Example—Lecithin in muscle.

	Sample placed in alcohol direct.		Sample dried in stream of warm air and then extracted.	
Lecithin..................	4.1	4.4	3.4	3.2

3. *Advantages of Adding Sodium Sulphate or Gypsum.*—(*a*) In cases, as for instance milk, where the material contains such a large amount of water that it would unduly dilute the alcohol or would be difficult to dry in a stream of hot air, a thin paste is made by adding about $^1/_3$ the weight of anhydrous sodium sulphate and the drying then proceeds rapidly.

(*b*) In special investigations such as those of Schryver[1] on autolysis and Rosenheim[2] in his estimation of cholesterol in the brain.

Disadvantages of Adding Sodium Sulphate or Gypsum.—(*a*) The fact that it is only possible to make a limited number of analyses with material preserved in this way.

In planning an investigation a consideration of the above factors will decide the choice of the preservative. In the work on the brain it has been found best to use alcohol and the methods to be subsequently described are based on that method of preservation.

Details of Method of Collecting and Preserving Material.—30–100 grams of the tissue, ground fine and thoroughly sampled as discussed above, are placed in a large mouthed, preferably glass stoppered, bottle and sufficient absolute alcohol added to bring the concentration up to at least 85 per cent. The amount of alcohol to be added depends upon the moisture content of the tissue. Thus 100 grams of tissue containing 25 per cent. of moisture, would require from 200–300 cc. of alcohol. After adding the alcohol the material should be thoroughly shaken up at intervals and the next day should be warmed to 75° by placing in a basin of water and gradually raising the temperature. It is better not to begin the analysis less than two weeks or more than 3 months after collecting the sample. It is well to make a note as to the time which elapses between the collection and analysis of the material, as some changes take place. (See alcohol preservation, disadvantage, *e*.)

[FROM THE HULL PHYSIOLOGICAL LABORATORY, UNIVERSITY OF CHICAGO.]

III. ESTIMATION OF THE PROXIMATE CONSTITUENTS.[3]

BY W. KOCH AND EMMA P. CARR.

Received October 12, 1909.

The six main groups of proximate constituents into which we have provisionally divided the constituents of a tissue are (1) Lipoids, (2) Ex-

[1] Schryver, S. B., *Biochem. J.*, **1**, 131 (1906).

[2] Rosenheim, O., *J. Physiol.*, **34**, 105 (1906).

[3] For introduction, see preceding papers.

tractives, (3) Ash, (4) Proteins, (5) Fat, (6) Glycogen. Although in some of these groups there may be found a number of chemical individuals which differ quite markedly in their structure, in their more general properties as well as physiological rôle, members of the same group more or less resemble one another. As a preliminary step to the more careful investigation of the individual members of the groups this arrangement, although only in a limited sense meeting the requirement of chemical accuracy, seems justified.

A brief description of these groups follows:

1. *Lipoids.*—Compounds containing C, H, O, often N and P, sometimes S. Soluble pretty generally in most of the fat solvents. Behave like colloids towards water. Play a rôle in the control of the chemical activities of the cell by the formation of semipermeable membranes. Their fatty acid radicles serve only incidentally as sources of energy.

2. *Extractives.*—Compounds containing C, H, O, often N, P and S. Soluble in water and to a certain extent in dilute alcohol. Non-colloidal molecules. Represent the watery solution in which the colloids of the cell are suspended and in which most of the chemical activities of the cell probably take place.

Inorganic Constituents.—Cations: K, Na, Ca, Mg, Fe. Anions: SO_4, PO_4, CO_3, Cl; very inadequately represented by the ash, especially the anions. Exist as free ions and in combination with the colloids and organic radicals. Have the function of controlling the state of aggregation of the colloids and thus play a rôle in the control of the chemical activities.

Proteins.—Simple proteins: Compounds containing C, H, O, N and S built up by dehydration from simple and substituted amino acids. Behave towards water like colloids. Insoluble in alcohol. Share with the lipoids the function of forming semipermeable membranes. The complexity of their structure makes possible a high degree of differentiation and specificity in different tissues.

Compound proteins: Contain C, H, O, N, S and P compounds of simple proteins with some radicals not an amino acid. Have a very complex state of aggregation and are more probably present as a rule as colloidal suspensions rather than colloidal solutions. The nucleoproteins are supposed to play a rôle in the oxidations of the cell.

