CLXXIV. THE DETERMINATION OF CYSTINE. USE OF THE ZEISS PHOTOMETER.

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MOST available methods for the determination of cystine depend upon its reduction to cysteine which in turn reduces a reagent with the production of a coloured solution. The methods suffer from the disadvantage, more or less general to all colorimetric reactions, that the accuracy is liable to be vitiated by factors only partly understood, since the conditions of the production of the colour have been defined in an empirical manner.

Presumably the claims for specificity made by Sullivan [1926] for his method have resulted in the adoption of his technique in much work reported since 1926, but when some years ago (1927) we applied this method to hydrolysed proteins, its unreliability became at once apparent. Rimington, in a private communication [1929], confirmed this unreliability, and the question has since been studied by Lugg [1933] who has modified the Sullivan technique by buffering "standard" and "unknown" with glycine, thus controlling the hydrogen ion concentration of the resulting mixed solutions.

The work described in the present paper was undertaken in order to study the accuracy of the results obtained by the Sullivan method and by the modification of Lugg by means of the Pulfrich photometer, as it was thought that such an approach was obviously suitable to the study of a colour reaction¹. The advantage of the method is that the intensity of the colour can be expressed in absolute units as an extinction coefficient, thus enabling the rate of colour development, the stability and reproducibility of the colour formed and the range and accuracy of the proportionality between colour and cystine to be determined.

EXPERIMENTAL.

The photometer method.

The light transmission of the substance in solution is compared with that of the solvent in cells of the same thickness, and the formula for the calculation of extinction coefficient $E = \log (I_0/I)/d$ then becomes

$$E = K \frac{(\log I_0 - \log I)}{C},$$

where I_0 = intensity of incident light,

I = intensity of emergent light,

C = concentration of substance in solution,

K = a constant,

E = extinction coefficient.

¹ It may be remarked that other workers have had the same idea, for apparently concurrently with this work, Krijgsman and Bouman [1933] applied the photometer to the determination of cystine in blood.

It is evident that the term "solvent" includes all the substances in the system under examination with the exception of the component to be determined, a fact which is only of importance when such substances absorb light of the wave-length employed.

The choice of wave-length (colour filters).

In the first instance it was necessary to determine the wave-length of light absorbed by the coloured substance produced from cystine, and whether such light is absorbed by the reagents employed in the development of that colour.

It is important to note that in the development of the cystine-quinone colour the reagent solutions must be freshly prepared. In the case of the sodium β -naphthoquinonesulphonate this was emphasised by Folin [1922] in his original paper, but not by Sullivan [1926], while Rimington in the above-mentioned private communication [1929] and Prunty [1933] indicated the necessity of using freshly prepared hydrosulphite solution.

The colour was developed according to Sullivan's technique in a series of solutions containing 30 to 210 parts of cystine per million, and the extinction coefficients were determined using light (obtained by the aid of light filters fitted to the photometer) of wave-lengths varying from 430 to 720 $m\mu$.

Consideration of the results obtained (Fig. 1) shows that the widest and most even spacing of the curves occurs when wave-lengths 470, 500 and 530 $m\mu$

Fig. 1. *Sullivan technique.* The relationship between extinction coefficient and wave-length with varying concentrations of cystine.

are employed (light filters Nos. 47, 50 and 53 respectively). These three wave-lengths were therefore chosen for use in the initial experiments, from the results of which light of wave-length 530 $m\mu$ (filter No. 53) was finally shown to give extinction coefficients proportional to the amount of cystine present.

The reproducibility of the colour and the influence of the reagents on its determination.

Sullivan and Lugg add different volumes of reagents to the cystine solution to develop the colour. For this reason in these experiments standard cystine solutions¹ were used such that the final concentration of cystine should be approximately the same in the two methods. Table I shows that this is so when a standard cystine solution of 150 parts per million is used in the Sullivan method and one of 600 parts per million in the Lugg method.

Table I.

	Standard cystine sol.		Final vol. of coloured solution ml.	Conc. of cystine in coloured solutions (parts per million)
	Vol. used ml.	Conc. parts per million		
Sullivan's method	5	150	16	47
Lugg's method	2	600	25	48

A large number of determinations was made of the colour developed by the two methods in their respective standard solutions (Table I) by measuring the extinction coefficients at the three chosen wave-lengths. Since it was observed that the reagents alone produce a yellow colour (more intense in the case of Lugg's method) the extinction coefficients of this colour (the "blank") were determined in a similar manner.

