A Method for the Estimation of Thiol Esters

By R. CECIL AND W. FERDINAND*

Department of Biochemistry, University of Oxford

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1. A method is described for the estimation of thiol ester groups. The thiol ester is converted into the corresponding thiol by reaction with ammonia; the thiol is then titrated amperometrically with mercuric chloride. 2. The method may be used in the presence of SH and S-S groups. The SH groups are titrated at pH 3 in the presence of excess of chloride; under these conditions thiol esters do not react with mercuric chloride. Thiol ester plus thiol is then estimated by titration after reaction with ammonia. Finally, titration after reaction with ammonia and sulphite gives the thiol ester plus thiol plus disulphide. 3. The procedure has been applied to glyceraldehyde phosphate dehydrogenase. The enzyme was found to contain 15–16 SH groups/mol. and no S-S groups. After reaction with acetyl phosphate 1.8–3.5 thiol ester groups were detected, the number depending on the conditions of acetylation. In the absence of bound NAD, the number of thiol ester groups formed was 1.8/mol., although a value of 2.9 labile acetyl groups/mol. was given by the method of Lipmann & Tuttle (1945). The presence of thiol ester groups in the *S*-(*p*-3-phosphoglyceryl)-enzyme was also demonstrated.

In the past thiol esters have usually been estimated by allowing them to react with hydroxylamine and measuring the amount of hydroxamic acid formed (Lipmann & Tuttle, 1945). This method suffers from the disadvantage that it measures the amount of labile acyl groups present and so is not specific for thiol esters; acetylimidazole, for example, reacts readily with hydroxylamine. To overcome this difficulty a method has been developed which depends on the reaction of the thiol ester with ammonia followed by titration of the SH groups thus formed with mercuric chloride. Hydroxylamine cannot be used in this method as it will reduce the mercuric chloride to mercury.

The method was tried out with simple thiol esters and then applied to the enzyme glyceraldehyde phosphate dehydrogenase [*D*-glyceraldehyde 3-phosphate-NAD oxidoreductase (phosphorylating), EC 1.2.1.12], which is known to form an *S*-acyl intermediate (Racker, Klybas & Schramm, 1959; Harris, Meriwether & Park, 1963). As this enzyme contains free SH groups (Koepppe, Boyer & Stulberg, 1956), conditions have had to be found for determining the number of these groups in the presence of the thiol ester groups.

* Present address: Department of Biochemistry, University of Sheffield.

MATERIALS

N-Acetylhomocysteine thiolactone. This compound was purchased from Schwarz Laboratories Inc. (Mount Vernon, N.Y., U.S.A.) and stored over CaCl₂. Its melting point was 110°, with sublimation, in good agreement with the value of 111° found by Benesch & Benesch (1956).

Butyl thiolacetate. A sample of this compound:

which had been prepared by the method of Noda, Kirby & Lardy (1953), was kindly given to us by Dr G. D. Clarke-Walker. It was redistilled before use (b.p. 159–160°).

Phenylmercuric acetate. This compound (microanalytical reagent; British Drug Houses Ltd., Poole, Dorset) was dissolved in water to give a concentration of approx. 10 mM. Concentrations were checked by potentiometric titration against KBr (Allison & Cecil, 1958) and agreed with the concentration by weight.

Carboxymethyl thiolacetate. This compound:

was purchased from Eastman-Kodak Ltd. (Rochester 3, N.Y., U.S.A.) and distilled before use (b.p. 137–141°).

Glyceraldehyde phosphate dehydrogenase. This enzyme was prepared from rabbit skeletal muscle as described by Ferdinand (1964). Preparations 14, 15 and 16 were used. Preparations 16A and 16B, referred to in Table 2, were recrystallized in the presence of NAD and contained 3.9 and 4.0 mol./mol. respectively.

Guanidine hydrochloride. Solutions, approx. 7M, were prepared by neutralizing solid reagent-grade guanidine

carbonate with conc. HCl at 0° to approx. pH3. The solutions were boiled at reduced pressure to eliminate CO₂.

METHODS

Polarographic measurements. The apparatus described by Cecil & Snow (1962) was used both for measurements of wave heights and for amperometric titrations.

