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Note

Usefulness of N-ethylmaleimide in the identification of ^{75}Se-labeled selenocysteine

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The discovery of several catalytically active selenoproteins[1–5] necessitates methods for identifying the form of selenium in these proteins. Identification of selenol compounds is particularly difficult because they are unstable[6]. They tend to decompose to elemental Se, oxidize to diselenides, and to react with sulfhydryl compounds; as a result, problems arise in their chromatographic identification[7]. Therefore, reagents that bind the selenol group to provide stable derivatives would be advantageous.

Carboxymethylation has recently been described for the identification of selenocysteine[8], and in the selenium-containing subunit of glycine reductase from *Clostridium sticklandii*, this selenoamino acid has been identified by formation of Se-carboxymethyl, Se-carboxyethyl, and Se-aminoethyl derivatives[5]. The sulfhydryl reagent, N-ethylmaleimide (NEM), has proved useful in the study of thiols[9]; it combines with the sulfhydryl group of cysteine yielding a derivative with distinctive chromatographic properties[10,11]. The similarity between sulfhydryl and selenol groups prompted us to investigate the ability of NEM to provide a similar derivative of selenocysteine.

MATERIALS AND METHODS

L-Cysteine·HCl was obtained from Eastman-Kodak (Rochester, N.Y., U.S.A.); L-cystine from Nutritional Biochemicals (Cleveland, Ohio, U.S.A.); D,L-selenocystine and N-ethylmaleimide from Sigma (St. Louis, Mo., U.S.A.); N-ethylmaleimide (ethyl-1-^{14}C), 8.4 mCi/mmole, was obtained from New England Nuclear (Boston, Mass., U.S.A.). All reagents were analytical grade.

Conditions known to succeed with cystine were chosen to form the NEM adduct of selenocysteine. One milliliter of a D,L-selenocystine or L-cystine solution (6 μmoles/ml) was reduced by the addition of 1 ml of 0.6 *M* sodium borohydride prepared immediately before use. Reduction was carried out under nitrogen for 90 min. The reaction mixture was acidified to pH 5.0 with a solution containing 1.0 *M* HCl and 1 *M* KH_2PO_4 (ref. 12) and diluted to twice the initial volume with water. To an

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aliquot was added an equal volume of 6 m*M* NEM in 0.2 *M* phosphate buffer (pH 7.0); the mixture was allowed to react at room temperature for 1 h. L-cysteine·HCl was treated with NEM similarly. Aliquots were applied to Whatman No. 1 chromatography paper and developed in three different solvent systems[11,13]. Chromatograms were sprayed with 0.25% ninhydrin in acetone.

In experiments with ^{14}C-labeled NEM, 1 μCi of the radioactive NEM was added to the non-radioactive NEM. Ratemeter tracings of radioactive chromatograms were obtained with a Packard Model 7201 radiochromatogram scanner.

The reaction of NEM with cysteine or selenocysteine was monitored spectrophotometrically at 300 nm according to the method of Roberts and Rouser[14].

RESULTS AND DISCUSSION

Many selenium compounds exhibit the same paper chromatographic (PC) properties as their sulfur analogs[15]. It would be expected, therefore, that the R_F value of a selenocysteine–NEM adduct be identical to that of the cysteine–NEM adduct. The R_F values listed in Table I, obtained with three different solvent systems, show that the reaction between borohydride and selenocystine generated a product that reacted with NEM to give a ninhydrin positive spot with an R_F identical to that of the cysteine–NEM adduct. Radioactive NEM travelled close to the solvent front. The reaction mixture could be frozen for several weeks with no apparent decomposition of the NEM adduct.

TABLE I

PAPER CHROMATOGRAPHY OF CYSTEINE- AND SELENOCYSTEINE-[^{14}C]NEM ADDUCTS IN USING THREE SOLVENT SYSTEMS

Solvent systems: EBFW = ethanol–*tert.*-butanol–formic acid–water (60:20:5:15)[11]; BFW = *tert.*-butanol–formic acid–water (70:15:15)[11]; BAW = *n*-butanol–glacial acetic acid–water (60:15:25)[13].

Compound	*R_F values*		
	EBFW	*BFW*	*BAW*
Cysteine·HCl	0.56	0.44	0.24
Cystine	0.16	0.06	0.02
Selenocystine	0.17	0.06	0.03
Cysteic acid	0.20	0.08	0.03
Cysteine-[^{14}C]NEM	0.66	0.57	0.33
$NaBH_4$ reduced cystine-[^{14}C]NEM	0.67	0.56	0.33
$NaBH_4$ reduced selenocystine-[^{14}C]NEM	0.66	0.55	0.32
[^{14}C]NEM	0.92	0.95	0.94

Ratemeter tracings of radiochromatograms verified the formation of the selenocysteine–[^{14}C] NEM adduct. Fig. 1 shows the coincidence of radioactivity with the predominant ninhydrin positive spot. A second, radioactive spot of lesser intensity and with an R_F equal to that of cystine and selenocystine was also present. The NEM adduct travelled as a discrete spot with no evidence of streaking. The absence of radioactivity at the R_F of [^{14}C] NEM and with the presence of a single radioactive peak, associated with the single ninhydrin positive spot of the adduct in three different solvent systems, indicate a stoichiometric utilization of NEM.