The *average extinction coefficients* recorded in Table II are calculated from a series of determinations, usually six in number, while the figures recorded as *variations in extinction coefficient* are the maximum differences from the average figure for the series.

This maximum difference is calculated as a percentage of the average extinction coefficient and recorded as *percentage variations in extinction coefficient*. The "modified Lugg method" quoted in this table is described later.

The percentage variations in extinction coefficient in Table II demonstrate the decided superiority of the modified Lugg method over that of Sullivan.

The average extinction coefficients of the reagents alone show that they absorb a small amount of light of wave-lengths 470, 500 and 530 $m\mu$ and will therefore affect to a small extent a cystine determination. Since this "reagent colour" must be present in all determinations of cystine, it is clearly desirable to use a reagent "blank," instead of water, against which the light transmission of the colour developed by cystine is matched.

The effect of atmospheric oxygen on colour development.

While studying the rate of colour development in a cystine solution and the stability of that colour, it was noticed that if the coloured solution were transferred from a flask to the matching cell and left undisturbed (protected from the air by a thin glass plate) the intensity of the colour reached a maximum in about 10–20 minutes and then faded very slowly. When, on the other hand, the solution was poured backwards and forwards from the cell to the flask for each light measurement to be taken a progressive darkening of the solution

¹ The cystine employed in all experiments described herein was 95 % pure by nitrogen determination.

Table II. *Extinction coefficients of coloured solutions matched against water.*

Method	Reagents alone									
	Average extinction coefficient			Variation in extinction coefficient						
	470 $m\mu$	500 $m\mu$	530 $m\mu$	470 $m\mu$	500 $m\mu$	530 $m\mu$	470 $m\mu$	500 $m\mu$	530 $m\mu$	
	(wave-lengths)			(wave-lengths)			(wave-lengths)			
Sullivan (1)	—	—	—	—	—	—	—	—	—	
" (2)	—	—	—	—	—	—	—	—	—	
" (3)	0.139	0.064	0.049	0.066	0.047	0.034	0.032	0.027	0.027	
" (4)	0.114	0.048	0.045	0.029	0.015	0.008	0.016	0.014	0.014	
Lugg	—	—	—	—	—	—	—	—	—	
Lugg modified (1)	0.298	0.093	0.029	0.017	0.008	0.009	0.005	0.009	0.005	
" (2)	0.291	0.091	0.029	0.025	0.014	0.030	0.015	0.023	0.007	
" (3)	0.294	0.087	0.025	0.010	0.007	0.006	0.003	0.005	0.004	
Reagents + 47–48 pts./10 ⁶ of cystine										
Method	Average extinction coefficient			Variation in extinction coefficient						
	470 $m\mu$	500 $m\mu$	530 $m\mu$	470 $m\mu$	500 $m\mu$	530 $m\mu$	470 $m\mu$	500 $m\mu$	530 $m\mu$	
	(wave-lengths)			(wave-lengths)			(wave-lengths)			
Sullivan (1)	0.626	0.667	0.559	0.097	0.125	0.107	0.079	0.075	0.075	
" (2)	0.642	0.687	0.537	0.097	0.074	0.153	0.099	0.134	0.068	
" (3)	0.841	0.945	0.793	0.106	0.049	0.127	0.105	0.101	0.086	
" (4)	0.839	0.900	0.754	0.033	0.081	0.042	0.093	0.057	0.057	
Lugg	0.574	0.422	0.345	0.045	0.047	0.066	0.046	0.052	0.040	
Lugg modified (1)	0.731	0.653	0.497	0.039	0.032	0.021	0.051	0.018	0.034	
" (2)	0.753	0.668	0.513	0.017	0.030	0.045	0.031	0.017	0.025	
" (3)	0.757	0.677	0.513	0.013	0.013	0.030	0.021	0.027	0.017	
							Percentage variation in extinction coefficient			
							470 $m\mu$	500 $m\mu$	530 $m\mu$	
							+	+	+	
							15.5	16.0	14.1	13.6
							20.0	18.6	14.1	13.6
							15.1	22.3	25.0	12.7
							12.6	13.5	12.8	10.9
							3.9	4.7	7.6	7.6
							7.9	15.6	15.3	11.8
							5.4	3.3	3.6	6.8
							2.3	6.7	4.2	5.0
							1.8	4.4	3.1	3.5

occurred. This observation suggested that the darkening was due to the oxygen of the atmosphere.

