The Electrolytic Reduction of Proteins*

S. J. Leach,† Aviva Meschers, and O. A. Swanepoel‡

ABSTRACT: An electrolytic method is described by which proteins such as bovine plasma albumin, lysozyme, wool keratin, ribonuclease, and insulin may be reduced to various extents. It is usually necessary to add small amounts of a thiol compound, which acts as a current carrier. An improved type of "scanning potentiostat" has been employed, which allows the level of reduction to be automatically controlled. The high extent of reduction without the need for denaturants suggests that the difficulties experienced in reducing certain disulfide bonds in proteins are owing to their low reactivity rather than to their molecular inaccessibility.

Of the side-chain interactions which are known to stabilize protein conformations, disulfide linkages are undoubtedly the most stable and important. This accounts for the great interest which is now shown in methods for partial or complete disulfide bond fission. For example, the fall in biological activity of a protein or enzyme, which accompanies progressive reduction, should give some indication of the role of each disulfide bond in maintaining the unique native conformation. Currently, progressive disulfide bond fission is being used to study the role of cross-links in determining transition temperatures and the occurrence of thiol-disulfide interchange during conformational changes. In physicochemical studies on keratins, complete disulfide bond fission is a prerequisite for the extraction of soluble proteins (see, e.g., Crewther *et al.*, 1964).

Existing methods for disulfide bond fission have disadvantages. Oxidation with peracids requires the presence of 98% formic acid and can sometimes lead to partial oxidation products (Maclaren *et al.*, 1959). Reductive methods, on the other hand, give complete fission only in the presence of, say, 8 M urea or extremely high concentrations of thiol compounds. The need to use denaturants in these methods has led to the suggestion that some disulfide bonds are not "available" for reaction until "exposed." This viewpoint has been questioned by Leach and O'Donnell (1961), and Leach (1960), who have shown that the fission process may be regarded as an equilibrium reaction. Thus reduction with thiols proceeds further toward completion as the thiol concentration is increased (Thompson and O'Donnell, 1961), and reduction with sulfite may also be forced to completion by removal of one of the reaction products with a mercurial (Stricks *et al.*, 1954; Leach, 1960). The reaction with mercurial has the disadvantage, for preparative purposes, that it leads to an asymmetrical fission, the products being $-SSO_3^-$ and —SHgR groups. An alternative procedure is oxidative sulfitolysis (Swan, 1957; Bailey, 1957; Leach and Swan, 1962; Leach *et al.*, 1963), which leads to symmetrical fission, with only $-SSO_3^-$ groups as products. However, unless denaturants are present and alkaline solutions are used, this reaction is useful in only a limited number of cases.

If the extent of disulfide bond reduction in proteins is limited merely by equilibrium considerations (that is, by their reactivity), it should be possible to force reduction to completion by, say, cathodic reduction. Markus (1960) showed that insulin could be reduced electrolytically, and R. Cecil and P. D. J. Weitzman (paper in preparation) have reduced both insulin and ribonuclease by this method. However, even in cases where the protein itself cannot be reduced directly at an electrode surface owing to its size and complex shape, it should be possible to find a "carrier" which can mediate (like an enzyme) between the protein and the electrode. Even low concentrations of thiols should suffice as long as they are continuously regenerated in the reduced form. The carrier would have to be reducible at the electrode and of a sufficiently low redox potential to be reoxidizable by disulfide compounds.

Since disulfide bonds in proteins appear to vary in reactivity, the final redox level (and the number of bonds reduced) could in theory be limited in either of two ways. One might use a trace of a powerfully reducing thiol compound such as ω-toluenethiol (Maclaren, 1962), and control the cathode potential at a fixed value; alternatively, the reduction potential might be set at the most negative value possible and the final level of reduction limited by the choice of thiol.[1] In this initial study, the thiol compounds investigated

* From the Division of Protein Chemistry, C.S.I.R.O., Wool Research Laboratories, Parkville N.2 (Melbourne), Victoria, Australia. *Received September 17, 1964.*

† Present address: Chemistry Department, Cornell University, Ithaca, N.Y.

‡ Present address: South African Wool Textile Research Institute, Rhodes University, Grahamstown, S. Africa.

