| Physical group. | Chemical group. | In per cent. of solids-not-fat. |
|---|---|---|
| Colloids | Proteins | 79.2 |
| | Phosphatides | 5.6 |
| Water-soluble-non-colloidal. | Extractives | 9.8 |
| | Ash | 5.6 |

Results on different tissues to be comparable should always be calculated in per cent. of solids-not-fat.

The methods here outlined may appear slow and laborious, but when we consider that they permit the estimation of all the above constituents in one sample, the amount of labor involved in each is not very great.

---

[From the Hull Physiological Laboratory, University of Chicago.]

## IV. ESTIMATION OF THE ELEMENTS, WITH SPECIAL REFERENCE TO SULPHUR.[1]

By W. Koch and F. W. Upson.

Received October 12, 1909.

Among the elements which go to build up the complex molecules of the cell constituents, carbon, hydrogen, and oxygen are not available for quantitative study on account of the lack of convenient methods. Nitrogen, phosphorus and sulphur can be studied and have besides a special significance as has already been pointed out. Thus nitrogen enters and leaves the organism as an unoxidized derivative. Phosphorus enters and leaves as an oxidized derivative, while sulphur enters in an unoxidized form but leaves as the highest oxidation product. The distribution of these elements in the various groups outlined in the previous article is also of special significance. Before going on to discuss this a few general points in the estimation of these elements may not be out of place.

In the selection of the best method to be used a number of factors have to be considered. Thus speed and the possibility of doing a great many analyses at once are quite as important considerations as extreme accuracy. Thus if we had to use the very accurate Dumas method of nitrogen estimation instead of the sometimes, but not always, equally accurate method of Kjeldahl,[2] only a fraction of the work in agricultural chemistry could have been done. Another point to be considered in the determination of an element is the complete destruction of organic matter, for in the first place, even if phosphorus is present as inorganic phosphate, its precipitation by the usual method would become extremely inaccurate on account of the organic material present. In the second place it is also frequently in actual combination with the organic material, in which case it would very often not give the ordinary precipitation

[1] See preceding papers.

[2] Kjeldahl, *Z. anal. Chem.*, **22**, 366 (1883).

reactions at all. The considerations which thus guide us in the selection of a method are speed, a sufficient degree of accuracy, and the possibility of completely converting the carbon and hydrogen into carbon dioxide and water; in other words, the complete destruction of the organic material. The three principal methods used for the latter purpose are:

(1) Destruction of the organic matter with sulphuric acid alone or in the presence of a catalytic agent which does not contain nitrogen. (Used for nitrogen.)[1]

(2) Destruction of the organic matter by means of sulphuric acid with the addition of fuming nitric acid. (Used for phosphorus; not safe for nitrogen.)

(3) Destruction of organic matter by fusion with an alkali such as sodium carbonate or sodium hydroxide, with or without the presence of an oxidizing agent, potassium nitrate or sodium peroxide. (Used for sulphur.)

(4) Simple incineration of the organic material in a muffle. (Not safe for S, P, or Cl, not available for N. Useful for Ca, Fe, etc.)

As has already been pointed out the estimation of total nitrogen, phosphorus or sulphur in a tissue does not give results of much value to the physiologist. The separation of the tissue constituents into the different groups outlined in the previous article must make the starting point for the further study of the elements. Thus we can speak of protein and extractive nitrogen, lipoid, extractive and protein phosphorus. This still does not give us any knowledge of the chemical individuals into which these elements enter, but indicates rather the line of further advance. For the nitrogen the nearest approach to this goal is the method of Hausmann, elaborated by Osborne.[2] Then there are the methods for the purine bases carefully studied in Mendel's laboratory.[3] For phosphorus we have the very good methods of Plimmer and Scott[4] which, with the exception of the possibility of estimating phospho-proteins, do not possess any additional value over the methods of Grindley and one of us already published. The subject of special study to be treated in this article concerns the sulphur, which up to the present has received the least attention.

[1] Kutscher and Steudel, *Z. physiol. Chem.*, **39,** 12 (1903). Folin, O., *J. Biol. Chem.*, **3,** 84 (1907).

[2] Osborne and Harris, THIS JOURNAL, **25,** 323 (1903). Osborne, Leavenworth and Brantlecht, *Am. J. Physiol.*, **23,** 180 (1908).

