Submicrodeterminations of Thiols, Disulphides and Thiol Esters in Serum by using *o*-Hydroxymercuribenzoic Acid and Dithiofluorescein

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1. Methods are described for selective estimation of thiols, disulphides and thiol esters in standard solutions and in serum. The methods are based on the reaction with the excess of *o*-hydroxymercuribenzoic acid (HMB) in alkaline solution with subsequent addition of dithiofluorescein in excess and determination of the extinction at 588m μ . The sensitivity of the methods amounts to 1.5×10^{-9} g.equiv. in 5ml. of final solution. Of results obtained on standard solutions 80% have the errors within the range $\pm 4\%$. 2. It has been found that serum contains an unidentified substance (substance X) producing green complexes with dithiofluorescein which undergo decomposition on addition of formaldehyde. The correction for substance X must be estimated in a separate sample and taken into account. The concentration of substance X can be calculated from extinctions measured at 588m μ and 635m μ in the presence of dithiofluorescein in excess. 3. The selective determination of thiols and disulphides is based on different reaction rates with formaldehyde. The complexes between HMB and cysteine can be selectively decomposed by formaldehyde, and free glutathione can be selectively removed by formaldehyde in the presence of protein thiols. 4. Thiols are determined in the presence of triethylamine, thiols plus disulphides in the presence of triethylamine and sulphite, and thiols plus thiol esters in the presence of dimethylamine, with subsequent addition of ammonium sulphate.

Most analytical methods for the estimation of sulphur compounds of biological importance deal with thiol groups by using titration with mercury compounds (reviewed by Wroński, 1963*a*, 1965*a*), or titration with silver nitrate (Kolthoff & Eisenstädter, 1961). Other methods involve the application of the induced iodine-azide reaction (Kurzawa, 1961), consumption of the tri-iodide ion (Cunningham & Nuenke, 1959, 1960), reduction of the fluorescence of mercurated fluorescein (Wroński, 1961*a*), the reappearance of the colour of bleached dithiofluorescein (Dubouloz, Foundarai & Pavone-Mariville, 1962), the methylation and desulphuration with Raney nickel (Krstěva, Alexiev, Ivanov & Yordanov, 1965), and the reduction of the ultraviolet absorption of *N*-ethylmaleimide after reaction with thiol groups (Leslie, Williams & Gorin, 1962). The thiol content can be determined spectrophotometrically by evaluating the increase of absorption due to the formation of the mercury-sulphur bond after addition of *p*-chloromercuribenzoic acid (Boyer, 1954). The excess of the reagent can be spectrophotometrically back-titrated with thiols (Fernandez Diez, Osuga &

Feeney, 1964). An interesting method for selective determination of protein and non-protein thiol groups is based on the reaction with di-(3-carboxy-4-nitrophenyl) disulphide (Jocelyn, 1962).

Most of the methods mentioned above can be applied to the estimation of disulphides as well, after reduction to thiols or in the presence of sodium sulphite. On the other hand there are only a few methods dealing with the microdetermination of thiol esters. Cecil & Ferdinand (1965) have suggested a method based on the reaction with ammonia and amperometric titration of the resulting thiols with mercuric chloride. The author (Wroński, 1966*a,b*) has proposed a more active reagent, dimethylamine, for the decomposition of thiol esters and titration of resulting thiols with *o*-hydroxymercuribenzoic acid in the presence of dithiofluorescein as visual indicator.

The use of HMB* and dithiofluorescein has already been suggested for microdetermination of cysteine and cystine (Wroński, 1963*b*) and for glutathione and cysteine in the presence of each other (Wroński, 1965*b*). These methods have been

* Abbreviation: HMB, *o*-hydroxymercuribenzoic acid.

found, however, inapplicable for the analysis of serum. The present investigation has shown that essentially there are two reasons for the failure of the mentioned methods: the occurrence in serum of an unidentified compound capable of producing complexes with dithiofluorescein and the influence of the sequence of addition of reagents on the resulting colour.

The alkaline solution of dithiofluorescein has an intense blue colour which changes to green after addition of serum. The constituent forming that complex is designated substance X.

Dithiofluorescein and HMB form a colourless complex which on addition of thiols is decomposed with reappearance of the blue colour of the free dithiofluorescein. As far as simple thiols are concerned the sequence of addition of reagents has no influence on the resulting absorption, e.g. for cysteine and glutathione the same absorption has been found with the sequence (a) thiol + HMB + dithiofluorescein or (b) dithiofluorescein + HMB + thiol. On the other hand, when the sample contains protein thiol the resulting absorption after sequence (a) is much higher than after sequence (b). This can be explained by assuming that free HMB reacts easily with protein thiol, but the complex HMB-dithiofluorescein formed at first in the sequence (b) is unable to get to the internal thiol groups in protein because it is too large.

The methods presented in this paper are based on the action of HMB on the sample with subsequent addition of the dithiofluorescein and determination of the excess photometrically. The disulphides plus thiols are determined in the presence of sulphite, the thiol esters and thiols in the presence of dimethylamine. Moreover, the reaction of thiols with formaldehyde is suggested for some selective determinations.