[1] The exponential nature of the redox equation might suggest that extremely negative reducing potentials could be achieved even with a "mild" reducing agent, as long as the latter were kept fully reduced. In practice, the concentration of the thiol in the reduced form would be vanishingly small under these conditions and only "good" reducing agents would give useful rates of reduction.

have been limited to mercaptoacetic acid and 2-mercaptoethanol, and the proteins to bovine plasma albumin, lysozyme, wool keratin, ribonuclease, and insulin. These proteins range in reducibility from the highly reactive (insulin) to the most refractory (lysozyme) and each presents a different problem in solubility.

Materials

Buffer solutions used were: sodium acetate (0.1 M, with 0.1 M KCl, *p*H 5), Tris (0.2 M, with 0.2 M HCl and 0.1 M KCl, *p*H 7), Tris (0.05 M, with 0.006 M HCl and 0.1 M KCl, *p*H 9), potassium carbonate (0.05 M, with 0.01 M HCl and 0.1 M KCl, *p*H 10.5), all diluted with deionized water. Mercaptoacetic acid and 2-mercaptoethanol were freshly redistilled *in vacuo* and stored in a full bottle to avoid air-oxidation. Cystinylbisglycine and bisglycylcystine were substantially pure by paper chromatography. Hydrazine hydrate and other reagents were the purest available.

Bovine plasma albumin was from Armour Laboratories (crystallized, lot CA 2140) and Commonwealth Serum Laboratories (lot 695-0000-B001). Lysozyme was from Nutritional Biochemicals Corp. and Armour Laboratories (crystallized, lot ED 1990). Wool keratins (Merino 64's, lots MW 127, 129; Lincoln, lot MW 144) were washed with aqueous and organic solvents before use. Ribonuclease was from Armour Laboratories (bovine pancreatic, 5-times-recrystallized, lot DN 0252) and insulin from British Drug Houses Ltd. (bovine zinc, crystalline, batch 2189).

Methods

Analytical Methods. Thiol and disulfide estimations on the reduced proteins were carried out by amperometric titration with methylmercuric iodide at the dropping mercury electrode as described by Leach (1960, 1964).

Proteins which contained only a low content of *thiol* groups reacted readily with the mercurial and could therefore be titrated directly. However, proteins which were more extensively reduced were slower to react (probably owing to molecular aggregation). To avoid spuriously low thiol values, it was therefore necessary to leave the reduced proteins to react for 24 hours with the mercurial (20–80% excess). Under these conditions there was found to be no advantage in including urea (8 M) in the analytical reaction mixture.

The estimation of *thiol plus disulfide* content is normally carried out at *p*H 9 (e.g., Leach, 1960); with proteins which were extensively reduced, this procedure led to some air-oxidation. *Disulfide* estimations on such proteins were therefore carried out only after the thiol groups had been blocked in the course of a thiol estimation at *p*H 7 in the absence of urea (see Leach, 1964). Corrections were made in the disulfide estimations for the weight increase owing to mercurial. Consecutive estimations of this kind were also more economical of samples.

Reduction with Thiol Compounds. Since most of the electrolytic reductions of proteins were carried out in the presence of mercaptoethanol (0.07 M at *p*H 5, 7, 9, and 10.3), these conditions were used also in "control" experiments, to estimate the extent of reduction in the absence of electrolysis.

The proteins (100 mg of bovine plasma albumin, lysozyme, or wool keratin) were added to the buffer solution, stirred, and degassed with oxygen-free nitrogen gas. The globular proteins dissolved. 2-Mercaptoethanol (250 μl) was then added, and the flasks were stoppered securely and left overnight at 20°. The wool was then removed and washed with acetone–1 M HCl (39:1), then with acetone, and finally dried and stored *in vacuo* over anhydrous calcium sulfate until analyzed. The globular proteins were acidified to *p*H 2 with HCl, dialyzed repeatedly against HCl (0.01 M, *p*H 2, 0°) first in the presence and then in the absence of ethanol (5%). The solutions were then freeze-dried and stored *in vacuo* for analysis. Analyses were usually carried out as soon as the proteins were sufficiently dry, but storage under the foregoing conditions for several days did not lead to any appreciable amount of reoxidation.