[3] Mendel, L. B., and Leavenworth, C. S., *Am. J. Physiol.*, **20,** 101 (1907), and **21,** 79 (1908). Kruger and Schmid, *Z. physiol. Chem.*, **45,** 1 (1908). Levene, *Am. J. Physiol.*, **12,** 278 (1905).

[4] Plimmer and Scott, *J. Physiol.*, **38,** 247 (1909). Grindley and Emmett, THIS JOURNAL, **28,** 25 (1906). Koch, *J. Biol. Chem.*, **3,** 159 (1907). Neumann, A., *Z. physiol. Chem.*, **37,** 131 (1902). Koch and Woods, *J. Biol. Chem.*, **1,** 203 (1905).

*Estimation of Sulphur. Outline of Method.*—In searching for a method suitable for the estimation of sulphur in the moist fatty material with which we are dealing, and in which it is sometimes necessary to estimate accurately 5–10 mg. of sulphur in 2–3 grams of organic material, the following difficulties must be taken into account: (*a*) The possibility of loss from too rapid combustion, especially with dry material, and the danger of spattering with material in a semi-liquid state. (*b*) The risk of incomplete combustion or oxidation, due to caking of the fusion mixture.

In an excellent review of all the methods Folin[1] finally recommends the sodium peroxide method. This, however, did not prove satisfactory with the moist fatty material we have heen investigating, and the method used by Schmiedeberg in his laboratory has been adopted.[2] This method gives very good results in cases where large amounts of organic material have to be destroyed, but it requires much time and care. The principle of the method consists in the gradual charring with an alcohol burner of the material in a mixture of seven parts of sodium carbonate and one part of potassium nitrate. This proportion of potassium and sodium avoids the source of error, pointed out by Folin, in the precipitation of the barium sulphate. The final burning is made with a Barthel alcohol burner at a temperature just below the fusion point of the mixture. After acidifying with hydrochloric acid a few drops of bromine water are added to remove any nitrous oxide in the solution, and the precipitation is done in the usual way.

*Description of Method.* (*a*) *Method of Fusion.*—One gram of the material is mixed with at least 12–15 grams of the fusion mixture ($Na_2CO_3$ seven parts, $KNO_3$ one part) in a lipped silver crucible of 30 cc. capacity, and *very gradually* charred over an alcohol spirit lamp. This process should take several hours, and the fumes from the burning should never rise so rapidly as to discolor very much the top layer of the fusion mixture. Should the fumes come off too rapidly, the spirit lamp must be removed for a short time and fresh fusion mixture sprinkled on the top of the mixture. In this manner the fumes arising from the burning are made to pass through a layer of fresh fusion mixture, and any sulphur fumes are kept back. After the material has completely charred and no more fumes come off even on placing the flame in direct contact with the crucible, the mass after cooling is thoroughly powdered in an agate mortar with the addition of about 1–2 grams of fresh fusion mixture. It is then returned to the crucible, a layer of fusion mixture sprinkled on top, and heated with the Barthel alcohol burner at a temperature

[1] Folin, O., *J. Biol. Chem.*, 1, 131 (1905); 3, 81 (1907). Grindley, H. S., and Gill, F. W., THIS JOURNAL, 31, 52 (1909).

[2] Koch and Mann, *Archives of Neurology and Psychiatry*, 1909, 4.

sufficiently low to avoid caking of the fusion mixture. The burning must not be hurried. The combustion will be more complete after heating at a moderately low temperature for half an hour, than by trying to force the process, for any caking is apt to include black specks which are difficult to oxidize, and not only may contain unoxidized sulphur, but also actually reduce some of the sulphates already formed, *e. g.*, the Leblanc soda process. The fusion mixture, after cooling, is transferred to a 600 cc. Jena Erlenmeyer flask, and the crucible boiled out twice with hot water to dissolve any silver sulphate which may have formed through contact with the crucible. If the mixture has caked a little this process of boiling out becomes much more difficult, and almost invariably a black stain of silver sulphide will be seen on the crucible. The loss from this source of error may amount to 1 mg. of barium sulphate. The solution with washings is carefully acidified with hydrochloric acid and heated on a water bath to drive off the carbon dioxide evolved. It is then filtered and to the boiling filtrate 5–10 cc. of 10 per cent. barium chloride solution are added and the barium sulphate estimated in the usual manner.