Fat.—Compounds containing C, H, O exist to a large extent as large aggregates (fat droplets) which are usually inert, but can be drawn upon for chemical activities. Fat is one of the principal sources of energy of the organism.

Carbohydrates.—Compounds of C, H, O represented mainly by glycogen in animal tissues. Probably exist as granules, but can be very quickly

utilized. They represent the most easily available source of energy at the disposal of the tissues.

Water.—The most important constituent of all. Not mentioned in the above grouping as results are invariably reported in percentage of solids.

The methods to be described have been in use for some time in my studies on the chemistry of the brain[1] but are here put in a more general form so as to be applicable to all tissues.

Principles of Extraction of Constituents.—The six groups of constituents mentioned above, are separated into four general fractions by solvents according to the following outline: Alcohol has proven to be the most satisfactory solvent, and although it is desirable in a quantitative study of this kind to rely on methods of separation by solvents as little as possible, observations which are recorded later (p. 1347) indicate that the separation of lipoids and fats from the proteins is as good as can be found at present.

MOIST TISSUE.

Add alcohol and extract alternately with alcohol and ether.

Extracts.		Residue.	
Evaporate to dryness, emulsify with water, ppt. with $CHCl_3$ in 0.5 per cent. HCl soln.		Dry, weigh, extract with hot water.	
(1) Lipoids.	(2) Filtrate.	(3) Filtrate.	(4) Residue.
Phosphatides. Cerebrins. Cholesterol. Sulphur compound. Neutral fat.	Water-soluble extractives. Inorganic constituents (ash).	Water-soluble extractives. Inorganic constituents (ash).	Proteins. Nucleoproteins.

In this plan for the separation of the different groups of constituents the following fractions are first obtained:

1 and 2. Fraction soluble in alcohol (85–95 per cent.).

3. Fraction insoluble in alcohol, soluble in hot water.

4. Fraction insoluble in alcohol and hot water.

Although ether is used in the extraction following the first alcohol, it does not remove any considerable amount of material and need not be considered in the above scheme. The apparatus used for the extractions is a modification of the old form of Wiley extraction apparatus, and was designed for us by Gallenkamp and Co., London. The advantages of this form over the Soxhlet, especially for work with nerve tissue, consists in the fact that the extraction takes place at the boiling point of the solvent.

The apparatus (Fig. 1) consists of a wide mouthed, carbon dioxide flask

[1] Koch, W., *Am. J. Physiol.*, 11, 303 (1904).

of 300 cc. capacity into which is fitted, by means of a ground glass connection, a small double surface condenser. On the lower end of the condenser are fused two glass hooks, from which is suspended in a thin platinum wire sling a 40 cc. perforated cup. Each condenser is fitted with at least three interchangeable flasks. If dry heat is used for the extraction the above form of condenser answers admirably, but should a water bath be used, the escaping steam will condense on the external surface of the condenser and, creeping into the ground glass connection, will possibly cause it to stick. With a water bath, a Hopkins' condenser in which the cooling is accomplished from the inside prevents this source of annoyance. Electric hot plates have the disadvantage that they are heated irregularly, but we have found them, *when used carefully*, to effect a great saving in time, and also to eliminate all risk of fire. When once started they require but little supervision, and can be left running during the night for the final extractions.

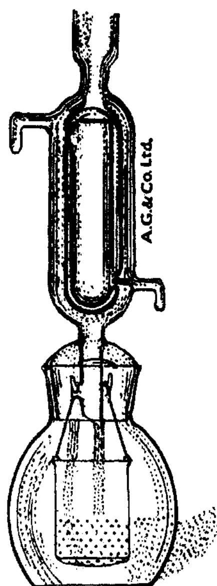

Fig. 1.

