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

I. THE REDUCTION OF PHOSPHO-18-TUNGSTIC ACID BY VARIOUS SUBSTANCES.

By JOSEPH W. H. LUGG.

From the Division of Animal Nutrition of the Commonwealth Council for Scientific and Industrial Research, University of Adelaide, South Australia.

(Received September 12th, 1932.)

Of the phosphomolybdates and phosphotungstates those containing eighteen atoms of the heavy metal to every P_2O_5 , members of the 1-18 series in our present nomenclature, have been exploited most in colorimetric estimations on account of the ease with which their pale-coloured solutions may be reduced by certain substances to give intense blue, greenish, or reddish tints. Some use has lately been made of the corresponding arsenic compounds.

Wu [1920] arranges the 1-18 and 1-24 phospho-molybdates and -tungstates in order of increasing sensitiveness to reduction, as follows: phospho-24-tungstic acid, B-phospho-18-tungstic acid, A-phospho-18-tungstic acid, phospho-24-molybdic acid, and phospho-18-molybdic acid. He further states that acids of the 1-17 and 1-22 series are quite inert. Salts of the 1-17 and 1-22 series are formed when those of the 1-18 and 1-24 series respectively are treated with excess of alkali hydroxide or carbonate. Salts of phospho-17-tungstic acid are thus precipitated as small white crystals difficultly soluble in large excess of alkali. These salts, together with others containing much more phosphorus which are supposed to exist, are said to regenerate 1-18 and 1-24 acids exclusively on treatment with mineral acids.

Wu concluded that two forms, A and B, of phospho-18-tungstic acid exist, and that his B form may be identified with the 1-18 acid of Kehrman [1887], who was the first to prepare such a compound. He notes however that Kehrman probably prepared both forms. The A and B forms are said to yield the same 1-17 acid on treatment with alkali, and the 1-17 acid regenerates exclusively the B 1-18 acid on treatment with mineral acids.

Some doubt has been thrown upon the existence of the A form by Folin and Marenzi [1929], who suggest the presence of molybdenum impurity in Wu's sodium tungstate and that the alleged A acid may have been a molybdotungsten complex.

Folin and Looney [1922] made use of the Folin and Denis [1912] uric acid reagent to determine the cystine content of protein hydrolysates. The uric acid reagent is essentially phospho-18-tungstic acid prepared by treating sodium tungstate with phosphoric acid, and if Wu's findings are correct one would expect it to contain both the A and B forms. The method is based upon the observation (apparently unpublished) of T. Zucker, that cystine *plus* sodium sulphite gives colours with the uric acid and phenol reagents, whereas cystine alone does not, and sulphite alone develops a comparatively feeble colour. Heffter [1907] had discovered that cystine may be reduced by sulphite to give cysteine.

Since 1922 many modifications of the Folin-Looney method have appeared. Hunter and Eagles [1927] warned against having standard and unknown at even slightly different degrees of alkalinity. Folin and Marenzi [1929], following the lead of Folin and Trimble [1924], carefully removed molybdenum from the reagent to avoid reactions with tyrosine, tryptophan and phenolic bodies generally. They also improved the technique in some directions. Rimington [1930], working upon an observation made by Folin, added urea to prevent precipitation of the 1-17 tungstate; an event that frequently ruins estimations under the Folin-Looney and Folin-Marenzi procedures. Folin and Marenzi themselves had tried to overcome this difficulty by adding large amounts of lithium salt to the reacting mixture. In their mixtures the solubility of lithium carbonate is so grossly exceeded that it is surprising its precipitation does not occur more frequently than it actually does. Tompsett [1931] reduced the alkalinity of the reacting mixture and thereby avoided precipitation of the 1-17 tungstate, reduced the effect of slight changes in alkalinity upon the colour developed, and greatly reduced interference by uric acid. Most of the solutions (*e.g.* hydrolysates of tissues) which have to be analysed for cystine contain other substances which contribute to the coloration developed. Uric acid gives much colour in both the Folin-Looney and Folin-Marenzi procedures in absence of sulphite, and more in presence of it. Those soluble products arising from the digestion of carbohydrate with mineral acid at the boiling point also reduce the reagent. It has become customary to subtract the blank obtained in absence of sulphite from the colour obtained in presence of sulphite, and to call the nett result "cystine." Inasmuch as the coloration developed by extraneous substances may be increased when sulphite is present, and perhaps reduced by the very presence of cystine, this procedure is unjustified. Furthermore, cysteine reduces the reagent in absence of sulphite, and this seems to have been neglected in nearly all interpretations of the results, the blank obtained in absence of sulphite being calculated nearly always as "uric acid."

In his study of the alkaline decomposition of cystine, Andrews [1928] used the Folin-Looney procedure for the estimation of both cysteine and cystine. With some concern he noted that the colour developed in presence of sulphite sometimes exceeded that which would have been obtained if none of the cystine had been destroyed, and ascribed this remarkable result to the presence of

some unknown strongly reducing substance produced in the treatment of cystine with alkali. His expectations were really based upon the accepted belief that sulphite reduces cystine to cysteine under the conditions of the cystine estimation. The fate of the cysteine during its oxidation by the reagent seems not to have interested anybody.

