CLX. THE DETERMINATION OF SULPHUR IN BIOLOGICAL MATERIAL

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THE first comprehensive paper on the determination of sulphur in biological material was published by Barlow [1904], who reviewed twelve methods which were then in common use and showed that all tended to give low results owing to loss of sulphur by volatilization. He described his own method in which organic material was burnt in a combustion tube and all the sulphur caught as SO_3 in a quartz-sodium carbonate column. This procedure appears to have been very accurate though a little cumbersome for routine use.

The destruction of organic matter without loss of sulphur always presents difficulty because sulphur and many of its compounds are very volatile. It may be done by fusion of the substance with an alkaline oxidizing mixture, by "wet" ashing or by hydrogenation.

Fusion methods

Early workers tried to prevent loss of sulphur during dry ashing by addition of alkaline salts, e.g. $Ba(OH)_2$, $CaCO_3$, Na_2CO_3 [see Barlow, 1904]. More recent investigators have fused the substance with an alkali such as Na_2CO_3, NaOH, or Na_2O_2. Of these, Na_2O_2 appears to have been the most successful and is still in use to-day.

Folin [1905] described the determination of total sulphur in urine by sodium peroxide fusion and Sherman [1902] a similar method for foods. These were the earliest applications of the method to biological material. Since its original introduction, many modifications of this procedure have been suggested, though the most serious difficulty encountered, i.e. the frequency with which explosions take place, has not yet been satisfactorily overcome, except by the use of a bomb [Parr, 1908]. This, however, involves the use of special and expensive apparatus [Parr Instrument Co., Booklets 115, 116, 117].

The fusion method has been adopted as the official method of the Association of Official Agricultural Chemists [1935] and the technique recommended by it has been found by the author to give accurate results, but to be unsuitable for the routine determination of sulphur. To prevent explosion or ignition of the fusion mixture, which ruins the estimation, constant care and attention have to be given to each individual fusion, every one of which has to be done separately and takes at least 1 hr.

Wet ash methods

A number of workers have attempted to apply the copper nitrate oxidation method of Benedict [1909], originally introduced for determination of total S in urine, to general biological analysis. Wolf & Osterberg [1910] and Halverson [1919] found preliminary treatment with HNO_3 to be necessary. Waelsch & Klepetar [1932] found that adjustment of the *p*H of the solution to about 7·2 by the addition of Na_2CO_3 gave better results, though Rutender & Andrews [1937] showed that the results varied according to the amount of Na_2CO_3 added. For

the determination of sulphur in pure organic compounds Hoffman & Gortner [1923] showed that the substance must be in solution so that finely divided particles come into contact with $Cu(NO_3)_2$.

Frear [1930] adapted the Benedict-Denis method for use on plants with apparent success but in the author's hands this method was found unsatisfactory. Painter & Franke [1936] have experienced similar difficulties which they explained by the failure of the Benedict-Denis mixture to oxidize methionine-sulphur quantitatively. This failure is further emphasized by work of Waelsch & Klepetar [1932], who found only 0·25% S in casein analysed by this method, whilst the accepted figure for casein-S is 0·8% [Sherman, 1937]; according to Baernstein [1936], 83·4% of the total S of purified casein is present as methionine. Painter & Franke also obtained lower figures for casein-S by the Benedict-Denis method than by the Parr bomb method; the author's results (Table VI) confirm the findings of these workers. For the analysis of many biological substances, therefore, the Benedict-Denis method is of little use.