For the extraction the material is transferred to the perforated cups and first extracted for 3–4 hours with 95 per cent. alcohol. As this removes about nine-tenths of the alcohol-soluble portion, it is better to discontinue heating on account of the danger of decomposing some of the compounds now in the alcohol solution. The alcohol extraction is followed with an ether extraction, and this, although it does not remove much material, has the advantage of rendering most tissues extremely brittle, so that they can be finely powdered, which procedure greatly aids the completeness of the later extraction. After powdering, the material is mixed with water and heated; absolute alcohol is then added to bring the concentration to at least 85 per cent. This procedure ensures the more complete extraction of these extractives, which are only slowly soluble in 95 per cent. alcohol, but which, if the extraction were continued long enough, would finally come out and really belong to the alcohol-soluble fraction. The material is again transferred to the cups and extracted for twelve hours with 95 per cent. alcohol. To continue the extractions longer than this is hardly necessary, for, although it is practically impossible to separate completely the constituents of any tissue by extraction with solvents, the following control experiments indicate that the error is not very great.

One hundred grams of brain material were extracted twice with alcohol and once with ether, as outlined above and described in detail later, and the amounts of sulphur and phosphorus removed estimated. The

material was then again ground up and remixed and subjected to another alcohol extraction for twelve hours, and the total sulphur and phosphorus in the extract estimated. The results are as follows:

3 hours alcohol, 2 hours ether, 12 hours alcohol extractions yielded	253.7 mg. $BaSO_4$
A further 12 hours alcohol extraction yielded	6.5 mg.
	260.2
Percentage extracted in first series	97.5

On the same sample phosphorus estimations were made and yielded:

First fractions	893.1 mg. $Mg_2P_2O_7$
Final fraction	6.0 mg. $Mg_2P_2O_7$
	899.1 mg. $Mg_2P_2O_7$
Percentage extracted in first series	99.3

Although the total amount of sulphur extracted in the final fraction is only 0.9 mg. as compared with 1.7 mg. phosphorus, it calculates to a little higher percentage of total on account of the smaller amount of total sulphur extracted with alcohol. The completeness of the extraction is, therefore, fairly satisfactory, especially as the error largely becomes eliminated in a series of comparative experiments such as we are dealing with here.

The residue insoluble in alcohol represents essentially proteins (the amount of glycogen in most tissues except liver is too small to be considered) plus a correction for water-soluble, alcohol-insoluble extractives (about 2 per cent. of the total residue). As these extractives, however, are rich in inorganic phosphates and sulphates, it is necessary, after weighing, to extract this residue with hot water. Eight extractions are usually sufficient. In these extractions it is well to exercise great care to avoid the danger of bacterial decomposition, as the results can be completely vitiated by this source of error.

Details of Method.—Material which has been preserved in 30 and 100 gram lots in 150 to 400 cc. of absolute alcohol and kept, with occasional heating to ensure complete coagulation of the proteins, for not less than two or more than four months, is transferred to the cups as follows: The perforations of three cups are covered with filter paper by cutting one round piece to fit the bottom end and one oblong piece to fold against the side. The filter paper is moistened with alcohol and pressed firmly against the sides with a glass rod. Even if all the perforations are covered with filter paper, small particles of material will pass through the cracks where the two pieces join, but if the adjustment of the filter paper has been carefully made, these cracks soon fill up and the filtrate is perfectly clear. The cups are then placed in large funnels which drain into

beakers placed below and the material carefully transferred. It requires usually three cups to hold 100 grams of material.

At first, as stated above, the filtrate is not clear, but when it begins to come through clear the beakers are changed and the first filtrate refiltered. After all the material has been transferred to the cups in this manner and the last particles washed in with hot alcohol, the cups are allowed to drain and then carefully transferred to the extraction apparatus.