MATERIALS AND METHODS

o-Hydroxymercuribenzoic acid. A portion (16g.) of *o*-hydroxymercuribenzoic acid anhydride (POCH, Gliwice, Poland) is dissolved in 200 ml. of *N*-sodium hydroxide and diluted to 1 l. The solution (approx. 0.05 *N*) is filtered after some days and standardized by titration with thioglycolic acid (Wroński, 1959), which is at the same time standardized against a solution of mercuric nitrate prepared from pure mercury and nitric acid. The solution of HMB is diluted to be 0.5 *mM* and 0.05 *mM*. The pH should be approx. 9, because at pH 6 a part of the HMB can precipitate.

Dithiofluorescein (thiofluorescein, $C_{20}H_{12}O_3S_2$). Approx. 4 mg. of dithiofluorescein (POCH, Gliwice, Poland) is dissolved in 2 ml. of solution containing 20 g. of EDTA (disodium salt) plus 20 g. of triethylamine in 1 l., and the solution is diluted to 100 ml. and filtered. 0.05 *mM*-HMB (2 ml.) is titrated with the solution of dithiofluorescein to a distinct blue colour. The concentration of dithiofluorescein is suitable when 1.2–1.7 ml. is required. As a result of air oxidation the concentration of dithiofluorescein decreases

at a rate of about 5%/24 hr. As the oxidation is accelerated by heavy metals and by light, only the purest reagents should be used and care taken that the glass used has never been in contact with concentrated solutions of salts of heavy metals. The solution of dithiofluorescein should be protected from light as completely as possible. The concentration of dithiofluorescein should always be determined in a blank sample parallel with the main estimation.

Solutions. (A) 0.4 *M*-Triethylamine plus 0.12 *M*-phosphoric acid; (B) *m*-dimethylamine plus 0.1 *M*-phosphoric acid; (C) 0.6 *M*-ammonium sulphate; (D) 0.5 *M*-monosodium sulphite ($NaHSO_3$) prepared from sodium pyrosulphite, controlled iodometrically; (E) 10% (w/v) EDTA (disodium salt) solution adjusted with sodium hydroxide to approx. pH 10; (F) 3% (w/v) formaldehyde; (G) 2 *M*-urea plus 0.5% (w/v) EDTA (disodium salt) solution adjusted with sodium hydroxide to pH 7.5.

Investigated substances. L-Cysteine hydrochloride, L-cystine, homocystine, glutathione (Fluka), ditosyl-L-cystine prepared after Swan (1955) and $\beta\beta'$ -dipropionamide disulphide ($-S \cdot CH_2 \cdot CH_2 \cdot CO \cdot NH_2)_2$ are prepared as follows. Sulphur (2.25 g.) is dissolved in 17 g. of melted sodium sulphide nonahydrate; the sodium disulphide so obtained is dissolved in 50 ml. of water and after filtration 10 g. of acrylamide is added. After some minutes a white precipitate is formed, which is filtered off and recrystallized from water. Yield, 10 g. A part of the disulphide was reduced with hydrogen selenide (Wroński, 1966c) to the corresponding thiol.

The standard solutions of thiols were prepared by solution of the thiol in water and dilution to a concentration approx. 0.05 *mM*. The final concentration was determined by titration of 50 ml. of that solution plus 2 ml. of *N*-ammonia with 0.5 *mM*-HMB in the presence of dithiofluorescein as indicator (Wroński, 1961b). The solution was used immediately after titration. No decrease of concentration due to air oxidation could be observed after 10 min.

The standard solutions of disulphides were prepared by solution of a known amount of disulphides, which had been dried at 105° under reduced pressure. Cystine and homocystine were dissolved in 0.1 *N*-sulphuric acid, $\beta\beta'$ -dipropionamide disulphide and oxidized glutathione in water, ditosylcystine in dimethylformamide. The concentration of the solutions (0.01–0.03 *M*) was controlled by titration with 0.01 *N*-HMB in the presence of sodium sulphite and dithiofluorescein (Wroński, 1963c). The solutions were diluted with water to make the concentration approx. 0.05 *mM*.

The standard solution of insulin was prepared from the solution for injection (10 ml.: 800 units, supplied by Polfa). A portion (2 ml.) of this solution was boiled with 2 ml. of conc. hydrochloric acid under reflux for 20 hr. and the excess of hydrochloric acid evaporated; the solution was diluted to 25 ml. and analysed for cystine content by the method described in this paper. The original solution was found to be 1.31 *mM* in respect to total cystine content.

The standard solution of *NS*-diacetylcysteine was prepared (Wroński, 1966a,b) as follows: To approx. 5 mg. of cysteine hydrochloride dissolved in 0.5 ml. of water plus 2 ml. of ethanol plus 1 ml. of triethylamine, 0.5 ml. of acetic anhydride was added and after 5 min. diluted with water to 50 ml. The concentration was determined by titration of 2 ml. of that solution plus 0.5 ml. of 40% (w/w) dimethylamine with 0.5 *mM*-HMB in the presence of dithiofluorescein as indicator (Wroński, 1966a,b).