Electrolytic Reductions. Both concentric- and H-type cells were employed but the latter were found to be most convenient, and a variant of that described by Meites (1961) was adopted. The conical cathode compartment[2] was separated from the cylindrical anode compartment by a horizontal bridge (18 mm i.d.) carrying B24 cones and coarse fritted disks of low electrical resistance. All three compartments could be filled and drained independently between experiments. The cathode was formed by adding mercury (33 ml, 40 cm^2 surface area) to the cathode compartment, and electrical contact was made via a platinum wire sealed into the base of the glass cell. The solution and cathode surface were stirred either by a magnetic stirrer floating on the mercury surface or by a motor-driven glass paddle. Usually, both methods were used simultaneously. The anode was a suspended platinum plate (14.4 cm^2).

All three compartments were filled to the same level with the supporting buffer, and hydrazine hydrate (0.07 M in cell) was added to the anolyte (30 ml). The hydrazine acted as a depolarizer so that excessively high applied voltages were not required to achieve a given cathode potential and also so that nitrogen was evolved at the anode instead of chlorine and oxygen. The catholyte (100 ml) was outgassed with pure nitrogen and 2-mercaptoethanol was added (0.5 ml). Before commencing electrolysis, a current-voltage curve was run with the rapid auto-"scanning" facility of the potentiostat (Wood, 1964). This feature provided a simple method for detecting traces of oxygen and, in the case of insulin[3] and cystine peptides, for following the course of the reduction. The protein (200 mg) was

[2] An accessory cylindrical cathode compartment of smaller capacity (25 ml) was easily fitted when required.

[3] Cystine-containing proteins larger than insulin do not show reduction waves in the polarograph, probably due to their small diffusion coefficients.

TABLE I: Electrolytic Reduction of Proteins in the Presence of 2-Mercaptoethanol (0.07 M).

pH	Without Electrolysis[a] SH (moles/mole of protein)	SS	After Electrolysis[a] SH (moles/mole of protein)	SS
Bovine plasma albumin				
5	3.6			
7	3.1		14.3	8.5
9	17.3	7.4	29.7	2.4
10.3[b]	19.3	6.1	29.6	1.6
Lysozyme				
5	0.2			
7	0.2		1.2	3.5
9	1.3	3.2	6.4	1.2
10.3	3.6	2.1	5.2	1.6

[a] It may be noted that the values for SH and SS do not always add up to the literature values for the two proteins (see, e.g., Leach, 1960). The values quoted are means for several experiments and, in addition to normal analytical variations, preparations varied slightly with respect to reduction efficiency as well as adventitious reoxidation. In individual experiments, when special care was taken to prevent air-oxidation during analysis, values of 33–35 and 8 moles/mole of (SH + 2 SS) were obtained for bovine plasma albumin and lysozyme, respectively. [b] The *p*H fell to 9.5 during prolonged electrolysis.

TABLE II: The Electrolytic Reduction of Wool in the Presence of 2-Mercaptoethanol (0.07 M) (Cathode Potential, 1.2 v).

pH	Without Electrolysis[a] SH (μmoles/g)	SS	After Electrolysis[a] SH (μmoles/g)	SS
5	280		390	250
7	375	265	700	10–40
9	305	295	710	0
10.3	275		670	0–35

[a] Initial values for SH and SS were 30 and 440 μmoles/g, respectively. While the precision of the analytical methods used for SS is usually ±10 μmoles/g, the results for different experiments varied by ±30 μmoles/g and the results, which are means for at least 4 experiments, are rounded off to the nearest 5 μmoles/g.

then added to the cathode compartment either in the solid form or dissolved in a few ml of buffer solution, and electrolysis was commenced.

Throughout electrolytic reduction, which proceeded for 2–24 hours, the cathode potential was maintained at a predetermined value using a potentiostat of novel design (Wood, 1964). This device allowed the potential to be set at any value between +0.5 and −3.0 v versus a saturated calomel electrode (SCE).[4] The potentiostat was capable of supplying a maximum current of 1 amp and a maximum voltage of 50 v. Its speed of response was sufficient to cope with the fluctuations caused by the rapid stirring of the mercury surface. The cathode potential, the voltage applied across the cell and the current flowing, could be observed throughout the reduction. The *p*H of the catholyte before and after electrolysis was measured with a pair of microelectrodes inserted in the cell.

As a rule, electrolysis was commenced at a cathode potential of −1.5 to −1.6 v (versus SCE) for 1–2 hours and then at −1.2 v overnight. The *p*H of the catholyte was then checked and HCl was added to give *p*H 2. Reduced wools were then washed with acetone–HCl, dried, and stored as already described for thiol reductions. Solutions of reduced lysozyme were cloudy after reduction, and reduced bovine plasma albumin became cloudy on acidification. Analyses showed that these precipitations did not usually fractionate reduced from unreduced protein, so the cloudy solutions were dialyzed against HCl (*p*H 2) and freeze-dried as already described, without removing the precipitate by centrifugation.