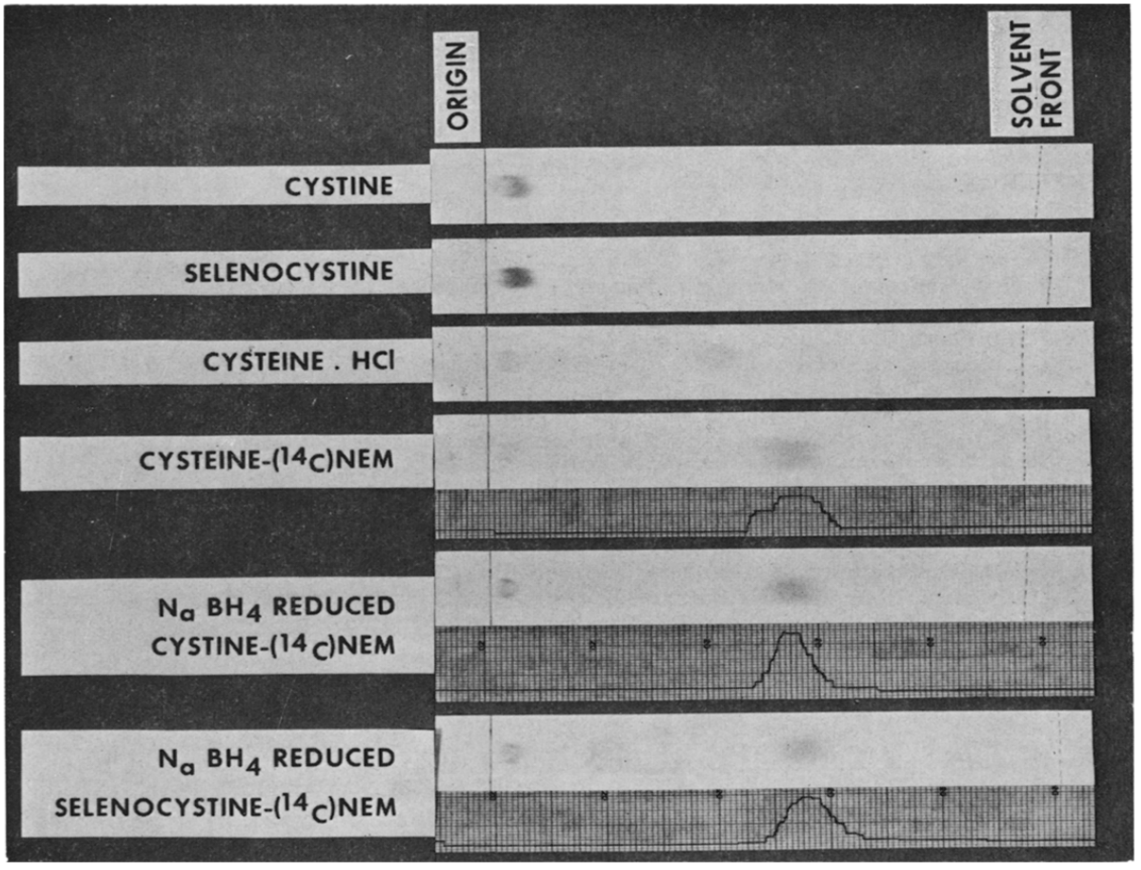

Fig. 1. Paper chromatograms of cysteine– and selenocysteine–[^{14}C]NEM adducts. Solvent: *tert.*-butanol–formic acid–water (70:15:15). R_F values are given in Table I.

The reaction of NEM with cysteine or selenocysteine was also monitored spectrophotometrically at 300 nm. However, quantitative determinations of selenol concentrations could not be obtained because the spectrophotometric data were variable (the cause of this variability is under investigation). Nevertheless, a decrease in absorbance was observed in all experiments, and is further evidence for the reaction between NEM and selenocysteine.

The binding of NEM to selenocysteine provides another approach to the identification of selenol compounds in cell extracts and proteins. A stabilized selenol group would be less likely to undergo oxidation during the extraction procedure; it is also likely that decomposition and streaking during chromatography would be eliminated. The NEM adduct should also be stable to enzymatic hydrolysis as has been found with the Se-carboxymethyl, Se-carboxyethyl, and Se-aminoethyl derivatives derived from digests of glycine reductase treated with the respective alkylating agents[5]. These advantages, we believe, will facilitate the identification of the selenium moiety in ^{75}Se-labeled selenoproteins.

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