

---

# Investigations of Ascorbate for Direct Labeling of Antibodies with Technetium-99m

D.J. Hnatowich, F. Virzi, P. Winnard, Jr., M. Fogarasi and M. Rusckowski

*Department of Nuclear Medicine, University of Massachusetts Medical Center, Worcester, Massachusetts*

---

Recently, a method for the direct labeling of antibodies with  $^{99m}\text{Tc}$  was described in which sulfhydryls were reportedly generated by reduction of antibody disulfides with ascorbic acid. Thereafter, these proteins may be labeled at high efficiency with  $^{99m}\text{Tc}$  following reduction of pertechnetate with dithionite. This investigation was initially conducted to evaluate the mechanism of the increased stability towards cysteine challenge reported for the label and subsequently to determine the role of ascorbate in the labeling process. **Methods:** It was possible to reproduce the reported high labeling efficiencies by increasing the dithionite concentration fivefold, presumably because of variabilities among lots of commercial sodium dithionite. **Results:** Despite success in labeling, it was not possible to confirm that antibody reduction followed the treatment with ascorbate. Using both Ellman's reagent and 2,2' dithiodipyridine as indicators, we were unable to detect sulfhydryls on one IgG antibody treated at ten times the suggested ascorbate-to-antibody molar ratio. It was estimated that the number of sulfhydryls generated could not have been more than 1% (dithiodipyridine) to 2% (Ellman's). Furthermore, radiolabeling efficiencies for two IgG antibodies and stabilities of the label to cysteine challenge were unchanged when the ascorbate was eliminated. The number of sulfhydryls generated by treatment of the antibody with dithionite at 1–2 times the concentration required for adequate labeling was about 1% (dithiodipyridine) to 5% (Ellman's). **Conclusion:** For the conditions of this investigation and for the antibodies employed, ascorbate apparently played no more than a minor role at best in the labeling process. If antibody reduction occurred, this most likely was a result of residual dithionite presented to the protein along with the reduced  $^{99m}\text{Tc}$ .

**Key Words:** Technetium-99m; antibodies; ascorbate

**J Nucl Med 1994; 35:127–134**

---

**B**ecause of its attractive properties,  $^{99m}\text{Tc}$  is often considered the label of choice for certain imaging studies with radiolabeled antibodies. Until recently, however, the existing methods for labeling were thought to be unsatisfactory because of label instabilities (1,2). In the past several years, a respectable number of new labeling methods have

been reported which, to judge by their popularity, provide a label suitable for in vivo use (3,4). In the majority of cases, these labeling methods do not involve exogenous chelators for binding but are thought to rely upon endogenous sulfhydryl groups generated by antibody reduction to provide the binding sites (5). Reducing agents that have been used include stannous ion (6), 2-mercaptoethanol (7) and dithiothreitol (8) among others. In addition to the use of a reducing agent for antibody reduction, these "direct" labeling methods often employ a weak complexing agent such as gluconate or methylenediphosphonate (9) which may serve to stabilize reduced  $^{99m}\text{Tc}$  for presentation to the reduced antibody.

In a comparison of five reducing agents, Thakur et al. have selected ascorbic acid as a preferred agent for antibody reduction in connection with direct labeling with  $^{99m}\text{Tc}$  (10). In the described method,  $^{99m}\text{Tc}$ -pertechnetate is reduced over 3–5 min with a freshly prepared and nitrogen-purged solution of sodium dithionite ( $\text{Na}_2\text{S}_2\text{O}_4$ ) at pH 11 and at a final concentration of about 0.7 mg/ml. This solution is then added to an antibody which had previously been exposed to a 3500:1 molar excess of ascorbate at pH 6.5 for 1 hr at room temperature such that the final sodium dithionite concentration is about 0.4 mg/ml. In a separate study, these investigators reported that no more than about 8% of the available disulfide bridges are reduced under these conditions based on 70 sulfhydryls per IgG molecule (11) or approximately 16% based on 36 sulfhydryls (12,13). It has been suggested that antibody fragmentation may be less likely because of the small number of disulfides reduced through this approach (11). Labeling efficiency was reported to exceed 95% and only about 20% of the label is lost by transchelation to cysteine at a 1000:1 molar excess during a 1-hr incubation at 37°C. Instability of the label to transchelation to cysteine has been determined to be one of the principal modes of in vitro and in vivo instability of  $^{99m}\text{Tc}$ -labeled antibodies (14,15).

An important advantage of the ascorbate/dithionite labeling method is that postlabeling purification is not required since labeling efficiencies exceed 95% and since the antibody reducing agent, ascorbate, is a natural vitamin and therefore nontoxic. A disadvantage may be the use of dithionite for pertechnetate reduction since dithionite is extremely sensitive to air oxidation (16). However, the potential advantage which was of most interest to this

---

Received Jan. 26, 1993; revision accepted Sept. 29, 1993.

For correspondence or reprints contact: D.J. Hnatowich, PhD, Department of Nuclear Medicine, University of Massachusetts Medical Center, Worcester, MA 01655.

laboratory was the increased stability reported for the label in the presence of cysteine. Accordingly, this investigation was conducted originally to evaluate the mechanism of this increased stability and subsequently to determine the role of ascorbate in the labeling process. As is shown below, for one IgG antibody, ascorbate did not reduce disulfides at detectable levels, but evidence for disulfide reduction was observed following treatment with dithionite. As expected, antibody labeling was observed only in the presence of dithionite, however, identical labeling efficiencies (and stabilities of the label to cysteine challenge) were achieved following the elimination of ascorbate. It is possible that if antibody reduction occurred in this investigation, it was achieved via dithionite rather than ascorbate, with the latter apparently playing no role in antibody labeling.