The extinctions of the samples containing the excess of the dithiofluorescein were determined at 588 m μ (max.) in a 1 cm. cell with a Spekol spectrophotometer (Carl Zeiss, Jena, Germany).

The equivalent extinction coefficient of dithiofluorescein has been estimated from measurements of the extinctions of the samples containing HMB and an excess of dithiofluorescein. As the complex HMB–dithiofluorescein is colourless, the measured extinction corresponds only to the excess of dithiofluorescein. If the total concentration of dithiofluorescein is c and total concentration of HMB (concentration of colourless complex) is b , then assuming the applicability of Beer's law:

$$E_1 = \epsilon(c_1 - b_1) \text{ and } E_2 = \epsilon(c_2 - b_2)$$

Inserting $c_1/c_2 = m$ (the ratio of volumes of the solution of dithiofluorescein taken at the same final dilution, 5 ml.):

$$\epsilon = \frac{E_1 - mE_2}{mb_2 - b_1}$$

The value b can easily be calculated from the volume of HMB taken.

It has been found with this procedure (extinction was maintained within the range 0.1–0.2) that the extinction coefficient of dithiofluorescein does not vary noticeably between pH 6 and 11, and for solutions containing (per 5 ml.): 0.5 ml. of A, 0.2 ml. of E, 0.5 ml. of A plus 0.1 ml. of D plus 0.2 ml. of E, 0.5 ml. of A plus 0.5 ml. of F, 0.5 ml. of B plus 0.3 ml. of C, the extinction coefficient is essentially the same, the mean value being 3.5×10^4 . On the other hand, in a solution containing sodium sulphite and formaldehyde, e.g. 0.5 ml. of A plus 0.1 ml. of D plus 0.2 ml. of E plus 0.5 ml. of F, the extinction coefficient was found to be 4.3×10^4 , and in solution without HMB 3.2×10^4 . The measurements should be repeated for every new sample of dithiofluorescein.

As an extinction of 0.01 can still be measured, the sensitivity of the determination of dithiofluorescein will amount to approx. $0.3 \mu\text{N}$, that is, with 5 ml. of solution 1.5×10^{-9} g. equiv. This can be taken as the sensitivity of the methods suggested in this paper.

EXPERIMENTAL AND RESULTS

Analysis of standard solutions

Method I: estimations of thiols. Thiol (10^{-5} – 10^{-4} m-mole) is added to the solution containing 2 ml. of 0.05 mN-HMB plus 0.5 ml. of A, and afterwards the solution of dithiofluorescein is added to a give distinct blue colour, diluted to 5 ml. and the extinction is determined, which should be within the range 0.1–0.2. For 10^{-6} – 10^{-5} m-mole use only 0.5 ml. of 0.05 mN-HMB. The blank determination is carried out with HMB, 0.5 ml. of A and dithiofluorescein in excess. The final solution is approx. pH 10.2.

The concentration of thiol (disulphides, thiol esters) can be calculated as follows. When V and V_0 are the volumes of dithiofluorescein taken in investigated and blank samples, and E and E_0 the corresponding extinctions, then the total concentra-

tion of dithiofluorescein in the investigated sample will be $[2 \times 10^{-5} + (E_0/\epsilon)](V/V_0)$. In the investigated sample the sum of the total concentrations of dithiofluorescein and thiol must amount to $2 \times 10^{-5} + (E/\epsilon)$. Hence the concentration of thiol may be written as:

$$[\text{RSH}] = 2 \times 10^{-5} + \frac{E}{\epsilon} - \left(2 \times 10^{-5} + \frac{E_0}{\epsilon}\right) \frac{V}{V_0}$$

For instance: $V = 0.8$ ml., $V_0 = 1.8$ ml., $E = 0.120$, $E_0 = 0.155$, $\epsilon = 3.5 \times 10^4$. Then:

$$[\text{RSH}] = 2 \times 10^{-5} + \frac{0.120}{3.5 \times 10^4} - \left(2 \times 10^{-5} + \frac{0.155}{3.5 \times 10^4}\right) \frac{0.8}{1.8} = 12.6 \mu\text{N}$$

The values demonstrated in Table 1 have been obtained by method I.

Method I has been found to be selective in the presence of an excess of disulphides and in the presence of amounts of thiol esters of the same order as that of the thiols. The selectivity can be increased by cooling the HMB solution to 0° before adding the sample.