It is usually considered advisable in making sulphate estimations to obtain a weighing of 100 mg. or more barium sulphate. As this would involve the destruction of very large amounts of organic material it was decided in these analyses to aim at 40–60 mg. of barium sulphate, considering that the error occasioned by a somewhat smaller weighing would be more than compensated for by the more complete extraction of the material and the decrease in the sources of error accompanying the destruction of large amounts of organic material. The 100-gram sample of moist brain material recommended in the collection of material accomplishes this.

*Estimation of Sulphur, Lipoid Sulphur* (special for nerve tissue).—The lipoids after filtration following the acid chloroform precipitation (see previous paper by Koch and Carr) represent a sticky mass adhering to the flask and filter paper. The mass on the filter paper can be washed off with hot alcohol. The destruction of all the lipoids from a 100-gram sample, however, involves an almost hopeless task, and would yield about 250 mg. barium sulphate. In order to keep the estimation within the limits of accuracy, adopted for the other fractions, it becomes necessary, therefore, to take an aliquot part. This can only be done by dissolving the mass in alcohol in the 1,000 cc. flask in which the precipitation was originally made and making up to the 1,000 cc. mark with alcohol; 200 cc. of this solution should then be used. This procedure, however, is complicated by the fact that the lipoids are only soluble in hot alcohol, which makes the taking of an aliquot part a matter of some difficulty. Before finally

adopting this method, therefore, it was necessary to try some control experiments. These were done as follows:

*Control Exp. I.*—A 50-gram sample of brain was treated in the usual way, and the lipoids, after dissolving in hot alcohol, made up to 500 cc. While warm 200 cc. were taken with a warm pipette and the sulphur estimated. The sulphur in the remaining 300 cc. of the solution was then also estimated. The yields were 24.0 mg. and 36.0 mg $BaSO_4$ respectively, which figures are in the proportion of 200 : 300.

*Control Exp. II.*—From three 100-gram samples of three brains the lipoids were made up to 1,000 cc. and 200 cc. aliquot parts taken. From these same brains 20-gram samples of material were collected, and the sulphur in the total lipoids estimated without taking an aliquot part.

The following table gives the results:

| | Barium sulphate. | | S. calculated in per cent. of dry matter. | |
|---|---|---|---|---|
| Case No. | Aliquot part. mg. | 20 g. sample. mg. | Aliquot part. | 20 g. |
| 19..... | 51.8 | 43.7 | 0.154 | 0.135 |
| 28..... | 33.4 | 31.6 | 0.192 | 0.097 |
| 42..... | 43.5 | 42.0 | 0.125 | 0.121 |

The agreement is better in the last samples which were estimated at a later time when more experience had been acquired in taking the aliquot part. There is a tendency for the aliquot part to come out higher; this can no doubt be accounted for by the cooling of the liquid in the pipette, and must be avoided as much as possible.

*Control Exp. III.*—Seventy-seven grams of blood which should contain no lipoid sulphur were treated in the same way. All the lipoid precipitate was destroyed and gave no weighable quantity of barium sulphate. Water-soluble organic sulphates do not, therefore, adhere to the lipoid precipitate in sufficient amount to account for the quantities found. This experiment serves as a negative control.

*Details of Method.*—After the lipoid precipitate has been allowed to drain and the volume of the filtrate recorded, the funnel is placed in the liter flask originally used and a hole punched in the bottom of the filter paper with a glass rod. By means of a hot alcohol wash bottle all the sticky mass adhering to the filter paper and to the glass rod is completely washed into the flask. It is better to use 95 per cent. alcohol for this. The amount of alcohol in the flask should now be 400–600 cc.; the bulk is made up to about 900 cc. with absolute alcohol and the whole warmed on a water bath until complete solution has taken place. Great care must be taken that the sticky mass, which has a tendency to adhere to the bottom of the flask and which may easily be missed, as it is rather transparent, has been completely dissolved. The shaking of the flask must also be carefully done, as too violent shaking may cause the liquid to boil over and thus spoil the analysis. When everything appears to have dissolved, enough warm alcohol is added to make the bulk, 1,000 cc. While keeping the flask on the water bath, a 100 cc. pipette is now introduced, and by carefully drawing up the liquid and allowing it to again flow back into the flask, at the same time turning the flask, a uniform mixture can be obtained. This treatment at the same time warms

the pipette. 200 cc. are removed as carefully and rapidly as possible, evaporated to a semi-pasty condition and then mixed with fusion mixture.