Estimation of free SH groups. Amperometric titration with HgCl₂ or phenylmercuric acetate (Allison & Cecil, 1958) was used.

Estimation of S-S groups. The methods described by Cecil & Wake (1962) were used, except that an ammonia-ammonium chloride buffer was used (see below).

RESULTS

Experiments with simple thiol esters

Reaction with ammonia. Conditions were sought under which the thiol ester group reacts rapidly with ammonia:

Since the reaction is with ammonia rather than with NH₄⁺ it had to be carried out at alkaline pH. Accordingly, it was studied in the polarographic cell with increasing concentrations of ammonia-ammonium chloride buffers at pH9.3 and 37°. The

amount of thiol formed was followed by measuring the height of the anodic (oxidation) wave at -0.3v.

The results with *N*-acetylhomocysteine thiolactone (Fig. 1) show that, with a buffer of composition 1.0M-ammonia-1.0M-ammonium chloride, the reaction is complete within 10min. Subsequent amperometric titration of the homocysteine amide with mercuric chloride at -0.3v gave a thiol titre corresponding to 99% of the theoretical.

The reaction of carboxymethyl thiolacetate with ammonia under the same conditions is complete in 30min. Subsequent titration with mercuric chloride gave a titre corresponding to 99.5% of the theoretical; however, 5% of this could be accounted for as free thioglycollic acid, which was present as an impurity (see below).

The estimation of butyl thiolacetate was complicated by the fact that, being rather insoluble in water and somewhat volatile, it tends to be lost from the polarographic cell when nitrogen is passed through (to remove oxygen). Nevertheless, it was shown that the reaction with ammonia is complete in 30min. Subsequent titration with mercuric chloride gave a titre corresponding to 85-90% of the theoretical.

In view of the ease with which thiols are oxidized at alkaline pH and of the difficulty of excluding all traces of oxygen, it is advisable to carry out a second titration in which a small excess of mercuric chloride is added at the start of the reaction [as described by Allison & Cecil (1958) for denatured proteins].

Estimation of the free SH groups. Thiols are likely impurities in thiol esters, and proteins that contain thiol ester groups are almost certain to contain some free SH groups. It is necessary to know the number of free SH groups present initially so that the titres obtained after reaction with ammonia can be interpreted. Conditions were therefore sought under which the free SH groups can be titrated with the minimum of decomposition of the thiol ester groups.

Table 1 (columns 2 and 3) shows the extent of reaction that occurs with the three thiol esters at pH9 and pH3 in 20min. at 37°; this was measured as before by observing the height of the anodic thiol wave at -0.3v. The amount of hydrolysis at pH3 is small, so that titration at this pH (which can be carried out at room temperature or below) should not be in error.

Titration were accordingly carried out with mercuric chloride at pH3 and it was found that homocysteine thiolactone and butyl thiolacetate contained no free thiol but that carboxymethyl thiolacetate contained 5%, expressed as thioglycollic acid.

Estimations of total free SH groups in proteins

Fig. 1. Reaction of *N*-acetylhomocysteine thiolactone with ammonia at pH9.3 and 37°. The amount of homocysteine amide formed was measured polarographically (as described in the text) and is expressed as a percentage of the initial concentration of the thiolactone (approx. 1mM). The buffers used were: A, 0.15M-NH₃-0.15M-NH₄Cl; B, 1.0M-NH₃-1.0M-NH₄Cl.

Table 1. *Rate of hydrolysis of thiol esters under various conditions*

The extent of reaction after 20 min. at 37° was measured polarographically as described in the text and is given as a percentage of the initial concentration (approx. 1 mm). The pH 3 buffer was 0.1M-formic acid adjusted to pH 3 with NaOH, and the pH 9 buffer was 0.1M-Na₂B₄O₇.

