Short Communications

Determination of sulfhydryl and disulfide groups by specific proton displacement*

In the quantitative determination of sulfhydryl groups with "specific reagents", the end-point is usually determined either by ascertaining the point at which the thiol concentration reaches zero, or by measuring the concentration of excess reagent.

A method has now been devised by which the proton of the –SH group itself is displaced quantitatively and specifically and then determined by titration with alkali, according to the reaction:

$$RSH + ICH_2CONH_2 = RSCH_2CONH_2 + H^+ + I^-$$

In order to utilise the liberation of hydrogen ions by this reaction analytically, the following conditions have to be met:

(1) A pH must be chosen, at which a whole proton is liberated from the –SH group, *i.e.* where this group is completely un-ionized.

(2) Quantitative reaction should occur within a reasonable time.

(3) The reaction must be carried out under conditions which preclude interfering side reactions, such as alkylation of amino groups[1,2,3,4].

(4) For maximum sensitivity, the end-point should be in a pH region in which buffering by other groups in the molecule (*e.g.* amino and carboxyl) is at a minimum.

To satisfy these conditions, the following procedure was adopted:

The solution of the –SH compound is brought to pH 5.5. At this pH the –SH group is completely un-ionized and buffering is at a minimum, since the amino group is in the NH_3^+ and the carboxyl group is in the COO^- form. A 2- to 5-fold excess of iodoacetamide over –SH is now added and the solution is brought to pH 9 by the addition of a known amount of standard alkali. This is done to insure rapid reaction between the alkylating reagent and the thiol group. A reaction time of 2 min at pH 9 was found to be sufficient for complete reaction with –SH groups without any reaction with either α or ε amino groups. This is illustrated in Fig. 1. After two minutes

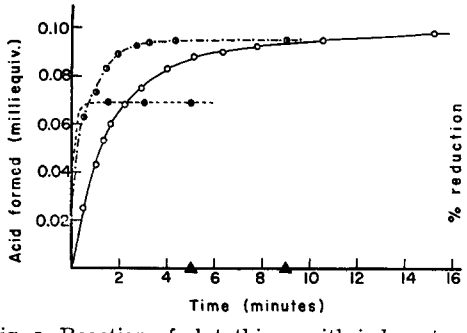

Fig. 1. Reaction of glutathione with iodoacetamide. 0.10 milliequivalent glutathione in 5 ml water was brought to the stated pH. 0.2 mequiv. iodoacetamide in 1 ml water was added at zero time and the pH was kept constant by the addition of alkali. ○——○ pH 7.10; ◑-·-·-◑ pH 7.96; ●---● pH 9.00; ▲——▲ pH 9.00; 0.10 mequiv. lysine instead of glutathione. At pH values greater than 7, the number of protons liberated per mole of glutathione is less than one, because part of the glutathione is in the GS^- form.

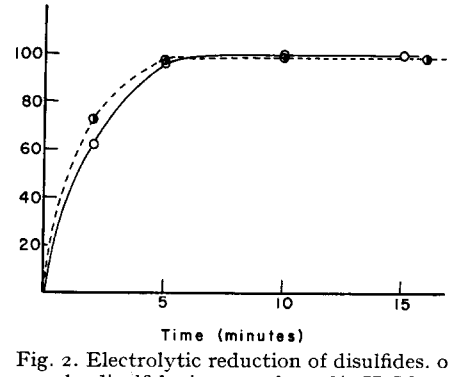

Fig. 2. Electrolytic reduction of disulfides. 0.10 mmole disulfide in 10 ml 0.2% H_2SO_4 was reduced with a current of 0.5 A. Percent reduction for oxidized glutathione is calculated on the assumption that the molecule contains two moles of ethanol[8]. ◑---◑ homocystine; ○——○ oxidized glutathione.

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at pH 9, the solution is titrated back to pH 5.5 with standard acid. The difference between the equivalents of alkali and acid added is equal to the hydrogen ions liberated and therefore equal to the –SH content of the original solution.

The actual titration is carried out as follows: 0.02 milliequiv. of thiol in a volume of 1 or 2 ml are placed in a 10 ml beaker. One drop of phenolphthalein and one drop of methyl red indicator are added and nitrogen is bubbled through the solution for the rest of the titration to provide mixing and prevent absorption of CO_2. The solution is now brought to the methyl red end-point (*ca.* pH 5.5). 0.2 ml of 0.2 *M* recrystallized iodoacetamide is now added and the solution brought to the permanent phenolphthalein end-point with standard alkali (pH 9). After remaining at this pH for 2 min, the solution is brought back to pH 5.5 with standard acid. A syringe-microburet, manufactured by Micrometric Instrument Co., Cleveland, Ohio, was found to be very convenient for these titrations. Table I illustrates some results obtained by this procedure:

TABLE I

Compound	*mequiv. added*	*mequiv. NaOH pH 5.5 → 9*	*mequiv. HNO_3 pH 9 → 5.5*	*mequiv. $H^+ = SH$*	*% –SH*
Cysteine	0.108	0.189	0.085	0.104	96
Cysteinylglycine	0.0380	0.0709	0.0350	0.0359	95
Glutathione	0.0177	0.0226	0.0052	0.0174	98
Glutathione	0.100	0.146	0.049	0.097	97

Disulfides can also be determined by this method after electrolytic reduction to the –SH form. It was first shown by DOHAN AND WOODWARD[5] that disulfides can be quantitatively converted to the corresponding thiols in a very simple electrolysis cell, employing a mercury cathode. For the present work the "Electrolytic Desalter", designed by CONSDEN *et al.*[6] was used for this purpose. It was found that not only soluble disulfides, such as oxidized glutathione, but even insoluble compounds such as cystine, are reduced rapidly and quantitatively to the –SH form. This is analogous to the reduction of insoluble mercaptides described in another paper[7]. In this way, the thiol is obtained in a salt-free condition, free from any excess reducing agent and is ready for the titration as described above. The course of the electrolytic reduction of homocystine and oxidized glutathione, as determined by the specific proton displacement method, is shown in Fig. 2.

This method has proved very useful for determining the purity of various amino acids and peptides containing thiol and disulphide groups, and for following the electrolytic reduction of mercaptides[7]. It might be emphasized that the method is an absolute one, being independent of the purity of the iodoacetamide (within reasonable limits) and depending only on the alkali and acid as primary standards. It therefore does not require a pure –SH compound for standardization, unlike many other methods. On the other hand, the presence of buffers seriously affects the accuracy of the method for two reasons. If the buffering is pronounced in the vicinity of pH 5.5, the sharpness of the end-point is, of course, affected. If the buffering occurs in the region between the two end-points, *i.e.* pH 5.5–9, the volume of both titrants automatically increases with consequent loss of accuracy. The method is therefore not recommended for use in highly buffered solutions of biological origin, such as microbial extracts, plasma, *etc.*

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