There is reason to believe that any sufficiently soluble sulphhydryl compound will reduce the reagent, and the same may be said of any disulphide which will react with sulphite to form a sulphhydryl compound. It is clear then that the reaction with phospho-18-tungstic acid is not only unspecific for cysteine among the sulphhydryl compounds, but is further unspecific for sulphhydryl compounds themselves. This lack of specificity has led many to abandon phosphotungstic acid in favour of a far more specific colour reaction discovered by Sullivan [1926], and, in turn, the enormous difficulties attending the application of Sullivan's reaction to quantitative analyses have thrown the list of estimations in the literature into a rather chaotic state.

The present paper constitutes an analysis of the phospho-18-tungstate colour reactions, and includes among other things, investigations of the nature of the reaction between cysteine and the reagent, the reactions between cystine and sulphite, and the degree to which the reagent is reduced by substances other than cysteine but likely to occur in a mixture to be analysed for cysteine or cystine.

EXPERIMENTAL.

The phospho-18-tungstic acid was prepared free from molybdenum according to the method of Folin and Marenzi [1929]. Some of the reducing substances examined were carefully prepared in this laboratory. I am indebted to Mr H. R. Marston of this Division for the supply of cystine.

The coloration developed by a reducer may vary with the time allowed for the reaction, the concentration and amounts of the reacting materials, the p_H of the reacting mixture, salt concentration and type, the order in which the materials are added, and sometimes with small amounts of substances which may react with a particular reducer under examination. With the more powerful reducers the effect of temperature seems to be almost nil.

Except in alkaline solutions, the order of adding the different materials has but little effect upon the colour developed in 5 minutes. In alkaline regions slightly greater colorations result if the reagent is first made alkaline and the reducers are then added, than if the reagent and reducer are first mixed and then made alkaline. This effect increases slightly with the time for which the alkaline reagent is allowed to stand, provided this is limited to a few minutes. Solutions containing cystine *plus* sulphite, cysteine, or ferrous salt must never be made alkaline before the reagent is added, or there will be a decided loss in coloration developed (*cf.* the Folin-Looney and Folin-Marenzi procedures for the estimation of cystine). Na_2S as reducer cannot be added to an acid solution and made alkaline afterwards. Such procedure would result in a loss of H_2S .

Fig. 1 expresses graphically the variation with p_H of the blue coloration developed by some of the reducers and mixtures, under conditions to be described. Different amounts of reagent were used in obtaining different curves, but for each curve the only important independent variable was the p_H . The free phosphate in the reagent acted as a buffer in certain regions but most of the buffering was done by acetate, citrate, and borate mixtures added for that purpose. In the more alkaline regions sodium carbonate was used to "neutralise" the reagent after the reducer had been added, and in the more acid ones sodium acetate was used. Temperature varied very little from 25°.

Fig. 1. 1, cystine + sulphite; 2, cysteine; 3, cysteine + sulphite; 4, Fe^{++} ; 5, Fe^{++} + sulphite; 6, Na_2S ; 7, Na_2S + sulphite; 8, uric acid; 9, furfuraldehyde; 10, furfuraldehyde + sulphite; 11, laevulinic acid; 12, laevulinic acid + sulphite; 13, sulphite.

Short of recording in a lengthy table the actual amounts of carbonate, citrate, acetate, *etc.*, used it would be impossible to state the actual salt concentrations, but it is believed that the reduction in the activity of the water in the final diluted solutions would correspond approximately with that observed in a 0.4 *M* NaCl solution. In so far as salt type and concentration may greatly influence the activities of the species taking part in the colour reactions, the point is however not without importance. Two sets of solutions were used in obtaining each curve. They differed only in that the members of the set used in estimating the coloration received reducing substance whilst the corresponding members of the other set, used in p_H determination, did not. p_H was determined colorimetrically with the aid of a wide range of indicators and recorded values may be in error by 0.1 unit or more in some instances.

The colour reactions were carried out in 100 cc. standard flasks. Colour development was allowed to proceed for 5–6 minutes in a volume not exceeding 30 cc. and not less than 15 cc., the volume being kept constant for any one

substance under investigation whatever the p_H or buffers employed. After this interval had elapsed the solutions were diluted to the mark with pure water and within 5 to 15 minutes were compared with some arbitrary and constant standard. It was found necessary to have some citrate present in alkaline solutions containing iron, tin, *etc.* to avoid formation of precipitates. Lithium sulphate was never added. The p_H beyond which one cannot proceed without precipitating the 1-17 tungstate is 8.5. The curves are for colorations obtained with:

1. 2 mg. (1/120 millimol.) of cystine *plus* 1 millimol. of Na_2SO_3 with 5 cc. of reagent.
2. 2.017 mg. (1/60 millimol.) of cysteine alone with 2 cc. of reagent.
3. 2.017 mg. of cysteine *plus* 1 millimol. of sulphite with 2 cc. of reagent.
4. 0.93 mg. (1/60 millimol.) of Fe^{++} alone with 2 cc. of reagent.
5. 0.93 mg. of Fe^{++} *plus* 1 millimol. of sulphite with 2 cc. of reagent.
6. 1.30 mg. (1/60 millimol.) of Na_2S alone with 10 cc. of reagent.
7. 1.30 mg. of Na_2S *plus* 1 millimol. of sulphite with 10 cc. of reagent.
8. 2 mg. (1/64 millimol.) of uric acid alone with 5 cc. of reagent.
9. 20 mg. (1/4.85 millimol.) of furfuraldehyde alone with 2 cc. of reagent.
10. 2 mg. of furfuraldehyde *plus* 1 millimol. of sulphite with 2 cc. of reagent.
11. 10 mg. (1/11.6 millimol.) of laevulic acid alone with 2 cc. of reagent.
12. 10 mg. of laevulic acid *plus* 1 millimol. of sulphite with 2 cc. of reagent.
13. 1 millimol. of Na_2SO_3 with 5 cc. of reagent.

