$[\alpha]_D^{20}$, —34.19°; the acetate melted at 124°; (b) m. p., 134–135°; $[\alpha]_D^{20}$, —33.61°; the acetate melted at 119°.

The complete separation of these fractions by crystallization is very difficult, if not impossible, and we do not believe that either of the above preparations was homogeneous.

3. Linseed oil contains at least two phytosterols which differ in melting point and optical rotation. As in the case of cotton seed oil 2 fractions of phytosterol were separated by fractional crystallization: (a) m. p., 138°; $[\alpha]_D^{20}$, —34.22°; the acetate melted at 129–130°; (b) m. p., not sharp, 134°; $[\alpha]_D^{20}$, —31.16°; the acetate melted at 124°.

None of the phytosterol preparations that we isolated contained 1 molecule of water of crystallization. The loss in weight on drying was somewhat irregular but corresponded more nearly to $^1/_2$ molecule of water of crystallization.

Mixtures of phytosterols such as are obtained from cottonseed oil and linseed oil are so nearly alike in solubility in the usual solvents that it is practically impossible to effect a complete separation by simple fractional crystallization.

GENEVA, NEW YORK

[CONTRIBUTION FROM THE HULL LABORATORIES OF PHYSIOLOGICAL CHEMISTRY AND PHARMACOLOGY, THE UNIVERSITY OF CHICAGO]

A QUANTITATIVE METHOD FOR THE DETERMINATION OF TOTAL SULFUR IN BIOLOGICAL MATERIAL[1]

BY MABEL STOCKHOLM AND FRED C. KOCH

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The estimation of total sulfur in biological materials has been the subject of many investigations. The most generally recognized source of error, the difficulty of forming a pure barium sulfate, has been largely eliminated by the now well-known modifications introduced as a result of the careful studies by Folin,[2] Allen and Johnston,[3] Johnston and Adams[4] and Krieble and Mangum.[5] There are, however, several other factors which have not been sufficiently emphasized. These are complete retention of the sulfur, complete oxidation to sulfate and complete precipitation of sulfate in blanks and in materials low in sulfur. The first two factors appear to be well controlled in the special methods for total sulfur in urine as developed by

[1] The greater part of the experimental work of this paper was carried out by Miss Mabel Stockholm in 1918 as part of the requirement for the degree of Master of Science.

[2] Folin, *J. Biol. Chem.*, **1**, 130 (1905).

[3] Allen and Johnston, THIS JOURNAL, **32**, 588 (1910).

[4] Johnston and Adams, *ibid.*, **33**, 829 (1911).

[5] Krieble and Mangum, *ibid.*, **41**, 1317 (1919).

Folin,[2] Benedict,[6] Denis[7] and Modrakowski.[8] Urine, however, is a very special case in which by far the greater part of the sulfur is already in the sulfate form and, in which we are dealing with relatively large amounts of sulfur as compared with the amount of organic matter to be oxidized. It is a very different matter when it is necessary to oxidize several g. of dry tissue in order to obtain a weighable amount of barium sulfate. This at once introduces the three difficulties referred to.

The incomplete retention of the total sulfur, due either to a loss by volatilization or to incomplete oxidation or both appears to be the most logical source of error in the usual fusion methods with dry sodium carbonate and potassium nitrate or dry sodium peroxide. The same is true for the wet, acid combustion methods. These predictions have been confirmed in this study and in part by earlier studies. Not until after the completion of this work did the authors come across the excellent and almost completely ignored work of Barlow[9] who showed the loss of sulfur in the usual dry fusion and wet, acid combustion methods to be due to volatilization. The method which Barlow finally developed is no doubt a reliable one, but it is too cumbersome for general biological application. In view of the impossibility of retaining all sulfur in the usual methods here considered the only alternative is one of two procedures. It is either a complete oxidation of the material by fusion in the presence of an excess of base in a closed vessel or the oxidation of the sulfur in an aqueous alkaline solution to a less volatile form, with the subsequent oxidation of the organic matter by fusion or by a wet, acid oxidizing method. The former involves the use of a specially devised Parr bomb as now employed by the Association of Agricultural Chemists.[10] By the use of this modification investigators working on sulfur problems in plants have found that seeds and plants contain much more sulfur than had previously been reported. This method is not likely to be generally adopted on account of the necessity of the special bomb.

