in the wash bottles proved to be 0.0294 g. per 100 cc. when cadmium chloride was used; 0.0245 when lead acetate was used; 0.0277 when sodium peroxide was used and 0.0302 when potassium hydroxide was used; in a number of determinations 0.0302 g. of total sulfur per 100 cc., on the basis of 3 g. of total solids exclusive of potassium chloride per 100 cc., had been found present in the blanks. The indications were, therefore, that potassium hydroxide stopped and held all of the volatile sulfur compounds evolved, while the other substances failed to do so, although cadmium chloride was almost as efficient.

**Determinations of Hydrogen Sulfide with Shorter Incubation.**—To eliminate the possibility of analytical errors having crept into this important determination, another exactly similar set of inoculations and sulfur determinations (with the exception that the cultures were incubated 48 hours instead of 72 hours) was made. The results are given in Table IV.

In this second set of determinations the results in all ways confirmed those of the previous work. The sum of the total sulfur remaining in the medium, plus the sulfur found as hydrogen sulfide in the wash bottles, proved to be 0.0304 g. per 100 cc. when cadmium chloride was used; 0.0243 when lead acetate was used; 0.0302 when sodium peroxide was used; and 0.0299 when potassium hydroxide was used: showing that cadmium chloride, sodium peroxide and potassium hydroxide were all efficient reagents to use, while lead acetate was not. In subsequent work, the three reagents named were all employed, although potassium hydroxide is preferable because the precipitate of cadmium sulfide tends to stick to the sides of the wash bottles, from which it must be dissolved by the potassium chlorate-nitric acid reagent with a possibility of sulfur being lost as hydrogen sulfide before it can be oxidized by the reagent, while there is a bad tendency toward spattering in the alkaline fusion when sodium peroxide is present, with possibility of loss. Neither of these difficulties is encountered when potassium hydroxide is used to catch and hold the volatile sulfur compounds.

It was very gratifying to have the sums of the volatile and nonvolatile sulfur compounds in the different cultures approximate so closely to the amount of total sulfur found in the blanks, as it showed that the analytical methods which had been adopted are capable of giving results which are surprisingly accurate when one considers the large number of operations, transferences and filtrations incident to the methods.

**Mercaptans.**—If mercaptans are formed by the action of the bacteria of sewage, they would pass over into the wash bottles, there to be held as metallic derivatives just as hydrogen sulfide would be held, and would be included under what has been designated sulfur as hydrogen sulfide. The amounts of mercaptans formed, if any, would undoubtedly be very

TABLE IV.

| All results are computed to the basis of 3.0 g. of total solids excl. of KCl per 100 cc. in the blank, 0.0302 g. being taken as the amount of total S. | Absorbent. | G. per 100 cc. of total solids excl. of KCl. | G. per 100 cc. of unattacked S. | G. per 100 cc. of S as non-vol. sulfide. | G. per 100 cc. of S as $H_2S$. | Unattacked S plus S as $H_2S$. | % of S accounted for. | % of total solids, excl. of KCl, destroyed. | % of total S converted into $H_2S$. | % of total S converted into non-vol. sulfide. |
|---|---|---|---|---|---|---|---|---|---|---|
| Peptone, KCl blank..... | ... | 3.0000 | 0.0301 | 0.0002 | .. | 0.0301 | 99.67 | ... | ... | .. |
| Culture in Fernbach. Air passed 48 hrs..... | $CdCl_2$ | 2.8136 | 0.0210 | 0.0008 | 0.0094 | 0.0304 | 100.66 | 6.21 | 30.46 | 1.65 |
| Culture in Fernbach. Air passed 48 hrs..... | $Na_2O_2$ | 2.9064 | 0.0222 | 0.0014 | 0.0080 | 0.0302 | 100.00 | 3.12 | 26.49 | 3.64 |
| Culture in Fernbach. Air passed 48 hrs..... | KOH | 2.7296 | 0.0189 | 0.0007 | 0.0110 | 0.0299 | 99.01 | 9.01 | 37.42 | 1.32 |
| Culture in Fernbach. Air passed 48 hrs..... | $PbAc_2$ | 2.8055 | 0.0209 | 0.0013 | 0.0034 | 0.0243 | 80.46 | 6.48 | 30.79 | 3.31 |

TABLE V.