In order to ensure a better distribution of the alcohol, as it drips into the cup from the condenser, a perforated plate is placed on top of the material in the cup; the extraction is started with 95 per cent. alcohol at the medium temperature of the electric plate and continued for 3–4 hours. At this temperature the alcohol boils, and about every two seconds one drop falls from the condenser and thus keeps the material thoroughly soaked. After 3–4 hours the apparatus is allowed to cool, clean flasks substituted, and an ether extraction continued for 2–3 hours. The apparatus is again allowed to cool and the material carefully ground up to as fine a powder as is possible in an agate mortar. When removing the material from the cups, care must be taken not to disturb the filter paper, as it is to be used again when the material is returned to the cups. The powdering of the material may be a slow and laborious process, but should be carefully and completely done in order to ensure complete extraction later. The powdered material is transferred to a 600 cc. Jena flask, moistened with 50 cc. water and heated on a water bath for one hour. At the end of this time 450 cc. absolute alcohol are added, the mixture is warmed, well shaken and allowed to stand over night. The material is again filtered into the cups, as previously described. and extracted for twelve hours with 95 per cent. alcohol. The flasks containing the alcoholic extracts, the beakers containing the filtrates, and the flasks containing the ether extract are put aside, and their manipulation will be described later under the separation of the lipoids. The residue in the cups is now transferred to a weighed beaker or platinum basin and dried at 105–110° to constant weight. This weight represents the total protein plus about 2 per cent. of extractives and ash (not removed by the alcohol), and is designated as Fractions 3 and 4. It is transferred to a 300 cc. Jena flask and moistened with 100 cc. water; the flask is plugged with a loose cotton-wool stopper and heated on a water bath for one hour. This eliminates all risk of bacterial decomposition until next day, when the fluid is filtered into a 600 cc. Jena beaker. The residue is washed back into the flask and 100 cc. of water again added and the process repeated on each of eight successive days. It is imperative that after the cotton-wool stopper has been removed, the material is not kept too long before it is again sterilized. The *precautions* are

merely those which will occur to any one familiar with *bacteriological technique.* One point, however, may escape notice. In case it is not possible on account of press of work to start the water extractions the next day after the residue has been dried, it is not safe to allow it to stand. The amount of moisture which is absorbed from the air is sufficient to enable bacteriological growth to commence, and we have frequently noticed samples which, although they were dry when transferred to a dry flask, develop a decided odor of putrefaction on standing. It is advisable in such a case to thoroughly soak the residue in 15–20 cc. chloroform and carefully stopper it before putting it aside. It is also well to add a little chloroform each time before placing the cotton stopper in the flask.

The filtrates as obtained from day to day are best evaporated to avoid the danger of bacterial decomposition. After the last extraction has been evaporated, the whole residue is again dissolved in about 150–200 cc. of hot water, filtered hot, evaporated, weighed and ashed as will be described later. The slight amount of residue which remains insoluble in the hot water represents coagulable protein, which either had not been hardened by the original alcohol treatment, or was again rendered soluble by the hot water extractions.

Separation of Lipoids.—The alcohol-soluble portion (Fractions 1 and 2) is now contained in three flasks from the first alcohol extraction, three flasks from the ether extraction, two flasks from the second alcohol extraction, and five beakers, three from the first filtrate in which the sample was originally preserved, and two from the treatment after grinding the material in an agate mortar. In the first set of these flasks a considerable amount has separated on cooling. All the material extracted with alcohol and ether must then be transferred to two or at most three Jena dishes of a suitable size for weighing. The evaporation must be carried on at a low temperature, and in no case must the samples be evaporated to complete dryness on the water bath or electric plate. After they have been reduced to small volume, the residual liquids are allowed to cool and the evaporation finished in a vacuum desiccator. In applying the vacuum great care must be taken to avoid spattering. All the alcohol must be removed as completely as possible, as it interferes with the later procedure. After about one week the samples are dry and free from alcohol and must be weighed to constant weight. They are then moistened with water, allowed to stand over night and the next day transferred to a graduated 250–1000 cc. flask. If the material has been well moistened it can easily be removed from the dishes as a watery emulsion. With fatty material this transfer is greatly aided by the use of chloroform, which, however, must be measured (see below). After all has been transferred to the liter flask it is well shaken and 10 cc.

conc. hydrochloric acid added. After again shaking, 15–25 cc. chloroform are added (see above) and the bulk made up to the mark. The flask is again well shaken and allowed to stand in a cool place for three to seven days, when a perfectly clear filtrate can be obtained. The danger of decomposition of the lipoids from this acid treatment has already been investigated[1] and occasional control experiments made in the course of the work have confirmed these negative results. If the filtrate is not perfectly clear, it can be shaken up again with chloroform and allowed to stand in a cool place; the solution is then again filtered through a fresh filter paper. In order to obtain a complete separation of the lipoids and the water-soluble extractives it would be necessary to wash the lipoid precipitate with acid water. This, however, has not been found practicable, but instead the filter paper containing the lipoid precipitate is allowed to drain thoroughly and the volume of the filtrate recorded. The difference between this volume and the total volume minus the chloroform approximately represents the amount of liquid adhering to the lipoid precipitate. In order to make this figure more accurate it is necessary to start the filtration through a dry filter paper. The filtrate from the lipoids is evaporated to constant weight and then ashed as will be described later. The following example will illustrate the case:

27.99 grams of moist muscle extracted with alcohol and ether gave residue of fraction (1 and 2) soluble in alcohol 3.3464 grams, residue of fraction (3 and 4) insoluble in alcohol 4.8980 grams. This gives total solids 8.2444 grams or in per cent. 29.4.