The use of HNO_3 for the destruction of organic matter. Many methods of "wet" ashing have been introduced in recent years, all of which involve the use of HNO_3 generally in conjunction with another oxidizing agent. Thus Stockholm & Koch [1923] used a "wet" ash technique employing H_2O_2 and HNO_3 in preference to a dry fusion. Blanck *et al.* [1937] compared results by ashing, with and without addition of alkali, with three wet ash techniques (digestion of material with HNO_3 and $KClO_3$, with aqua regia and $KClO_3$ or with NaOH, H_2O_2 and Br); ashing without addition of alkali always resulted in losses of S. The other methods all yielded similar values for S, but digestion with HNO_3 and $KClO_3$ was found to be the most satisfactory as it was quicker and simpler. Bertrand & Silberstein [1929] destroyed the organic matter and oxidized the S in plants by heating them with HNO_3, followed by an alkaline fusion; a similar process was used by Rippel & Nabel [1937] and by Blanck & Sachse [1938] and was found by the latter authors to be more reliable than five other wet ash methods investigated for the analysis of taurine, cystine and allyl *iso*thiocyanate. Cherbuliez & Meyer [1933] mixed the material with an equal weight of $NaNO_3$ and 30 ml. fuming HNO_3 for the oxidation, whilst Echevin & Crépin [1928] mixed the substance to be analysed with $Mg(NO_3)_2$ and subsequently heated it with HNO_3. Revol & Ferrand [1935] used a mixture of HNO_3 and perhydrol and passed the escaping fumes into a NaOBr solution to guard against any loss of S. Warth & Krishnan [1935] gave the material (urine, faeces and feeding stuffs) a preliminary oxidation with HNO_3, and followed this by fusing the evaporated filtrate with NaOH in a silver basin. Stotz [1937] found that fusion of the material with peroxide with or without preliminary digestion with HNO_3 might fail to convert S into $SO_4^=$ and stated that ashing in presence of KOH and $KMnO_4$ effected complete oxidization.

The nitric-perchloric acid method. This method was first introduced by Kahane [1927] for the determination of S in rubber; in the same year Le Matte *et al.* [1927, 1, 2] used it for the analysis of animal tissues. Wolesensky [1928] also employed it for estimating S in rubber, and it was later used on biological material by Toepfer & Boutwell [1930], who preferred it for large samples to the Parr bomb. Kemmerer & Boutwell [1932] used the method for destroying organic material before estimating S in a large number of foodstuffs. Gieseking *et al.* [1935] used HNO_3 and $HClO_4$ for the destruction of organic matter in plants.

Balks & Wehrmann [1937], however, digested the material with HNO_3 and $HClO_4$ only after it had been given a preliminary heating with 10% aqueous NaOH and treated with H_2O_2, since they found the method was only satis-

factory for taurine and allyl *iso*thiocyanate under these conditions; these substances, however, are not widely distributed in biological material. Kägi [1937] used this mixture of acids for estimating S in milk.

Present investigation of the nitric-perchloric method

Method of digestion adopted. The material to be analysed (1–5 g.) was weighed out, brushed into a 100 ml. long-necked Kjeldahl flask and 5 ml. of conc. HNO_3 added. A glass bulb was placed in the neck of the flask to prevent loss by sputtering. The mixture was kept on a sand bath at a low temperature till fumes of HNO_3 no longer came off. Care had to be taken during this initial reaction to prevent the contents frothing out of the flask. When effervescence had ceased a further 3 ml. of HNO_3 were introduced, and as soon as solution was complete, 2 ml. of $HClO_4$ were added and the temperature raised. Additions of HNO_3 and $HClO_4$ were made at intervals until a clear colourless solution resulted, usually after some 16 hr. By this time all the HNO_3 had been boiled off and the solution contained $HClO_4$ and salts only. The later stages of the digestion sometimes had to be taken more slowly when analysing fatty materials to minimize effervescence and bumping.

The solution was then heated on the sand bath for at least 12 hr., more $HClO_4$ being added as necessary. Finally, it was allowed to evaporate to a volume of 2 ml. When the residue was allowed to cool, a small white precipitate occasionally formed which sometimes dissolved on addition of water. In this case it was assumed to be $KClO_4$. At other times it remained insoluble, when it was assumed to be SiO_2 and was filtered off. Many workers [Kemmerer & Boutwell, 1932; Toepfer & Boutwell, 1930; Wolesensky, 1928; Kahane & Kahane, 1934] have evaporated the solution to dryness with HCl after adding NaCl to fix the free H_2SO_4 in order to remove the excess HNO_3 and $HClO_4$. HNO_3, when present, interferes with the precipitation of $BaSO_4$ [Treadwell & Hall, 1935]; the author found this refinement unnecessary as all the HNO_3 had already been boiled off, and only 1 or 2 ml. of $HClO_4$ remained, which did not appear to interfere in any way with the precipitation of $BaSO_4$. The results shown in Table I demonstrate this point.

Table I. *Analysis of substances containing known amounts of S by nitric-perchloric method*

Substance	Amount taken mg.	S calc. mg.	S found mg.	% recovery
Na_2SO_4	4·42	1·0	1·03	103·0
			0·96	96·6
,,	8·84	2·0	2·03	101·5
			1·95	97·5
,,	13·26	3·0	3·00	100·0
			3·03	101·0
,,	17·7	4·0	4·00	100·0
			4·00	100·0
$Na_2S_2O_3, 5H_2O$	6·08	1·57	1·61	102·6
Cystine	50·00	13·385	13·46	100·8
Methionine	50·00	10·75	10·90	100·8

N.B. Estimations of N on the samples of cystine and methionine used for analysis showed them to be 96 and 93% pure respectively. Allowance was made for this in the above calculations.