Substance	pH 3	pH 9	pH 3 + HgCl ₂	pH 3 + HgCl ₂	pH 3 + HgCl ₂
			(1 equiv.)	(1 equiv.) + KCl (0.2M)	(1 equiv.) + KCl (3.0M)
N-Acetylhomocysteine thiolactone	0	20	30	8	2
Carboxymethyl thiolacetate	0.3	2	100	12	< 1
Butyl thiolacetate	1.2	2	< 1	12	< 1

(e.g. reactive plus unreactive groups; Cecil, 1963) have normally to be carried out in the presence of denaturing agents, when the oxidation of SH to S·S groups can be rapid. To avoid errors from this cause the titrations are normally carried out in the presence of a small excess of mercuric chloride (Allison & Cecil, 1958). Table 1 (column 4) shows that, even at pH 3, the reaction of thiol ester with mercuric chloride can be rapid but that this is greatly diminished in the presence of chloride (Table 1, columns 5 and 6), when the mercury is present as [HgCl₄]²⁻. Provided therefore that the chloride concentration is high, and the excess of mercuric chloride small, this source of error can be kept within acceptable limits.

Estimation of S·S groups. It may be necessary to estimate S·S groups in molecules containing both SH and thiol ester groups. Once the number of both these types of groups is known a further reaction is carried out in the ammonia buffer together with sodium sulphite (0.2M) and a small excess of mercuric chloride. With proteins the presence of 3M-guanidine hydrochloride will also be required. Under these conditions the S·S groups will react according to the equations:

The resulting titre will be the sum of SH, S·S and thiol ester groups. This method was used with glyceraldehyde phosphate dehydrogenase (see below).

Experiments with glyceraldehyde phosphate dehydrogenase

Estimation of free SH groups. Attempts to measure the number of reactive SH groups by amperometric titration with phenylmercuric acetate at pH 9 and at -0.6 v were bedevilled by the ease with which the enzyme precipitated when nitrogen was passed through the solution to remove oxygen. This tendency was greatly decreased if the titrations were carried out at 0°, when it was found that

Table 2. *Estimation of the number of SH groups in denatured glyceraldehyde dehydrogenase*

Titration of the denatured protein were carried out with HgCl₂ at -0.3 v and expressed as mol. × 2 of HgCl₂/mol. of enzyme. The pH 3 buffer was 3M-guanidine hydrochloride in 0.1M-formic acid adjusted to pH 3 with NaOH; the pH 9.3 buffer was 3M-guanidine hydrochloride in 0.1M-Na₂B₄O₇-0.05M-Na₂S₂O₃. The results are expressed as the number of groups/mol. (mol.wt. 140000).

Enzyme preparation	Denatured protein at 37°	
	pH 9.3	pH 3
no.		
14	15.7	15.8
15	14.8	15.1
16	15.0	15.0
16A	15.3	15.1
16B	14.7	14.8

2 SH groups reacted/mol. (mol.wt. 140000). This value can only be regarded as approximate because a slow reaction takes place even with the small excess of phenylmercuric acetate present during titration.

Since there appeared to be no sharp distinction between 'reactive' and 'unreactive' SH groups as was found in haemoglobin (Ingram, 1955; Allison & Cecil, 1958), it was necessary to determine the total number present in the presence of a denaturing agent. Precipitation was experienced in the presence of urea and sodium dodecyl sulphate, but satisfactory results were obtained with mercuric chloride in the presence of 3M-guanidine hydrochloride at 37°; these are summarized in Table 2 (columns 2 and 3). The titres obtained at pH 9 were slightly higher than those at pH 3 unless thiosulphate was added; this is presumably because the mercaptides had a greater tendency to bind additional Hg²⁺ at pH 9 than at pH 3 (Cecil, 1963). There was a small variation between different preparations but the results were consistent within each preparation.

Estimation of S·S and thiol ester groups. Titrations were carried out on the same samples of the

Table 3. *Reaction of acetyl phosphate with glyceraldehyde phosphate dehydrogenase*

The reactions with acetyl phosphate were carried out at 0° in 5mm-EDTA adjusted to pH6.5 with NaOH. The SH and thiol ester groups were estimated as described in the text.

Enzyme preparation no.	Acetyl phosphate (mol./mol. of enzyme)	NAD (mol./mol. of enzyme)	Reaction time (min.)	SH (mol./mol. of enzyme)	Thiol ester (mol./mol. of enzyme)
15	20	0	30	13.3	1.8
15	20	1.5	5	13.0	2.1
15	20	7.5	5	12.6	2.7
15	20	7.5	30	12.2	3.1
15	100	10	5	13.2	2.5
16	70	1.1	15	11.8	3.5
16B	70	4	35	13.2	2.1

enzyme in the presence of both sulphite and ammonia. The titres were the same as those obtained in the absence of these reagents, showing that these samples contained neither S-S nor thiol ester groups.