## MATERIALS AND METHODS

All reagents used in this investigation, L-cysteine HCl, sodium L-ascorbate, sodium tartrate, sodium phthalate, 2,2'-dithiodipyridine (Sigma Chemical Co., St. Louis, MO), L-ascorbic acid (ICN Biochemicals, Cleveland, OH), Ellman's Reagent (Pierce Chemical Co., Rockford, IL) and Sandoglobulin IgG (Sandoz Pharmaceuticals, East Hanover, NJ) were used without purification. The B72.3 IgG antibody was a gift from Cytogen Corp. (Princeton, NJ). Technetium-99m-pertechnetate was obtained from a <sup>99m</sup>Tc generator (NEN Dupont, N. Billerica, MA).

Sodium dithionite was purchased under nitrogen (Aldrich Chemical Co., Milwaukee, WI) for this investigation and was stored in a desiccator at -20°C. This reagent was dispensed within a nitrogen-filled glove bag into small tared vials which were crimp-sealed for storage at -20°C. Solutions of dithionite were prepared by injecting into each vial the correct volume of nitrogen-purged 0.05 M carbonate buffer, pH 11.5, to provide a 10- or 50-mg/ml solution of sodium dithionite. The solutions were never exposed to air and were always used within 10 min of solubilization. Because of its tendency towards air oxidation, dithionite must be carefully stored and used (16).

Solutions of ascorbic acid (20 mg/ml, 0.11 M) and sodium ascorbate (20 mg/ml, 0.10 M) were freshly prepared in distilled, deionized water and the pH of the ascorbate solution (originally 7.3) was adjusted to 6.5 with small volumes of the ascorbic acid solution. Because the pKa of ascorbic acid is 4.0 (17), it was not practical to adjust the pH of the ascorbic acid solution (originally around 2.2) to 6.5 with the ascorbate solution. The pH-adjusted solution of sodium ascorbate was added to the antibody within 2 min of the ascorbate solubilization. Although less sensitive to air oxidation than dithionite, ascorbate in solution will auto-oxidize (17).

### Antibody Radiolabelings

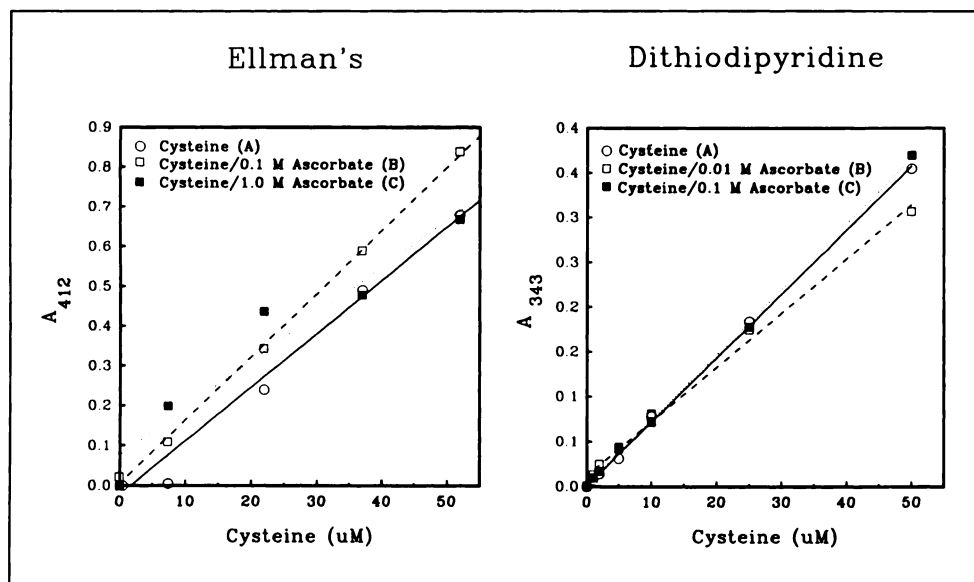
Both B72.3 and Sandoglobulin IgG were radiolabeled with <sup>99m</sup>Tc using mercaptoethanol and tin as reducing agents as previously reported (14,15). For mercaptoethanol, the antibodies were first reduced for 30 min at a 1000:1 mercaptoethanol-to-antibody molar ratio and then purified over Sephadex G50 using as eluant nitrogen-purged 0.2 M PBS, pH 6.0, containing 0.1 to 1.0 mM mercaptoethanol to prevent disulfide reformation. The fractions containing protein were pooled. The reduced antibodies were radiolabeled with <sup>99m</sup>Tc-glucoheptonate (Glucoscan, NEN Dupont) and the preparation was purified over a Sephadex G50 column. Following purification, radiochemical purities were gen-

erally 98% or better as determined by size-exclusion HPLC using a Superose 12 column (Pharmacia, Piscataway, NJ). In the case of tin reduction, a 0.005 M solution of stannous ion in 0.01 M tartrate and 0.04 M phthalate was degassed by boiling under nitrogen and adjusted with NaOH to pH 5.6. The antibody and stannous tartrate-phthalate solutions were mixed such that the weight ratio to antibody of SnCl<sub>2</sub>·2H<sub>2</sub>O was 0.71 and the solution was allowed to incubate overnight at room temperature and under nitrogen. Radiolabeling was accomplished by adding <sup>99m</sup>Tc-pertechnetate solution and purifying the antibody over a Sephadex A25 anion-exchange column (Aldrich). Radiochemical purities were generally in excess of 90%.