In order that the figure might not be unduly complicated, many of the data have been left out of it, and some of these will be recorded below.

When carefully purified and fresh, pyruvic acid (10 mg.) does not reduce the reagent at any p_H . In acid regions the coloration developed in presence of sulphite is practically that of the sulphite itself, but in more alkaline regions the coloration is somewhat greater than that due to the sulphite alone. Pyruvic acid polymerises on standing and old, impure samples may reduce the reagent.

Creatinine only slightly reduces the reagent, the least reduction being observed in acid solutions. Quinol reduces the reagent strongly in alkaline regions and fairly well in acid solutions. Phenylhydrazine and "metol" are powerful reducers. Cuprous chloride reduces the reagent after the manner of ferrous salt. Ferric chloride *plus* sulphite gives a colour corresponding with that of the sulphite alone. Stannous chloride alone gives a slight colour in acid solution but a stronger one in alkaline. With sulphite the colour developed is much greater than mere addition would allow, and the effect is therefore reminiscent of that observed with furfuraldehyde or laevulic acid. Ethyl sulphide reduces only slightly in acid solution and rather more strongly in alkaline. It is very sparingly soluble. The addition of sulphite intensifies the colour somewhat. It is probable that the sample contained a trace of mercaptan or of a disulphide, or both. Oxalates, malates, and tartrates have no effect upon the reagent. Tetrathionates seem to be without effect.

Stability.

Colorations developed by cysteine, cystine *plus* sulphite, cysteine *plus* sulphite, ferrous salt, and ferrous salt *plus* sulphite are extremely stable in the region p_H 5.0–6.5. As a rule they increase by 1 or 2 % in the course of several hours and then begin to fall. Using 5 cc. of reagent at p_H 5.7, the coloration with 2 mg. of cystine *plus* 1 millimol. of Na_2SO_3 is reduced by only 5 % in 18 hours, whereas at p_H 8.35 the loss may be 30 %. After 48 hours the losses might be 20 and 50 % respectively. Below p_H 0.4 the development of colour by cystine *plus* sulphite is extremely slow and the depth of coloration increases considerably during several hours. The coloration developed by cysteine alone or ferrous salt, on the other hand, comes up rapidly in acid mixtures, but in the case of ferrous salt, with or without sulphite, the solutions fade during dilution and for some time thereafter. In acid solutions, colorations given by Na_2S increase considerably with time.

Colorations developed by sulphite alone, laevulic acid with and without sulphite and furfuraldehyde with and without sulphite increase relatively much more in the first few hours and fade much more slowly.

The coloration developed by quinol is curious in that it fades extremely rapidly. At p_H 6.5 for example, the loss may be 50 % within 30 minutes.

The coloration developed by uric acid alone progressively increases in acid solution during 18 hours and longer, whereas in alkaline solution it fades much more rapidly than in the case of cystine *plus* sulphite. Besides greatly increasing the depth of coloration with uric acid, sulphite improves the stability in alkaline solution.

The effects of varying the amounts of reagent, etc.

A series of cystine *plus* sulphite colorations developed by only 2 cc. of reagent, yielded a curve rather less than 2 % below the one in Fig. 1 over the range p_H 5.0–7.5. Below p_H 5 the curve approached the abscissa more steeply than the one in the figure and above p_H 7.5 the curve rose less steeply, thereby following more closely the curves for cysteine and ferrous salt (both also obtained with 2 cc. of reagent) in this region and down to p_H 5. The reverse variations from the curve in the figure were observed when 8 cc. of reagent were employed. The amount of sulphite could be doubled or halved without affecting the coloration developed with cystine by more than 2 %, provided the reactions were carried out above about p_H 5.2 and that sufficient time, the usual 5–6 minutes, was allowed for colour development. The rate of colour development however was greatly influenced by varying the quantity of sulphite, rate and quantity of sulphite decreasing together, and more so the lower the p_H . Below p_H 5 the curve became steeper as the quantity of sulphite employed was decreased. The shape of the curve below p_H 5 therefore depends largely upon the amounts of sulphite and reagent employed and the

time allowed for the reaction, as it tends to flatten out if the 5-6 minute interval is increased.

The colorations developed by cysteine and ferrous salt do not vary much with the amount of reagent; the effects at each end of the curves being less pronounced than they are with cystine *plus* sulphite.

