A Sulfhydryl-Specific Fluorescent Label, *S*-Mercuric *N*-Dansylcysteine. Titrations of Glutathione and Muscle Proteins†

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ABSTRACT: A new fluorescent reagent, *S*-mercuric *N*-dansylcysteine, has been developed which can be used both to estimate sulfhydryl groups and to label proteins. The reagent reacts readily with small molecules and proteins to form mercury-bridged mercaptides with extremely high association constants. For small molecules, the fluorescence of the attached label is enhanced 2X without spectral shift, while larger enhancements (2.8, 7, and 20X) and spectral shifts (5, 13, and 20 nm) are observed when the label is bound to the muscle proteins tropomyosin, troponin C, and actin, respectively. These latter changes result from differences in the protein surface environments at the label binding sites. Troponin C binds 1.0 mol of label/20,000 daltons and G- or F-actin binds 1.25 mol/42,000 daltons, in agreement with the composition and known reactivity of the SH groups of these proteins. Tropomyosin binds 0.8 mol/34,000 daltons, suggesting that only 1 SH/chain is freely exposed.

The labeling of proteins with fluorescent molecules has been a useful approach for the study of protein structure and interactions. In order to obtain maximum information from such studies, however, it is desirable to have labels that can be conveniently attached to specific sites on the surface of the protein under conditions which will not alter the protein's native properties.

A large number of SH-directed reagents have been developed in recent years which satisfy these requirements to varying degrees. A partial listing of these includes didansylcystine (Wu and Stryer, 1972; Cheung *et al.*, 1971), the iodoacetamide naphthylaminesulfonates (I-AEDANS)[1] (Hudson and Weber, 1973), dansylaziridine (Scouten *et al.*, 1974), the fluorescein derivatives: difluorescein cystine (Wu and Stryer, 1972; Bunting and Cathou, 1973), fluorescein mercuric acetate (Karush *et al.*, 1964; Heitz and Anderson, 1968), and mercurichrome (Weltman *et al.*, 1972); 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-chloride) (Birkett *et al.*, 1970; Allen and Lowe, 1973), *N*-(1-anilinonaphthyl-4)-maleimide (ANM) (Kanaoka *et al.*, 1973), *N*-(*p*-(2-benzimidazolyl)phenyl)-maleimide (BIPM) (Kanaoka *et al.*, 1970), and pyrenemaleimide (Weltman *et al.*, 1973).

While most of these compounds have proved useful in the study of some protein systems, disadvantages such as limited solubility in aqueous solutions (dansylaziridine, ANM, pyrenemaleimide), lack of specificity (NBD-Cl), the necessity for long reaction time and/or excess reagent (didansylcystine, dansylaziridine, difluorescein cystine, I-AEDANS, ANM, BIPM), complexities in their syntheses (ANM, I-AEDANS, BIPM, pyrenemaleimide, dansylaziridine), and low stability (I-AEDANS) can in some cases limit their suitability for general use. Other problems involve limitations imposed by the spectral properties of the label; *e.g.*, low quantum yields (NBD-Cl-sulfhydryl derivatives), changes in spectra in the neutral pH region (fluorescein), and fluorescence heterogeneity (I-AEDANS).

In this study, we present data on the synthesis and use of a new SH-specific label which compares favorably with the others as a reagent for labeling proteins. It can, in contrast to the others, also be used for the fluorometric estimation of SH groups. The reagent *S*-mercuric *N*-dansylcysteine (Dns-Cys-SHg^+) is easily prepared, stable, and water soluble. It reacts rapidly and stoichiometrically with SH groups over a wide range of pH to form mercury-bridged mercaptides with a high affinity. The dansyl moiety is incorporated as the fluorophore because of its well-known, useful fluorescence properties. It is a sensitive probe of the chemical environment (Steiner and Edelhoch, 1965; Chen, 1967), has a high intrinsic polarization and a reasonably long lifetime (Weber, 1952), and has spectral properties that permit efficient energy transfer from intrinsic protein fluorophores (Stryer, 1959). Finally, while the label is bound strongly to the protein (*e.g.*, it can be used for fluorescence identification of protein bands in sodium dodecyl sulfate polyacrylamide electrophoresis), the native protein molecule can easily be regenerated by adding an excess of any small thiol compound. A preliminary report of this work has been presented (Leavis and Lehrer, 1973).

Experimental Section

Preparation of Dns-Cys-SHg^+. A saturated solution (~5×10^{-3} M) of either didansyl-L-cystine or dansyl-L-cysteine (Sigma) was allowed to react with a tenfold excess of dithiothreitol for 12–18 hr at room temperature in 50 mM Tris-acetate buffer (pH 8.0). This treatment was found to be necessary to ensure that all of the dansylamino acid was in the reduced cysteine form. After the incubation, the pH was adjusted to 3 with concentrated HNO_3 to prevent oxidation of SH

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[1] Abbreviations used are: EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(aminoethyl ester); NEM, *N*-ethylmaleimide; PCMB, *p*-chloromercuribenzoate; DTT, dithiothreitol; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); ATP, adenosine triphosphate; I-AEDANS, iodoacetamide naphthylaminesulfonate; NBD-chloride, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole; ANM, *N*-(1-anilinonaphthyl-4)-maleimide; BIPM, *N*-(*p*-(2-benzimidazolyl)phenyl)maleimide.

groups, and the excess dithiothreitol was separated from the dansylcysteine by chromatography on Sephadex G-10, employing 1 mM HNO_3 as the eluent. Under these conditions the dithiothreitol is eluted in the included column volume, while the dansyl compound, which is adsorbed to the gel, is eluted in a broad peak in approximately three column volumes.