Residue 1 and 2 (alcohol extract) was emulsified with water, transferred to a 250 cc. flask with 15 cc. of chloroform and 5 cc. of conc. hydrochloric acid, made up to mark and allowed to stand six days. Filtered through a dry filter, 229 cc. of filtrate were obtained. Considering that 15 cc. of the 250 cc. total were represented by chloroform, this would leave 6 cc. ($250 - 15 - 229 = 6$) filtrate clinging to the lipoids. The bulk of the lipoids could at a maximum be only 3 cc., which is neglected as it just about counterbalanced the chloroform lost by manipulation and soluble in the filtrate. The residue from the 229 cc. filtrate dried to constant weight at 105° was 0.6804 gram. This becomes 0.6984 gram by correction for the 6 cc. clinging to the lipoids.

Residue (3 and 4) insoluble in alcohol yielded on eight water extractions a residue weighing 0.1946 gram.

To sum up we have then:

Fraction 1 = 2.6480 grams includes lipoids and neutral fats, groups 1 and 5.
Fraction 2 = 0.6984 gram includes extractives and ash, groups 2 and 3.
Fraction 3 = 0.1946 gram includes extractives and ash, groups 2 and 3, also gelatin.
Fraction 4 = 4.7034 grams includes proteins, glycogen and some ash, groups 4, 6 and 3.

8.2444 grams total solids.

[1] Koch, W., *J. Physiol.*, **11**, 303 (1904).

It is apparent then that the alcohol extracts only part of the extractives but should extract all the lipoids and fats. Whether or not there are any qualitative differences between the extractives soluble in the alcohol and those insoluble is doubtful. This point will be taken up again in a later paper. The glycogen is, of course, only present in Fraction 4, if no glycolysis has been allowed to take place, otherwise it would be present in Fractions 2 and 3 as soluble sugar.

Estimation of Groups.—In the above four fractions the six general groups of constituents—lipoids, extractives, inorganic constituents, proteins, fats, glycogen—can now be estimated.

Estimation of Lipoids. Outline of Method.—The lipoids are all found in Fraction 1 (p. 1347) which precipitates with acid chloroform water. They consist mainly of cholesterol, cerebrins, phosphatides, and lipoid sulphur.

The method for *cholesterol* devised by Ritter[1] has been found inadequate. The method of Rosenheim[2] cannot be used in these estimations. The subject is very much in need of revision.

The method for the estimation of *cerebrin* has already been described by S. A. Mann[3] and one of us, and considering the fact that only minimal amounts are found in any tissue except the brain need not be here again considered. (*Lipoid sulphur* will be taken up in a later article.)

The methods of estimating *phosphatides* are still in a very unsatisfactory state. The attempt of Woods[4] and one of us to separate them into two main groups, the *lecithins* and *kephalins*, by means of their lead salts had to be abandoned on account of the large number of apparently uncontrollable factors which influenced the results. The usual methods of determining the phosphatides is to extract the tissue with alcohol, evaporate, take up the dry residue with dry ether and determine phosphorus. As will be pointed out in the estimation of fat by this method there are certain sources of error involved in this procedure due to the fact that it is almost impossible to work under anhydrous conditions. We have preferred, therefore, to estimate the phosphorus in the lipoid precipitate (Fraction 1) and correct for the extractive phosphorus in the fluid (Fraction 2) clinging to the lipoid precipitate. This phosphorus is then multiplied by 25.8 on the assumption that the average molecular weight of the phosphatide is 800. The details of the method of estimating the phosphorus have already been given in the paper by Woods and one of us.