On the other hand the coloration with sulphite alone increases with increasing amounts of reagent but not linearly. These remarks apply also to furfuraldehyde and laevulic acid, alone and with sulphite, but for uric acid alone the depth of coloration is nearly proportional to the amount of reagent over the entire p_H range.

The coloration developed by Na_2S in the 5-6 minute time interval increases with the amount of reagent when only a few cc. are used. With 5 or 10 cc. there is not much difference in the amount of colour developed at high p_H values.

The rate of colour development decreases as the volume of the reacting mixture is increased. With large volumes, full colour development may be attained with cysteine or ferrous salt in 5 or 6 minutes, but with cystine *plus* sulphite or with Na_2S this may not be so, particularly in acid regions.

Proportionality.

The depth of coloration developed by cysteine, cystine *plus* sulphite, ferrous salt, and Na_2S , the last subject to certain restrictions in the p_H range, is strictly proportional to the amount of reducer employed within the usual limits imposed by the nature of colorimetry, provided that the chosen p_H be maintained and that sufficient reagent be present. Thus in presence of 2 cc. of reagent, 4 mg. of cystine *plus* sulphite give approximately 3.95 times as great a coloration as is developed by 1 mg. of cystine *plus* sulphite at any fixed p_H in the range 5.0-8.4.

At any chosen p_H cysteine *plus* sulphite gives almost exactly (to within 1 %) twice the coloration developed by the cysteine alone. The curves showing this relationship may be seen in Fig. 1.

At any chosen p_H the depth of coloration developed by uric acid is far from proportional to the amount present, reagent concentration remaining the same. Thus with 2 cc. of reagent at p_H 8.4, 2 mg. of uric acid give a coloration only 20 % greater than does 1 mg. If sulphite is present and/or the amount of reagent be increased, a deeper coloration is developed and the poor proportionality is improved. Thus under the same conditions as before, but with 1 millimol. of sulphite present, 2 mg. of uric acid give a 40 % greater coloration than does 1 mg.

Other variables remaining the same, the depth of coloration developed by sulphite alone is approximately proportional to the amount of sulphite present.

Furfuraldehyde alone and laevulic acid alone, give colorations varying more or less directly with the amounts present when other variables are unaltered. The colorations developed in presence of a fixed amount of sulphite however are by no means proportional to the amounts present. There

appears to be no maximum with laevulic acid, but with 2 cc. of reagent at p_H 5.7 and in presence of 1 millimol. of sulphite, 3 mg. of furfuraldehyde give a greater coloration than any other amount. In presence of 1 millimol. of sulphite at p_H 5.7, 0.25 and 0.5 millimol. of SnCl_2 develop about the same degree of coloration.

Inhibition and enhancement of colour development.

Sufficient acetone, if added before reagent and sulphite, will completely inhibit the production of colour by cystine for some distance on either side of p_H 5.7. 2 mg. of cystine *plus* 1 millimol. of purified acetone-bisulphite compound will produce at p_H 5.7 about 80 % of the coloration obtained by using sulphite instead of acetone-bisulphite, and 1 millimol. of acetone-bisulphite will give only a fraction of the coloration developed by 1 millimol. of sulphite alone.

In presence of sufficient furfuraldehyde (*e.g.* 150 mg.) cystine *plus* sulphite fails to reduce the reagent appreciably.

When a fairly large amount (1 or 2 millimol.) of cadmium chloride is added to a solution containing cystine and followed by sulphite and reagent, the coloration develops extraordinarily slowly so that at the end of 5 minutes only 60 % of the full colour value may be attained at p_H 5.7. CdCl_2 also reduces the depth of coloration developed by laevulic acid *plus* sulphite and by furfuraldehyde *plus* sulphite.

Zinc chloride in quantity (4 millimol.) will reduce the coloration developed by cystine *plus* sulphite at p_H 5.7 by several %. 2 millimol. of ZnCl_2 do not seem to alter the coloration given by sulphite alone. The coloration developed by cysteine is very slightly decreased and that by ferrous salt not at all. 2 millimol. of ZnCl_2 will reduce the coloration given by 10 mg. of laevulic acid *plus* sulphite by as much as 35 %. The coloration given by furfuraldehyde *plus* sulphite is also greatly reduced.

Mercuric chloride does not alter the coloration given by sulphite alone, but in the presence of even such a small amount as 0.1 millimol. of HgCl_2 , cysteine alone fails to develop a trace of colour with the reagent at p_H 5.7, and cystine *plus* sulphite yields only the colour which would be expected of the sulphite alone. The effect extends beyond p_H 5.7 in either direction. When solutions of cysteine hydrochloride and HgCl_2 are mixed, a white precipitate of the mercaptide forms immediately. It is readily soluble in citrate buffer at p_H 5.7 and in strong acids. HgCl_2 does not affect the coloration developed by ferrous salt. 0.2 millimol. of HgCl_2 will reduce the coloration given by laevulic acid *plus* sulphite by about 20 %. HgCl_2 gives a white precipitate with uric acid at p_H 5.7. The precipitate is not soluble in citrate buffer but is readily dissolved by sulphite, by thiosulphate, by CdCl_2 , by ZnCl_2 and by large quantities of NH_4Cl .

The heavy metals do not appear to influence the colorations given by laevulic acid or furfuraldehyde alone to a marked extent.

