CCLIV. THE APPLICATION OF PHOSPHO-18-TUNGSTIC ACID (FOLIN'S REAGENT) TO THE COLORIMETRIC DETERMINATION OF CYSTEINE, CYSTINE AND RELATED SUBSTANCES.

II. THE DETERMINATION OF SULPHYDRYL COMPOUNDS AND DISULPHIDES ALREADY EXISTING IN SOLUTION.

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THE principles underlying the new methods will be indicated briefly, but to form an idea of the magnitudes and likelihood of errors and for a proper understanding of the principles involved, it will be necessary to refer to Part I of the series [1932].

By estimating cysteine and cystine in acid solution all the disadvantages of alkaline colour development may be avoided. The disadvantages of acid colour development include slight loss in colour and diminution in rate of development. For all round utility a p_H of 5.7 has been selected, and to minimise colour development by extraneous reducers and for the sake of economy, only small amounts of reagent are employed. A cystine *plus* sulphite standard is used for comparison.

The cysteine is allowed to develop its coloration in absence of sulphite and its estimation in absence of extraneous reducers is quite simple. In coloration developed, 1 mol. of cystine = 2 mol. of cysteine, or 2 mg. of cystine = 2.017 mg. of cysteine. When extraneous reducers are present their effects must be found by artifice. In the presence of mercuric chloride cysteine gives no coloration at all, and ferrous salt is used to find the contribution of the extraneous reducers to the total coloration developed. Since $HgCl_2$ will form a precipitate with uric acid, and because it influences slightly the coloration developed by some extraneous reducers, it is necessary to have a fairly large amount of zinc chloride present to prevent precipitation and to provide an initial "heavy metal" effect upon the weaker reducers so that the effect of the $HgCl_2$ will be diminished. To nullify the inhibiting effect of the $ZnCl_2$ upon the rate of colour

development by cysteine, it is desirable that some NH_4Cl be present also. The ZnCl_2 and NH_4Cl are incorporated with the citrate buffer used in preparing standard and unknowns.

Cystine is estimated with sulphite present and the estimation is simple when extraneous reducers are absent. The same buffer as is used in estimating cysteine is employed. When extraneous reducers are present, mercuric chloride and ferrous salt are used as in the corresponding cysteine estimation.

Provided extraneous reducers are absent, the estimations of cysteine and cystine together are not difficult. In absence of sulphite the coloration is due to cysteine alone. The cystine develops its usual colour in presence of sulphite and the cysteine develops twice as deep a coloration and hence, subtracting twice the value obtained in absence of sulphite from that obtained in presence of sulphite, the nett amount represents the cystine. In so far as the cysteine in the unknown solution may be converted fairly rapidly into cystine by aerial oxidation, it might be preferable to record the "total cystine" (cysteine *plus* cystine) content by subtracting the value of the coloration obtained in absence of sulphite from that obtained in its presence and calculating the nett amount on the basis of 2 mg. of cystine = 2 mg. of "total cystine." The solution which develops its coloration in presence of sulphite should develop most of its cysteine coloration in absence of the sulphite, which is added after a short interval. By this precautionary measure the "total cystine" might be estimated quite accurately even if a considerable amount of the cysteine were to suffer aerial oxidation during colour development. Provision is made for this in the method, but tests so far have shown that the precaution is not necessary.

When extraneous reducers are present they may contribute different amounts of colour in absence and presence of sulphite. Mercuric chloride and ferrous salt are used in absence and presence of sulphite to find the two contributions.

The use of mercuric chloride renders these methods highly specific for sulphydryl compounds and disulphides, and in analysing any solution it is wise to assume that extraneous reducers are present and to proceed accordingly.

Neither cysteine nor cystine is extracted from acid aqueous solution (p_{H} less than 3) by ether, but part of the cysteine may be oxidised to cystine in the process of extracting a solution. On the other hand laevulic acid, pyruvic acid, furfuraldehyde, and similar substances may all be extracted though never completely. Their contributions to the coloration developed may thus be greatly reduced and the possible error in a cystine estimation thereby diminished. Four extractions with twice the volume of ether each time will remove approximately four-fifths of the laevulic and pyruvic acids and practically all the furfuraldehyde. Ether extractions introduce a decided degree of specificity for cysteine and cystine among the sulphydryl compounds and disulphides. The condition that these compounds should remain in the aqueous acid solution is probably that they contain a strongly basic group like $-\text{NH}_2$.