The difficulties of the second alternative, that of wet oxidation in an alkaline solution, we overcame by the use of hydrogen peroxide in concentrated form. Here, also, we later found numerous confirmations as to the value of this reagent for oxidizing reduced sulfur. Thus, as early as 1884 Classen and Bauer[11] used hydrogen peroxide for the complete oxidation of sulfides and sulfites. This was confirmed by many other studies and especially by Klemmer[12] and Petersen.[13] The former uses it for the

[6] Benedict, *J. Biol. Chem.*, **6**, 363 (1909).
[7] Denis, *ibid.*, **8**, 401 (1910).
[8] Modrakowski, *Z. physiol. Chem.*, **38**, 562 (1903).
[9] Barlow, THIS JOURNAL, **34**, 342 (1904).
[10] Latshaw, *J. Assoc. Official Agr. Chem.*, **5**, 136 (1921).
[11] Classen and Bauer, *Ber.*, **16**, 1061 (1884).
[12] Klemmer, *Chem.-Ztg.*, **46**, 79 (1922).
[13] Petersen, *Z. anal. Chem.*, **42**, 406 (1903).

complete oxidation of sulfur in gas and Petersen reports the quantitative oxidation of sulfur in the forms of sulfides, thio-urea, thiocarbanilide and of carbon disulfide by hydrogen peroxide in alkaline solutions. Folin,[2] in fact, uses essentially the same principle in evaporating the urine after the addition of several g. of sodium peroxide and subsequently oxidizes other constituents by fusion with the addition of more sodium peroxide. As stated before, this method is reliable for urine, but the subsequent peroxide fusions are not applicable to tissues in general unless conducted in a bomb. Unless this precaution is taken the chances are that the analyst will lose many estimations due to the explosive combustion, especially when the material contains considerable carbohydrate. We therefore devised our method so as to follow the oxidation by hydrogen peroxide by a second process of acid oxidation with nitric acid and bromine. The results are very satisfactory, as indicated in the table, and as found by experience in the use of the method in other studies. The presence of nitrate in sulfate precipitations has been severely criticized, but the very high results reported as due to the presence of nitrates have been obtained under conditions where the precipitations were carried out in small volumes with relatively high concentrations of nitrates. Under the conditions employed here, that is, of precipitating from a volume of 500 to 600 cc. and digesting on the steam-bath for 10 to 12 hours before filtering and washing, the error is a very slight one. In fact the slight difference of 0.5 to 1.0 mg. of barium sulfate in a total of 0.1125 g. may easily be due to a trace of sulfate in the relatively large amounts of reagents employed. Furthermore, this is corrected by the introduction of a true blank estimation as practiced in this Laboratory for the last 8 or 10 years.

The basis of this true blank test is as follows. It is a very common experience to find no sulfate precipitated in the usual length of time in blank estimations and in other cases where it is known that small amounts of sulfur must be present. If the solution is permitted to stand several days these traces of sulfate may be precipitated. This is especially true when such small amounts of sulfate are to be precipitated from a 500 to 600cc. volume containing 25 to 30 g. of soluble salts. When, however, a known amount of sulfuric acid is added to such solutions, the usual rapid and complete precipitation of barium sulfate is observed. In view of the very satisfactory results obtained in this and other laboratories by the use of this procedure, we have adopted the plan of consistently adding 10 cc. of 0.1 *N* sulfuric acid to every sulfur estimation including the blank tests. The value obtained in the blank tests is, of course, subtracted from the unknowns and the differences give values truly corrected for blank sulfur in the reagents and procedure.

Experimental Part

In view of the nitric acid oxidation methods suggested by Wolf and Oster-

berg,[14] by Konschegg[15] and by Halverson,[16] we tried various modifications of these acid oxidation methods on pure cystine and in no case could we obtain theoretical values. In all estimations careful blank tests were made. Oxidation by bromine in alkaline solution with subsequent oxidation by nitric acid gave the highest results, that is 22.26 to 25.06% of sulfur as compared with the calculated value of 26.69%.

A few preliminary trials in which we attempted first to oxidize cystine in an alkaline solution by means of hydrogen peroxide and later complete the oxidation by nitric acid indicated possibilities by this method of oxidation. After a number of attempts with various modifications we finally devised the new Perhydrol-nitric acid method by means of which we obtained theoretical values for cystine after the proper correction for carefully run blank estimations. The method thus developed was then applied in parallel with the ordinary sodium carbonate-potassium nitrate fusion method to various types of biological material containing different forms of organically combined sulfur. The ordinary fusion method and the new Perhydrol-nitric acid method as used in these studies are given in detail below, and the results obtained after proper corrections for blanks are given in Table I.