The coloration developed by sulphite alone may be increased considerably in the more acid mixtures by addition of much salt. Thus at p_H 5.7, 50 millimol. of Na_2SO_4 may nearly treble the coloration developed by the sulphite.

Ammonium chloride in quantity (5 millimol.) at p_H 5.7 increases the rate at which the coloration given by cystine *plus* sulphite develops, whereas large amounts of citrate buffer rather diminish the rate. NH_4Cl does not influence the coloration developed by ferrous salt but it may increase that given by cysteine alone by about 1 %. 5 millimol. of NH_4Cl increase the coloration given by 10 mg. of laevulic acid *plus* sulphite by about 18 % at the same p_H . It also greatly increases the coloration given by furfuraldehyde *plus* sulphite.

NH_4Cl does not appear to influence the colorations developed by laevulic acid or furfuraldehyde alone to a marked extent.

Reduction of the reagent by mixtures of substances.

The type example is the reaction by which cystine may be estimated. Cystine alone does not reduce the reagent. Sulphite reduces but slightly. A mixture of the two reduces strongly and the final coloration is virtually proportional to the cystine present provided this is not infinitesimal and that sufficient reagent is employed. Assuming that the sulphite contributes its full individual share to the total coloration developed in presence of cystine, one may calculate the departure to be expected from linearity of depth of coloration with cystine, assuming that only a small amount of sulphite is involved in converting the cystine to the same degree each time into something that will reduce the reagent. The calculated departure from linearity is several times in excess of that actually found. In other words the sulphite appears not to contribute its own coloration completely.

The same phenomenon is more easily appreciated from the curves in Fig. 1, showing the colorations developed by ferrous salt in absence and presence of sulphite. Here it is obvious that superimposing a sulphite curve on one given by ferrous salt alone will not lead to a curve of the type given by ferrous salt *plus* sulphite. As the displacement between the curves for ferrous salt with and without sulphite is small, and about the same at all p_H values, it is believed to be due to the sulphite precluding aerial oxidation of the ferrous salt rather than anything else.

Under certain conditions of volume, *etc.* and in the presence of 2 mg. of cystine, 1 millimol. of sulphite, and 2 cc. of reagent at p_H 5.7, 10 mg. of pyruvic acid, 10 mg. of laevulic acid, and 2 mg. of furfuraldehyde increased the coloration developed, by 0, 1 and 1.5 % respectively; whereas if the colorations had been additive the increases would have been 1, 8 and 11 % respectively.

Similarly the products of acid digestion of carbohydrates have been found to contribute only a fraction of the coloration they develop with sulphite to the total coloration obtained when fair quantities of cystine, ferrous salt, or cysteine are present.

Analysis and discussion of the experimental data.

The effects noticed when NH_4Cl and the heavy metal chlorides are added to laevulic acid or furfuraldehyde in presence of sulphite are really enhancement and inhibition of the rate of colour development. If the colour reactions are allowed to proceed for 5 hours instead of 5 minutes, the effects mentioned are almost lost.

The same might be said of the effects these substances exert in the case of cystine and cysteine. The actions of CdCl_2 as an inhibitor and of NH_4Cl as an enhancer are certainly upon the rate of colour development rather than upon the final equilibrium that might be attained, though this also might be affected. It is not unlikely that the action of HgCl_2 , apparently protection of the $-\text{SH}$ group by mercaptide formation, is of the same type, the effect being so pronounced however that no noticeable colour is developed even after some hours. The effects of these three heavy metal chlorides thus increase with their tendency to behave as non-polar substances and this is the order of the appearance of the metals in the same sub-group of the periodic system.

It is commonly stated that bisulphite reacts with only the lower members of ketonic series to form bisulphite compounds. The increased colour development noticed when furfuraldehyde and laevulic acid reduce the reagent in presence of sulphite cannot be explained. It can hardly be due to bisulphite compound formation since, for example, if a large excess of furfuraldehyde be used, extremely little colour is developed. This inhibiting effect, however, like that of acetone, is believed to be due to bisulphite compound formation.

As Wu points out in his examination of the complex acids, the "reducibility" of these substances by most reducers is small. He concludes that very few of the heavy metal atoms in the whole bulky complex are reduced as a rule, and that phospho-18-tungstic acid loses one atom of oxygen when reduced by ferrous salt in alkaline solution.

The colour reaction may be written:

but this form involves the assumption that a true equilibrium may be reached. Until some small amount of the reagent is reduced the oxidation potential of the reagent system is virtually infinite.

In referring to oxidation-reduction potentials of the reagent and reducer systems we really imply that the potential of the one system is linearly related to $\ln \frac{[\text{reagent}]}{[\text{reduced reagent}]}$ and that of the other to $\ln \frac{[\text{reducer}]}{[\text{oxidised reducer}]}$. These relationships might not apply, but assuming that they do it is not difficult to see why different reducers might give different amounts of colour and why weak reducers fail to develop their full colorations in presence of strong ones. When cysteine, ferrous salt, cystine *plus* sulphite or Na_2S (the last in alkaline solution) is the reducer, the balance represented by the arrows is well in favour of the right-hand side of the equation. With other substances the balance

seems to be in favour of the left-hand side. This is evidenced by the greater colour developed when more reagent is used, as in the case of uric acid; the lack of proportionality between colour and amount of reducer, the poor reduction effected by large amounts of potential reducer, *etc.* The actual magnitudes as well as the ratios of the forward and backward velocity constants must be important factors in some instances.