Calculation.—27.99 grams (moist muscle) (29.4 per cent. solids) yielded 14.0 mg. phosphorus in the lipoid precipitate and 19.5 mg. phosphorus in filtrate from the lipoid

[1] Ritter, E., *Z. physiol. Chem.*, **34**, 456 (1901).
[2] Rosenheim, O., *J. Physiol.*, **34**, 105 (1906).
[3] Koch, W., and Mann, S. J., *Archives of Neurology and Psychiatry*, **4**, 31 (1909).
[4] Koch, W., and Woods, U. S., *J. Biol. Chem.*, **1**, 203 (1906).

precipitate. By calculation given previously 6 cc. of this filtrate were found to be clinging to lipoid precipitate. $19.5 \times 6/229 = 0.5$ mg. Phosphorus represents the correction due to this, $14.0 - 0.5 = 13.5$ mg. lipoid phosphorus corr.

$$(13.5 \times 25.8)/(27.99 \times 29.4) = 4.2 \text{ per cent. of phosphatides in solids.}$$

Sources of Error.—A low result may be due: (1) To incomplete extraction, especially in case tissue has been dried in a stream of air before adding alcohol. (2) To carelessness in burning lipoid phosphorus with acids. This is especially the case in samples which contain large amounts of fat and are consequently apt to spatter and char a good deal. (3) The possibility that lipoid phosphorus may be split off by the acid treatment, has been shown to be slight, at most two per cent. by total lipoid phosphorus. (4) Inaccuracies involved in the factor 25.8.

Estimation of Extractives and Ash. Outline of Method.—Members of this group are found in Fractions 2 and 3 (see p. 1351). For the determination of this group, the method devised by Grindley,[1] namely extraction of the fresh tissue direct with cold water, coagulation of the proteins and evaporation to constant weight would possess many advantages over the one employed by us, if it were not for the fact that it is quite useless for tissues like the liver or the brain, which contain a large percentage of lipoids. The only method found so far that enables the separation of the extractives from a large quantity of lipoids consists of extraction with alcohol, evaporation, emulsification and separation of the lipoids by 0.5 per cent. hydrochloric acid saturated with chloroform. With a little experience and care it is possible by this method to obtain a perfectly clear filtrate which can then be evaporated and dried to constant weight. One difficulty encountered in this evaporation is the charring of the organic matter, which no amount of care seems to be able to obviate. Previous neutralization of the acid would necessitate a correction due to the sodium chloride formed, which might in some cases be greater than the actual weight of extractives. There seems to be, however, no appreciable loss from the charring and a constant weight can be obtained.

The protein residue insoluble in alcohol yields some material on water extraction. Except for the fact that this fraction (3) is richer in inorganic constituents, there is no reason for supposing that it differs essentially from Fraction 2. Wherever a tissue is rich in cells yielding gelatin this will be found in this fraction. Proliferation of connective tissue always leads to increase in the organic matter of this fraction due to an increase of gelatin.

The ash obtained by incineration of the residues of Fractions 2 and 3 represents the inorganic constituents, as far as the cations are concerned, fairly accurately. The anions PO_4 and SO_4 are partly derived

[1] This Journal, **26**, 1086 (1904).

from the incineration of the organic material. Another fraction which yields a slight amount of ash is the protein residue. The ash is here only considered as an approximation to the inorganic constituents. In a later paper it is the intention to take up the subject of the estimation of the inorganic constituents more in detail. The advantage of this method over the method involving incineration of the whole sample is that the sources of error due to lipoids and protein phosphorus are largely avoided.

Details of Method. Fraction 2.—The clear filtrate from the lipoid precipitate is evaporated to dryness in a platinum dish and dried to constant weight at 105. The residue is incinerated to a white ash and weighed. To prevent heating at too high a temperature the small dish is surrounded by a larger one, which is covered with a lid and heated to a full capacity of a Bunsen burner.

Fraction 3.—The collected filtrates from the hot water extractions are evaporated to dryness as they are obtained. Keeping them dry prevents danger of bacterial decomposition. The dry residue is dissolved in 200 cc. hot water, filtered from coagulated protein, evaporated to dryness in a platinum dish, incinerated like the above and weighed.

Fraction 4.—An aliquot part 0.5–1.0 gram of the dry residue is incinerated to a white ash in a platinum crucible.