Both B72.3 and Sandoglobulin IgG antibodies were also radiolabeled with <sup>99m</sup>Tc, with and without ascorbate treatment. Labelings were performed at the suggested sodium dithionite concentration (approximately 0.7 mg/ml during pertechnetate reduction and 0.4 mg/ml during protein labeling) and at approximately five times this concentration. The effect of increasing the dithionite concentration was to increase the labeling efficiency from approximately 20% to greater than 90%. Both antibodies were first prepared in saline at concentrations of 5.5 to 30 mg/ml. One hour prior to antibody labeling, 26 μl of a 0.10 M ascorbate, pH 6.5 solution was added to one-half the protein solution while the same volume of saline was added to the remainder so that the final protein concentration was 2.3 mg/ml in 50 μl. For ascorbate samples, the molar ratio of ascorbate-to-protein was 3500:1. Three minutes before the addition of radioactivity to these solutions, 125 μl (~2 mCi) of <sup>99m</sup>Tc-pertechnetate solution (eluted not more than 2 hr previously) was mixed with 8 μl of 10 or 50 mg/ml solution of sodium dithionite freshly prepared in nitrogen-purged 0.05 M carbonate buffer, pH 11.5. The final pH was 11.0 and the final concentration of sodium dithionite was 0.6 or 3 mg/ml during <sup>99m</sup>Tc reduction and 0.4 to 2 mg/ml in the antibody solutions. After the addition of the radioactivity, the samples were allowed to incubate at room temperature for an additional hour before analysis. Labeling efficiency was determined by Sephadex G50 column chromatography performed simultaneously on identical 1.5 × 15-cm columns eluted with saline and by measuring the radioactivity retention following centrifugation (Ultrafree MC, Millipore Corp., Boston, MA). Aliquots of each labeled protein solution were also subjected to cysteine challenge after purification by two centrifugations.

Sandoglobulin IgG antibody was used to perform a number of radiolabelings under different conditions to investigate further the importance of ascorbate, dithionite, etc. on this technique. For each preparation, strip chromatography was performed with Whatman no. 1 paper (VWR Scientific, Boston, MA) using both 100% acetone eluant to measure the percentage of label migrating and therefore present as pertechnetate, and with normal saline eluant to measure soluble forms of label other than labeled antibody, which, along with radiocolloids, will remain at or near the origin. After development, each 1 × 8-cm strip was cut in half and each half placed separately in a dose calibrator to measure its radioactivity. Labeling efficiency was determined by Sephadex G50 chromatography as the fraction of applied radioactivity appearing in the void volume after passage through 1.5 × 15-cm columns.

In the first set of experiments, the labeling procedure was identical to that described above: <sup>99m</sup>Tc-pertechnetate was reduced for 3 min with dithionite; the antibody had been added to ascorbate at pH 6.5 1 hr earlier. As controls, solutions were also prepared in which saline replaced either the ascorbate solution or



**FIGURE 1.** Curves showing the relationship of cysteine concentration and absorbance determined in this investigation with Ellman's reagent (left) and dithiodipyridine (right). Each graph presents results for ascorbate-free cysteine solutions (curves A) and solutions of cysteine in the presence of ascorbate (curves B and C).

the antibody solution. The final dithionite concentration was always 0.4 mg/ml. After mixing, each solution was incubated for 30 min and then analyzed by G50 and paper chromatography. Solutions without antibody were not analyzed by G50 chromatography.

In the second set of experiments, solutions of antibody with ascorbate were allowed to incubate for either 5 min or 1 hr before the addition of the  $^{99m}\text{Tc}$ -pertechnetate. Immediately thereafter, dithionite was added to the same final concentration as before. In half the samples, the ascorbate-to-antibody molar ratio was 3,500:1, whereas it was 35,000:1 for the remainder. As a control, the antibody solution was replaced with saline in several samples. The solutions were left undisturbed for 30 min before analysis as above.

### Cysteine Challenge

The cysteine challenge assay involved incubating aliquots of labeled antibody in 0, 0.64, 16, 32, 48 and 64 mM solutions of cysteine in saline at room temperature. A fresh 640-mM solution of l-cysteine HCl in 2 M phosphate buffer, pH 7, was successively diluted with the buffer to prepare solutions at the desired molarities. To 10  $\mu\text{l}$  of each cysteine solution was added 90  $\mu\text{l}$  of each labeled antibody solution so that the cysteine-to-antibody molar ratio was a maximum of 30,000:1. Each solution was incubated at room temperature for 1 hr before analysis on 2.5  $\times$  20-cm strips of Whatman no. 1 paper using 0.1 M PBS, pH 7, as an eluant. After development and drying, several of the strips were scanned on a radiochromatographic scanner. The results showed that each strip should be cut at between one-third and one-half the distance between the origin and solvent front. In this system, labeled antibody remains at or near the origin; labeled cysteine migrates with an Rf of about 0.8–1.0. Strips were therefore cut in half and each half was counted separately in a dose calibrator.

## RESULTS

### Determination of Sulfhydryl Concentrations

By plotting the absorbance for solutions of free cysteine, after subtraction of the background due to Ellman's reagent, a straight line relationship passing through the origin was obtained for concentrations of cysteine above about 22

$\mu\text{M}$  (equivalent to an absorbance of about 0.24 (s.d., 0.003;  $n = 5$ )) absorbance units. As shown in Figure 1, left (curve A), absorbance was no longer linear with decreasing cysteine concentrations. In the linear range, the error in the quantitation of cysteine solutions of known concentrations averaged 8%. The assay was applied to samples of Sando globulin IgG treated with solutions of ascorbate at five concentrations in the range 0.1 to 1 M (before dilution with the antibody solution). The final ascorbate-to-antibody molar ratios were 3,500 to 35,000:1. Each solution was purified by G50 chromatography prior to analysis. The absorbance values ranged from 0.012 (s.d., 0.004;  $n = 5$ ) and 0.027 (s.d., 0.003;  $n = 5$ ). As these values are below the upper limit of quantitation of 0.24 absorbance units for this analysis (Fig. 1), a concentration of cysteine at the upper limit of the assay (22  $\mu\text{M}$ ) was assumed in the following calculation. For the fractions of G50 with the highest concentration of protein (about 1.3 mg/ml after dilution), the upper limit on the number of cysteine groups generated at a 35,000:1 ascorbate-to-antibody molar ratio was calculated to be 1.7 per antibody molecule and therefore less than 7% of the 36 sulfhydryl groups possible for a typical IgG antibody (12,13). Presumably, fewer sulfhydryls would be generated at lower molar ratios. Thakur and DeFulvio report about 8% at a 17,500:1 ascorbic acid-to-antibody molar ratio based on 70 possible cysteine residues (11) or 16% based on half that number, as in the above calculation.