The concentration of dansyl-L-cysteine was determined from $\epsilon_{350\,nm} = 3980\ M^{-1}$ (Cheung *et al.*, 1971) at pH 7.0, and from the sulfhydryl concentration determined with DTNB at pH 7.0 (Ellman, 1958). These values, which were in agreement, were used to determine the amount of $Hg(NO_3)_2$ to add to the system (pH 3) to produce *S*-mercuric *N*-dansylcysteine. Both the purity of the starting substance and the products of Hg^{2+} binding were evaluated by thin-layer chromatography on Eastman 6060 silica gel plates using a solvent system of chloroform–methanol–acetic acid (75:25:5) (Seiler and Wiechmann, 1967). No SH groups were detected on the product when assayed at pH 7.0 with DTNB.

The final product, in solution, was stored in a brown bottle at 4° and appeared to be stable for at least several months at pH 3.0.

In separate experiments, the spectral properties of the reaction intermediates were studied. Dansyl-L-cysteine was mixed with various mercury salts at molar ratios of Hg/Dns ranging from 0 to 3 at pH 7.0.

Protein Preparations. Troponin C, the Ca^{2+}-binding component of troponin, was prepared by a modification (Potter and Gergely, 1974) of the method of Greaser and Gergely (1971). The protein was incubated with 10 mM DTT in Tris-HCl (pH 7.0) and dialyzed to remove the DTT prior to use. Protein concentrations were determined using the microbiuret method employing bovine serum albumin (Pentex) as the standard.

Tropomyosin was prepared by the Bailey method as modified by Greaser and Gergely (1971). It was lyophilized and stored at −10°. For these experiments, the protein was dissolved in a solution containing 20 mM DTT. The DTT was removed after 12–18 hr by dialysis. The tropomyosin concentration was determined by measuring the absorbance at 277 nm employing an $A_{1cm}^{1mg/ml} = 0.25$ (Ooi, 1967). The purity of the preparation was confirmed by the lack of tryptophyl fluorescence in a solution excited at 280 nm.

Actin was prepared as previously described (Lehrer and Kerwar, 1972). G-actin solutions always contained 0.1–0.2 mM ATP to protect against denaturation.

Myosin was prepared by a modification (Nauss *et al.*, 1969) of the method of Tsao (1953) and stored as the $(NH_4)SO_4$ precipitate at −20°. Prior to use it was homogenized in 0.5 M KCl, 10 mM Tris-HCl (pH 7.5), 10–20 mM DTT, and 1 mM EDTA, then dialyzed against 0.5 M KCl and 10 mM Tris-HCl (pH 7.5). Myosin concentrations were measured by the microbiuret method and ATPase activities were determined by measuring the P_i released from ATP (Fiske and Subbarow, 1929) upon incubation for 5 min at 25° with 0.1 mg/ml of myosin. Incubation media contained 50 mM Tris-HCl (pH 7.5), 10 mM Ca^{2+}, 50 mM KCl, and 5 mM ATP for K^+-activated ATPase. Reactions were terminated by the addition of 10% trichloroacetic acid.

All protein solutions were made fresh from rabbit back and leg muscles and stored at 0–4°. They were used within a few days. Sodium dodecyl sulfate polyacrylamide gel electrophoresis was used to evaluate the homogeneity of the protein preparations (Weber and Osborn, 1969). The following molecular weights were used in the calculations: actin, 42,000; tropomyosin, 68,000; troponin C, 20,000; myosin, 500,000.

Titrations. Two types of titrations were performed. The first

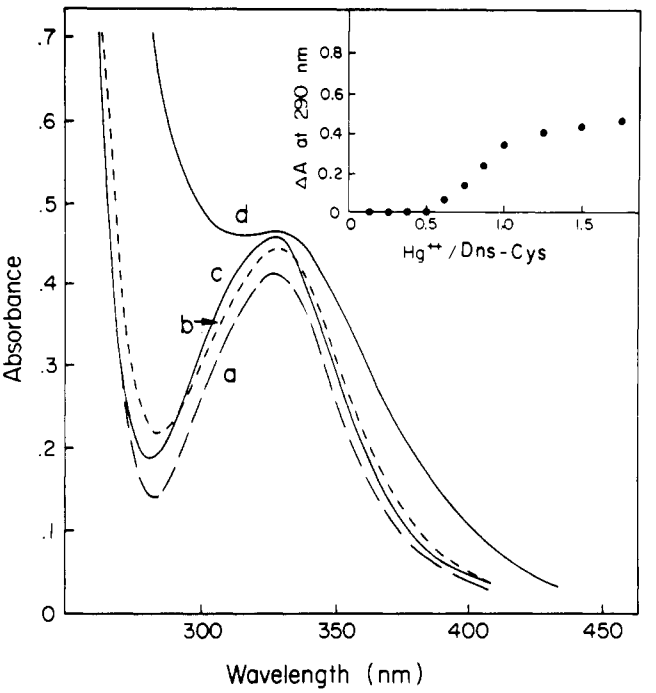


FIGURE 1: Absorption spectra of Dns-Cys compounds: a, $(Dns\text{-}Cys\text{-})_2$; b, Dns-Cys; c, $(Dns\text{-}Cys\text{-}S\text{-})_2Hg$; d, $Dns\text{-}Cys\text{-}SHg^+$. The chromophore concentration was identical for all spectra (1.0×10^{-4} M). Inset: titration of Dns-Cys with Hg^{2+}. Breaks in the titration curve occur at Hg^{2+} to Dns ratios of 0.5 with the formation of $(Dns\text{-}Cys\text{-}S\text{-})_2Hg$ and 1.0 with the formation of $Dns\text{-}Cys\text{-}SHg^+$. Tris-acetate buffer, 20 mM, pH 7.0.