Calculations.—Fraction 2 yielded 0.6984 gram residue corrected (see p. 1351).
This gave ash 0.2214 gram corrected.
Fraction 3 yielded 0.1946 gram (see p. 1351).
This gave ash 0.0900 gram.
Fraction 4 0.5 g. 0.0024 gram.

Fraction 2. $\frac{0.6984}{27.99 \times 29.4}$ = 8.5 extractives and ash in per cent. of dry matter.

Fraction 2. $\frac{0.2214}{27.99 \times 29.4}$ = 2.7 ash in per cent. of dry matter.

5.8 extractives in per cent. of dry matter.

Fraction 3. $\frac{0.1946}{27.99 \times 29.4}$ = 2.4 extractives and ash in per cent. of dry matter.

Fraction 3. $\frac{0.0900}{27.99 \times 29.4}$ = 1.1 ash in per cent. of dry matter.

1.3 extractives in per cent. of dry matter.

Fraction 4. $\frac{24 \times 4.7034}{0.5 \times 27.99 \times 29.4}$ = 0.3 ash in per cent. of dry matter.

Total extractives in per cent. of solids = 7.1.
Total ash in per cent. of solids = 4.1.

Sources of Error.—Difficulty in drying the charred residue from Fraction 2 to constant weight. Danger of bacterial decomposition in extraction of protein residue with water from Fraction 3.

Estimation of Proteins. Fraction 4.—The residue in alcohol and hot

water corrected for the slight amount of ash (see above) is called the protein.

The nitrogen of this residue = 16.5 per cent.

$(4.898 - 0.1946 - 0.0225)/(27.99 \times 29.4) = 56.9$ protein in per cent. of solids.

Estimation of Fat. Outline of Method.—An attempt to give even a bibliography of the literature on this subject would carry us beyond the scope of this article. A very complete discussion and comparison of the main methods, which have been employed, for the estimation of fat is given in a paper by Kumagawa and Suto.[1] The gist of the whole matter may be summarized in the statement, that the Soxhlet method yields results which are too low, while the Rosenfeld method yields results which are too high. Also none of the methods take the error introduced by the phosphatides of the tissues sufficiently into account. The phosphatides cannot be counted as fat even if they are completely extracted by fat solvents, as they have an entirely different physiological function.

As the result of their very exhaustive studies Kumagawa and Suto suggest a new method, which in principle has already been used by Noel Paton,[2] namely the saponification of the fat and the estimation of the total fatty acid. This method again does not take into account the fact that some of the fatty acid may come from lecithin, as well as the fact that it is very difficult to completely saponify all the lecithin. To test this source of error in the otherwise excellent method of Kumagawa and Suto the following experiments were undertaken:

4.0372 grams dry muscle tissue yielded by the method of Kumagawa and Suto 1.0582 grams fatty acid.

3.3918 grams dry muscle tissue +0.393 gram brain lecithin (phosphorus = 3.5 per cent.) yielded by the same method (heated for a longer time than they direct) 1.0737 grams fatty acid.

	Calculated.				Error calculated per cent. of lecithin fatty acid.
	Muscle.	Lecithin.	Total.	Found.	
Fatty acid in muscle plus lecithin...	0.889	0.249	1.138	1.0737	25.7

The lecithin added was saponified to the extent of about 75 per cent. although the heating was continued for very much longer than they suggest. It may be possible to so modify this method as to obviate this source of error in some tissues but in other tissues richer in phosphatides than muscle this will be attended with great difficulty and for nerve tissue will be quite impossible. If the method of Kumagawa and Suto had been found to give a complete saponification of all the fats

[1] Kumagawa and Suto, *Biochem. Z.*, 8, 212 (1908).

[2] Noel Paton, D., "Report of Investigations on the Life History of the Salmon in Fresh Water, Fishery Board for Scotland," p. 143 (1898).

and lipoids it might have been possible by estimating the amount of fatty acid derived from lecithin to obtain a fairly accurate figure for the fatty acids derived from neutral fat. It is necessary to look for another method.