The same result was obtained with preparations 14, 15, 16 and 16B. When these preparations were analysed (for labile acetyl groups) by the method of Lipmann & Tuttle (1945), values for apparent thiol ester of 0.6, 0.4, 0.1 and 0 mol./mol. respectively were obtained.

Experiments with the acetyl-enzyme. The acetyl-enzyme was prepared from the apoenzyme by the method of Krinsky (1955). Titrations with mercuric chloride were carried out at pH3 and pH9.3 in the presence of ammonia; the difference amounted to 1.8 mol. (of thiol ester)/mol. (mol.wt. 140000). Analysis of the same sample for labile acetyl groups by the method of Lipmann & Tuttle (1945) gave 2.9, so that there was a difference of 1.1 mol. of apparent thiol ester/mol. between the two methods.

Since Krinsky (1955) reported that the acetyl enzyme is unstable in the presence of NAD, experiments were carried out in which the enzyme was allowed to react with excess of acetyl phosphate in the presence of different amounts of NAD. The excess of acetyl phosphate was not removed (as in the first experiment) before estimating the content of thiol ester, as it does not interfere with the mercuric chloride titrations. The results (summarized in Table 3) show that the amount of acetyl-enzyme formed was no less in the presence of NAD.

Experiments with the D-3-phosphoglyceryl-enzyme. In the presence of NAD D-glyceraldehyde 3-phosphate reacts with the enzyme to form the S-(D-3-phosphoglyceryl) derivative. This is unstable and is hydrolysed rapidly to phosphoglyceric acid. Nevertheless, when preparation 16B, which contains 4 mol. of bound NAD/mol., was treated

with a tenfold molar excess of D-glyceraldehyde 3-phosphate (i.e. a 20-fold excess of the DL-derivative), it was found to contain 1.3, 0.8 and 0.6 mol. of thiol ester/mol. after $\frac{1}{2}$, 5 and 10 min. reaction time respectively.

DISCUSSION

Reaction with ammonia provides a rapid and specific method for converting thiol esters into thiols. The reaction is best carried out in the presence of a small excess of mercuric chloride to prevent oxidation of the thiol formed.

The formation of S-acyl groups as intermediates in the reaction of glyceraldehyde phosphate dehydrogenase is well established (Koeppe *et al.* 1956; Racker *et al.* 1959, Harris *et al.* 1963) and has been confirmed in the present work. The S-acetyl derivative, produced by reaction with acetyl phosphate (Krinsky, 1955), is reasonably stable and was used to compare the values for thiol ester content obtained by this method and by the method of Lipmann & Tuttle (1945). This difference amounted to as much as 1 mol./mol. of enzyme and could be due either to acetyl groups combined with, say, imidazole groups in the protein or even to occluded acetyl phosphate (although the precipitated acyl-enzyme was washed twice with saturated ammonium sulphate to remove excess of acetyl phosphate).

The continued formation of acetyl-enzyme obtained in the presence of NAD differs from Krinsky's (1955) findings. This difference is likely to be due to the fact that, in Krinsky's (1955) work, the excess of acetyl phosphate had to be removed before estimation of the S-acetyl groups by Lipmann & Tuttle's (1945) method, whereas in the present work the excess (20–100 mol./mol.) was present throughout.

The value for total SH groups of 15–16/mol. agrees well with previous determinations (e.g.,

Koepe *et al.* 1956) and with the 16 half-cystine residues/mol. found by Harris & Perham (1963). The molecule consists of identical sub-units, each of mol.wt. 35 000–40 000 (Harris & Perham, 1963), and on the basis of an overall mol.wt. 140 000 each sub-unit will contain 4 SH groups.

The value of 2 reactive SH groups/mol. is probably not significant. In some proteins, such as the haemoglobins, there is a marked difference in the behaviour of the 'reactive' and 'unreactive' SH groups (Cecil, 1963). In glyceraldehyde phosphate dehydrogenase there is less difference and the values obtained depend on variables such as the time of titration and the extent of denaturation consequent on bubbling nitrogen through the solution.

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