Explosions. Occasionally there have been explosions during the wet digestions. Kahane [1937] described a similar experience and stated that the action of

$HClO_4$ on organic substances may become explosive in the absence of a diluent such as H_2SO_4 or excess $HClO_4$. Kägi [1937] also found excess of $HClO_4$ to be necessary because the mixture exploded if the solution was evaporated to dryness before all the organic matter had been destroyed. The author, however, found that the digestion mixtures were liable to explode both before and after the addition of $HClO_4$; but that they did not do so if the preliminary stages were taken slowly. These explosions may be due to the formation of nitro-compounds.

Estimation of sulphate. The solution in the Kjeldahl flask was washed out into a 500 ml. beaker, and the sulphates resulting from the oxidation were estimated gravimetrically as $BaSO_4$ in the usual way [Treadwell & Hall, 1935].

Effect of iron on the precipitation of $BaSO_4$. Tervalent metals may interfere with the precipitation of $BaSO_4$ [Treadwell & Hall, 1935]. In consequence some authors [Evans, 1931; Cherbuliez & Meyer, 1933] remove iron before precipitating the sulphate.

Varying amounts of $FeCl_3$ were added to solutions of Na_2SO_4 containing 4 mg. S. The sulphate was estimated gravimetrically and the results are shown in Table II.

Table II. *Effect of iron on $BaSO_4$ precipitates*

Amount of Fe added mg.	Theoretical amount $BaSO_4$ mg.	Amount $BaSO_4$ recovered mg.	Difference mg.	
0·3	29·0	29·3	+0·3	Filter pad brown
0·2	29·0	28·9	−0·1	
0·1	29·0	28·0	−1·0	
0·05	29·0	28·8	−0·2	
0·01	29·0	28·5	−0·5	Filter pad uncoloured
0·005	29·0	28·6	−0·4	

When the solution contained 0·05 mg. Fe or more the asbestos filter pad in the crucible was coloured brown. In the many estimations performed by this method coloration of the filter never occurred. Hence it would appear that in no case had there been more than 0·05 mg. Fe present in the solution. Table II shows that such amounts do not affect the result beyond the limits of experimental error ($\pm$0·5 mg. of $BaSO_4$).

Analysis of substances containing known amounts of S. Analysis of standard solutions of Na_2SO_4, $Na_2S_2O_3$ and of cystine and methionine showed the method to give results accurate to within 3% (Table I) and greater accuracy was obtained when larger quantities were analysed, because the precipitates could be weighed more precisely.

Determination of S in casein. The determination of S in casein has been studied and its oxidation has been found to present more difficulty than that of

Table III. *Effect of prolonged digestion on the determination of S in casein*

Sample	Duration of heating after solution became clear hr.	% of S found
A	0	0·700
		0·645
B	1	0·716
		0·722
C	6	0·745
		0·724
D	12	0·750
		0·770
E	24	0·750
		0·770

cystine or similar compounds; at first low figures were obtained and good duplicates were rare. However, if boiling was continued after the solution in the Kjeldahl flask had become clear, the method gave higher and more consistent results (Table III); heating for 12 hr. appeared to be sufficient. The figures are still lower than those quoted earlier, but nevertheless are in good agreement with those obtained by the fusion method (Table VI).

Loss of S during oxidation. To avoid the possibility of loss of S by volatilization during oxidation, bombs have frequently been used [Sherman, 1902; Parr, 1908; Evans, 1931; Painter & Franke, 1936; Godden, 1937]. A bomb, however, is tedious to use when many estimations have to be made. Revol & Ferrand [1935] prevented loss by distilling into bromine in alkali, which converted SO_2 into SO_3. Kahane & Kahane [1934] estimated the S in pure organic substances with a mixture of I_2O_5, $HClO_4$ and HNO_3, trapping the escaping gases in a solution of I_2O_5 to prevent any loss of H_2S or SO_2.