A similar assay was performed for samples not purified from ascorbate prior to analysis because of concerns that purification may have contributed in the above assay to disulfide reformation and therefore an erroneous low value for sulfhydryl generation. As shown in Figure 1, left (curve B), at a starting ascorbate concentration of 0.1 M, a linear relationship passing through the origin was obtained for free cysteine concentrations in the range 7.4 to 52  $\mu\text{M}$  after subtraction of the contribution due to ascorbate and Ell-

man's reagent. Also as shown in the figure (curve C), a linear relationship with a different slope was obtained at a starting ascorbate concentration of 1.0 M, which in this case did not pass through the origin. Samples of Sandoglobulin IgG were assayed at five starting ascorbate concentrations between 0.1 and 1.0 M. The corrected absorbances fell between 0.021 (s.d., 0.011; n = 5) and zero for all five concentrations. By using the upper limit for this assay of 7.4  $\mu$ M cysteine and at the protein concentration of 2.0 mg/ml, it may be calculated that a maximum of 0.6 sulfhydryls were generated under these conditions. This represents less than 2% of the 36 possible sulfhydryls.

A similar analysis was applied to protein samples exposed to dithionite. Sandoglobulin IgG antibody was treated at final concentrations of dithionite of 0, 0.4 and 2.2 mg/ml, the latter being five times higher than that recommended (10,11). After correcting for the background due to Ellman's reagent, the absorbance was determined to be 0.06 (0.003, s.d.; n = 5), 0.10 (0.004, s.d.; n = 5) and 0.23 (0.007, s.d.; n = 5) for the three solutions, respectively. From Figure 1, left (curve A, referring to purified antibody), it is evident that 0.23 absorbance units is, within experimental error, in the linear range and therefore may be used to provide an accurate value for the sulfhydryl concentration of 20  $\mu$ M. At an IgG concentration of 1.9 mg/ml, this is equivalent to 1.6 cysteine groups per IgG molecule or 5%.

Figure 1, right presents the calibration curve for the determination of cysteine concentration with dithiodipyridine over the range of 0 to 50  $\mu$ M as determined by the measurement of absorbance at 343 nm. As shown in the figure (curve A), absorbance was linear with cysteine concentration for ascorbate-free solutions throughout the entire range. When corrected for the absorbance due to ascorbate at this frequency, the relationship is also linear and passes through the origin for cysteine concentration in 0.01 M (curve B) and 0.1 M (curve C) ascorbate. The absorbance at 343 nm for solutions of Sandoglobulin IgG after treatment with ascorbate at a 3,500 and a 35,000:1 molar ratio to antibody but without purification of the antibody from ascorbate was found to be identical within experimental error with or without the addition of the dithiodipyridine indicator. It was therefore possible to set an upper limit of 1  $\mu$ M on the concentration of sulfhydryls generated by the treatment of the antibody with ascorbate at either molar ratio. At a protein concentration of 0.45 mg/ml, this concentration translates into a maximum of 1% of 36 possible sulfhydryls.

The results were more positive after the treatment of the antibody with dithionite. The increase in absorbance following the addition of dithiodipyridine averaged 0.05 absorbance units, corresponding to 8.3  $\mu$ M (0.22  $\mu$ M s.d., n = 5) cysteine (Fig. 1, right). At an antibody concentration of 4.0 mg/ml, approximately 1% of 36 possible sulfhydryl groups were generated. The absorbance of antibody-free solutions of dithionite taken through the purification pro-

cedure were at background levels demonstrating that the procedure was removing all traces of dithionite.

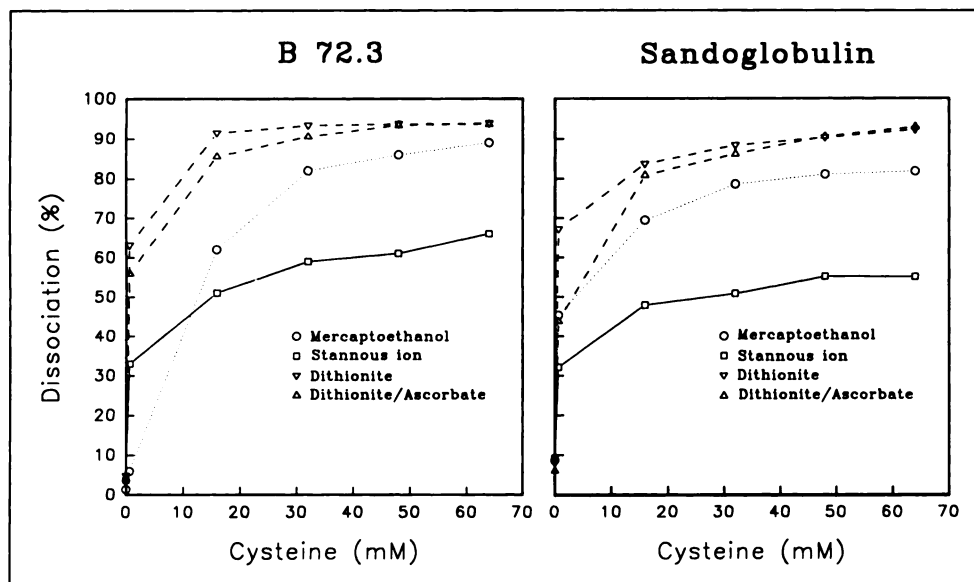
### Antibody Radiolabeling

The highest labeling efficiencies were obtained with samples containing dithionite at a final concentration of 2 mg/ml, about five times that recommended (10,11). When measured by centrifugation, the labeling efficiency was 95% (range 90–99%, n = 2) for both ascorbate-treated and untreated Sandoglobulin. By G50 chromatography, these values were 86% (5% s.d., n = 5) and 83% (4% s.d., n = 5), respectively. The results for the identical experiments on B72.3 IgG were 95% (range 92–97%, n = 2) and 93% (range 91–95%, n = 2) respectively by centrifugation and 83% (3% s.d., n = 5) and 68% (3% s.d., n = 5) by G50. For both proteins, therefore, lower values for labeling efficiency were obtained by G50 chromatography over centrifugation, suggesting that some radiocolloids may have been present in the final preparations.