| All results are computed to the basis of 3.0 g. of total solids excl. of KCl in the 100 cc. blanks. | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Peptone, KCl blank..... | ... | 3.0000 | 0.0303 | 0.0003 | .. | 0.0303 | 100.33 | .. | ... | .. |
| Culture in Fernbach. Air passed 48 hrs..... | $Na_2O_2$ | 2.7836 | 0.0242 | 0.0006 | 0.0059 | 0.0301 | 99.67 | 7.21 | 19.87 | 0.99 |
| Part of peptone insol. in alcohol, KCl control | ... | 3.0000 | 0.0324 | 0.0005 | .. | 0.0324 | 100.00 | .. | ... | .. |
| Culture in Fernbach. Air passed 48 hrs..... | $Na_2O_2$ | 2.8055 | 0.0299 | 0.0009 | 0.0024 | 0.0323 | 99.69 | 6.48 | 7.72 | 1.23 |
| Culture in Fernbach. Air passed 48 hrs..... | KOH | 2.8986 | 0.0303 | 0.0010 | 0.0009 | 0.0312 | 96.30 | 3.38 | 6.48 | 1.54 |

TABLE VI.

| All results are computed to the basis of 3.0 g. of total solids excl. of KCl in the 100 cc. blanks. | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Peptone, KCl blank..... | ... | 3.0000 | 0.0302 | 0.0002 | .. | 0.0302 | 100.00 | .. | ... | .. |
| Culture in Fernbach. Air passed 48 hrs..... | KOH | 2.8430 | 0.0242 | 0.0006 | 0.0064 | 0.0306 | 101.32 | 5.23 | 19.87 | 0.99 |
| Culture in Fernbach. Air passed 48 hrs..... | $CdCl_2$ | 2.8092 | 0.0224 | 0.0013 | 0.0072 | 0.0296 | 98.01 | 6.36 | 25.83 | 3.31 |
| Part of peptone sol. in alcohol, KCl control.... | ... | 3.0000 | 0.0228 | 0.0002 | .. | 0.0228 | 100.00 | .. | ... | .. |
| Culture in Fernbach. Air passed 48 hrs..... | KOH | 2.8825 | 0.0225 | 0.0003 | 0.0003 | 0.0228 | 100.00 | 3.92 | 1.01 | 0.44 |
| Culture in Fernbach. Air passed 48 hrs..... | $CdCl_2$ | 2.9220 | 0.0224 | 0.0003 | 0.0001 | 0.0225 | 98.68 | 2.60 | 1.75 | 0.44 |

small and consequently no attempt was made to separate and determine them.

**Sulfur in Alcohol Extract of Peptone and in Residue from Alcohol Extraction.**—In order to ascertain whether the bacteria present in artificial sewage produce hydrogen sulfide more energetically or less energetically from those portions of peptone which are soluble in alcohol (containing lipoid sulfur) and insoluble in alcohol (containing protein sulfur) than they do from peptone itself, culture media were made, in one of which the part of peptone insoluble in alcohol was substituted for peptone, and in the other of which the part of peptone soluble in alcohol was substituted for peptone. The portions of peptone soluble and insoluble in alcohol which were employed, were obtained in the course of the work described in the preceding article.

Hydrogen Sulfide Production from the Part of Peptone Insoluble in Alcohol.—Two Fernbach culture flasks were prepared by introducing into each 500 cc. from a mixture of 150 cc. of a 30% solution of the part of peptone insoluble in alcohol, 75 cc. of 10% potassium chloride solution and 1275 cc. of strained artificial sewage. The remaining 500 cc. were immediately sterilized and kept as a control to be analyzed. One of the Fernbachs was attached to a wash bottle containing sodium peroxide solution (15: 225) and the other was attached to a wash bottle containing potassium hydroxide solution (20: 225) arranged as previously described. One Fernbach flask to act as a check, was prepared by introducing into it 500 cc. from a mixture of 100 cc. of 30% peptone solution, 50 cc. of 10% potassium chloride solution and 850 cc. of strained artificial sewage. The remaining 500 cc. were immediately sterilized and kept as a blank to be analyzed. The Fernbach flask was attached to a wash bottle containing sodium peroxide solution (15: 225).