Complications arise when the unknown solution is coloured initially. Selective absorbents should never be used as their selectivity can never be perfect and is frequently very poor. Theoretically the compensating colorimeter is the instrument to be employed and the blank used with the standard must or must not contain sulphite according to its presence or absence in the unknown. There is always some uncertainty in the matter of the blanks because one cannot know what effect the reagent would have upon the adventitious coloration.

If the adventitious coloration is the same as that being developed its value may be subtracted from the readings obtained when an ordinary colorimeter is employed. Even when the adventitious coloration is at the other end of the spectrum, as in protein hydrolysates, an experienced operator may blind himself to it, if it is not extremely pronounced, and achieve excellent results with an ordinary colorimeter. It is the author's experience that most compensating instruments fail to survive even those simple tests to which an ordinary instrument is put before being used.

The reagents, which are listed below, should be filtered through sintered glass if necessary. The list is followed by directions for making estimations.

Phospho-18-tungstic acid reagent. This is prepared according to the directions of Folin and Marenzi [1929] with separation of any molybdenum present according to their plan. It may be looked upon as containing per litre 100 g. of the salt $\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$, 172 g. of H_3PO_4 , and 16.1 g. of LiOH . The sodium tungstate should be prepared by dissolving the necessary amount of precipitated WO_3 in the necessary amount of NaOH solution. If the reagent fails to comply with the following requirements it must be discarded. (1) It must give no coloration with tyrosine or tryptophan at any p_{H} . (2) At p_{H} 5.7 and 8.4, 4 mg. of cystine in presence of 2 cc. of reagent and 1 millimol. of sulphite must give not less than 3.95 times the coloration developed by 1 mg. of cystine. (3) At p_{H} 5.7 and 8.4 and in absence of zinc and ammonium salts, 80 mg. of cysteine with 2 cc. of reagent must give almost exactly seven times the coloration developed by 2 mg. of cysteine.

Sodium acetate solution: a 4 *M* solution containing 544 g. of the salt $\text{C}_2\text{H}_3\text{O}_2\text{Na}$, $3\text{H}_2\text{O}$ per litre.

Composite citrate buffer. This contains 105 g. of crystalline citric acid $\text{C}_6\text{H}_8\text{O}_7$, H_2O , 52.5 g. of NaOH , 13.6 g. of ZnCl_2 and 26.8 g. of NH_4Cl per litre. It is best prepared by shaking the citric acid and NaOH with about 500 cc. of water with constant cooling to avoid boiling, until entirely dissolved. The ZnCl_2 is dissolved in 20 cc. of water and added to the cold solution, and this is followed by the NH_4Cl dissolved in about 200 cc. of water. The mixture is diluted to 1 litre.

Buffered sulphite. 9.5 g. of sodium metabisulphite ($\text{Na}_2\text{S}_2\text{O}_5$) are dissolved in about 70 cc. of water, 15 cc. of 4 *M* sodium acetate solution are added and the mixture is diluted to 100 cc. This *M* sulphite solution will keep for months in a stoppered bottle.

Mercuric chloride: a 0.1 *M* solution containing 2.72 g. of HgCl_2 per 100 cc. of solution.

Ferrous salt: a $M/12$ Fe^{++} solution containing 3.268 g. of Mohr's salt ($\text{FeSO}_4 \cdot (\text{NH}_4)_2\text{SO}_4 \cdot 6\text{H}_2\text{O}$) and 8.5 cc. of $N \text{H}_2\text{SO}_4$ per 100 cc. It will keep for months in a well-stoppered bottle and may be titrated against potassium permanganate to check alteration. The solution is diluted 1 in 8, 1 in 9, 1 in 10, etc., before use in the estimations.

Standard cystine solution. 0.5 g. of pure cystine (*l*-cystine is most easily obtained) is dissolved in 50 cc. of *N* hydrochloric acid, and diluted to 500 cc. to provide a $M/240$ solution in 0.1 *N* HCl , containing 1 mg. of cystine per cc. The solution will keep indefinitely.

Estimations in general.

All estimations are carried out in standard 100 cc. flasks. Standard and unknown should be of approximately the same colour intensity (70–150 %) and must be prepared together. In reference to the 5–10 minute interval allowed for colour development, one of about 7 minutes is to be preferred. The solutions should be read within 30 minutes of being diluted to the mark.