was carried out in order to characterize the spectral changes induced in the probe by its reaction with SH groups. For this, small increments of either glutathione (GSH) or cysteine were added to solutions containing constant amounts of $Dns\text{-}Cys\text{-}SHg^+$ in Tris-acetate buffer at pH 7.0. In other titrations, the probe was added to solutions of the muscle proteins at concentrations of $1\text{–}5 \times 10^{-6}$ M. In the case of troponin C and tropomyosin, fluorescence measurements were made immediately after each addition of titrant. With actin, however, the reaction was slower at low ratios of reagents to protein. The reaction was therefore allowed to proceed for at least 10 min before the fluorescence was measured in order to achieve stoichiometry. In some experiments with actin, NEM (Worthington) was reacted with the protein (2:1 mole ratio) at pH 7.0 for 1 hr to block sulfhydryl groups (Lehrer *et al.*, 1972). In all cases, titrations were performed directly in fluorescence cuvets, and titrants were added with a Metrohm-type E457 microsyringe. In parallel studies using higher reactant concentrations, the SH concentrations were monitored using DTNB.

Spectral Measurements. Absorbance measurements were made using either a Cary Model 15 or a Zeiss Model PMQ II spectrophotometer. Fluorescence measurements were made as previously described (Lehrer and Kerwar, 1972). Fluorescence was measured at room temperature (23 ± 1°) at right angles to the exciting source. The absorbance at the exciting wavelength was always less than 0.1. Fluorescence spectra were not corrected for variations in monochromator and photomultiplier sensitivity with wavelength.

All chemicals not listed above were commercial preparations of reagent grade quality. Water was deionized using mixed-bed resins and glass distilled.

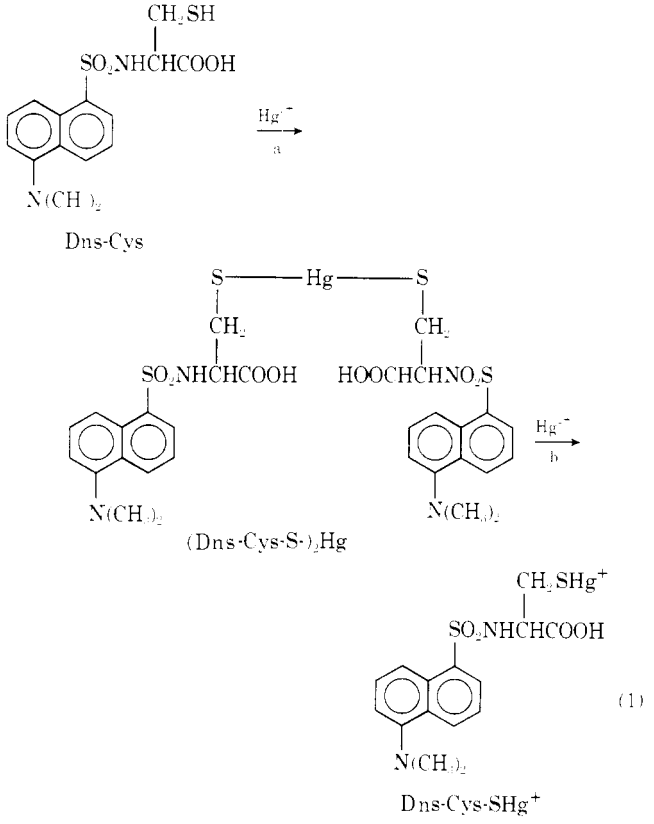
Results

Spectral Properties of the Dansyl Compounds. Absorption and fluorescence spectra were studied in order to obtain infor-

mation on the stoichiometry and spectral characteristics of the reaction intermediates in the preparation of Dns-Cys-SHg^+. Absorption spectra are shown in Figure 1. When didansyl-L-cystine was reduced to dansyl-L-cysteine (Dns-Cys), there was a small increase in absorbance (Figure 1, curves a and b). With the addition of $Hg(NO_3)_2$ to Dns-Cys to a mole ratio of 0.5, there was a small, but reproducible spectral shift with isosbestic points at 292 and 338 nm (Figure 1, curve c). Further addition of $Hg(NO_3)_2$ to a mole ratio of 1.0 resulted in more pronounced absorbance changes as shown in curve d in Figure 1. A plot of the absorbance at 292 nm as a function of Hg/Dns (Figure 1, inset) shows breaks in the titration at ratios of 0.5 and 1.0.

The corresponding fluorescence spectral changes for these solutions are shown in Figure 2, with the inset showing a fluorescence titration at 525 nm. As was the case with the absorbance, breaks in the titration occurred at Hg/Dns ratios of 0.5 and 1.0. The uncorrected fluorescence maximum for all the dansyl compounds was at 535 nm, and the quantum yields decreased in the following sequence: $(Dns\text{-}Cys)_2$ > Dns-Cys > $(Dns\text{-}Cys\text{-}S)_2Hg$ > Dns-Cys-SHg^+ (see eq 1 below for abbreviations).