[4] Abbreviation used in this work: SCE, saturated calomel electrode.

Results and Discussion

Reduction without Electrolysis. The reduction of bovine plasma albumin and lysozyme by 2-mercaptoethanol (0.07 M) proceeds further as the *p*H is raised (see Table I, first three columns). As noted by numerous investigators, reduction becomes extensive only at *p*H 9 and above. At these higher *p*H values there is a build-up of negative charges along the protein chain which favors swelling and therefore disulfide bond fission. In addition, the reducing thiol ionizes in this *p*H range and the thiol anion is believed to be the reactive species in the thiol-disulfide interchange reaction (Jensen, 1959, Spackman *et al.*, 1960). Even at *p*H 10.3, however, reduction is far from complete for either protein.

Table II (first three columns) shows that the reduction of wool by 2-mercaptoethanol (0.07 M) is optimal at *ca.* *p*H 7. This apparent anomaly, which occurred in every series of experiments with wool, has not been explained, but might be associated with Donnan membrane effects. These effects produce an "internal" *p*H within a fibrous protein which can differ from the measured external *p*H by as much as 2 *p*H units (see, e.g., Crewther *et al.*, 1964).

Within the limits of experimental variations in analysis (see footnote to Table I), each disulfide bond "lost" in the reduction of the three proteins produced two thiol groups in the products.

Electrolytic Reduction Without Thiols. The electrolytic reduction of bisglycylcystine and cystinylbisglycine at *p*H 7 and 9 presented no problems. Each showed characteristic current-voltage curves before electrolysis, which disappeared after reduction for two hours at a cathode potential of −1.2 v. Titration of the reduced solutions with methylmercuric iodide showed that reduction was complete.

Insulin did not appear to be reducible at *p*H 2.9 (glycine buffer, cathode potential −1.25 v), although slow and incomplete reduction occurred at lower *p*H values. More extensive reduction occurred at *p*H 9 (Tris buffer, cathode potential −1.2 v, then −2.0 v) and after 18 hours, 4 thiol groups were detectable per mole of insulin. Lysozyme was partly reducible at *p*H 7 (Tris buffer, cathode potential −0.8 v, then −1.3 v) and the precipitated material had 3.8 thiol and 2.1 disulfide groups per mole, corresponding to 50% reduction. Ribonuclease showed evidence of reduction of one disulfide bond at *p*H 7. Neither bovine plasma albumin nor wool were reducible electrolytically under the above range of conditions.

Electrolytic Reduction in the Presence of 2-Mercaptoethanol (0.07 M). Of the two thiols chosen as current carriers, mercaptoacetate was found unsuitable since its oxidized form was unstable at *p*H values above 7. 2-Mercaptoethanol gave satisfactory results.

Attempts were made to eliminate the need for outgassing the catholyte with nitrogen gas; oxygen was removed by electrolytic reduction before addition of the protein and continuation of reduction. This procedure was unsuccessful since *p*H changes during electrolysis were always excessive. Oxygen was therefore removed by passing nitrogen *through* the solution before reduction commenced and *over* the solution during the electrolysis. In this way *p*H changes were limited to less than 0.1 unit except at *p*H 10.35, where prolonged electrolysis caused a fall to 9.5.

Tables I and II (last two columns) show that electrolysis drives the reduction of bovine plasma albumin, lysozyme, and wool further toward completion than does the use of 2-mercaptoethanol alone. This is true even at *p*H 5 and 7 although reduction is much farther from completion in this *p*H range. The extent of reduction was found to decrease as the cathode potential was set at less negative values. For example, prolonged electrolysis of wool at *p*H 7 produced maximal thiol contents of only 140 μmoles/g when the cathode potential was −0.7 v (compared with 700 μmoles/g at −1.2 v). This dependence of reduction level on the cathode potential was noted also for lysozyme and ribonuclease, and suggests that it should be possible to select the final reduction level in this way. Two disulfide bonds per ribonuclease molecule were reducible at *p*H 9 using a cathode potential of −1.5 v.