Cysteine and the other strong reducers certainly seem to achieve some sort of balance. At p_H 5.7 with 2 cc. of reagent, 1, 2 and 4 mg. of cystine *plus* sulphite or of cysteine alone, or 1 and 2 mg. of cysteine *plus* sulphite, give amounts of colour almost strictly proportional to the amounts of cystine or cysteine present. With 6 mg. of cystine *plus* sulphite or 6 mg. of cysteine or 3 mg. of cysteine *plus* sulphite, the departure from linearity is slight, but with say 10 mg. of cysteine or a corresponding amount of ferrous salt for example the departure is considerable (20 %). With 80 mg. of cysteine the departure is about 800 %. The relation between colour and amount of cysteine follows approximately the same trend at p_H 8.4, though the balance would appear to be rather more in favour of the right-hand side of the equation than it is at p_H 5.7.

The reagent is probably almost exhausted when such large amounts of cysteine are employed. Assuming that all the tungsten present in 2 cc. of reagent exists in the complex and that each molecule of the complex may lose only 1 atom of oxygen to cysteine or ferrous salt, then if 80 mg. of cysteine could reduce all the reagent the departure from linearity would be 670 % instead of 800 %. If Wu's findings are correct then at least 80 % of the tungsten present in the reagent employed here exists in the complex. But the balance cannot be so completely in favour of the right-hand side of the equation (*cf.* the departure with 10 mg. of cysteine) and hence it is possible that nearly 100 % of the tungsten exists in the complex.

It will be seen later that cysteine is oxidised to cystine by the reagent. Whatever the nature of the balance achieved by cysteine may be, it cannot at present be interpreted in known physico-chemical terms. The potential of a cysteine-cystine system at an inert electrode cannot be described by Peter's equation. In the equation that describes the potential,

$$E = E_0 - \frac{RT}{F} \ln [\text{cysteine}] + \frac{RT}{F} \ln [H^+],$$

the concentration of cystine finds no place at all, and attempts to explain it in terms of Wieland's theory of hydrogen acceptors have recently been discredited by Michaelis and Flexner [1928].

Those reducers which seem to establish an early balance with the reagent practically all give increasing colorations at p_H values increasing up to about 7.5. Above p_H 6.5 the predominating sulphite ion in a sulphite solution will be the SO_3^- ion. With the exception of such substances as uric acid the colorations thereafter decline. This effect is offset by that observed with the stronger reducers as p_H increases above about 7.5. That the colorations with ferrous

salt and the other strong reducers should increase, strongly suggests that the reagent is subject to a different reduction from that occurring in the acid solutions or that the reagent is of different form in the two zones. The former possibility seems to be ruled out by the discussion on the extent to which 2 cc. of the reagent are reduced by 80 mg. of cysteine at p_H 5.7 and p_H 8.4. The latter possibility, if accepted, revives interest in Wu's claim that the 1-18-acid may exist in two forms A and B. The A form which is said to give nearly 30 % more colour than the B on total reduction is also said to be more easily reduced than the B form. With this in mind, the curves in Fig. 1 might suggest that the two forms are in equilibrium at any p_H , the B form predominating at low p_H and the A at high, though one would expect the weak reducers to give more instead of less colour at high p_H if this were so, and that a new equilibrium is only slowly attained when the active mass of one form is altered. This view is supported by the observations made on the effects of the order of adding the materials in alkaline solution.

The very rapid fading encountered with quinol as reducer cannot be explained. Excess bromine readily destroys the blue colour developed by the substances described in this paper, but H_2O_2 is usually almost without effect. Saturation with acetone sometimes causes complete bleaching in a few seconds.

The slow fading observed with cysteine, cystine *plus* sulphite, *etc.*, as reducers seems to be associated in some way with decomposition of the 1-18-acid. Its rate increases with the p_H and to some extent with salt concentration and type. Thus large amounts of $ZnCl_2$ or $CdCl_2$ very considerably increase the rate. The stock reagent used in all this work is buffered at about p_H 2.1 and remains stable for years. If some reagent is brought to p_H 8.4 and buffered it will be found to lose practically all its reducibility after some hours. Buffered at p_H 5.7 the reagent will "work" for a few days. Thereafter it loses its pale apple-green colour, which becomes pale sky-blue, and is not reduced by cysteine or cystine *plus* sulphite. Ferrous salt reduces it slightly giving a blackish-green tint. Nothing is precipitated from the reagent kept at p_H 5.7 even after standing for 5 months at room temperature. This form of decomposition of the reagent is not at present understood.

In reference to the colorations given by ferrous salt, cysteine and the other members of that group it has been concluded that the reducers are virtually fully oxidised to other substances by a relatively small excess of reagent, and from the colorations developed by ferrous salt during its oxidation to the ferric state one may associate a certain amount of colour with a certain amount of reduction.