If this is the case, an excess of the reducing substance, the alkaline sodium hydrosulphite, might retard the oxidation of the reactive substances in the system by preferentially absorbing any atmospheric oxygen, thus delaying the darkening effect.

In an experiment the colour was developed in one portion of a standard cystine solution according to Lugg's technique and in another using the same method but with a concentration of sodium hydrosulphite three times the usual strength. A portion of each coloured solution was transferred to a matching cell and left undisturbed, while the remainder of each solution was shaken periodically. The results of determinations of the extinction coefficients obtained at all three wave-lengths were similar; those at wave-length $530m\mu$ are shown in Fig. 2.

Fig. 2. *Lugg technique*. The relationship between extinction coefficient and time with and without aeration.

<i>a</i>	Standard amount of hydrosulphite—No shaking		
<i>a'</i>		"	—Shaken
<i>b</i> 3 ×		"	—No shaking
<i>b'</i> 3 ×		"	—Shaken

It will be noted that a slow fading takes place in the solutions not shaken with air while if the solution containing the normal amount of hydrosulphite is shaken it darkens rapidly. This change is dramatic and may be observed by eye. Where three times the usual concentration of hydrosulphite was used the darkening was delayed.

Similar experiments have been made employing the Sullivan method of colour development. The results which are shown in Fig. 3 indicate that changes in extinction coefficient do occur with and without shaking but that, in distinction from the results when the Lugg technique was employed, such changes vary with different wave-lengths.

These results appear to be particularly interesting but it is not proposed at the moment to pursue the matter further. Suffice it to say that the colour developed by the methods both of Sullivan and of Lugg is affected by shaking in the presence of air.

Fig. 3. *Sullivan technique.* The relationship between extinction coefficient and time with and without aeration using light of different wave-lengths.

a	Solution not shaken—light wave-length	470 $m\mu$
b	" " "	500 $m\mu$
c	" " "	530 $m\mu$
a'	" shaken — "	470 $m\mu$
b'	" " — "	500 $m\mu$
c'	" " — "	530 $m\mu$

The determination of cystine in pure solution based on its relationship with the colour developed by the modified Lugg technique.

Lugg developed the colour from cystine solutions in 25 ml. stoppered cylinders. It is obviously advantageous to use 25 ml. graduated flasks in order that the coloured solution may be accurately diluted to the mark with water after the addition of hydrosulphite, rather than to rely on about ten pipette measurements to aggregate 25 ml.

The slight modifications of the Lugg technique employed in this work are as follows:

In order to eliminate the effect of atmospheric oxygen (already demonstrated) the column of air above the liquid in the 25 ml. flask was displaced by passing in a rapid stream of purified nitrogen for half a minute immediately before and after the addition of the sodium hydrosulphite reagent. The quinone, sodium sulphite and sodium hydrosulphite solutions were freshly prepared,

the last in an atmosphere of purified nitrogen. The reagents were stored and mixed in flasks held in a water-bath maintained at 21°.

A series of solutions containing 240–960 parts per million of cystine in 0.1 *N* hydrochloric acid were prepared, the solution containing 600 parts per million being regarded as the “standard” and the others as “unknowns.” Colour was developed simultaneously in each “unknown” cystine solution, in the “standard” and in the “blank” (reagents alone).

With each set of three solutions corresponding to each concentration of cystine, extinction coefficients were obtained by matching both the “standard” and the “unknown” against the blank (using matching cells 5 mm. thick).

The results are shown in Fig. 4 where mean straight lines have been drawn through the experimental points. Since the colour was developed in the standard (600 parts per million of cystine) at the same time as in each of the unknowns,

Fig. 4. The relationship between cystine content and extinction coefficient (matching against reagent “blank”).