As noted already for nonelectrolytic reduction, each disulfide bond in the soluble proteins (Table I) produced two thiol groups on electrolytic reduction. The same is true for the electrolytic reduction of wool but only at *p*H 5 (Table II). At *p*H 7, 9, and 10.3, where few disulfide bonds remain unreduced, the number of thiol groups detectable should be between 830 and 910 μmoles/g. The highest value recorded was only 710 μmoles/g.

This discrepancy is illustrated also in Figure 1, which shows the number of disulfide groups remaining and thiol groups produced after electrolytic reduction at *p*H 7.3 for various lengths of time. A discrepancy in the SH-SS balance appears to occur when reduction affects about 50% or more of the disulfide bonds in wool. This discrepancy cannot be due to air-oxidation during thiol estimations since there would be a corresponding increase in the subsequent disulfide estimations (these were always carried out on the same sample). In any case, thiol values were no higher when stringent precautions were taken to exclude oxygen during analysis. Thiol analyses carried out at *p*H 5 were unsatisfactory owing to the slowness of the reaction with methylmercuric iodide. The ultimate values were no higher than at *p*H 7, whether in the presence or absence of urea.

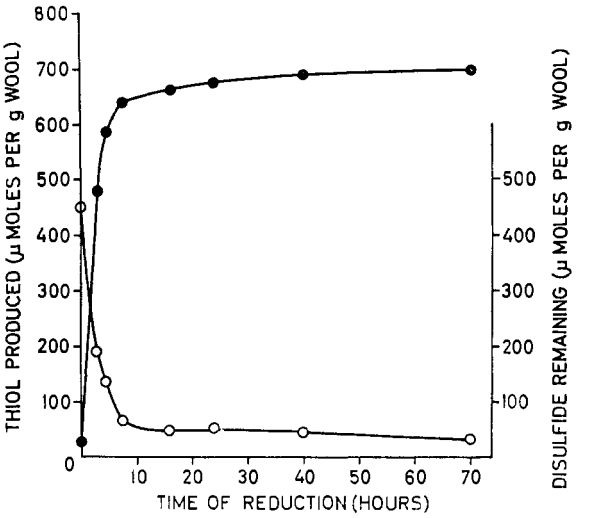

FIGURE 1: The electrolytic reduction of wool at *p*H 7 in the presence of 2-mercaptoethanol (0.07 M). ●, thiol groups detectable; ○, disulfide groups remaining. Each point is a mean of 2–4 analyses.

Of the proteins investigated, only wool showed anomalously low thiol contents in the reduced state. Mercury analyses on these wools showed that there was no uptake of Hg(II) from the cathode. Nor was there any production of lanthionine[5] (—CH_2—S—CH_2—) cross-links, which could account for the disappearance of thiol or disulfide groups. There was a small loss of elemental sulfur from the wool; for the points shown in Figure 1, the sulfur analyses (starting at zero time) were 3.35, 3.22, 3.20, 3.15, 3.27, 3.19, 3.16, and 3.19%, respectively. The associated weight losses were less

[5] It was necessary to alkylate the reduced wool with iodoacetate before acid hydrolysis and chromatography, so as to avoid the production of lanthionine from free thiol groups during hydrolysis.

than 1%. Electrolytic reductions at *p*H 9 produced wools with 700–710 μmoles/g of thiol groups and a sulfur content of 3.07%. Under these conditions there was a weight loss of only 1–2%. Some of the dissolved material could be precipitated by acidifying the catholyte. The sulfur content of this material was 4.0%. The loss of thiol (or disulfide) is therefore not caused by the dissolution of a sulfur-rich component of wool.

The analytical discrepancy is not peculiar to one type of wool; it was observed with three types (two Merino and one Lincoln wool). Nor does it appear to be peculiar to the electrolytic method of reduction. Reduction with ω-toluenethiol alone, using the procedure of Maclaren (1962), gave a product with 725 μmoles/g thiol groups and 50 μmoles/g disulfide groups. Finally, the amino acid analysis of electrolytically reduced wool, after alkylation with iodoacetate and acid hydrolysis, showed that no changes had occurred to amino acids other than cystine. The cystine, *S*-carboxymethylcysteine, and cysteic acid contents were 132, 595, and 18 μmoles/g, respectively, showing that recovery of sulfur amino acids was virtually complete. The large amounts of cystine present in the hydrolysate may result from the breakdown of *S*-carboxymethylcysteine (Crestfield *et al.*, 1963).