In extreme alkaline regions and with plenty of reagent, the coloration given by Na_2S corresponds with the oxidation of both atoms of hydrogen in the H_2S molecule. Unless sulphite is present the solution is clouded by free sulphur and as the sulphite does not alter the colour, as far as one may judge between cloudy and clear solutions, it is believed that it merely combines with the free sulphur to form thiosulphate. At lower p_H values nothing like

full colour development is obtained in 5 minutes but it is approached with longer periods of standing. The solutions always smell of H_2S during colour development and unless plenty of reagent is used to diminish the time necessary for almost complete oxidation appreciable amounts of H_2S may be lost. At still lower p_{H} values the H_2S and sulphite must react in some measure to form thionic acids.

The cysteine hydrochloride used was not perfectly dry and in consequence the amounts weighed out for standard solutions were probably deficient in cysteine by 1 or 2 %. It had been prepared by reducing cystine with tin and hydrochloric acid and strict precautions were taken to prevent its re-oxidation in the course of separation. It certainly contained only the minutest trace of cystine, if any.

Now if the reagent, in being reduced by cysteine, oxidises the cysteine to cysteic acid, then, focusing attention purely upon the amounts of colour given by cysteine and by cystine *plus* sulphite, one may say that the facts do not disagree with the view that the sulphite reduces the cystine to cysteine. But if this were so, why should cysteine *plus* sulphite develop just twice the colour given by cysteine alone? The hypothesis that the sulphite reduces the cystine to cysteine and that this is oxidised by the reagent to cysteic acid, is rendered untenable by that fact alone. The second part of the hypothesis is independently invalidated by a consideration of the amounts of colour given by cysteine alone and by ferrous salt. The colour given by cysteine alone corresponds to within a few units % with the oxidation of the cysteine to cystine, not to cysteic acid. And according to this fact just established, the first part of the hypothesis must be incorrect, for if not, the colours developed by cysteine *plus* sulphite and cystine *plus* sulphite should continually increase in intensity until the sulphite is exhausted. The rôle played by cystine would then be that of catalyst to the reduction of the reagent by the sulphite, and this obviously is not the case.

The anomaly is bound up with the belief that sulphite reduces the cystine to cysteine under the conditions obtaining in the estimations. It seems that this is quite incorrect and must be abandoned. Other evidence which might appear to be against it, but is not necessarily so, is the following. Sulphite reduces the reagent only slightly, yet cysteine during its oxidation to cystine reduces strongly. Is it likely therefore that the sulphite could reduce cystine appreciably under the same conditions? The extent of reduction unfortunately cannot be calculated because the sulphite-sulphate system is irreversible under these conditions and the cysteine-cystine system is anomalous.

The following table (p. 2157) summarises the position with respect to colour development by ferrous salt, cysteine, cysteine *plus* sulphite, cystine, cystine *plus* sulphite, Na_2S , and Na_2S *plus* sulphite, the last two only in alkaline solution. The unit of colour is to be associated with 1 atom of reducing hydrogen. RSH and R-S-S-R represent cysteine and cystine respectively.

When cysteine is first carefully oxidised to cystine at p_{H} 5-6 by chromate,

or by H_2O_2 *plus* a trace of ferrous salt, it gives on subsequent addition of sulphite only half the colour it would have given if it had not first been oxidised.

Amount and nature of reducer	Units of colour developed
I $2r \text{ Fe}^{++}$	$2r$
II $2r \text{ RSH}$	$2r$
III $2r \text{ RSH} + \text{sulphite}$	$4r$
IV $r \text{ R}-\text{S}-\text{S}-\text{R}$	0
V $r \text{ R}-\text{S}-\text{S}-\text{R} + \text{sulphite}$	$2r$
VI $2r \text{ H}_2\text{S}$ (alkaline)	$4r$ cloudy
VII $2r \text{ H}_2\text{S} + \text{sulphite}$ (alkaline)	$4r$

The equation

is, as we have seen, quite unable to account for the facts.

Let it now be supposed that the action of sulphite on r molecules of cystine is to produce r molecules of cysteine *plus* s molecules of a substance X which is unable to reduce the reagent. The following formulation will suffice:

It has also been established that:

the balance being found almost completely in favour of the right-hand side of the equation. The fact that the colour given by cysteine is doubled in presence of sulphite now admits of easy explanation.

Starting with equation (4) and with case II in the table, $2r$ of cysteine first give $2r$ units of colour and are converted into r of cystine. If sulphite is present then r of cystine according to equation (3) yield another r of cysteine which react with the reagent to give another r units of colour and $\frac{1}{2}r$ of cystine, and so on. The total colour developed is therefore

$$2r + r + \frac{1}{2}r + \frac{1}{4}r + \frac{1}{8}r + \dots\dots$$

which, in the limit, becomes $2r + 2r$ or $4r$.

The colour developed by r of cystine alone is 0, and by r of cystine *plus* sulphite is $2r$. The case admits of similar explanation to that given above. The initial $2r$ of colour is of course missing and the total colour developed is merely that of the summation, $r + \frac{r}{2} + \frac{r}{4} + \text{etc.}$, becoming $2r$ in the limit.