It is perfectly futile to attempt the destruction of this organic matter with less than 40 grams of fusion mixture distributed between three silver crucibles of the size previously described. The difficulties experienced at this point will soon convince any one how practically impossible it would be to try to burn all the lipoid precipitate in this manner instead of taking an aliquot part. There seems to be an unnecessary amount of detail in this description, but we feel convincedthat any one attempting to repeat these analyses with any aim at accuracy would wish there had been more, as the factors which from time to time tended to spoil analyses seemed almost infinite.

*Estimation of* $S_2$ (*Extractive Sulphur*).—The water solution filtered from the lipoid precipitate is evaporated to moist dryness, mixed with 10 grams fusion mixture and the sulphur estimated. In adding the fusion mixture care must be taken to prevent excessive spattering due to the liberation of carbon dioxide by the acid present. It is also well *not* to heat this estimation to as high a temperature with the Barthel burner as the others, on account of the fact that the larger amount of sodium chloride present is apt to cause it to cake, also the sodium chloride is apt to attack the silver crucibles and bring about the formation of a colloidal form of silver chloride in the final solution, and this spoils the neatness of the barium sulphate precipitation.

*Estimation of* $S_2^I$ (*Inorganic Sulphates*).—The filtrate from a second 100-gram sample is evaporated to about 300 cc., filtered, if necessary, and the sulphate estimated directly by barium chloride. The weighings of barium sulphate here amount to about 12–15 mg. Too much confidence must therefore not be placed in this result; it is better to regard it in the nature of a correction, by means of which it is possible to estimate the organic part of the $S_2$ fraction. Sulphates appear to be eliminated from the tissues as rapidly as they are formed, so that the amount present at any time is never very great and the variations are of comparatively little significance (3–4 per cent. of total sulphur).

*Estimation of* $S_3$ *and* $S_4$.—The protein residue insoluble in alcohol is dried, weighed, and allowed to come into equilibrium with the moisture of the air as previously described. One gram is then taken, mixed with 12 grams fusion mixture and the sulphur estimated. Great care must be taken *not* to hurry the preliminary burning, as sulphur may be lost on account of the dry nature of the material. The result represents $S_{3+4}$. The remainder of the protein residue is then extracted with hot water (as directed in the previous article), the extracts evaporated, dissolved in 15 cc. hot water and 85 cc. absolute alcohol added. The dilute alcohol solution is filtered, evaporated to dryness, and sulphur estimated. This

sulphur in reality belongs to the $S_2$ group as it represents sulphur compounds soluble in water not precipitated by 85 per cent. alcohol. It usually amounts to 1.5 per cent. of the total sulphur, or in the case of an adult brain to about 15 per cent. of the $S_2$ fraction. The result is therefore added to the $S_2$ and subtracted from the $S_{3+4}$.

*Estimation of* $S_3^I$ (*Inorganic Sulphates*).—From another 100-gram sample the eight hot water extracts of the alcohol-insoluble residue are evaporated with 5 cc. conc. hydrochloric acid to 300 cc., filtered if necessary, and the sulphates estimated directly with barium chloride. This result plus the above alcohol-soluble fraction are subtracted from the result for the $S_{3+4}$ and the resulting figure is considered to represent the protein or protein-like sulphur.

We have so far considered the estimation of the following groups of sulphur compounds in a given tissue: Lipoid, extractive, protein, and sulphates. With the exception of the sulphates these groups probably contain the sulphur in more than one kind of combination. There should be especially considered the ethereal or $R\text{-}SO_2OH$ and the cystine or R–S–H type of grouping. In a previous publication by one of us[1] it was found that the lipoid sulphur is in the form of an ethereal sulphate, a result which was again confirmed. A slight amount of ethereal sulphate was also found in the protein group (1.7 per cent. of total S). The greater part of the protein sulphur consists, however, of the cystine-like grouping. In the $S_2$ group a small amount of a sulphur-containing compound is precipitated by tannic acid. The amount is however too small to be of much significance. The study of the SH grouping seemed of especial interest.

*Estimation of SH Sulphur. Outline of Method.*—The two principal investigations of this subject are by Schulz[2] and Osborne.[3] The main difficulty to be overcome in devising a method for estimating this group quantitatively consists in finding an oxidizing agent sufficiently strong to separate the sulphur from its combination with carbon, which at the same time does not oxidize the sulphur. The method finally adopted is essentially the one used by Osborne and consists in splitting off the SH by sodium hydroxide at a temperature of 150° in the absence of oxygen. The hydrogen sulphide is then liberated by acidification and driven over into sodium carbonate solution. This is liberated with 0.01 *N* iodine solution. From a sample of pure cystine this method splits off about 2/3 of the sulphur, so that it can only be considered as of a qualitative nature.