○ ——— ○ 470 *mμ*   □ — — — □ 500 *mμ*   △ — — — △ 530 *mμ*

several such standards (six in all) were prepared and examined. The average values for the extinction coefficients of the standards are shown in the above-mentioned figure as solid points, while the range is shown by the vertical lines.

Fig. 4 shows that when using this technique there is an approximately linear relationship between the cystine present and the extinction coefficient of the coloured solution obtained therefrom.

It is evident that the proximity of the line to the origin indicates with what success the colour developed by the cystine alone has been determined. It follows therefore that the results shown in Fig. 4 indicate that wave-lengths 530 and 470 are preferable to 500 *mμ*.

When the colours are matched against "blank reagents" the interfering colours produced in the solutions are largely eliminated thus enabling reasonable results to be obtained at all three wave-lengths (Fig. 4). When however matching is made against "water," instead of the blank reagents, the wave-lengths at which "reagent colours" are also determined become evident by showing less proportionality between extinction coefficient and the cystine present. Such results were obtained from the solutions of the above experiment and are shown in Fig. 5 where it is evident that at wave-length $470 m\mu$ colours not derived from cystine interfere.

Fig. 5. The relationship between cystine content and extinction coefficient (matching against water).

○——○ 470 $m\mu$ □-----□ 500 $m\mu$ △---△ 530 $m\mu$

Examination of Figs. 4 and 5 therefore enables the conclusion to be drawn that when employing light of wave-length $530 m\mu$ there is best proportionality between the determined extinction coefficient of the solution and the cystine present. This, therefore, is the wave-length $530 m\mu$ (filter No. 53) which should be employed in the determination of cystine, and at which matching against the blank reagents should, for reasons already stated, be employed.

It follows that cystine may be determined in an "unknown" by simple proportion from the determined extinction coefficient of the coloured solution (prepared by the modified Lugg technique) and that of a standard cystine solution prepared at the same time.

When such calculations are made from the results recorded in Fig. 4, employing as the "standard" the 600 parts per million cystine solution in which the colour was developed at the same time as in the "unknown," the results

shown in Fig. 6 were obtained. The percentage errors of the determinations are calculated from the results plotted in Fig. 4 and are shown in Table III A. These errors are obtained by expressing the deviations of the individual points from the mean straight line as a percentage of cystine present.

It will be noted from Figs. 4 and 6 that occasionally, and for no apparent reason, an abnormal result may be obtained (e.g. 840 pts./ 10^6 , Fig. 4; 720 pts./ 10^6 , Fig. 6). It therefore follows that there are disturbing factors other than that of oxidation at present incompletely controlled.

Fig. 6. Relationship between actual and calculated cystine content (from Fig. 4).

○ 470 $m\mu$ □ 500 $m\mu$ △ 530 $m\mu$

The determination of cystine in the presence of hydrolysed caseinogen based on its relationship with the colour developed by the modified Lugg technique.

A series of solutions each containing 10 % hydrolysed caseinogen (Hammarssten's casein) and different amounts of added cystine was prepared. Using the modified Lugg technique, the colour was developed in each of these solutions, and the extinction coefficients were determined.

The results are plotted in Fig. 7 (matched against the blank reagents). It is evident that the experimental points fall more nearly on a straight line than did those obtained using pure cystine. This is perhaps more readily demonstrated by the percentage errors shown in Table III B, where it is shown that the error is approximately $\pm 8\%$, using a cystine concentration greater than 200 pts./ 10^6 .

Fig. 7 also demonstrates that a cystine determination employing wave-length 570 $m\mu$ would, owing to the slope of the curve, be much less accurate than one employing 530 $m\mu$ wave-length; the former was the wave-length of

Table III.

B Estimation of cystine in the presence of hydrolysed caseinogen						C Estimation of cystine in pure solution		
A Estimation of cystine in pure solution		Cystine equivalent of hydrolysed caseinogen solution pts./10 ⁶				Cystine present pts./10 ⁶	Percentage error	
Cystine present pts./10 ⁶	Percentage error		Cystine added pts./10 ⁶	Total cystine present pts./10 ⁶	Percentage error		Exp. I	Exp. II
240	+ 5.8	100	0	100	-12.0	60	0.0	+11.6
360	0.0	100	100	200	+15.0	120	-4.2	+ 6.7
480	+ 4.7	100	200	300	0.0	180	-1.7	- 4.2
600	max. + 3.3	100	300	400	+ 8.2	240	+0.8	+ 4.2
	av. - 2.1	100	400	500	+ 7.4	300	0.0	- 1.7
	min. -10.3	100	500	600	+ 1.2	360	+1.0	0.0
720	+ 4.9	100	600	700	- 5.0	360	-3.5	+ 0.8
840	-13.7	100	700	800	- 7.5	420	-2.6	+ 3.3
960	- 1.2	100	900	1000	+ 3.0	480	+0.4	+ 1.3
						540	+0.2	- 1.3