The data in Figures 1 and 2 indicate that the formation of the Dns-Cys-SHg^+ occurs in two steps:



The first step (a) results in the formation of a mercury-bridged dimer. This is supported by the absence of SH groups as detected by DTNB at a mole ratio of 0.5. Thin-layer chromatography analyses of the species at 0.5 and 1 Hg/Dns also demonstrated two distinct species.

The quantum yield for the Dns-Cys-SHg^+ is estimated to be 0.026 in aqueous solution, using the known value of 0.087 for $(Dns\text{-}Cys\text{-}S\text{-})_2$ (Chen, 1967).

Binding of Dns-Cys-SHg^+ to GSH. Studies were done to see if a fluorescence change is produced when Dns-Cys-SHg^+ binds to small SH compounds to form mixed mercury-bridged mercaptides. The results with reduced glutathione are shown in Figure 3. In this case, the fluorescence and SH concentration were determined during a titration of Dns-Cys-SHg^+ with GSH. The break in the titration curve at Dns/GSH = 1 indicated the formation of bridged mercury mercaptides according to

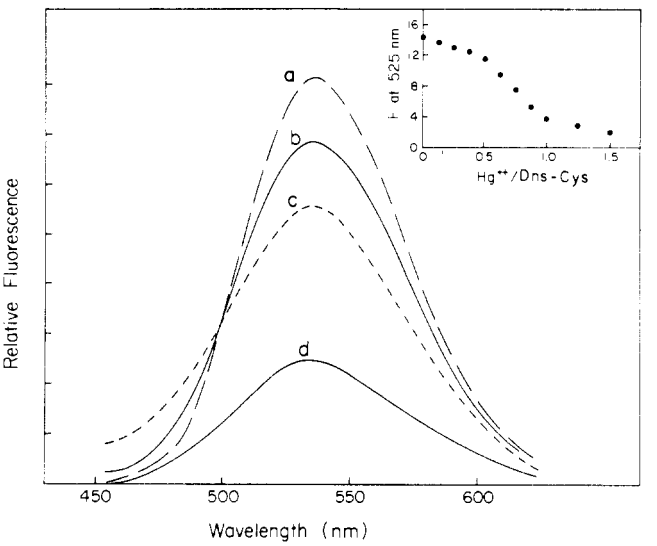


FIGURE 2: Uncorrected fluorescence spectra of Dns-Cys compounds: a, $(Dns\text{-}Cys\text{-})_2$; b, Dns-Cys; c, $(Dns\text{-}Cys\text{-}S\text{-})_2Hg$; d, Dns-Cys-$SHg^+$. These spectra were obtained using 1:10 dilutions of the corresponding solutions in Figure 1. Inset: fluorescence titration of Dns-Cys with Hg^{2+}. λ_{ex} = 330 nm.

$$Dns\text{-}Cys\text{-}SHg^+ + RSH \rightarrow Dns\text{-}Cys\text{-}SHgS\text{-}R + H^+ \qquad (2)$$

with a resulting twofold enhancement of fluorescence. Free SH groups were detected when GSH was added in excess of the label; however, no further changes in fluorescence occurred.

The twofold fluorescence enhancement associated with this reaction appears to be attributable to the formation of the bridged mercaptide and is not influenced by the nature of the R group in RSH, because similar enhancements occur in all cases without wavelength shift, when Dns-Cys-SHg^+ reacts with other small SH-containing compounds such as cysteine, or dansyl-L-cysteine (Figure 2, curves c and d). This observation is in contrast to the case of proteins (see below).

Binding of Dns-Cys-SHg^+ to Muscle Proteins. TROPONIN C. Troponin C, which is the calcium binding subunit of the troponin complex, contains one sulfhydryl group per molecule (Greaser and Gergely, 1973). The fluorescence titration of troponin C with Dns-Cys-SHg^+ presented in Figure 4 was performed in the presence of 5×10^{-4} M EDTA since the SH group is not reactive when Ca^{2+} is present (Potter *et al.*, 1973).

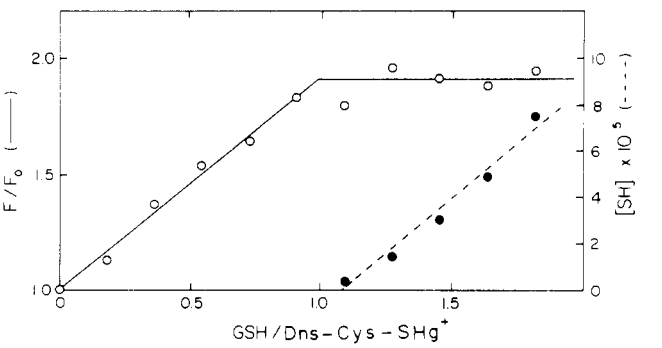


FIGURE 3: Fluorescence titration of Dns-Cys-SHg^+ (1.0×10^{-4} M) with reduced glutathione (O——O). The appearance of SH groups when excess glutathione is present (●- - -●). Buffer is 20 mM Tris-HCl (pH 7.0).