The standard cystine solution is acid and every 1 cc. requires 0.25 cc. of the sodium acetate solution to be brought to p_{H} 5.7. Aliquots of the unknown cysteine, cystine, and mixed solutions must be titrated to p_{H} 5.7 with the sodium acetate solution, using bromocresol purple indicator. The appropriate amounts of acetate must be included with the larger amounts mentioned in the schedules below when preparing the standards and unknowns.

Estimation of cysteine.

Unknown. 10 cc. of citrate buffer, 2 cc. of reagent and 2 cc. of sodium acetate solution are mixed in a flask in that order, and to the mixture is added the chosen aliquot of unknown cysteine solution.

Standard. To a mixture of the same composition as that used for the unknown is added the necessary amount of cystine solution and this is followed by 1 cc. of buffered sulphite.

After 5–10 minutes the solutions are diluted and compared. The apparent cysteine content will be $x + a$ mg., where x is the true amount; in absence of extraneous reducers a will be zero. If extraneous reducers are present a is found as follows. Another unknown is prepared just as before, but, prior to adding the unknown cysteine solution, 1 cc. of the mercuric chloride solution is added to the mixture. The amount of coloration developed, calculated as cysteine, will correspond with A mg. By trial and error in a series of solutions, the amount of ferrous salt necessary to make the total coloration agree closely with the original apparent $x + a$ mg. of cysteine, is found. A new unknown is prepared containing the mercuric chloride and unknown cysteine solutions followed by the necessary amount of ferrous salt, and compared with a new

standard containing precisely the same amount of ferrous salt but none of the unknown cysteine solution. The contribution of the extraneous reducers to the colour developed by the ferrous salt is found to be a_1 in terms of mg. of cysteine. a_1 may be different from A but it is nearly the same as a , and hence x is found from $x + a - a_1$.

Estimation of cystine.

The standard and unknown are prepared in precisely the same manner as the standard cystine *plus* sulphite solution used in estimating cysteine. The apparent amount of cystine found will be $y + b$ mg., where y is the true amount, and b is zero when extraneous reducers are absent. If extraneous reducers are present then b is found as follows. Another unknown is prepared just as before and containing sulphite, but prior to adding the unknown cystine solution 1 cc. of the mercuric chloride solution is introduced. The coloration will correspond with b mg. of cystine. By trial and error the amount of ferrous salt necessary to make the total coloration agree closely with the original apparent $y + b$ mg. of cystine is found. A new unknown is prepared containing mercuric chloride, the unknown cystine solution, and sulphite, and after colour development has proceeded for about 1 minute the necessary amount of ferrous salt is added. Colour development is allowed to continue for the remainder of the 5-10 minutes and on dilution the solution is compared with a new standard containing mercuric chloride, sulphite, and the same amount of ferrous salt, but none of the unknown cystine solution. The contribution of the extraneous reducers to the colour developed by the ferrous salt in presence of sulphite is thus found to correspond with b_1 mg. of cystine. Identifying b_1 with b , y is found from $y + b - b_1$.

Estimation of cysteine and cystine when present together.

The procedure is merely a combination of those used in estimating cysteine and cystine. Suppose there are x mg. of cysteine and y mg. of cystine present in a chosen aliquot. In absence of extraneous reducers the colour developed without sulphite will correspond with the x mg. of cysteine. With sulphite the colour will correspond with $2x$ mg. of cysteine *plus* y mg. of cystine. Hence both x and y are found. If extraneous reducers are present the coloration in absence of sulphite will correspond with $x + a$ mg. of cysteine, and in presence of sulphite it will correspond with $2x$ mg. of cysteine *plus* $y + b$ mg. of cystine. In absence of sulphite mercuric chloride is used first to find a and later with ferrous salt to find a_1 . In presence of sulphite it is used first to find b and then with ferrous salt to find b_1 . a_1 is assumed identical with a , and b_1 with b , and hence both x and y may be found.

None of the coloured solutions used in estimating cysteine, cystine, and mixtures of the two, should exceed in intensity the coloration developed by 4 mg. of cystine in presence of sulphite.

SUMMARY.

Colorimetric methods involving the use of phospho-18-tungstic acid (Folin's reagent) are described for the estimation of sulphydryl compounds and disulphides, with especial reference to cysteine and cystine.

The estimations may be performed in the presence of large amounts of extraneous reducers. Under such conditions the methods are rather tedious but have been found to yield reasonably accurate results.

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REFERENCES.

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