For selective determination of thiols the reaction with formaldehyde has proved to be very useful (Wroński, 1966d). The thiols with a free β - or γ -amino group react rapidly to form products stable towards HMB; glutathione reacts relatively slowly and thiols with an acetylated amino group react with formaldehyde in alkaline solution very slowly, if at all. An interesting phenomenon observed is that the complexes containing HMB and thiols with a free β - or γ -amino group are liable to decomposition in the presence of formaldehyde and dithiofluorescein according to the scheme:

As a result of the decrease of concentration of free dithiofluorescein the extinction decreases. This is indicated in Fig. 1. The decrease of extinction of the solution containing only dithiofluorescein and formaldehyde is very slow (curve 1), but in the presence of the complex cysteine–HMB the decrease is very rapid and after approx. 5 min. the resulting extinction attains the same value as in the sample containing no cysteine at all (curve 2). The complex glutathione–HMB is also decomposed (curve 3), but in comparison with the complex cysteine–HMB relatively slowly. On this basis glutathione can be determined in the presence of cysteine as suggested in the next method.

Method II: estimation of cysteine (homocysteine, cysteamine) and glutathione (or thiols without amino group) in the presence of each other. The total of both

Table 1. *Estimation of thiols by method I*Results are expressed as concn. (10^{-5} M) of final solution (5 ml.).

Cysteine	Calc.	0.26	0.52	0.78	1.04	1.30	1.45
	Found	0.23	0.53	0.79	1.03	1.26	1.53
Cysteine	Calc.	0.036	0.072	0.108	0.144	0.180	0.216
	Found	0.037	0.082	0.110	0.142	0.173	0.207
Reduced glutathione	Calc.	0.33	0.65	0.98	1.30	1.62	1.94
	Found	0.34	0.68	0.98	1.29	1.60	1.89
β -Mercaptopropionamide	Calc.	0.31	0.63	0.94	1.25	1.57	1.88
	Found	0.34	0.64	0.95	1.22	1.55	1.82

Fig. 1. Course of decomposition of complexes HMB-cysteine and HMB-glutathione by formaldehyde in the presence of dithiofluorescein followed by the decrease of extinction at $588\text{m}\mu$. Every sample contains (in 5 ml.): 2 ml. of 0.05mM -HMB plus 0.5 ml. of solution A (0.4M -triethylamine plus 0.12M -phosphoric acid) plus 0.5 ml. of 3% formaldehyde. Total concn. of dithiofluorescein: 1 and 2, $23.6\text{ }\mu\text{N}$; 3 and 4, $16.5\text{ }\mu\text{N}$. Concn. of cysteine: 1, 0; 2, $18.6\text{ }\mu\text{N}$; 3, 0; 4, $3.72\text{ }\mu\text{N}$. Concn. of glutathione: 1, 0; 2, 0; 3 and 4, $8.65\text{ }\mu\text{N}$.

thiols is determined by method I. A second sample is added to the solution, containing 2 ml. of 0.05mM -HMB plus 0.5 ml. of A, and then 0.5 ml. of 3% formaldehyde and dithiofluorescein (in excess) are added; after 1 min., if the blue colour disappears, more dithiofluorescein is added and the solution is diluted to 5 ml. and the extinction is determined as a function of time. The extinction corresponding to the complete decomposition of the cysteine complex is estimated graphically according to Fig. 1. The cysteine content is calculated by difference.

The method has proved satisfactory for the mixtures of cysteine and glutathione.

Free glutathione reacts with formaldehyde relatively quickly. In a solution containing 0.6% of formaldehyde at pH 10, after 30 min. at room temperature 97% of glutathione is converted into compounds resistant towards HMB, and after 50 min. free glutathione cannot be detected, whereas such thiols as β -mercaptopropionamide or tosylcysteine react approx. 1% under the same

conditions. This great difference in the rates of reaction of various thiols has been evaluated by method III.

Method III: estimation of glutathione and thiols without a free amino group in the presence of each other. The total of the thiols is determined by method I. A second sample (1–2 ml.) is added to the solution, containing 0.5 ml. of A plus 0.5 ml. of 3% formaldehyde, and after 30–50 min. 2 ml. of 0.05mM -HMB and dithiofluorescein (in excess) are added; the solution is diluted to 5 ml. and the extinction determined. The cysteine, if any, is determined by method II and is subtracted from the glutathione content.

Method III has been tried for the mixtures containing glutathione and β -mercaptopropionamide, and satisfactory results have been obtained.

Method IV: estimation of disulphides. 1.8×10^{-5} m-mole of disulphide is added to the solution containing 2 ml. of 0.05mM -HMB plus 0.5 ml. of A plus 0.1 ml. of 0.5M -monosodium sulphite; when the reaction has proceeded to completion, 0.2 ml. of 10% EDTA (soln. E) is added to prevent the rapid decolorization of dithiofluorescein which takes place in the presence of sulphite. Dithiofluorescein is added in excess, the solution is diluted to 5 ml. and the extinction is determined. The blank is run under the same conditions without disulphides. The pH of the final solution should be approx. 10.0. One mole of consumed HMB corresponds to 1 mole of disulphide according to the reaction:

The thiol content, if any, should be estimated by method I and subtracted from the result. The thiol esters, if present in concentration less than the concentration of disulphide, do not interfere; at greater amounts a correction should be taken into account.