A number of labeling studies were also performed to further investigate the influence of ascorbate, dithionite, timing and the order of addition, using both G50 column chromatography and paper chromatography for analysis. Regardless of the procedure, the elimination of dithionite essentially led, as expected, to quantitative migration of the label in acetone and less than about 4% of the activity eluting with protein on G50. Since ascorbate is incapable of reducing pertechnetate at the concentrations employed herein (18), the elimination of dithionite resulted in no pertechnetate reduction and therefore no antibody labeling. As before, no significant differences were observed in labeling efficiency with or without ascorbate; labeling efficiency averaged 20% (8% s.d., n = 6) with and 20% (10% s.d., n = 6) without ascorbate. About 20% of the remaining activity migrated in acetone and 60% in saline in both cases. Thus, the low labeling efficiency in these experiments was probably due in part to incomplete reduction of pertechnetate (which migrates in acetone) and possibly to incomplete reduction of the antibody with the result that the majority of the activity was present as pertechnetate and as a dithionite complex (both of which migrated on paper) incapable of transferring its label to the antibody. Eliminating the antibody increased the percentage of activity migrating in both acetone and in saline. Changing the order of addition also had little effect; labeling efficiency was 29% (11% s.d., n = 4) when the antibody was incubated with  $^{99m}$ Tc-pertechnetate and ascorbate for 5 min prior to the addition of dithionite with analysis 30 min later and 25% (11% s.d., n = 4) when the incubation time was extended to 1 hr.

### Cysteine Challenge

Figure 2 presents the results of subjection to cysteine challenge B72.3 (left) and Sandoglobulin (right), labeled both with and without ascorbate. Included in the figure are the results of identically challenging the respective antibody after labeling with mercaptoethanol and tin reduction. The shape of each curve is typical of that routinely



**FIGURE 2.** Curves showing the percentage dissociation during incubation with increasing concentrations of cysteine for  $^{99m}\text{Tc}$  bound to B72.3 IgG (left) and Sandoglobulin IgG (right). Each graph presents results for the respective antibody radiolabeled with ascorbate/dithionite, dithionite alone and radiolabeled after antibody reduction with mercaptoethanol and stannous ion.

observed in this laboratory: the change in label instability is greatest at low cysteine concentrations with little additional dissociation occurring as cysteine concentration is increased. The instability to cysteine challenge is identical with and without ascorbate for both antibodies and was greater than that observed for either mercaptoethanol or tin reduction in these experiments.

## DISCUSSION

Ascorbic acid has not been previously considered for the reduction of antibody disulfides (10). As a reducing agent, it may not be particularly strong; in a comparison of the reduction rate of nine electron acceptors under identical conditions, equal or more rapid reduction was reported with dithionite relative to ascorbate in each case (19). No evidence was found in this investigation to suggest that ascorbate was capable of reducing either of two IgG antibodies. In particular, labeling efficiencies were unchanged when the ascorbate was eliminated. This is in contrast to the results of Thakur et al. who report that antibody labeling efficiencies decrease to about 20% in the absence of ascorbate (10). Since only two IgG antibodies were studied in this work, it is possible that other antibodies are more susceptible to reduction with ascorbate.

Additional evidence from this study for the inability of ascorbate to reduce antibody disulfides resulted from the quantitation of sulfhydryls. With both Ellman's reagent and dithiodipyridine as indicators, the concentrations of sulfhydryls generated by the treatment of Sandoglobulin IgG with ascorbate were below detectable limits for both assays despite a 35,000:1 ascorbate-to-antibody molar; i.e., a 10-fold excess over that recommended (10,11). It was estimated that no more than 1% (dithiodipyridine) to 2% (Ellman's reagent) of the 32-36 possible sulfhydryls in these IgG antibodies were generated. This is in contrast to Thakur and DeFulvio who report about 16% for a human IgG antibody at an ascorbate-to-antibody molar ratio of

17,500:1 (11). Although this discrepancy may be attributed to the study of different antibodies, it is also possible that it may be due to the selection of different indicators. Thakur and DeFulvio selected ninhydrin at acidic pH as their indicator because of its large molar extinction coefficient and its potential for a more sensitive determination (11). Ninhydrin has not been previously employed for the quantitation of protein-bound sulfhydryls, having been used primarily to detect alpha-amino acids including free cysteine (20,21). Ninhydrin has also been shown to be sensitive to sialic acids in sialoglycoproteins (21), a property which may complicate its use in the determination of antibody sulfhydryls. One obvious disadvantage to the ninhydrin assay is the need to subject the protein to concentrated acetic and hydrochloric acid, absolute ethanol and a 10-min boiling step (11); procedures which may introduce artifacts.

By contrast, Ellman's reagent, although less sensitive than ninhydrin (acid) to judge by extinction coefficients (11), is used under mild conditions and is a popular indicator for the determination of free and antibody-bound sulfhydryls (8,9,22). Ascorbate interferes with Ellman's reagent, however, the assay may still be successfully performed; in this investigation sulfhydryl concentrations were determined both for antibody purified from ascorbate and in the presence of ascorbate. The results were essentially the same with both at undetectable levels. Furthermore, these measurements were repeated with dithiodipyridine as indicator. This assay has been previously employed to determine the number of sulfhydryl groups in biological materials (23). Because ascorbate does not interfere in this assay, sulfhydryl concentrations in antibody solutions could be measured with good sensitivity. However, the results were the same as that obtained with Ellman's reagent, i.e., concentrations of sulfhydryls below detectable limits.