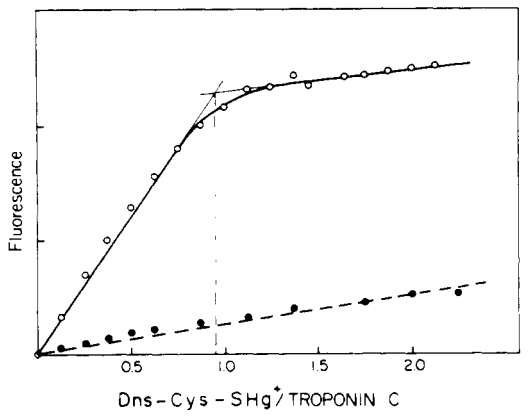


FIGURE 4: Fluorescence titration of troponin C with Dns-Cys-SHg^+: troponin C (O——O); buffer alone (●- - -●). [Troponin C] = 0.1 mg/ml; buffer is 50 mM KCl, 0.5 mM EDTA, 10 mM Tris-HCl (pH 7.0); λ_{ex} = 330 nm, λ_{em} = 520 nm.

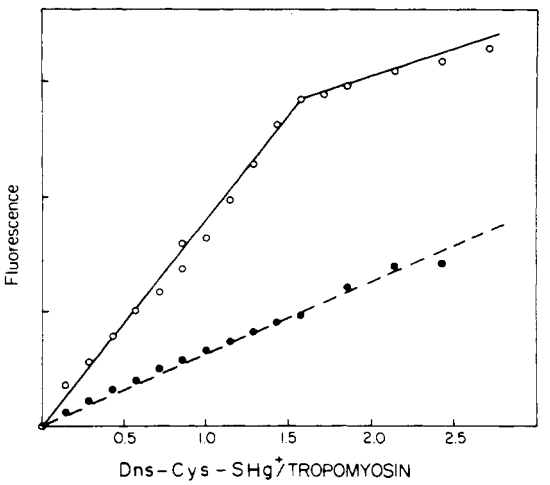


FIGURE 5: Fluorescence titration of tropomyosin with Dns-Cys-SHg^+: tropomyosin (O——O); buffer alone (●- - -●). [Tropomyosin] = 0.18 mg/ml; buffer is 0.1 M KCl and 20 mM Tris-HCl (pH 7.5); λ_{ex} = 330 nm, λ_{em} = 520 nm.

In our experiments 0.8–0.95 mol of Dns-Cys-SHg^+ was found to bind to 1 mol of troponin C. The DTNB assay for SH groups was negative at the fluorescence end points. The bound dansyl label showed an approximately sevenfold fluorescence enhancement and a peak blue shift of 13 nm as compared to the free label in buffer. This is consistent with an environmental change in which the bound dansyl fluorophore is located in a less polar environment than when free in the solvent (Chen, 1967).

The high affinity of the fluorescent label for the SH group of troponin C is evident in that the latter is labeled even in the presence of a 200-fold excess of EDTA, which competes with SH groups for Hg^{2+}. Also, titrations in the presence of 0.1 M Cl^-, as compared to titrations performed at low Cl^- concentrations, gave identical results despite the strong affinity of $RSHg^+$ for Cl^-, which should compete with groups other than SH (Sillen and Martell, 1964) for mercury.

TROPOMYOSIN. The tropomyosin molecule is made up of two helical chains (Woods, 1966), which strongly interact by noncovalent bonds to form a coiled-coil structure of 68,000 daltons (Hodges and Smillie, 1972; Hodges *et al.*, 1972). Figure 5 shows a fluorescence titration of tropomyosin with Dns-Cys-SHg^+ in 0.1 M KCl, where interactions between tropomyosin molecules are minimized (Ooi *et al.*, 1962). It can be seen that 1.65 mol of Dns-Cys-SHg^+ bind per mol of tropomyosin, or a little less than 1 mol of label/chain of 34,000 daltons. An assay for SH groups using DTNB on the same sample (done at a higher concentration) also gave 1.65 SH groups/molecule of tropomyosin. At the end point, there was a 2.8-fold enhancement of the fluorescence and a 5-nm spectral blue shift compared to the free label.

ACTIN. Although actin contains 5 SH groups (Elzinga and Collins, 1972), only an average of 1.2–1.3 react preferentially with NEM (Lehrer *et al.*, 1972), PCMB (Tonomura and Yoshimura, 1967; Lehrer *et al.*, 1972), and with disulfide reagents (Drabikowski and Bitney-Szlachto, 1964). Lehrer *et al.* (1972) have shown that one SH group is predominantly involved, and studies with radioactive NEM have shown this to be Cys-373 (Elzinga *et al.*, 1973). Figure 6 shows titrations of G- and F-actin with an end point at Dns-Cys-SHg^+/actin = 1.25. On binding, there is about a 20-fold fluorescence enhancement and a 20-nm spectral blue shift of the dansyl probe. The results are identical with G- or F-actin. Denaturation, which would expose additional SH groups, did not occur, as evidenced by a lack of shift in the tryptophyl fluorescence spectrum (Lehrer and Kerwar, 1972).

The specificity of the label was checked by titrating G-actin that had previously been allowed to react with NEM in twofold molar excess at pH 7.5 for 1 hr. This reaction, as was discussed above, blocks Cys-373. Subsequent DTNB assay showed no available SH groups, and no fluorescence enhancement occurred when NEM-G-actin was titrated with Dns-Cys-SHg^+ (Figure 6). Therefore, Dns-Cys-SHg^+ binds predominantly to Cys-373.