The time necessary for the quantitative conversion at room temperature for cystine, oxidized glutathione, $\beta\beta'$ -dipropionamide disulphide is 15 min., for homocystine it is 30 min., for ditosylcystine 90 min. and for insulin 150 min. The most

Table 2. *Estimation of disulphides by method IV*

Results are expressed as concn. (10^{-5}M) of final solution (5ml.). Times are those of conversion with HMB and sulphite.

Cystine (25 min.)	Calc.	0.28	0.55	0.83	1.10	1.37	1.66
	Found	0.26	0.52	0.81	1.07	1.39	1.66
Oxidized glutathione (25 min.)	Calc.	0.26	0.52	0.78	1.04	1.30	1.56
	Found	0.30	0.55	0.79	1.03	1.27	1.50
$\beta\beta'$ -Dipropionamide disulphide (25 min.)	Calc.	0.28	0.56	0.83	1.11	1.39	1.67
	Found	0.29	0.58	0.83	1.10	1.37	1.62
Homocystine (45 min.)	Calc.	0.25	0.50	0.74	0.99	1.24	1.48
	Found	0.28	0.51	0.72	1.00	1.24	1.42
Insulin (220 min.) (total disulphide bond content)	Calc.	0.32	0.63	0.94	1.26	1.57	1.89
	Found	0.36	0.59	0.91	1.24	1.57	1.82

suitable time of reaction was selected at about 50% in excess. As the air oxidation of sulphite amounts to approx. 5%/hr., there is no need to work under inert gas atmosphere.

The results obtained are summarized in Table 2.

Some disulphides can be determined in the presence of each other by using selective decomposition of the complexes of the corresponding thiols and HMB by formaldehyde, after the reaction of disulphides with sulphite and HMB has proceeded to completion. The reaction of formaldehyde (in excess with regard to sulphite) with complexes of HMB produced from disulphides and sulphite is essentially the same as indicated on Fig. 1. Cystine and homocystine after quantitative conversion with sulphite and HMB can be afterwards removed quantitatively by reaction with formaldehyde, whereas oxidized glutathione reacts very slowly, and ditosylcystine or dipropionamide disulphide under the same conditions do not react at all. This is demonstrated in the following method.

Method V: estimation of cystine (homocystine) and oxidized glutathione (or disulphides without a free amino group) in the presence of each other. The total of the two disulphides is determined by method IV. The second sample is added to solution containing 2ml. of 0.05M-N-HMB plus 0.5ml. of A plus 0.1ml. of 0.5M-monosodium sulphite, and time allowed for complete conversion to be effected; 0.2ml. of 10% EDTA, 0.5ml. of 3% formaldehyde and dithiofluorescein in excess are then added. After 1 min., to ascertain whether any more dithiofluorescein should be added, the mixture is diluted to 5ml., the extinction is determined as a function of time and the extinction corresponding to the final decomposition of the cysteine complex is determined from the results.

Method VI: estimation of thiol esters. 1.8×10^{-5} m-mole of thiol ester is added to solution containing 2ml. of 0.05M-N-HMB plus 0.5ml. of B (resulting pH 11.3). After 20 min. (for NS-diacetylcysteine) 0.3ml. of 0.6M-ammonium sulphate is added

Table 3. *Estimation of NS-diacetylcysteine by method VI*

Results are expressed as concn. (10^{-5}M) of final solution (5ml.).

Calc.	0.23	0.45	0.67	0.90	1.13	1.35
Found	0.23	0.46	0.67	0.92	1.15	1.38

(resulting pH 10.1), with dithiofluorescein in excess, the mixture is diluted to 5ml. and the extinction determined. The blank is run under the same conditions.

Results are given in Table 3.

Thiols, if any, should be estimated by method I and subtracted from the results. A disulphide content of the same order as that of the thiol esters can be tolerated. In the presence of an excess of disulphides, their decomposition should be taken into account. This can be simply done by estimation of the consumption of HMB according to method VI, after 30 and 60 min.; then

$$R_{\text{corr.}} = R_{30} - (R_{60} - R_{30}) (R_{\text{corr.}}, \text{corrected result}).$$

Analysis of bovine serum

In the course of investigation of bovine serum by the methods used for standard solutions some complicating facts had to be taken into account. As has been mentioned above, the blue colour of dithiofluorescein changes to green on addition of serum, which can be attributed to formation of complexes between dithiofluorescein and an unidentified compound described in this paper as substance X. The amount of dithiofluorescein in complex decreases with increasing pH. Above pH 11 the complexes are decomposed; below pH 5 the complexes and dithiofluorescein become colourless. The addition of formaldehyde results in a rapid decomposition of the complexes with the reappearance of the colour of free dithiofluorescein. On addition of HMB in excess the green colour disap-

Fig. 2. Influence of bovine serum (substance X) on the extinction of dithiofluorescein. 1, 2, 3, 4: pH 10.2, 588 $m\mu$. 5: pH 5.5, 635 $m\mu$. Concn. of dithiofluorescein: 1 and 2, increasing; 3, 9.9 μN ; 4, 5.9 μN ; 5, 15 μN . Concn. of serum: 1, 0; 2, 0.2 ml.; 3, 4, 5, increasing.