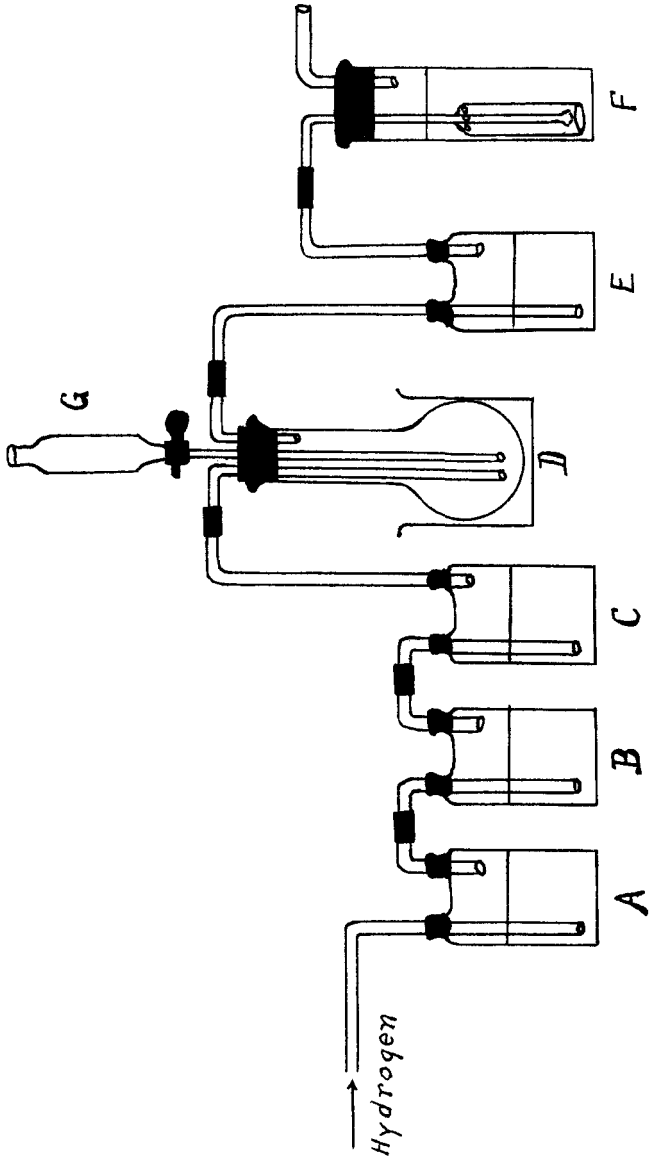
*Details of Method.*—The form of apparatus used is shown in the diagram.

[1] Koch, *Z. physiol. Chem.*, **53**, 497 (1907).

[2] Schulz, *Ibid.*, **25**, 16 (1898).

[3] Osborne, THIS JOURNAL, **24**, 140 (1902).

The fusion with sodium hydroxide is carried out in flask D in an atmosphere of hydrogen. The flask is heated by means of an oil bath. The wash bottles A and B, respectively, contain a strong alkaline solution




of lead acetate and potassium permanganate. These are necessary to remove any impurities, such as hydrogen sulphide, which may be contained in the hydrogen. Bottles C and E contain dilute sulphuric

acid. F is a Folin absorption apparatus containing a saturated solution of sodium bicarbonate for absorbing the hydrogen sulphide. During the fusion E and F are not connected. In the case of protein residues approximately half-gram samples are placed in flask D together with about 30 cc. of 50 per cent. sodium hydroxide and heated at a temperature of 140–150° for one and one-half hours. A slow current of hydrogen is passed through to prevent bumping, as well as to keep the apparatus free from oxygen. At the end of this time, after cooling, connection is made with the absorption apparatus and a moderate excess of sulphuric acid added through the dropping funnel, G. The contents of the flask are again heated nearly to boiling and hydrogen is bubbled through for forty-five minutes. This is sufficient to drive the hydrogen sulphide over into the absorption apparatus. The contents of F are then titrated with 0.01 *N* iodine solution, using starch as the indicator. (1.0 cc. of this solution is equivalent to 0.16 mg. S.) Several blank determinations showed that not more than 0.1–0.2 cc. of the iodine solution were necessary to produce the blue color with the starch.