In contrast to troponin C and tropomyosin, it was found necessary to include low concentrations of CN^- or SCN^- in the actin solution to prevent apparent binding of the label to protein side chains other than cysteine. In the absence of these anions, the titrations of actin did not show definite endpoints.

In the case of actin, one can follow the binding of Dns-Cys-SHg^+ by monitoring the loss of tryptophyl fluorescence, which results from energy transfer to the dansyl. About half of the fluorescence of tryptophan is quenched by the bound dansyl (Figure 6). A larger enhancement of the bound dansyl fluorescence over free dansyl was also observed with excitation at 290 nm (not shown). Addition of 1 mM DTT to the labeled actin solutions caused an immediate increase in tryptophyl fluores-

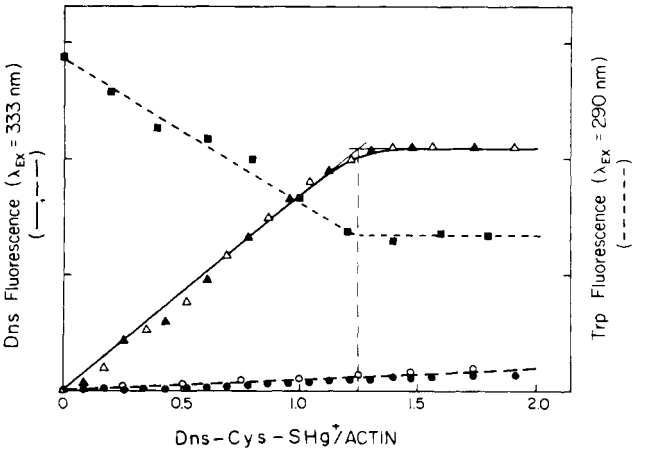


FIGURE 6: Fluorescence titration of actin with Dns-Cys-SHg^+: Dns fluorescence (λ_{ex} = 333, λ_{em} = 520) with G-actin (△——△); F-actin (▲——▲); G-actin previously treated with NEM (O- - -O); buffer (●- - -●). Tryptophyl fluorescence (λ_{ex} = 290, λ_{em} = 330) with G-actin (■- - -■). [Actin] = 0.1 mg/ml; buffer is 5 mM Tris-HCl (pH 7.5), 0.2 mM ATP, 0.2 mM Ca^{2+}, and 1 mM $NaCN^-$ (0.1 M KCl for F-actin).

TABLE I: Peak Fluorescence Enhancements and Wavelength Shifts Due to Binding of Dns-Cys-SHg^+.

System	Peak Enhancement (F_{bound}/F_{free})	Peak Wavelength Shift (nm)
Glutathione	1.9	0
Tropomyosin	2.8	5
Troponin C	7	13
G- or F-actin	20	20

cence and loss of dansyl fluorescence owing to the removal of the bound dansyl.

MYOSIN. It is well known that myosin contains two classes of functionally important SH groups. Blocking the most reactive (S_1) class results in an enhancement of the Ca^{2+}-activated ATPase and inhibition of K-EDTA-stimulated ATPase (Kielley and Bradley, 1956), while blocking the second (S_2) class produces a total loss in ATPase activity (Kielley and Bradley, 1956; Sekine and Yamaguchi, 1963). In an effort to determine whether Dns-Cys-SHg^+ would react selectively with either of these two classes, we have titrated myosin with the label. On binding the label, there was a progressive loss in both Ca^{2+} and K-EDTA-activated ATPase, which was complete at a ratio of 8 Dns-Cys-SHg^+/myosin molecule. At the same ratio, another mercurial, PCMB, enhances the Ca^{2+}-activated ATPase (Kielley and Bradley, 1956). With the addition of 12 Dns-Cys-SHg^+/myosin, the protein appeared to undergo denaturation, suggested by the appearance of turbidity and subsequent precipitation upon standing. This behavior was not observed with PCMB.

Discussion

Spectral studies, SH analysis, and tlc data all support the suggested binding scheme of mercury to the SH groups of Dns-Cys as shown in eq 1. This scheme had been used to explain the binding of Hg^{2+} to mercaptalbumin by Edelhoch *et al.* (1953). Their study, in fact, generated the idea for our work. In other studies (Sperling *et al.*, 1969; Arnon and Shapira, 1969), mercuric ions have been inserted between two sulfhydryl groups normally forming a disulfide bond, thus modifying the protein by taking advantage of the extremely high association constant for the formation of RS-Hg-SR′ (Gurd and Wilcox, 1956). Such high constants would be expected to produce the sharp titration end points observed in our study both for the reaction of Dns-Cys with $Hg(NO_3)_2$ and for the reaction of the label with GSH and the muscle proteins.

The origin of the spectral changes (Figures 1 and 2) associated with the binding of mercury to Dns-Cys is not clear. Chen (1971) has shown that the formation of a 1:1 mercury–GSH complex generates a mercaptide absorption band, the tail of which extends into the 300–330-nm region. This tail may partially contribute to the small shift in the absorption spectrum seen upon formation of the (Dns-Cys-S-)$_2$Hg complex (Figure 1, curve c). However, both the shape and magnitude of the difference spectra accompanying formation of this complex as well as the 1:1 complex, Dns-Cys-SHg^+ (Figure 1, curve d) suggest that other mechanisms are involved in producing the observed changes.