pears. On the other hand the sulphite does not notably influence the formation of the complexes. The absorption maximum of the complexes was found at 635 $m\mu$, whereas that of the dithiofluorescein was at 588 $m\mu$. The extinction of dithiofluorescein decreases at 588 $m\mu$ and increases at 635 $m\mu$ on addition of serum. This has been shown on Fig. 2: curve 1 represents the relationship between concentration and extinction at 588 $m\mu$; curve 2 the same relationship in the presence of 0.2 ml. of serum in 5 ml. of solution. The addition of serum to the excess of dithiofluorescein (curves 3 and 4) results in a decrease of extinction at 588 $m\mu$. The curves are composed of the two intersecting lines, the point of intersection changes with the initial concentration of dithiofluorescein but the lines remain parallel with regard to each other. Curve 5 indicates the increase of extinction at pH 5.5 measured at 635 $m\mu$.

For detection of substance X the following procedure may be useful. To some drops of approx. 0.1 mN -dithiofluorescein the solution of 0.5 m -monosodium phosphate is added until the colour becomes slightly blue. On addition of 0.02–0.1 ml.

of serum containing substance X the colour changes to distinct green.

No substance X could be detected in egg proteins or urine.

The presence of substance X must be taken into account by using the analytical methods based on the excess of dithiofluorescein, with exception of the procedures using formaldehyde. The correction can easily be found as the difference of the extinctions of two samples containing equal amounts of dithiofluorescein and solutions used in analysis but without HMB. The second sample contains 0.05–0.1 ml. of serum. The concentration of dithiofluorescein should be in excess with regard to substance X.

The estimation of thiols in serum by method I is possible, but more reproducible results were obtained when the sample was cooled to 0° and set aside for 5 min. before adding dithiofluorescein.

As might be expected in a solution containing 0.5 ml. of A plus 0.5 ml. of 3% formaldehyde the thiol content decreases noticeably in the period of approx. 30 min., but the further decrease is very slow. The typical decrease in thiol content can be represented as follows: 5 min., 28%; 20 min., 32%; 40 min., 33%; 60 min., 33%; 3.5 hr., 36%. This evidently corresponds to the reaction between formaldehyde and glutathione, whereas the unreactive thiols can be attributed to the thiol proteins. Cysteine, if any, reacts instantaneously. On the other hand somewhat low recoveries of glutathione from serum were found. This can be explained assuming that a part of glutathione produces complexes with formaldehyde and serum proteins, which are afterwards decomposed by HMB. All the values for glutathione thiol obtained by the formaldehyde method have therefore been adjusted by a factor 1.25.

The disulphide bonds in serum react with sulphite and HMB at different rates, which can be evaluated by kinetic differential analysis. As a part of the disulphides is very resistant to the action of HMB and sulphite, increased concentrations had to be used.

The investigation concerning the thiol esters content in fresh bovine serum has showed a rapid increase in consumption of HMB during approx. 20 min. followed by a slow linear increase: e.g. 10 min., 1.0; 20 min., 1.7; 30 min., 1.9; 45 min., 2.2; 60 min., 2.5 μN (0.1 ml. of serum in 5 ml.). The corrected value for thiol esters content is $[1.9 - (2.5 - 1.9)]10^{-6} = 1.3 \mu N$. On the other hand, in serum inactivated for 30 min. at 57° no thiol esters were detectable, the consumption of HMB being: 10 min., 0.4; 30 min., 1.0; 60 min., 1.9 μN . In both examples cited above the consumption of HMB for thiols was subtracted.

The procedures for analysis of serum are given

Table 4. *Estimation of total thiols in bovine serum by method (1)*

Results are expressed as concn. (10^{-5}M) of final solution (5ml.).

Vol. of serum (ml.)	0.10	0.15	0.20	0.25		
Concn. of SH found	0.73	1.02	1.32	1.60		
Vol. of serum (ml.)	0.10	0.10	0.10	0.10	0.10	0.10
Concn. of added cysteine	0.18	0.36	0.54	—	—	—
Concn. of added glutathione	—	—	—	0.24	0.48	0.72
Increase of concn. of SH found	0.19	0.36	0.51	0.24	0.47	0.67

below. Extinctions due to colour or turbidity should be determined in a separate sample and subtracted. To spare the serum one of the samples containing serum and dithiofluorescein can be decolorized by a drop of 0.01N-HMB and the remaining extinction measured. To estimate the concentration of dithiofluorescein a blank should be run immediately after the main determination, with only the serum omitted.

(1) Estimation of total thiol content. The solution containing 2ml. of 0.05mN-HMB plus 0.5ml. of A is placed in ice-water, 0.1–0.2ml. of serum is added and, after 5min., dithiofluorescein is added to give a distinct blue colour; the mixture is diluted to 5ml. and the extinction is measured. In a separate sample the decrease of extinction due to substance X content is determined and added to the measured extinction. The results are given in Table 4.