Wolesensky [1928], who used the nitric-perchloric method for the estimation of S in rubber, stated that he obtained good recoveries and hence no S appeared to be lost as spray. If, however, the digestion mixture was strongly heated so that oxidation took only 7–8 min., as originally recommended by Kahane, much S was lost. The present author found that when the substance was oxidized slowly, no S was detectable in the escaping gases when these were drawn through a trap which contained distilled water. (Glass joints were used in this apparatus because the fumes of HNO_3 attacked the rubber bungs, resulting in S contamination.) Any gases containing S escaped into an atmosphere of HNO_3 and $HClO_4$; hence it was not considered necessary to add any further oxidizing agent to the trap. This was checked by analysing one substance twice, the first time with distilled water, and then with NaOBr in the trap. The results were compared and showed no difference.

The contents of the flask and trap were washed out separately, and $BaCl_2$ added to each. Eleven different substances were analysed in duplicate in this way. In eight of them no precipitate was observed when a solution of $BaCl_2$ was added to the washings from the trap. Where a precipitate did occur it was confined to one of a pair of duplicates, and was in any case almost imponderable (i.e. less than 0·2 mg. S). The S in the samples taken for analysis varied from 1·5 to 13·6 mg.

Casein was the only substance which persistently gave a precipitate in the trap. The total weight of $BaSO_4$ obtained from the washings of flask and trap was the same, however, as that obtained from the flask when no trap was attached. This phenomenon was thought to be explained by the fact that the gases containing volatile S compounds would normally be prevented from escaping by the refluxing action of the long-necked flask. When the trap was attached to the flask and suction employed this action was lost and the gases were drawn over into the water. It was concluded that a trap was not necessary.

Recovery of added S. S in the form of Na_2SO_4, Na_2SO_3 and cystine was added to a number of foodstuffs. A study of Table IV shows all the recoveries to be between 95 and 105%, the majority being very much closer to 100%. As often only 10–30 mg. $BaSO_4$ were obtained from the material used, results of greater accuracy could not be expected.

Table IV. *Recoveries of added S by the nitric-perchloric method*

Substance	Amount of S in sample taken mg.	S compound added	S added mg.	S recovered mg.	% recovery
Butter beans	1·17	Na_2SO_4	2·00	2·02	101·0
				2·00	100·0
Chocolate	1·50	Na_2SO_4	2·00	2·08	104·0
				2·03	101·5
Almonds	3·10	$Na_2S_2O_3$	2·99	2·87	96·0
				2·99	100·0
Biscuits (mixed plain)	2·50	$Na_2S_2O_3$	2·99	3·00	100·5
			1·51	1·48	98·1
Malted milk	3·24	$Na_2S_2O_3$	2·99	2·97	99·5
Sausages	2·90	$Na_2S_2O_3$	2·99	3·00	100·5
				2·85	95·5
Barcelona nuts	3·75	Cystine	5·10	5·16	101·1
				5·05	99·4
Peas (split, dry)	2·00	Cystine	5·10	5·19	101·5
				5·25	102·8
Oranges	1·35	Cystine	5·10	5·08	99·5
				5·01	98·0
Sago	0·20	Cystine	5·10	5·19	101·5
				5·44	105·2
Salsify	2·68	Cystine	5·10	4·88	95·5
				5·18	101·5
Vitaweat	3·05	Cystine	5·10	5·09	99·8
				5·07	99·5

Conclusions

This procedure appears to have many advantages. The ashing can be done in a pyrex Kjeldahl flask, and therefore large quantities of materials with low S content can be taken. When possible, enough material was taken to give at least 50 mg. of $BaSO_4$, as the balance used weighed to 0·1 mg. The oxidation requires very little attention, and so many estimations can be made simultaneously.

Hydrogenation method

All the methods so far described depend on the *oxidation* of S to sulphate. ter Meulen [1922] introduced a method of estimation for use with organic compounds, depending on the *reduction* of S to H_2S, which was estimated iodimetrically; as yet this method has not been much used. A very similar method is described by Kubota & Hanai [1928].

ter Meulen only applied the method to pure organic substances, oils and coal gas. There appeared to be no reason why it should not be applied to biological material such as foods, urine and faeces. Investigation showed this to be possible, and the technique finally developed follows.

Reagents. A cylinder of hydrogen (a Kipp's apparatus is unsatisfactory).

Platinized asbestos.

B.D.H. A.R. pellet NaOH. 2 pellets = 0·25 g.

A strong solution of NaOH.

Solution A. 2 % $CdCl_2$.