The doubled fluorescence quantum yield of (Dns-Cys-S)$_2$Hg (Figure 2, curve c) as compared with Dns-Cys-SHg^+ (Figure 2, curve d) appears to be characteristic of the mercury-bridged species. As previously pointed out, enhancements of the same magnitude result from formation of mixed mercaptides of the type Dns-Gys-SHgS-R. Changes in both the absorption and fluorescence spectra may be related to the ability of the sulfhydryl-bound mercury to interact with the dansyl ring. If such interactions quench the fluorescence *via* a heavy atom effect (*e.g.*, Chen, 1971) in Dns-Cys-SHg^+, then further sterically restricting Hg^{2+} in the dimercaptide complex may enhance fluorescence by decreasing the accessibility of mercury to the ring. When mercury in excess of 1:1 is added to Dns-Cys-SHg^+, further changes occur in both absorption and fluorescence. Similar spectral changes were seen when mercury was added to (Dns-Cys)$_2$. Thus, it is likely that this is the result of additional mercury binding, probably to the dimethylamino group. This is suggested by similar spectral changes occurring on protonation of this group (Lagunoff and Ottolenghi, 1965). Binding of mercury to this site can be inhibited by including 0.1 M Cl^- in the solution. The Cl^- ion competes favorably with the dimethylamino nitrogen for mercury without competing with the interaction between SH groups and mercury. In the presence of 0.1 M Br^- or I^-, however, competition with SH groups was observed, since titration of Dns-Cys with mercury did not give sharp end points even at 1:1, presumably due to the relatively high affinities of these anions for Hg^{2+} (Sillen and Martell, 1964).

The basis for the use of Dns-Cys-SHg^+ to estimate SH groups in small molecules is the twofold increase in fluorescence yield associated with the labeling reaction (eq 2). In the case of the muscle proteins, additional enhancements and spectral shifts occur owing to changes in the dansyl fluorophore environment upon interaction with the protein surface after binding *via* the Hg–S reaction. The differences in fluorescence enhancement and spectral shift seen from one protein to another (Table I) reflect the different environments near each group with which the excited dansyl interacts. Thus the label functions as a fluorescence probe of surface environment making it useful in studies of conformational changes of proteins.

In all systems studied, the titration end points using Dns-Cys-SHg^+ agreed with SH estimations by the DTNB technique. Using fluorescence, however, the sensitivity can be increased 100–1000 times, and the titration requires as little as 1 nmol of protein.

One potential problem in the use of Dns-Cys-SHg^+ for labeling proteins is the possibility of binding to groups other than SH. This can be minimized or completely eliminated by titrating in the presence of 0.1 M Cl^- or millimolar concentrations of $EDTA^{3-}$, SCN^-, or CN^-. The stability constants for the complexes between mercury and these ions are greater than those of any Hg^{2+}–protein side-chain complex except Hg–SR. Thus, these anions successfully prevent Hg^{2+} from binding to the protein after all available SH groups are bound.

The possibility of nonspecific binding of the label to the protein *via* the dansyl moiety alone, which might cause a fluorescence enhancement, was discounted because, in all cases, the enhancement was completely lost upon the addition of 1 mM DTT. This is expected for complexes involving Hg^{2+} binding, but not for nonspecific binding. This DTT effect also provides a quick and convenient method to remove the label.

In the case of troponin C, the fluorescence titration showed that the single SH group is inaccessible to Dns-Cys-SHg^+ or DTNB, except in the presence of EDTA or EGTA, apparently owing to the protective effect afforded by a tightly bound Ca^{2+} (Potter *et al.*, 1973). On the other hand, once the protein is labeled, a large fluorescence enhancement accompanies the binding of Ca^{2+} (Potter *et al.*, 1974).

Recent studies on tropomyosin have shown it to consist of 2

types of chains present in the ratio of 4:1 and containing 1 and 2 SH groups per chain, respectively (Cummins and Perry, 1973). Furthermore, the coiled-coil model of Hodges *et al.* (1972) locates one cysteine (49) of each chain on the protein surface. Our data suggest that this cysteine is almost completely available for reaction with Dns-Cys-SHg^+ or DTNB. The small fluorescence enhancement (2.8-fold) and spectral shift (5 nm) observed at the titration end point supports the idea of a highly polar surface environment near the binding site of the label. The other SH groups in tropomyosin are apparently inaccessible to these reagents.

From the studies reported above, it appears that Dns-Cys-SHg^+ binds predominantly to actin at Cys-373. Addition of Dns-Cys-SHg^+ to G- or F-actin up to 5 mol/mol with incubation up to 1 hr did not produce any additional enhancement showing that no further binding occurred. The similarities in the spectral and titration results between G- and F-actin are to be contrasted with the observations of Cheung *et al.* (1971) who noted spectral differences between dansylcysteine labeled G- and F-actin. This may be due to differences in the labeling specificity and environment of the different SH-directed dansyl labels employed.

Since actin contains tryptophyl side chains, in contrast to tropomyosin and troponin C, energy transfer from tryptophan to dansyl is possible. This is fairly efficient for actin as seen by the fact that about half of the tryptophyl fluorescence is quenched when 1 mol of Dns-Cys-SHg^+ is bound. The course of the SH titration could be followed in this case either by monitoring the directly excited dansyl fluorescence, the dansyl fluorescence sensitized by tryptophan, or the corresponding loss of tryptophyl fluorescence.