(2) Estimation of cysteine. The solution containing 2ml. of 0.05mN-HMB plus 0.5ml. of A is placed in ice-water, 0.1–0.2ml. of serum is added and, after 5min., 0.5ml. of 3% formaldehyde and dithiofluorescein in excess are added, the mixture is diluted to 5ml. and the extinction after 5min. at room temperature is measured. Correction for substance X is not made. The cysteine content is calculated as a difference of the results (1) and (2).

(3) Estimation of reduced glutathione. At room temperature 0.1–0.2ml. of serum is added to the solution containing 0.5ml. of A plus 0.5ml. of 3% formaldehyde, and after 40min. it is cooled in ice-water; 2ml. of 0.05mN-HMB is added and, after 5min., dithiofluorescein is added in excess, the mixture is diluted to 5ml. and the extinction is determined. From the decrease of thiol content (result 1 minus result 3) subtract the cysteine content and multiply by 1.25. Correction for substance X is not made. The results obtained by methods (2) and (3) are given in Table 5.

Table 5. *Estimation of cysteine by method (2) and glutathione by method (3)*

Results are expressed as concn. (10^{-5}M) of final solution (5ml.).

Vol. of serum (ml.)	0.10	0.15	0.20			
Cysteine, found	0.01	0.02	0.02			
Glutathione, found	0.30	0.46	0.60			
Vol. of serum (ml.)	0.10	0.10	0.10	0.10	0.10	0.10
Cysteine, added	0.18	0.36	0.54	—	—	—
Glutathione, added	—	—	—	0.24	0.48	0.72
Increase of cysteine	0.19	0.37	0.54			
Increase of glutathione				0.24	0.50	0.70

(4) Estimation of (a) total disulphides and (b) cystine. (a) Serum (0.05–0.2ml.) is added to a solution containing 1ml. of 0.5mN-HMB plus 1.5ml. of A plus 0.3ml. of 0.5M-monosodium sulphite. After 2hr. at room temperature, 0.5ml. of 10% EDTA is added and the mixture diluted to 10ml.; 2ml. is measured, dithiofluorescein is added in excess, diluted to 5ml. and the extinction determined. Take into account the correction for substance X. Subtract from the result the consumption of HMB corresponding to the thiol content.

(b) To another 2ml. sample of the solution, 0.5ml. of 3% formaldehyde and dithiofluorescein in excess are added, the mixture is diluted to 5ml. and the extinction is determined after 5min. Correction for substance X is not made. Subtract from the total consumption of HMB after (a) the total consumption of HMB after (b) and consumption corresponding to cystine content. The result corresponds to the cystine content plus mixed cysteine disulphides, if any. The results are summarized in Table 6.

(5) Estimation of thiol esters. To the two samples containing 2ml. of 0.05mN-HMB plus 0.5ml. of B, at room temperature, is added 0.1–0.2ml. of serum. To the first sample 0.3ml. of C is added after 30min., and to the second sample after 60min. Dithiofluorescein is added in excess, the mixture is diluted to 5ml. and the extinction determined. The correction for substance X is determined in a separate sample and added to the extinctions measured. The consumption of HMB corresponding to the thiol content is subtracted from the results. The difference in consumption of HMB after 60 and 30min. is subtracted from the result obtained after 30min. Table 7 shows the results obtained. [The extinction coefficient of dithio-

Table 6. *Estimation of total disulphides and cystine in bovine serum by methods (4a) and (4b)*

Results are expressed as the concn. ($10^{-5}M$) of final solution (5ml.).

Vol. of serum (ml.)	0.02	0.03	0.04
Total S-S, found	0.76	1.18	1.40
Cystine, found	0.05	0.08	0.09
Vol. of serum (ml.)	0.02	0.02	0.02
Cystine, added	0.28	0.55	0.82
Increase of total S-S	0.29	0.48	0.76
Increase of cystine	0.31	0.49	0.72

Table 7. *Estimation of thiol esters in bovine serum by method (5)*

Results are expressed as concn. ($10^{-5}M$) of final solution (5ml.).

Vol. of serum (ml.)	0.10	0.10	0.10	0.10
NS-Diacetylcysteine, added	—	0.23	0.46	0.69
Thiol esters, found	0.01	0.25	0.46	0.64

fluorescein used in method 4(b) is 4.3×10^4 ; in all remaining methods it is 3.5×10^4 , at $588m\mu$.]

(6) Estimation of substance X. Serum (0.1 ml.) is diluted to 5 ml. with water and three 1.5 ml. samples are taken. To each sample is added 0.5 ml. of solution G and increasing amounts of dithiofluorescein in excess, with regard to substance X. The mixture is diluted to 5 ml. and the extinction at $588m\mu$ (E_1) and at $635m\mu$ (E_2) determined. The concentration of the complex dithiofluorescein-X (C_x) and concentration of the free dithiofluorescein (C_d) are calculated from the equations:

$$C_x = \frac{E_2 - 0.16 E_1}{2.6 \times 10^4}$$

$$C_d = \frac{E_1 - 1.7 \times 10^4 C_x}{3.2 \times 10^4}$$

The value C_d should be within the range of $2-6\mu N$. Within this range the value C_x increases insignificantly with increasing C_d . To make the comparison possible C_x should be adjusted by interpolation to the value corresponding to $C_d 3\mu N$.