Fig. 7. The relationship between extinction coefficient at various wave-lengths and cystine in the presence of hydrolysed caseinogen.

□-----□ 500 $m\mu$ Δ---Δ 530 $m\mu$ ◇-----◇ 570 $m\mu$

the light chosen by Krijgsman and Bouman for cystine determination (see footnote p. 1293).

It may be mentioned that since extinction coefficients determined at wave-length 530 $m\mu$ are directly proportional to the cystine present, by producing back that line (Fig. 7) until it cuts the abscissa, a measure of the cystine present

in the hydrolysed caseinogen solution used is obtained. This result, the cystine equivalent of the hydrolysed caseinogen solution, is shown in Table III B (*i.e.* 100 parts per million). The amount indicated by this curve does not represent the cystine in caseinogen since during the hydrolysis no precautions were taken to prevent destruction of that amino-acid.

Comparison of Figs. 4 and 7 and also of the results given in Table III A and B indicates that more consistent results are obtained in the presence than in the absence of hydrolysed caseinogen. Lugg has already stated that p_H is an important factor in the colour development, and it is suggested that the improved results in the presence of hydrolysed caseinogen are due to its added buffer capacity which would reduce errors in the adjustment of the p_H during the development of the colour.

One other factor which is believed to be an important one, and which may not be adequately controlled in the modified Lugg method, is the reduction of cystine to cysteine. If such a factor exists then curves constructed similarly to those of Fig. 4 employing cysteine instead of cystine should show an improved straight line relationship.

Fig. 8. The relationship between extinction coefficient and cysteine present.

a. Exp. 1.

b. Exp. 2.

The relationship between the cysteine present and the colour developed by the modified Lugg technique.

A series of solutions containing 60 to 530 pts./10⁶ cysteine was prepared and the colours developed from them were matched against a "blank" using light of wave-length 530 mμ.

The results are plotted in Fig. 8, from which the percentage errors have been calculated as previously described and recorded in Table III C, where it will be noted that above a concentration of 120 pts./10⁶ of cysteine the error is approximately $\pm 4\%$.

It is evident that there is a considerable improvement in the accuracy of the determination of cysteine over that of cystine (Fig. 4, Table III A and B), from which it is concluded that there is a variable factor concerned with the reduction of the cystine in the modified Lugg method.

SUMMARY.

1. The criticisms of the Sullivan method by Rimington [1929] and by Lugg [1933] have been confirmed by the aid of photometric absolute measurements of the colour produced.

2. Photometric determinations of extinction coefficients have shown that the best proportionality between them and the cystine content is obtained when light of wave-length 530 *mμ* is used (filter No. 53).

3. The colour produced by the reagents, particularly those of Lugg, is affected appreciably by the presence of air (oxygen). When the colour is developed by the Sullivan method from a cystine solution the extinction coefficient of that solution varies up to $\pm 25\%$ from the mean value; by employing the Lugg technique with suitable precautions against oxidation that variation is reduced to $\pm 8\%$.

4. This modified Lugg technique enables cystine in pure solution to be determined with an error of approximately $\pm 5\%$, but with an occasional error of $\pm 15\%$, indicating it is believed, that some factor or factors are not sufficiently controlled.

5. In the presence of hydrolysed caseinogen, when the solution is more highly buffered, the outside limit of error of cystine determination becomes $\pm 8\%$.

6. An indication has been given of a method of determining cystine in hydrolysed caseinogen and presumably in any hydrolysed protein.

7. Cysteine in pure solution may be determined with an error of only $\pm 4\%$, thus indicating that one other factor affecting cystine determination is introduced by the process by which the cystine is reduced to cysteine.

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