The reason for the discrepancy remains unknown but the most likely possibility seems to be the loss of thiol groups by rapid reactions with other (nonsulfur) side chains in the wool. One would have to postulate that such reaction is peculiar to those disulfide bonds which are the most difficult to reduce and that the products break down on acid hydrolysis to give back cystine and the other (unchanged) amino acid.

Conclusions. Electrolytic reduction of peptides and proteins containing disulfide groups may be conveniently carried out with the potentiostat. Most proteins will probably require the use of thiols such as 2-mercaptoethanol, 2-mercaptoethylamine, or ω-toluenethiol to "catalyze" the reduction. However this should present no problems even in conformation studies, since the thiol need be present only in low concentrations and the reduction proceeds smoothly to completion without the need for high concentrations of denaturants. By choosing appropriate thiols and/or cathode potentials, it appears possible to reduce disulfide bonds selectively.

It may be assumed that the "catalyzed" electrolytic reduction of proteins proceeds in two steps, the first being the reduction of protein disulfide bonds by the thiol catalyst and the second being the electrolytic reduction of the disulfide form of the catalyst. Since the latter step is known to be rapid, the rate-determining step in the "catalytic" reaction appears to be the first. That is, with the comparatively low concentrations of thiol used, reduction of the protein disulfide bonds is slow.

One of the implications of the results which have been described is that virtually *all* disulfide bonds in these proteins are "available" for reduction. If any disulfide bonds can be described as truly "inaccessible," they must be very few in number. The limiting factor in chemical reductions is their reactivity toward the reducing agents which are at present available. The efficacy of denaturants such as urea, formic acid, and detergents in promoting chemical reductions is probably their effect on protein conformation. All of these substances are known to favor the random-coil conformation. Where this conformation is not possible owing to disulfide bond constraints, the cross-links must be under a state of stress. Disulfide bonds which are subjected to such stresses will have effectively lower bond strengths (and less negative reduction potentials) than in the unstressed state, and the activation energy required for rupture will be correspondingly smaller.

Acknowledgments

The authors wish to thank Mr. I. J. O'Donnell for help in the early stages of this work, Mr. A. S. Inglis for sulfur and amino acid analyses, Mr. L. Dowling for lanthionine analyses, Dr. R. Zimmerman for mercury analyses, Dr. W. E. Savige for gifts of cystine peptides, and the Armour Laboratories for a gift of crystalline ribonuclease.

References

Bailey, J. L. (1957), *Biochem. J. 67*, 21 P.

Crestfield, A. M., Moore, S., and Stein, W. H. (1963), *J. Biol. Chem. 238*, 622.

Crewther, W. G., Fraser, R. D. B., Lennox, F. G., and Lindley, H. (1964), *Advan. Protein Chem. 19* (in press).

Jensen, E. V. (1959), *Science 130*, 1319.

Leach, S. J. (1960), *Australian J. Chem. 13*, 520.

Leach, S. J. (1964), Laboratory Manual of Analytical Methods in Protein Chemistry, Vol. IV, P. Alexander and H. Lundgren, Oxford, Pergamon (in press).

Leach, S. J., and O'Donnell, I. J. (1961), *Biochem. J. 79*, 287.

Leach, S. J., and Swan, J. M. (1962), *Australian J. Chem. 15*, 365.

Leach, S. J., Swan, J. M., and Holt, L. A. (1963), *Biochim. Biophys. Acta 78*, 196.

Maclaren, J. A. (1962), *Australian J. Chem. 15*, 824.

Maclaren, J. A., Leach, S. J., and O'Donnell, I. J. (1959), *Biochim. Biophys. Acta 35*, 280.

Markus, G. (1960), *Federation Proc. 19*, 340.

Meites, L. (1961), *Record Chem. Progr., Kresge-Hooker Sci. Lib. 22*, 81.

Spackman, D. H., Stein, W. H., and Moore, S. (1960), *J. Biol. Chem. 235*, 648.

Stricks, W., Kolthoff, I. M., and Tanaka, N. (1954), *Anal. Chem. 26*, 299.

Swan, J. M. (1957), *Nature 180*, 643.

Thompson, E. O. P., and O'Donnell, I. J. (1961), *Biochim. Biophys. Acta 53*, 447.

Wood, K. I. (1964), *Anal. Chem. 36* (in press).