*Sources of Error.*—In the first place a high result is usually due to a contamination of the hydrogen gas with hydrogen sulphide. The hydrogen should be passed through slowly and the washing solutions in A and B must be changed frequently. On the other hand, too low results may arise from incomplete fusion due to insufficient heating or from the use of too dilute alkali. After acidification care must be taken to sweep over all the hydrogen sulphide into the sodium bicarbonate solution.

This method admits of the determination of very small amounts of sulphur, much smaller than could be determined by splitting off as lead sulphide and converting to the sulphate. In the judgment of the writers 1–2 mg. of sulphur can be determined with a fair degree of accuracy.

*Estimations of SH Sulphur in the $S_2$ Fraction.*—In obtaining the $S_2$ fraction a slight change from the usual procedure is made. After the emulsification of the residue from the alcoholic extracts sulphuric acid instead of hydrochloric acid is used in the precipitation of the lipoids. The hydrochloric acid might interfere in the SH estimation later. Five cc. of sulphuric acid and 15 cc. of chloroform are added and the whole made up to 1000 cc.; after standing for several days the lipoids are filtered out in the usual manner. The watery filtrate is made slightly alkaline with sodium hydroxide and evaporated in vacuum in an atmosphere of hydrogen, the final evaporation being made in the flask in which the fusion is to be carried out. The sodium hydroxide is added to this residue and the estimation of the SH sulphur carried out as above described.

*Estimations of the SH Sulphur in the $S_4$ Fraction.*—The protein residue after the final extraction with water and absolute alcohol is dried at

100°, finely powdered, and allowed to come into equilibrium with the moisture of the air and weighed. Half-gram samples of this are used for the estimation of the SH sulphur.

## Summary.


DISTRIBUTION OF SULPHUR IN PER CENT. OF TOTAL SULPHUR IN BRAINS AT DIFFERENT AGES.

| | | | $S_2$. Extractive. | | | $S_3$ Extractive. | | $S_4$. Protein. | |
|---|---|---|---|---|---|---|---|---|---|
| | | | Organic, soluble in water and 85 per cent. alcohol. | | | | | | |
| Age. | Sulphur in per cent. of solids. | $S_1$ Lipoid $(RO)_2SO_2$. | Unoxidized R—SH. | Partially oxidized R—$SO_2OH$. | $S_2^I$. Oxidized $SO_2(OH)_2$. | Organic, soluble in water and 85 per cent. alcohol. | $S_3^I$. Oxidized. $SO_2(OH)_2$. | Unoxidized R—SH. | Partially oxidized in unknown combination.[1] |
| At birth... | 0.58 | 3.8 | 7.8 | 16.6 | 4.4 | 1.4 | 2.5 | 38.5 | 24.9 |
| 43 years... | 0.50 | 24.0 | (2.0) | 5.8 | 1.9 | 1.5 | 2.3 | 43.5 | 19.0 |
| 64 years... | 0.49 | 18.6 | 1.8 | 3.7 | 2.7 | 1.9 | 2.5 | 42.0 | 26.9 |

*The Variation* in the total sulphur is not very great. The greatest variations are: An increase in the lipoid sulphur with age, which later again decreases. A decrease in the water-soluble organic sulphur with age. The presence of SH sulphur among the group of extractives is of especial interest and will be taken up more in detail in a later paper. The physiological interpretation of these results will be reserved until more data can be collected.

The methods here outlined are then capable of detecting differences in the same tissue at different ages, or in different states of physiological activity (growth).


---

[FROM THE DEPT. OF AGR. CHEMISTRY OF THE UNIVERSITY OF WISCONSIN.]

# THE QUANTITATIVE ESTIMATION OF LACTIC ACID IN CHEESE.

BY S. SUZUKII AND E. B. HART.

Received October 9, 1909.

In connection with chemical studies of cheese ripening that are being conducted at the Wisconsin Experiment Station, in co-operation with the Dairy Division of the Bureau of Animal Industry, United States Department of Agriculture, it has been necessary to employ some quantitative method for the estimation of lactic acid. In order to fix upon the most reliable method for this work, preliminary examination of the accuracy and applicability of several methods already proposed was undertaken.

[1] By difference.

[2] Expert in dairy chemistry, Dairy Division, United States Department of Agriculture. Engaged in co-operative work between the Division and the Station.