Details of Method and Calculation.—On page 1348 is given the weight of lipoids and fat present in Fraction 1 as = 2.6480 grams. This figure is obtained by subtracting from the total alcohol-soluble residue the weight of the water-soluble-non-colloidal extractives, 0.6984 conc. As far as we know the only lipoids found in muscle besides cholesterol are the phosphatides. If we estimate their quantity by multiplying the lipoid phosphorus by 25.8 we get 0.3483 gram. This leaves for neutral fat and cholesterol 2.2799 grams, or in per cent. of dry tissue 27.6 per cent. neutral fat.

Sources of Error and Comparison with Other Methods of Fat Estimation.—The result may be too high (1) on account of inaccuracies in the drying of the alcohol-soluble residue. (2) Loss of organic material due to the charring of the extractives on evaporation. (3) The presence of cholesterol (in muscle this error is not very great). (4) Inaccuracies involved in the factor 25.8 for calculating phosphatides. Most sources of error tend to bring the result too high. A comparison of the above result with an analysis made by the method of Kumagawa and Suto, corrected on the supposition that all the lecithin was saponified and with an estimation by the method of Soxhlet corrected for lecithin on the basis of its phosphorus content follows:

Method.	Neutral fat in percentage of dry matter.
Koch and Carr	27.6
Kumagawa and Suto	25.8
Soxhlet	24.8

This discrepancy between our result and the results of Kumagawa and Suto is probably accounted for by the fact that our result is a little too high due to the sources of error pointed out and their results too low on account of incomplete saponification of lecithin. The cholesterol content enters as a constant source of error into all three methods.

Estimation of Glycogen.—The method of Pflüger[1] can be readily adjusted and the scheme of analysis outlined in this paper.

Estimation of Water.—The amount of blood in the tissues at death and the accidental and almost uncontrollable variations in the amount of drying during the collection of the material are apt to introduce a difference in the water content of various tissues, and for these reasons not much importance has been attached to the comparatively slight variations which occur. It is, however, necessary to estimate the water content in investigations of this kind in order to determine the various con-

[1] Pflüger, E., *Arch. ges. Physiol.* (*Pflüger*), 114, 231 (1906).

stituents in percentage of the total solids. Lack of attention to this point is the reason why it is almost impossible to use a great many published analyses.

It has been already demonstrated that it is difficult to dry to constant weight material of colloidal nature, and that the best method consists in drying *in vacuo* below the coagulation point of the colloid. This method has been adopted. Weighed quantities of minced brain matter have been spread out on one of two tares (paired watch-glasses fitted with a clip), allowed to remain in a vacuum desiccator for some time and then dried to constant weight in a vacuum oven at 40–42°. When constant weight is attained, it is found that raising the temperature to 100° does not materially affect the result.

Benedikt[1] has since devised a method which in principle resembles ours, but offers no special advantages. The method of Shackell[2] published while this article was in preparation does appear to possess decided advantages.

The amount of material taken should be as near as possible 2 grams; a larger quantity than this makes the drying a very long and tedious operation, while smaller amounts on account of their smallness may introduce error. A number of moisture determinations on brain material made in duplicate are given below, and the results not only serve as a check on the accuracy of the method, but also indicate that the mincing process gives a uniform mixture of the gray and the white matter, as the samples were taken in each instance from quite distinct portions of the minced material.

No. of case.		28	40.	41.	29.	42.
Per cent. of moisture ...	(a)	78.61	77.41	78.38	77.52	76.69
	(b)	78.74	77.50	78.55	77.49	76.83

Another method of estimating moisture is by calculation from weight of protein residue and the weight of the alcohol-soluble residue obtained in the extraction of the large sample as outlined under the estimation of fat.

Summary.

Results of an analysis of a sample of meat by the above methods:

	Groups.	In per cent. of solids.	
27.99 grams of moist tissue yielded.....	Fraction 1: 2.6480 grams	Neutral fat..........	27.6
		Phosphatides........	4.2
	Fraction 2: 0.6984 gram	Extractives.........	5.8
		Ash................	2.7
	Fraction 3: 0.1946 gram	Extractives.........	1.3
		Ash................	1.1
	Fraction 4: 4.7034 grams	Proteins............	56.9
		Ash................	0.3

[1] Benedikt, *Am. J. Physiol.*, 13, 309 (1905).

[2] Shackell, *Ibid.*, 24, 325 (1909).

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).