Solution B. 1 % $CdCl_2$ in 20 % acetic acid.

20 % acetic acid.

$N/100$ $Na_2S_2O_3$ standardized against $N/100$ $KMnO_4$.

$N/100$ I_2 dissolved in KI, standardized against $N/100$ $Na_2S_2O_3$.

Apparatus. The apparatus is shown in Fig. 1. The five-litre jar *A* contains hydrogen, and a steady stream of gas can be procured at any desired pace by running water into the bottle from a tap.

The wash bottle *B* contains a strong solution of NaOH. A blank estimation showed no further purification of the hydrogen to be necessary. The glass connexion between the wash bottle and the combustion tube is drawn out to a fine jet to prevent the gas from blowing back.

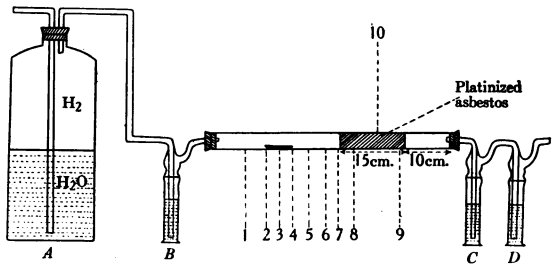

Fig. 1. Apparatus employed for the determination of S by hydrogenation.

The combustion tube is made of quartz, and transparent or opaque tubing can be used with equal success. The latter is to be recommended, for it is cheaper, and also frequent heatings soon render a transparent tube opaque. The tube employed is of 10 mm. diameter, and 60 cm. long. A small diameter is most successful, as it is easier to displace all the gas.

The combustion tube is loosely packed with platinized asbestos for a length of 15 cm. as shown in the diagram; corks are used in preference to rubber bungs since contamination from the former is less likely.

The mixture of gases from the heated combustion tube is passed through two wash bottles, *C* and *D*, each of which contains two pellets of NaOH and 1 ml. of solution A.

Kubota & Hanai used a tube with wire coiled round it so that all portions of the tube were heated equally. In the present investigation the tube was heated by means of Bunsen burners with spreaders attached.

Procedure for hydrogenation. (1) The substance was introduced into the quartz tube in a boat, and the corks inserted and waxed over.

(2) Hydrogen was passed through the tube rapidly, until sufficient time had elapsed (i.e. 5–10 min.) for all the air to have been displaced. The rate of flow of hydrogen was then adjusted to one bubble per sec., which experiment had shown to be most satisfactory for H_2S formation.

(3) The platinized asbestos was heated to a dull red heat by Bunsen burners placed in positions 8, 9 and 10 (Fig. 1). Meanwhile, the tube was gradually heated from positions 1 to 7 (Fig. 1) by two more Bunsen burners. One without a spreader was placed in position 1, and then moved towards position 7, at approximately 5 min. intervals, so that the total time of heating was about 35 min. As the first burner was moved along the tube, a second one, with spreader, was introduced behind it. The region under the boat was heated very gradually. In each position the tube was heated, gently at first, and then the intensity of the flame gradually increased to its maximum. This technique was adopted so that the substance was volatilized slowly.

Position 7 having been reached, heating was continued for another 15 min. to ensure complete hydrogenation.

(4) The Bunsen burner in position 9 was moved along to the right (Fig. 1). Experiment showed that some substances would volatilize and condense in this part of the tube, as often a considerable quantity of H_2S came over when this portion was heated.

(5) The rate of flow of hydrogen was gradually increased to make sure that all the H_2S present was displaced. When H_2S had ceased to come over (*vide infra*) the hydrogenation was ended and the CdS estimated.

During hydrogenation carbon is deposited on the catalyst and walls of the tubes. The catalyst is thereby poisoned and, if the tube is not cleaned, low results are obtained, as H_2S is absorbed by the carbon. The latter is removed and the tube made ready for another analysis by displacing the hydrogen and then heating to redness in a current of air. ter Meulen [1922] recommended that substances that charred excessively should be mixed with platinum black before hydrogenation to prevent absorption of H_2S by carbon. The tube should also be cleaned by rinsing it with HCl after it has been used for a number of estimations.

Estimation of H_2S. When the NaOH and $CdCl_2$ were mixed in the wash bottles, a white precipitate of $Cd(OH)_2$ was thrown down. As soon as H_2S commenced to come over yellow CdS was formed. Since no yellow precipitate ever appeared in the second wash bottle, it was concluded that all the H_2S was absorbed in the first one, and the second bottle was later discarded. This colour change was also used to indicate the end point of the hydrogenation. The wash bottle in which H_2S was being collected was replaced by a fresh one. If the precipitate in this jar remained white, heating was terminated.