Fusion Method

Reagents.—The fusion mixture was prepared by thoroughly grinding and mixing 225 g. of potassium nitrate with 750 g. of anhydrous sodium carbonate. 0.1 *N* Sulfuric acid and 10% barium chloride solution were used.

Procedure.—A sample of 0.5 to 2.0 g. is thoroughly mixed with 15 g. of the fusion mixture in a 50 × 70 mm. nickel crucible. Over this mixture is placed a cover of 10 g. of fusion mixture. The crucible is covered and heated very gradually over an alcohol lamp. If smoke escapes from the crucible the flame is lowered and the heating is continued and gradually increased until, with the flame almost touching the crucible, very little or no smoke is developed after heat has been applied for an additional hour at that temperature. When thus thoroughly charred, without loss due to visible "smoking," the contents of the crucible are thoroughly mixed, the crucible is covered and then heated for 10 minutes in a medium sized flame on a Roger's burner.

After the fusion mass has cooled, it is dissolved in about 400 cc. of water in a flask and 40 cc. of concd. hydrochloric acid is added. This is boiled in a flask to remove the carbon dioxide and then evaporated to dryness in a disk on the steam-bath. When completely dry, the mass is moistened with 20–25 cc. of concd. hydrochloric acid and again evaporated to dryness. This same treatment is repeated once more.

The mass of salts is next dissolved in 300 to 400 cc. of distilled water,

[14] Wolf and Osterberg, *Biochem. Z.*, **29**, 429 (1910).

[15] Konschegg, *Arch. ges. Physiol.* (*Pflüger's*), **123**, 274 (1908).

[16] Halverson, THIS JOURNAL, **41**, 1494 (1919).

10 cc. of concd. hydrochloric acid added, and the whole transferred to a 500cc. volumetric flask and accurately made up to 510 cc. The well-mixed solution is then filtered through a dry filter into a dry 500cc. volumetric flask and, after exactly 500 cc. has been collected, it is transferred to a liter beaker together with 100–200 cc. of rinsing water. To this is now added 10 cc. of 0.1 *N* sulfuric acid solution and after the mixture has been heated to boiling, the 10 cc. of a 10% barium chloride solution is added, drop by drop. The boiling is continued for 10 to 15 minutes and then the mixture is heated for 10 to 12 hours before it is filtered and washed as usual. The filter paper and precipitate are cautiously burned and weighed. Blank estimations are made in exactly the same way. After the value found in the blank has been subtracted from the total weight the difference represents, of course, only 500/510 of the original sample.

Perhydrol-Nitric Acid Method

(1) **Reagents.**—(a) A 25% solution of sodium hydroxide by volume; (b) Perhydrol or Superoxol, a 30% solution of hydrogen peroxide; (c) fuming nitric acid; (d) bromine; (e) 0.1 *N* sulfuric acid; (f) a 10% solution of barium chloride.

(2) **Procedure.**—Into a 100cc. nickel crucible (50 × 70 mm.) containing 10 cc. of the 25% sodium hydroxide solution is transferred 0.5 to 2.0 g. of the substance. The covered crucible is then heated on the steam-bath until the mass is almost dry. This requires several hours, but causes considerable decomposition of the complex substances present, so that the sulfur in particular can later be easily oxidized. In case the evaporation has proceeded too rapidly it is best to add again about 10 cc. of water and to repeat the slow evaporation. To the slightly moist material 5 cc. of Perhydrol is added very gradually. In some cases it is necessary to stir the mass with a glass rod or to add a few drops of water so as to distribute the reagent properly. During this treatment the heating is continued on the steam-bath.

The material thus partially oxidized is next transferred to a 300cc. Kjeldahl flask, acidified with nitric acid and concentrated over a free flame until salts begin to separate. This concentrated solution is then oxidized, while boiling, by the gradual addition of fuming nitric acid and bromine until 10 cc. of acid and 40 to 50 drops of bromine have been used. With material low in, or free from, fat this treatment is usually sufficient to bring about complete oxidation of the organic matter. The solution is next evaporated almost to dryness and after water has again been added evaporation is repeated to remove most of the nitric acid. When the water solution of this material is not absolutely clear it is filtered and after it has been neutralized with sodium hydroxide and diluted to about 600 cc. it is acidified by the addition of 10 cc. of concd. hydrochloric acid. The 10 cc. of 0.1 *N* sulfuric acid is added and the precipitation and estimation are con-

ducted as usual and as described for the fusion method. Blank estimations must be made in exactly the same way with the same amounts of reagents.