In this investigation, it was possible to reproduce the

high antibody labeling efficiencies reported by Thakur et al. for the ascorbate/dithionite method (10,11). To do so, however, necessitated increasing the concentration of dithionite fivefold. Thakur and DeFulvio have also reported that the concentration of dithionite required for efficient antibody labeling varied from about 0.7 to 5 mg/ml, depending on the commercial source of sodium dithionite (24). These observations illustrate a significant difficulty with dithionite, namely its strong oxygen-scavenging properties (16). Thus, different lots and, indeed, different samples of each lot may contain different amounts of the reducing agent.

Commercially obtained samples have been reported to vary in dithionite concentrations from 52% to 84% (26). In addition, samples of dithionite have been shown to be contaminated with sulfite (25), also a reducing agent but with different properties. When IgG antibodies were treated with sulfite at pH 6, the interchain disulfides were preferentially reduced (13). Dithionite has been previously used for the reduction of  $^{99m}\text{Tc}$ -pertechnetate (26–30). Adequate reduction was achieved at pH 7 (29,30), pH 9.8 (28) and pH 11.0 (30), however, increased concentrations of  $^{99m}\text{Tc}$ -colloids were observed at pH values of 11 or below if the dithionite-to- $^{99m}\text{Tc}$  concentration was too low (30).

To minimize air oxidation in this investigation, dithionite was handled only in a nitrogen atmosphere and solubilized only with nitrogen-purged buffer. Added precautions include purging the solution of pertechnetate with nitrogen prior to the addition of dithionite (30), a precaution not taken in this study nor in Thakur et al.'s (10,11). Difficulties in the use of dithionite may also have been responsible for variations in the stability of both the B72.3 and Sandoglobulin IgG to cysteine following labeling via dithionite. Although labeling efficiency was unchanged, the stability of both labeled antibodies to cysteine challenge was occasionally improved about a factor of two over that presented in Figure 2 for no apparent reason. Thakur et al. report a 20% loss of label at a cysteine-to-antibody molar ratio of 1000:1 after 1 hr at 37°C (10), which is considerably less than that observed in this work of about 60% at a 300:1 molar ratio after 1 hr at room temperature. This difference may therefore be the result of variability in the dithionite used.

In this investigation, antibody labeling was only achieved in the presence of dithionite. This is not surprising since only dithionite and not ascorbate was capable of reducing  $^{99m}\text{Tc}$ -pertechnetate; an essential step for antibody labeling. Our observation that cysteine residues are generated upon treatment of one IgG antibody with dithionite, albeit at a 1–2-fold higher concentration than that recommended, suggests that the role of dithionite may include antibody reduction. The report of Lees et al. may be particularly relevant here since dithionite was added in that study to reduce  $^{99m}\text{Tc}$ -pertechnetate in the presence of the lipoprotein LDL, with the result that one-third to one-half of the  $^{99m}\text{Tc}$  was protein bound in 10 min at room temperature (26). The mechanism of protein labeling was not established.

Two critical observations from this work were the appearance of sulfhydryls when Sandoglobulin IgG was treated with dithionite but not ascorbate, and the identical labeling efficiencies (and stabilities of the label to cysteine challenge) in the presence or absence of ascorbate. Under different ascorbate concentrations, order of addition and length of incubation times, no significant differences were observed in labeling efficiency with and without ascorbate. This is in contrast to the observations of Thakur et al. who report a decrease from 95% to 20% in labeling efficiency with the omission of ascorbate (10). It is therefore possible that ascorbate played at best a limited role in antibody reduction in this investigation. If antibody reduction did occur, it may have been accomplished by residual dithionite remaining after pertechnetate reduction. The stabilities were similar to that achievable by other direct labeling methods.

In conclusion, for the two antibodies investigated, ascorbate was found to play at best a minor role in the labeling rather than the expected essential role as a reducing agent of antibody disulfides. If antibody reduction was occurring, we believe it may have been due to residual dithionite. The label was found to be unremarkable in its stability to cysteine. Because of the difficulties in using dithionite as a reducing agent, there appears to be few advantages to the ascorbic acid/dithionite approach to labeling these antibodies relative to alternative direct labeling methods.

## APPENDIX

### Determination of Sulfhydryl Concentrations

The quantitation of sulfhydryl concentrations was accomplished both with Ellman's reagent (5,5'-dithio bis-(2-nitrobenzoic acid)), in which absorbance was measured at 412 nm (31), and with 2,2' dithiodipyridine with absorbance measured at 343 nm (23). Ellman's reagent has been used extensively to measure sulfhydryl concentrations in solutions of reduced antibodies (8,9). For analysis by Ellman's reagent, the protein solution was combined with an equal volume of a 0.3 mg/ml solution of the reagent, freshly prepared in 0.1 M sodium bicarbonate solution pH 8.0. Absorbance was measured using a recording UV spectrophotometer (U-2000, Hitachi Instruments, Inc., Danbury, CT). Because ascorbate itself will reduce Ellman's reagent to provide a background absorbance at 412 nm and because of concerns that if purified from ascorbate, antibody disulfides may reform before the analysis could be completed, the determination of antibody sulfhydryls was performed with and without purification of the antibody before analysis.

The procedure with purification was as follows: a stock solution of 1 M ascorbate was prepared at pH 6.5 and immediately diluted to prepare solutions at final concentrations of 0.1, 0.25, 0.50, 0.75 M, as well as 1.0 M. Each solution was readjusted to pH 6.5, a process requiring no more than about 2 min. Without delay, 1.1 ml of each solution was added to separate test tubes, each containing 5.0 mg of Sandoglobulin IgG in 0.17 ml of saline. Thus the ascorbate-to-antibody molar ratio varied from 3500 to 35,000:1. After incubation at room temperature for one hour, all solutions were simultaneously purified through individual 1.2 × 18-cm columns of Sephadex G50 (Sigma). One milliliter fractions were collected from each column using as eluant a solution of 0.1



M NaCl, 0.01 M EDTA, 0.05 M sodium phosphate, pH 6.5. EDTA was included to complex trace metals in the eluant which may interfere with the determinations by accelerating the rate of reoxidation (8). The protein concentrations determined for each fraction by absorbance at 280 nm were found to range between 2.5–3.9 mg/ml in the peak fractions (absorption coefficient 1.2). Immediately after purification, 0.2 ml of each peak fraction was combined with an equal volume of Ellman's reagent freshly prepared at 0.3 mg/ml in 0.1 M sodium bicarbonate, pH 8.0. Protein-free solutions were also analyzed as controls. After 15 min at room temperature, the absorbance of each sample was measured at 412 nm and the results applied to the standards curve.