The contents of the wash bottle were transferred to a 50 ml. centrifuge tube, and 2·5 ml. of solution B added. This either dissolved any residual $Cd(OH)_2$, or, on the other hand, ensured the complete precipitation of S as CdS should all the $Cd(OH)_2$ have been used up and some H_2S be held by the NaOH. After centrifuging for 5 min. the supernatant fluid was decanted and the precipitate washed with 10 ml. of 20 % acetic acid. The tube was again centrifuged, and the supernatant fluid removed. A known excess of $N/100$ I_2, varying with the size of the precipitate, was added, and the whole acidified wtih conc. HCl, until solution was complete. (It was essential to add an excess of HCl because CdS is insoluble in acid weaker than $1{\cdot}3N$.) The reaction proceeds according to the equations:

$$2HCl + CdS = CdCl_2 + H_2S,$$
$$H_2S + I_2 = 2HI + S.$$

The excess I_2 was titrated with $N/100$ $Na_2S_2O_3$. The amount of S was then calculated from the equation

$$1 \text{ ml. } N/100\ I_2 = 0{\cdot}16 \text{ mg. S.}$$

Very accurate and reliable results were obtained by this method (Table VI).

Discussion of hydrogenation method

The method, as given above, differs somewhat from ter Meulen's, although the principle remains the same.

1. *Apparatus.* The relative merits of hard glass and quartz tubing for the above estimation have been discussed by other workers. ter Meulen originally recommended quartz tubing, and platinized asbestos as catalyst, but later [1931] he stated that as good results had been obtained by using pure in lieu of platinized asbestos. Gauthier [1935] obtained low values when he employed a hard glass tube and pure asbestos, and said that satisfactory results were only

obtained if pumice stone was used as a catalyst. ter Meulen [1935] replied to this by pointing out that if pure asbestos was used, the tube had to be heated to at least 1000° and must therefore be of quartz. The present author found hard glass tubing unsatisfactory, both with pure and platinized asbestos. In both cases very low results were obtained, owing, no doubt, to the fact that the tubing could not be heated strongly enough without its beginning to collapse. Quartz tubing and platinized asbestos gave the best results. With pure asbestos results were not so reliable, in some cases being slightly too low; this was probably due to the fact that the tube was heated by Bunsen burners, and not by a furnace. In consequence, a sufficiently high temperature was not reached.

2. *Estimation of H_2S.* ter Meulen [1922], in his original method, absorbed the H_2S in NaOH. At the end of the hydrogenation, the NaOH was washed into a known excess of I_2 solution and acidified, and the S was estimated by a back titration of the I_2 with $Na_2S_2O_3$.

The author used this technique and obtained very satisfactory results with cystine and methionine. However, when more complex substances such as casein or foods were analysed in the same way, the results were irregular and high. No permanent end-point could be obtained and it was in any case difficult to discern, owing to the solution turning a greenish colour. For consistent and accurate results, H_2S has to be separated from any compound which might interfere with its estimation. This was achieved by precipitating it as insoluble CdS.

ter Meulen [1934] showed that when a platinum spiral was used as a catalyst and N was present in the compound analysed, $(CN)_2$ was formed, and interfered with the I_2 titration. HCN was liberated on acidification of the NaOH solution, and this absorbed I_2, thereby giving high results. He overcame the difficulty in a manner very similar to that of the author, though this was not known at the time when the present method was devised.

Unsaturated hydrocarbons would also interfere, if present, though this was considered unlikely. No doubt the unreliable results obtained by the present author for casein etc. were explained by the formation of $(CN)_2$ in the presence of platinized asbestos, though it is curious that a similar interference did not upset the estimation of S in cystine and methionine.

In all estimations $N/100$ I_2 and $N/100$ $Na_2S_2O_3$ solutions were used, which enabled amounts of S as low as 0·2 mg. to be estimated. ter Meulen [1922] has described a colorimetric micro-method for estimating quantities as low as 0·05 mg. S; the H_2S is converted into PbS and the colour compared with a standard preparation of PbS made from NaS. The chief difficulty of this method is to prevent the standard Na_2S solution from becoming oxidized, though Giberton [1933] has described a very simple means of standardizing the Na_2S before use.