The three Fernbachs were incubated for 48 hours at 38°, air in each case being sucked through a test tube containing the same solution as the respective wash bottle, then through the Fernbach flask, and finally through the wash bottle at as uniform a rate as possible. After 48 hours of incubation, each Fernbach was heated to boiling on a water bath for 30 minutes while air was sucked through it and its respective wash bottle. The solution from each Fernbach was then made up to 500 cc. (only 1 cc. to 2 cc. had been lost by evaporation in each case) and total solids, total sulfur, sulfur as nonvolatile sulfide and sulfur as hydrogen sulfide were determined in measured portions. In the determinations of sulfur as nonvolatile sulfide, the distillates were caught in 12% potassium hydroxide solution. The results of the analysis are given in Table V.

In both of the Fernbachs containing the medium made from the part of peptone insoluble in alcohol, much less hydrogen sulfide was produced than had been produced in media made from peptone itself. That this was not due to a less energetic bacterial flora in the artificial sewage than had been used in previous work, is proved by the fact that in the check Fernbach made up with the regular peptone medium and with the same sewage, the amounts of total solids exclusive of potassium chloride, total sulfur, sulfur as nonvolatile sulfide and sulfur as hydrogen sulfide, computed to the basis of 3 g. of total solids exclusive of potassium chloride per 100 cc. in the blank, were 2.7836, 0.0242, 0.0006 and 0.0059 g. per 100

cc., respectively, more than twice as much hydrogen sulfide having been produced as in either of the cultures containing the part of peptone insoluble in alcohol.

**Hydrogen Sulfide Production from Part of Peptone Soluble in Alcohol.**—A set of inoculations was made using a culture medium made from the part of peptone soluble in alcohol.

Two Fernbach culture flasks were prepared by introducing into each 500 cc. from a mixture of 150 cc. of a 30% aqueous solution of the part of peptone soluble in alcohol, 75 cc. of 10% potassium chloride solution and 1275 cc. of strained artificial sewage. The remaining 500 cc. were immediately sterilized and kept as a control to be analyzed. One of the Fernbachs was attached to a wash bottle containing cadmium chloride solution (10: 225) and the other was attached to a wash bottle containing potassium hydroxide solution (20: 225) arranged as previously described. Two Fernbach culture flasks to act as checks were also prepared by introducing into each 500 cc. from a mixture of 150 cc. of 30% peptone solution, 75 cc. of 10% potassium chloride solution and 1275 cc. of strained artificial sewage. The remaining 500 cc. were immediately sterilized and kept as a blank to be analyzed. One of the check Fernbachs was attached to a wash bottle containing cadmium chloride solution (10:225) and the other was attached to a wash bottle containing potassium hydroxide solution (20:225) arranged as previously described. The four Fernbachs were then treated in the manner described on page 620. The results of analysis are given in Table VI.

In both of the Fernbachs containing the medium made from the part of peptone soluble in alcohol, much less sulfur-containing material was broken down and very much less hydrogen sulfide was produced than had been produced in media made from the part of peptone insoluble in alcohol, which in turn had given smaller amounts than had been found in media made from peptone itself. That this was not due to a less energetic bacterial flora in the artificial sewage than had been present in previous tests is proved by the fact that in the check Fernbach flasks made up with the regular peptone medium and with the same sewage, the amounts of total solids exclusive of potassium chloride, of total sulfur, of sulfur as nonvolatile sulfide and of sulfur as hydrogen sulfide, computed to the basis of 3 g. of total solids exclusive of potassium chloride per 100 cc. in the blank, were, in the cases where cadmium chloride and potassium hydroxide, respectively, had been used in the wash bottles, 2.8092, 0.0224, 0.0013, 0.0072 g. per 100 cc.; and 2.8430, 0.0242, 0.0006, 0.0064 g. per 100 cc.

Hence, it is evident that a medium made with peptone as the sulfur-containing material is a better one to use for the detection of the bacteria producing hydrogen sulfide than are media made with the part of peptone soluble in alcohol or with the part of peptone insoluble in alcohol.

## Summary.

1. The percentage of sulfur present in the very small part of peptone which is insoluble in water is practically the same as in peptone itself. This explains why, in testing for bacteria which produce hydrogen sulfide, it makes no difference whether the medium is filtered or not.

2. The Liebig-Koch method for total sulfur and the potassium chlorate-nitric acid method for easily oxidized sulfur are as accurate for culture media made from peptone and potassium chloride as they are for peptone itself.