The procedure without purification of ascorbate from the reduced protein was as follows. The IgG antibody was incubated with ascorbate at the same concentrations and for the same time as described above. Without purification, each solution was combined with an equal volume of 0.3 mg/ml Ellman's reagent freshly prepared in 0.1 M sodium acetate, pH 6.0 (rather than pH 8.0 as before to reduce the background absorbance as Ellman's reagent is slowly reduced by ascorbate under these conditions). In each case, the absorbance was read after  $5.0 \pm 0.5$  min. The standards consisted of ascorbate solutions at 0.1 and 1.0 M, either alone or in the presence of free cysteine at 7.4 to 52  $\mu$ M.

Ellman's reagent was also used to quantitate the concentration of sulfhydryls following antibody reduction with dithionite. A solution of Sandoglobulin IgG was prepared in 0.1 M phosphate buffer, pH 6.8, at a concentration of 3.8 mg/ml. To 1.3 ml of this solution was added 50  $\mu$ l of a nitrogen-purged 0.05 M carbonate buffer, pH 11.5, containing either 0.0, 0.5 mg or 5.0 mg of sodium dithionite. The concentration of sodium dithionite in the protein solutions was 0, 0.4 and 4 mg/ml. After 30 min of incubation at room temperature, each solution was dialyzed overnight against 3.5 liters of 0.1 M NaCl, 1 mM EDTA and 0.05 M phosphate buffer, pH 6.5. Dialysis was found to be necessary in this case because of the rapid reduction of Ellman's reagent by even small traces of dithionite. After the concentration of protein was determined by the measurement of absorbance at 280 nm, Ellman's reagent was used as above to quantitate the sulfhydryl concentration of each dialyzed sample: to each solution was added an equal volume of 0.3 mg/ml Ellman's reagent in 0.1 M bicarbonate, pH 8.0. The absorbance of each sample at 412 nm was measured after 15 min.

In addition to Ellman's reagent, 2,2' dithiodipyridine was also used to quantitate sulfhydryls. Dithiodipyridine has absorbance maxima at 233 and 281 nm. Upon reduction with sulfhydryls, 2-thiopyridone is formed with absorbance maxima at 271 and 343 nm (19). Since proteins absorb strongly at 280 nm, the 343-nm peak was monitored. Even though dithiodipyridine and Ellman's reagent have similar extinction coefficients for sulfhydryls (18,19), the former indicator is less sensitive to interference by ascorbate to judge by the superior results obtained. It was not necessary, for example, to purify antibody samples from ascorbate prior to analysis with dithiodipyridine. This was not the case with dithionite, which readily reduces dithiodipyridine and consequently had to be removed by dialysis from the antibody solution before analysis.

Standard solutions of cysteine at concentrations between 1.0 and 50  $\mu$ M were freshly prepared in both 0.01 and 0.1 M solutions of ascorbate in distilled water. To 1 ml of each solution was added 16  $\mu$ l of a 1 mg/ml solution of dithiodipyridine freshly prepared in distilled, deionized water. The absorbance was measured after 5 min at room temperature. The absorbance of 0.01 M and 0.1 M

ascorbate solutions were also determined in an identical manner and the result subtracted from that of the appropriate cysteine solution.

For the determination of antibody sulfhydryl concentrations, 0.1 and 1.0 M ascorbate solutions were prepared in distilled water and added to a solution of Sandoglobulin IgG as described earlier for analysis with Ellman's reagent. After incubation at room temperature for 1–1.5 hr, 115  $\mu$ l of each solution was diluted to 1.0 ml (to reduce the absorbance blank of ascorbate), providing solutions 0.01 and 0.1 M in ascorbate. To 300  $\mu$ l of each solution was added 16  $\mu$ l of the 1 mg/ml solution of dithiodipyridine and the absorbance was determined immediately at 343 nm.

The influence of dithionite on antibody disulfides was likewise determined with dithiodipyridine. A solution of Sandoglobulin IgG was prepared at a concentration of 29 mg/ml in saline. To 0.17 ml of this solution was added 20  $\mu$ l of a nitrogen-purged 0.05 M carbonate buffer, pH 11.5, containing either 0.0 or 0.38 mg of sodium dithionite. The concentration of sodium dithionite in the protein solutions was therefore 0 or 2.2 mg/ml. After 1 hr of incubation at room temperature, each solution was purified by centrifugation using a 30,000 Dalton cut-off filter (Ultrafree). An antibody-free solution of dithionite was also filtered as a control. Each sample was reconstituted in 200  $\mu$ l of 0.1 M NaCl, 1 mM EDTA and 0.05 M phosphate buffer, pH 6.5, and its concentration determined by absorbance at 280 nm. To measure the sulfhydryl concentration, 150  $\mu$ l of each solution was diluted 1:2 in distilled water and 16  $\mu$ l of a 1 mg/ml solution of dithiodipyridine added. The absorbance of each sample was measured at 343 nm after 5 min.

## ACKNOWLEDGMENTS

The authors are grateful to their colleagues at Cytogen Corp. for providing the B72.3 antibody for this investigation. This work was supported in part by NIH grant AI25570.