In case considerable lipin material is present, such as in nerve tissue, egg yolk, etc., drops of lipin or fatty acid remain unoxidized by the above procedure. These, we find, can be filtered off after the mass has cooled without any loss of sulfur, but we consider it safer to modify the method so as to make the oxidation more efficient and prolonged. By conducting the oxidation by nitric acid and bromine in a flask provided with a glass-stoppered reflux condenser and continuing the boiling for 24 hours on an electric plate we were able to decompose completely egg yolk and a sulfo-lipin preparation from brain tissue.

TABLE I

ANALYTICAL RESULTS

Substance analyzed	Fusion method: Substance taken G.	Fusion method: Corrected $BaSO_4$[a] G.	Fusion method: S %	Perhydrol method: Substance taken G.	Perhydrol method: Corrected $BaSO_4$[a] G.	Perhydrol method: S %
Pure cystine (calc. 26.69% of S)	0.1416	0.2555	25.27	0.1541	0.2998	26.62
	.1494	.2627	24.56	.1120	.2171	26.61
Fat-free dried tissue No. 6.....	.8496	.0546	0.882	1.3718	.0916	0.909
	1.7334	.0998	.853	1.2004	.0780	.895
Fat-free dried tissue No. 4.....	1.7289	.1025	.814	1.2119	.0722	.816
	1.8028	.1033	.782	1.3459	.0783	.812
Fat-free dried tissue No. 7.....	1.7446	.0438	.345	1.5591	.0563	.496
	1.7180	.0465	.366	1.4235	.0520	.498
Fat-free dried tissue No. 1.....	1.4679	.0537	.507	1.5672	.0615	.538
	1.3614	.0491	.492	1.5690	.0605	.529
Sulfo-lipin from brain tissue....	1.3590	.0569	.575	1.3694	.0587	.581
	1.5446	.0614	.545	1.3989	.0591	.581
Air-dried egg yolk.....	1.1171	.0147	.181	1.2233	.0246	.276
				1.3828	.0278	.275
Crude bile salts.....	0.6524	.1859	3.91	0.6882	.1998	3.99
	.8930	.2512	3.86	.7282	.2341	4.08

[a] Corrected for the sulfuric acid added and the trace in the reagents introduced in the process.

The results in Table I clearly show that the fusion method tends to give low and irregular results. For cystine the values are distinctly low by the fusion method, but the Perhydrol method gives theoretical values and excellent checks. That the low results in the fusion method are due to the loss of sulfur by volatilization is shown by a combustion of a proper mixture of cystine and fusion mixture in a combustion tube with a slow, well-regulated and well-washed current of air passing over the material, and a final washing of the air through 2 wash bottles containing 0.5 *N* sodium hydroxide solution. The latter solution was next evaporated almost to dryness and analyzed for sulfur by the Perhydrol method. Blank estimations were made in the same way with fusion mixture alone, in the combustion tube instead. By thus heating 2 g. of cystine with 75 g. of fusion mixture and

gradually raising the temperature in the course of $2^1/_2$ hours, we obtained 2.8% of the cystine sulfur in the washing solution, after due allowance had been made for a careful blank test. This, to be sure, is a rapid heating but repetition of the same experiment with a very gradual heating over a period of 6 hours gave 1.3% of the cystine sulfur in the washing solution. Furthermore, when the gases from the tube at the close of this period were passed into a lead acetate solution, a precipitate of lead sulfide was obtained at once.

The completeness of oxidation of cystine sulfur is easily shown by observation of the rapid loss of reduced sulfur in an alkaline solution of cystine when it is treated with hydrogen peroxide. Such solutions can no longer be reduced to cysteine by sodium sulfite as suggested by Folin and Looney[17] in their recent method for the estimation of cystine. However, such oxidized solutions do not yield calculated values for sulfate unless the alkaline peroxide oxidation is followed by the usual nitric acid and bromine combustion.

Summary

1. The dry fusion method used in these studies tends to give low results when properly corrected for blanks.

2. These low and irregular results are due to the loss of reduced sulfur by volatilization during the heating period of the dry mixture. This loss is greater the more rapidly the temperature is increased.

3. The new Perhydrol method gives calculated values for cystine. The results are uniform and in general higher than by the fusion method on different forms of organically combined sulfur found in biological material.

4. Blank estimations for sulfates are made easily and accurately by adding a known amount of sulfate (10 cc. of 0.1 *N* solution) to the solution in order to hasten the complete precipitation of traces of sulfate.

CHICAGO, ILLINOIS

[17] Folin and Looney, *J. Biol. Chem.*, **51**, 427 (1922).