Thus equations (3) and (4) account satisfactorily for the known facts. The reaction expressed by (4) is quite rapid over the p_{H} range 5–8.4, the greatest rate of colour development occurring in the most alkaline solutions. From the very low rate of colour development with cystine *plus* sulphite at low p_{H} values compared with the rate at higher values, it has been concluded that (a) the reaction velocities in equation (3) are very small in the acid region,

¹ The double reaction arrows in this and following equations must be taken only to represent a possible equilibrium of some sort, not necessarily of the type such equations ordinarily represent.

or (b) the velocities may be high but with equilibrium well in favour of the left-hand side in acid solutions, or (c) both (a) and (b) might apply.

From the point of view of interest in the X compound of equation (3), the following considerations and experiments are appended.

The simplest form equation (3) may take is

in which sX becomes RHS_2O_3 .

Equation (5) represents a reaction somewhat similar to that given for the action of cyanides upon cystine

An alternative to equation (5) as a particular case of equation (3) would be

in which the X compound would occur as a mixture of tetrathionic and cysteic acids, both of which are fairly stable and not reactive toward the reagent. On the evidence thus far submitted equations (5) and (7) would perhaps have equal claims to being the correct one.

Organic thiosulphates of the form postulated in equation (5) are by no means unknown. Purgotti [1892], investigating the thiosulphonyl derivative of glycollic acid, prepared it by treating chloroacetic acid with sodium thiosulphate. Aqueous solutions of the salts are not readily decomposed by boiling, but, in contrast to the action of mineral acids upon inorganic thiosulphates, boiling hydrochloric acid converts the barium salt of the thiosulphonyl derivative of glycollic acid, for example, into barium sulphate and thioglycollic acid.

It was found that a stream of SO_2 could be blown into a solution of cystine in excess of hydrochloric acid without any chemical change occurring. No sulphate was produced and none of the cystine was affected in any way. On boiling to expel SO_2 the solution was found to contain the original cystine and hydrochloric acid and nothing else. The original cystine could be accounted for colorimetrically and the total sulphur content corresponded with the amount of cystine present.

A mixture containing cystine and $NaHSO_3$ was made alkaline at p_H 12-13, then acid, alkaline again, and acid again, all in the cold. No sulphate was produced. An aliquot was allowed to develop its colour with the reagent in presence of more sulphite and the colour was compared with that of a standard cystine + sulphite solution containing the same amount of cystine as that originally present in the aliquot. There was a 7 % loss of colour. Another aliquot was boiled to expel SO_2 and then allowed to reduce the reagent in presence of sulphite. There was a gain in colour developed of 77 %. In Part II of this series, methods for estimating cysteine and cystine will be discussed in detail. Applying them to analysis of the boiled aliquot it was found that

81 % of the cystine had been converted into cysteine, 13 % remained unchanged, and the balance, 6 %, had been lost to colorimetric estimation. Gravimetric analyses of sulphate and total sulphur confirmed these results and revealed that the sulphur of the 6 % cystine lost to colorimetry remained in the solution, probably as cysteic acid.

These experiments throw a little light on the nature of the *X* compound which is certainly elusive but not necessarily chimerical. If the thiosulphonyl derivative of α -aminopropionic acid is the *X* compound and reacts with boiling mineral acid as does Purgotti's thiosulphonyl derivative of glycollic acid, the observations are automatically explained. There can really be little doubt that equation (5) is the correct form of equation (3).

SUMMARY.

1. The reduction of solutions of phospho-18-tungstic acid by different types of substances has been investigated colorimetrically under varying conditions, especially in respect to varying p_H . Some attempt has been made to describe the results in general terms of reaction velocities and equilibria. Reductions by cysteine have been examined in some detail.

2. Evidence has been adduced to show that the reaction between cystine and sulphites in cold aqueous solution is not a "reduction" in the ordinary sense of the term, but that it is most probably expressed by the equation, $R-S-S-R + H_2SO_3 \rightleftharpoons RSH + RHS_2O_3$, where $R-S-S-R$ and RSH represent cystine and cysteine respectively.

I wish to express my indebtedness to Sir Charles J. Martin for his interest in this work and for correcting the manuscript and arranging for its publication.

REFERENCES.

- Andrews (1928). *J. Biol. Chem.* **80**, 191.
- Folin and Denis (1912). *J. Biol. Chem.* **12**, 239.
- and Looney (1922). *J. Biol. Chem.* **51**, 421.
- and Marenzi (1929). *J. Biol. Chem.* **83**, 103.
- and Trimble (1924). *J. Biol. Chem.* **60**, 473.
- Heffter (1907). *Chem. Zentr.* **2**, 822.
- Hunter and Eagles (1927). *J. Biol. Chem.* **72**, 177.
- Kehrmann (1887). *Ber. deutsch. chem. Ges.* **20**, 1805.
- Michaelis and Flexner (1928). *J. Biol. Chem.* **79**, 689.
- Purgotti (1892). *Gazzetta*, **22**, 422.
- Rimington (1930). *Biochem. J.* **24**, 1114.
- Sullivan (1926). *U.S. Pub. Health Serv. Reports*, **41**, 1030.
- Tompsett (1931). *Biochem. J.* **25**, 2014.
- Wu (1920). *J. Biol. Chem.* **43**, 189.