3. The ratio of total solids, exclusive of potassium chloride, to total sulfur is very nearly the same in the soluble and insoluble portions of cultures which have been inoculated with artificial sewage, containing bacteria which produce hydrogen sulfide, and incubated at 38° for 48 hours, and hence the indications are that the insoluble material is mostly peptone.

4. More material is broken down by the bacteria and more hydrogen sulfide is produced in flasks having much surface of the culture exposed to air than in flasks having comparatively little surface exposed.

5. It is noteworthy that, when sterile air was passed over the cultures, about 50% more total sulfur was converted into hydrogen sulfide than when they were exposed to quiescent air; and about 100% more than when sterile carbon dioxide was passed over them. Therefore, in the case of the flora which was used at least, the organisms were most active and produced the most hydrogen sulfide when supplied most freely with air. This fact would appear to be of great importance in connection with work directed toward the elimination of odors arising from septic tanks for sewage disposal.

6. From 25–30% of the total sulfur was convered into hydrogen sulfide when cultures were incubated 48 hours with sterile air passing over the culture, and from 50–60% when incubated 72 hours.

7. The volumetric iodine method for the determination of hydrogen sulfide is not available in the analysis of the gases given off by bacteria inoculated into the simple peptone medium, presumably because of the interference of volatile unsaturated organic compounds.

8. The use of potassium hydroxide is to be preferred for absorbing the hydrogen sulfide generated.

9. The sum of the sulfur remaining in the cultures after incubation plus the sulfur in gaseous form, which is practically all present as hydrogen sulfide, equals the total sulfur present in the cultures before incubation, when the determinations are made by the methods adopted in this investigation.

10. In media made from the part of peptone soluble in alcohol much less sulfur-containing material was broken down and very much less hydrogen sulfide was produced than was found in the media made from the part of peptone insoluble in alcohol, the ratios being about 3 : 5 and 1 : 6. In both of these media, very much less sulfur-containing material was broken down and very much less hydrogen sulfide was produced than in the simple peptone medium, the ratios being, respectively, about 1 : 2 and 1 : 20 in the soluble part of peptone, and 2 : 3 and 1 : 4 in the insoluble part of peptone.

11. As shown by the percentage of total solids exclusive of potassium chloride destroyed, and by the percentage of total sulfur converted into hydrogen sulfide, a larger percentage of sulfur-containing material than of total peptone was broken down, the ratio being about 3 : 1. The fact that the bodies of the bacteria were included in the total solids exclusive of potassium chloride, would not materially influence the ratio.

12. A larger percentage of easily oxidized sulfur than of total sulfur was converted into hydrogen sulfide by the bacteria, the ratio being about 4 : 3. It is evident that if a synthetic medium is to be prepared for the detection of the bacteria producing hydrogen sulfide, work upon which is herewith promised, that the sulfur should be introduced in an easily oxidized form.

13. A larger percentage of loosely bound sulfur than of total sulfur was converted into hydrogen sulfide by the bacteria, the ratio being about 3 : 2.

14. A very slightly greater percentage of loosely bound sulfur than of easily oxidized sulfur was convered into hydrogen sulfide by the bacteria, the ratio being about 10 : 9. There was about twice as much easily oxidized sulfur as there was loosely bound sulfur, both before and after the bacteria had acted upon the medium.

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[CONTRIBUTION FROM THE CHEMICAL LABORATORIES OF COLUMBIA UNIVERSITY, NO. 245.]

# STUDIES ON AMYLASES. VIII. THE INFLUENCE OF CERTAIN ACIDS AND SALTS UPON THE ACTIVITY OF MALT AMYLASE.

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The reaction most favorable to the activity of malt amylase has been given as acid, neutral and alkaline, by different investigators, and there are also striking discrepancies in the statements regarding influence of salts. To some extent, though not entirely, the confusion has arisen through lack of (or failure to use) adequate or uniform criteria of neutrality.

Baswitz[1] observed that carbon dioxide increased the diastatic activity of malt extract. Later Mohr[2] confirmed the observation and found that very small amounts of lactic acid could also be used with beneficial effects.

Kjeldahl[3] found that very small additions of sulfuric acid (making the reaction of the medium equivalent to 0.0005 $N$) increased the saccharo-

[1] *Ber.*, **11**, 1443 (1878); **12**, 1827 (1879).

[2] *Ibid.*, **35**, 1024 (1903).

[3] Dingler's *Polytech. Jour.*, **235**, 379, 452 (1880).