In conclusion, these studies have shown that Dns-Cys-SHg^+ can be readily used both to estimate SH groups of small molecules and proteins and to label accessible SH groups of proteins with a sensitive fluorescence probe. The relative advantages of this labeling agent over other SH-directed labels have been discussed. In many cases, parallel studies on proteins labeled with different fluorophores may be useful. For example, a comparison of the fluorescence properties of proteins labeled both with didansylcystine and Dns-Cys-SHg^+ will be of great interest, since fluorescence differences would be interpreted in terms of the environmental change imposed by the extra length associated with a Hg^{2+} inserted between the S atoms of a disulfide bond. Finally, the presence of the electron dense mercury atom could facilitate X-ray studies on Dns-Cys-SHg^+-labeled protein crystals. This could additionally lead to information which may help correlate the dansyl environment with its fluorescence properties.

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Amino Acid Sequence Determination with Radioactive Proteins†

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ABSTRACT: A procedure is described for quantitative analysis of amino acids based only on their radioisotope content. The effluent from a conventional amino acid analyzer is collected so that each amino acid is placed in a discrete fraction for subsequent scintillation counting. This procedure is applied to the analysis of the hydrolytic products of thiazolinones formed by the automatic degradation of proteins in the Edman–Begg sequenator. Sequence data derived solely from the radioisotope content of the protein under investigation are then obtained. We tested the procedure for 30 degradations with a uniformly ^{14}C-labeled peptide of 47 residues in length obtained from Brome Mosaic virus coat protein. The radioisotope sequence agreed completely with that derived from conventional chemical analysis. The radioisotope sequencing procedure can also be used with ^{3}H labels. The procedure is insensitive to the presence of nonradioactive proteins introduced accidentally or deliberately during the purification of the radioactive protein.

Determining the primary structure of proteins using the automated Edman–Begg sequenator is currently restricted to proteins available in milligram quantities. These sequence determinations are limited by the sensitivity of the methods used to identify and quantitate the resulting phenylthiazolinones or the amino acids obtained from them by hydrolysis.

In contrast, a method for sequencing radiolabeled proteins would not effectively be limited by the availability of any fixed amount of protein but rather by its specific activity. With such a method, amino acid sequence information could be obtained on proteins available in only trace quantities provided that sufficient radiolabel could be introduced. An important additional advantage inherent in procedures using radiolabeled protein is that the protein under study need be purified only to radiochemical homogeneity; the presence of adventitious nonradioactive contaminants is unimportant.

This paper presents a method for identification and quantitation of radioactive amino acids and illustrates how these methods can be automated and applied to sequencing radiolabeled proteins.

Materials and Methods

Proteins. The [^{14}C]Brome Mosaic virus peptide ([^{14}C]BMV-D)[1] was prepared from a cyanogen bromide digest of [^{14}C]Brome Mosaic plant virus coat protein (BMV) which was uniformly labeled by growing a virus-infected plant in a $^{14}CO_2$ atmosphere (Shih and Kaesberg, 1973). This 47 residue peptide was a generous gift of Mr. Joe Moosic (Biophysics Laboratory, University of Wisconsin). The human λ Bence-Jones protein (Eddy) which has a free α-amino group was prepared by diethylaminoethyl-cellulose chromatography of the patient's urinary proteins. The preparation of [^{3}H]*S*-carboxymethyl T_1 ribonuclease has been previously described (McKean and Smith, 1974).

Sequence Procedures. Uniformly radiolabeled [^{14}C]BMV-D (0.076 μCi in 32 nmol of protein, corresponding to 150,000 cpm ^{14}C) was combined with 1 mg of unlabeled carrier protein (λ chain), 0.53 μCi of [^{3}H]T_1 ribonuclease, 1 mg of Braunitzer's III reagent (Pierce Chemical Co., Rockford, Ill.), and 1 mg of dithioerythritol (Pierce Chemical Co.). The [^{3}H]T_1 ribonuclease, with radiolabeled cysteines at positions 2, 6, 10, and 103, was added to the sequenator sample to permit monitoring of the sequenator chemistry efficiency; its radiolabel was not involved in determining the sequence of [^{14}C]BMV-D. The combined protein sample was sequenced with an Edman–Begg sequenator (Illitron Division, Illinois Tool Works, Chicago, Ill.) using procedures and chemicals previously described (Edman and Begg, 1967; Smithies *et al.*, 1971).

A portion (5%) of the thiazolinone sample from each degradative step was counted for ^{3}H and ^{14}C to determine the degradative efficiency of the [^{3}H]T_1 ribonuclease and the total amount of ^{14}C in each sample, respectively. A known amount of [^{14}C]-β-alanine (7×10^{-3} μCi) was then added to each of the thiazolinone samples as in internal standard to control for sample loss and dilution. The thiazolinone samples were dried and hydrolyzed with HI (Smithies *et al.*, 1971). Unlabeled amino acid standards (Pierce Chemical Co., Rockford, Ill.), 20 nmol each, supplemented with 20 nmol each of α-aminobutyric acid, β-alanine, *allo*-isoleucine, and ornithine, were added to all hydrolyzed samples. These amino acids were added to act as carriers for the radioactive amino acids and to produce enough ninhydrin color to be detected by the analytical system of the amino acid analyzer. The hydrolysates were then subjected to amino acid analysis on a Durrum Model D500 amino acid ana-

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[1] Abbreviations used are: BMV-D, Brome Mosaic virus peptide; BMV, Brome Mosaic plant virus coat protein.