DISCUSSION

As may be seen from Tables 1-7, the suggested methods work satisfactorily, 80% of results included in Tables 1-3 give recoveries in the range 96-104%. The agreement between the amounts of estimated substances added to the samples and those found analytically may be considered good. On the other hand, the results calculated for 11. of serum decrease

slightly with the increasing amounts of serum taken for analysis. Constant amounts of serum should therefore be used to make possible an exact comparison. The following amounts of human serum can be suggested for analysis: total thiols, 0.10 ml.; correction for substance X, 0.05 ml.; total thiols minus cysteine, 0.15 ml.; glutathione, 0.15 ml.; total disulphides and cystine, 0.05 ml.; correction for substance X, 0.05 ml.; thiol esters, 0.10 ml. plus 0.10 ml. plus correction for substance X, 0.05 ml.; substance X, 0.10 ml. It is thus possible to use for all estimations 0.9 ml. of serum only. The correction for substance X should be adjusted in accordance with the volume of serum taken in the main estimation. It is convenient to express the results in equivalents or moles/l. of serum (N or M).

The normal concentrations in human serum (10 samples, women) were found to be: thiol content, 0.32-0.46 mN; glutathione, 1.2-2.2 mN; disulphides, 4.2-5.0 mM; substance X, 1.2-1.5 mN. The concentrations of free cysteine, cystine and thiol esters in normal serum are not much above the sensitivity of the analytical methods, taking into account that they are calculated by difference.

The attempts to determine the oxidized glutathione in serum by using the kinetic method based on different rates of reaction of disulphide bond in oxidized glutathione and proteins with sulphite and HMB were not successful, for the velocity constant decreases continuously. By kinetic methods, however, one can get the 'spectrum' of reactivity of disulphide bonds. The other approach to this problem can be based on the paper electrophoresis of serum, treatment with hydrogen selenide and photometric evaluation of separated selenium (Wroński, 1966e). It would be possible to determine in this way the disulphide content in various fractions of serum. The reduction of disulphide bonds in serum by hydrogen selenide with subsequent analysis of resulting thiols is also possible.

The analytical methods suggested in this paper can easily be adapted for analysis of urine and other biological fluids.

REFERENCES

- Boyer, P. D. (1954). *J. Amer. chem. Soc.* **76**, 4331.
- Cecil, R. & Ferdinand, W. (1965). *Biochem. J.* **97**, 403.
- Cunningham, L. W. & Nuenke, B. J. (1959). *J. biol. Chem.* **234**, 1447.
- Cunningham, L. W. & Nuenke, B. J. (1960). *J. biol. Chem.* **235**, 1711.
- Dubouloz, P., Foundarai, J. & Pavone-Mariville, R. (1962). *Analyt. chim. Acta*, **26**, 241.
- Fernandez Diez, M. J., Osuga, D. T. & Feeney, R. E. (1964). *Arch. Biochem. Biophys.* **107**, 449.
- Jocelyn, P. C. (1962). *Biochem. J.* **85**, 480.
- Kolthoff, I. M. & Eisenstädter, J. (1961). *Analyt. chim. Acta*, **24**, 83.

- Krstěva, M., Alexiev, B. V., Ivanov, C. P. & Yordanov, B. (1956). *Analyt. chim. Acta*, **32**, 465.
- Kurzawa, Z. (1961). *Chem. analit.* **6**, 813.
- Leslie, J., Williams, D. L. & Gorin, G. (1962). *Analyt. Biochem.* **3**, 257.
- Swan, J. M. (1955). *Proc. int. Wool Text. Res. Conf., Australia*, C-25.
- Wroński, M. (1959). *Z. anal. Chem.* **169**, 351.
- Wroński, M. (1961a). *Z. anal. Chem.* **180**, 185.
- Wroński, M. (1961b). *Chem. analit.* **6**, 859.
- Wroński, M. (1963a). *Wiad. chem.* **187**, 1.
- Wroński, M. (1963b). *Chem. analit.* **8**, 467.
- Wroński, M. (1963c). *Z. anal. Chem.* **192**, 294.
- Wroński, M. (1965a). *Chem. Listy*, **59**, 1079.
- Wroński, M. (1965b). *Analyst*, **90**, 697.
- Wroński, M. (1966a). *Chem. analit.* **11**, 1153.
- Wroński, M. (1966b). *Analyst*, **91**, 745.
- Wroński, M. (1966c). *Roczn. Chem.* **40**, 327.
- Wroński, M. (1966d). *Aust. J. Chem.* **19**, 341.
- Wroński, M. (1966e). *J. Chromat.* **24**, 480.