3. *Interference by metals.* In his original paper ter Meulen [1922] stated that when alkali salts were present, some S remained behind, attached to the metal. This S could be recovered by fusion of the residue in the boat with borax. Gauthier [1935] used this technique, but only recovered half the S when analysing alkali sulphates, and recommended instead that after a preliminary hydrogenation, the boat should be withdrawn, a drop of HCl added, the boat replaced and heating continued.

The author intended to use this method for the analysis of substances which contained alkali salts (urine, foods etc.) and the matter was further investigated. Three foods and a specimen of urine were analysed with and without the addition of borax and HCl. Table V shows that the results were often lower

and more inconsistent when borax was added, than those obtained when no additions were made. With HCl the results tended to be very slightly higher, but not significantly so. From this it would appear that the addition of either borax or HCl to the residue in the boat is unnecessary, and that the method may be used as it stands for the analysis of biological material.

Table V. *Effect of adding HCl or borax to substance in the boat*

	mg. S/100 g. or 100 ml. Found after addition of		
Substance	Nil	Borax	HCl
Meat, dried	752·5	660·0	757·5
Pearl barley, A	108·0	111·0	109·7
Peas, split, dried	166·0	156·0	170·5
Urine	46·6	34·7	46·1

Comparison of investigated methods

Table VI gives a comparison of results obtained by all four methods investigated. Two points are at once apparent:

(1) The Benedict-Denis technique fails entirely with methionine and casein, and cannot therefore be recommended as a general method.

(2) The agreement between the remaining two oxidation methods and the hydrogenation method is, on the whole, good.

Table VI. *S determinations by four different methods*

	% S by the various methods				
Substance	Benedict-Denis	Sodium peroxide fusion	Nitric-perchloric oxidation	Hydro-genation	Theoretical %
Cystine	27·00	26·55	26·90	26·80	26·67
Methionine	9·10	21·70	21·80	21·60	21·50
Casein	0·47	0·77	0·76	0·77	—
Biscuit, dried	—	0·102	0·102	0·117	—
Meat, dried	—	0·625	0·630	0·600	—
Pearl barley, B	—	0·135	0·124	0·117	—
Peas, dried	—	0·178	0·186	0·166	—
Sausages	—	0·157	0·150	0·165	—
Urine (24 hr. specimen) A	—	—	0·047	0·047	—
Urine (24 hr. specimen) B	—	—	0·068	0·071	—

All three methods give figures in good agreement with the theoretical S values of cystine and methionine. With casein, and also with other foods, agreement is close, though greater variations are found between the figures obtained for these substances than for the simpler compounds.

Disadvantage of the hydrogenation method. The method has one minor disadvantage. It is not easy to run several estimations concurrently. The author found it possible, however, to carry out two estimations at the same time by placing the combustion tubes side by side and heating them both with the same set of Bunsen burners.

Advantages of the hydrogenation method. The hydrogenation method has many advantages when compared with oxidation methods.

(1) The final stage is a titration. This enables smaller quantities of S to be estimated more accurately than can be done by the oxidation-gravimetric methods. This in turn means that smaller quantities can be taken for analysis.

(2) The method is considerably quicker than any oxidation process. A complete estimation can be done in 2–2½ hr., whereas all the other methods take at least 24 hr. and often longer.

(3) There is no risk of losing S by volatilization.

Summary

1. Four methods of estimating sulphur in biological material have been investigated. Three of these methods, i.e. the Benedict-Denis, the sodium peroxide fusion and the nitric-perchloric methods, were oxidation processes. The fourth involved hydrogenation.

2. The sodium peroxide fusion method gave accurate results, but frequent explosions made it unsatisfactory for routine use.

3. The Benedict-Denis method was found to be unreliable, probably because of its failure to determine methionine-sulphur accurately.

4. The nitric-perchloric method, in which destruction of the organic material by a mixture of HNO_3 and $HClO_4$ was followed by a gravimetric estimation of the resultant sulphates, appeared to be reliable. Large quantities of material could if necessary be taken, and the oxidation required very little attention, so that many estimations could be made simultaneously.

5. The hydrogenation method was applied to biological material, and was found satisfactory after certain modifications had been made. The results by this method compared favourably with those given by the fusion and nitric-perchloric methods. It was found to be a quicker and more sensitive method than any oxidation process investigated, and seemed to be the best available for the routine determination of sulphur.

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