## REFERENCES

1. Zimmer AM, Kazikiewicz JM, Rosen ST, et al. Pharmacokinetics of  $^{99m}\text{Tc}(\text{Sn})$ - and  $^{131}\text{I}$ -labeled anti-carcinoembryonic antigen monoclonal antibody fragments in nude mice. *Cancer Res* 1987;47:1691–1694.
2. Arano Y, Yokoyama A, Furukawa T, et al. Technetium-99m labeled monoclonal antibody with preserved immunoreactivity and high in vivo stability. *J Nucl Med* 1987;28:1027–1033.
3. Granowska M, Mather SJ, Britton KE, et al. Technetium-99m radioimmunoscinigraphy of colon cancer. *Br J Cancer* 1990;62:30–33.
4. Baum RP, Hertel A, Lorenz M, et al. Technetium-99m-labelled anti-CEA monoclonal antibody for tumor immunoscintigraphy: first clinical results. *Nucl Med Commun* 1989;10:345–352.
5. Rhodes BA. Direct labeling of proteins with  $^{99m}\text{Tc}$ . *Nucl Med Biol* 1991; 18:667–676.
6. Hawkins EB, Pant KD, Rhodes BA. Resistance of direct Tc-99m-protein bond to transchelation. *Antibod Immunol Radiopharm* 1990;3:17–25.
7. Schwarz A, Steinstrasser A. A novel approach to Tc-99m labeled monoclonal antibodies [Abstract]. *J Nucl Med* 1987;28:721.
8. Pak KY, Nedelman MA, Tam SH, et al. Labeling and stability of radiolabeled antibody fragments by a direct  $^{99m}\text{Tc}$ -labeling method. *Nucl Med Biol* 1992;19:669–677.
9. Mather SJ, Ellison D. Reduction-mediated technetium-99m labeling of monoclonal antibodies. *J Nucl Med* 1990;31:692–697.
10. Thakur ML, DeFulvio J, Richard MD, et al. Technetium-99m-labeled monoclonal antibodies: evaluation of reducing agents. *Nucl Med Biol* 1991; 18:227–233.
11. Thakur ML, DeFulvio JD. Determination of reduced disulfide groups in monoclonal antibodies. *Biotechniques* 1990;8:512–516.
12. Turner MW. Structure and function of immunoglobulins. In: Glynn LE, Steward MW, ed. *Immunochemistry*. London: Wiley; 1977:1–57.
13. Cecil R, Stevenson GT. The disulfide bonds of human and rabbit gamma-globulins. *Biochem J* 1965;97:569–572.
14. Mardirossian G, Wu C, Rusckowski M, et al. The stability of  $^{99m}\text{Tc}$  directly

- labelled to an Fab' antibody via stannous ion and mercaptoethanol reduction. *Nucl Med Commun* 1992;13:503-512.
15. Hnatowich DJ, Mardirossian G, Rusckowski M, et al. Pharmacokinetics of the FO23C5 antibody fragment labelled with <sup>99m</sup>Tc and <sup>111</sup>In: a comparison in patients. *Nucl Med Commun* 1993;14:52-63.
  16. Rinker RG, Gordon TP, Mason DM, et al. Kinetics and mechanism of the air oxidation of the dithionite ion (S<sub>2</sub>O<sub>4</sub>) in aqueous solution. *J Phys Chem* 1960;64:573-581.
  17. Taqui Kahn MM, Martell AE. Kinetics of metal ion and metal chelate catalyzed oxidation of ascorbic acid. IV. Uranyl ion catalyzed oxidation. *J Am Chem Soc* 1972;94:9062-9073.
  18. Stern HS, Zolle I, McAfee JG. Preparation of technetium (Tc<sup>99m</sup>)-labeled serum albumin (human). *Int J Appl Rad Isotope* 1965;16:283-288.
  19. Dixon M. The acceptor specificity of flavins and flavoproteins II. Free flavins. *Biochim Biophys Acta* 1971;226:259-268.
  20. Friedman M, Siegel CW. A kinetic study of the ninhydrin reaction. *Biochemistry* 1966;5:478-485.
  21. Yao K, Ubuka T, Masuoka N, et al. Direct determination of bound sialic acids in sialoglycoproteins by acidic ninhydrin reaction. *Anal Biochem* 1989;179:332-335.
  22. Habeeb AFSA. Reaction of protein sulfhydryl groups with Ellman's reagent. *Meth Enzymol* 1972;25:457-464.
  23. Grassetti DR, Murray JF Jr. Determination of sulfhydryl groups with 2,2'- or 4,4'-dithiodipyridine. *Arch Biochem Biophys* 1967;119:41-49.
  24. Thakur ML, DeFulvia JD. Technetium-99m-monoclonal antibodies for immunoscintigraphy. Simplified preparation and evaluation. *J Immunol Method* 1991;137:217-224.
  25. McKenna CE, Gutheil WG, Song W. A method for preparing analytically pure sodium dithionite. Dithionite quality and observed nitrogenase-specific activities. *Biochim Biophys Acta* 1991;1075:109-117.
  26. Lees RS, Garabedian HD, Lees AM, et al. Technetium-99m low density lipoproteins: preparation and biodistribution. *J Nucl Med* 1985;26:1056-1062.
  27. Fritzberg AR, Lyster DM, Dolphin DH. Evaluation of formamidine sulfinic acid and other reducing agents for use in the preparation of Tc-99m-labeled radiopharmaceuticals. *J Nucl Med* 1977;18:553-557.
  28. Jones AG, Orvig C, Trop HS, et al. A survey of reducing agents for the synthesis of tetraphenylarsonium oxotechnetiumbis(ethanedithiolate) from [<sup>99</sup>Tc]-pertechnetate in aqueous solution. *J Nucl Med* 1980;21:279-281.
  29. Vilcek S, Kalincak M, Machan V. Technetium-99m-labelled compounds prepared with sodium dithionite as reducing agent. *Nucl Med* 1981;20:283-289.
  30. Khaw BA, Strauss HW, Carvalho A, et al. Technetium-99m labeling of antibodies to cardiac myosin Fab and to human fibrinogen. *J Nucl Med* 1982;23:1011-1019.
  31. Ellman GL. Tissue sulfhydryl groups. *Arch Biochem Biophys* 1959;82:70-77.