(Tyson and Martell, 1972), and Fe^{3+} promoted the destruction of Amadori rearrangement products in the oxygen-dependent browning system of a glucose–diglycine mixture (Hashiba, 1975).

The metal-binding proteins, such as conalbumin from egg white (Azari and Feeny, 1961) and transferrin from serum (Koechlin, 1952), have a greater stability to proteolytic hydrolysis and thermal denaturation. In the stored ovalbumin–Cu^{2+} system (OV–Cu-IV), few effects on the denaturation and solubility of ovalbumin could be found even if metal bound to the ovalbumin in any form. Although copper catalyzes oxidation steps involving cystines–cysteines (Feeney et al., 1956), which might be involved in the aggregation phenomenon of denatured protein, this action might be negligible in the OV–Cu-IV system used.

The difference in the capacity to accelerate the Maillard reaction between Fe^{2+} and Fe^{3+} reflects the possibility that the first step of metal catalysis is "oxidation activation" involving the reduction of metal.

It has been clearly demonstrated that there are many species of minerals like calcium, copper, iron, magnesium, manganese, potassium, sodium, and zinc in egg white (Cotterill et al., 1977). In the present study, the effects of only three kinds of metals on the browning reaction in the ovalbumin–glucose mixture were studied. However, it is considered that Cu^{2+} and Fe^{3+} might accelerate the browning reaction in the egg white solid–glucose system reported in the previous study (Kato et al., 1978). The addition of a mere 0.003 ppm of manganese was found to inhibit the rate of browning reaction of the glucose–glycine system in air or oxygen (Bohart and Carson, 1955). Attention must be paid to the existence of trace metals in the studies on the rate of the overall browning reaction in the complex system such as egg white.

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Binding of Methylmercury to Ovalbumin as Methylmercuric Cysteine

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After the administration of methylmercury in the form of $CH_3{}^{203}HgCl$ to laying hens, the egg ovalbumin was isolated, and the binding of ^{203}Hg was determined. Enzymic hydrolysis of ovalbumin, followed by covalent chromatography of the hydrolysate on 2-pyridyl-S-S-propyl-Sepharose, enabled the separation of a ^{203}Hg-labeled digestion product. Amino acid analysis of the latter, after acid hydrolysis and performic acid oxidation, produced only cysteic acid. The hydrolyzed, but unoxidized, sample had an elution time different from that of cysteine or cystine. The ^{203}Hg-labeled, Sepharose-bound fraction showed a higher R_f value than either cysteine or cystine but a value less than that of methionine. It had a mobility similar to that of methylmercuric cysteine prepared in vitro. It was suggested that the fraction separated from egg ovalbumin contained methylmercuric cysteine and that the binding of the ^{203}Hg to the cysteine probably involved the SH group.

The interactions of mercurials with proteins usually involve the SH and S–S groups, and such interactions have been linked to the toxic effects of mercury (Hg) (Vallee and Ulmer, 1972; Webb, 1966). Despite much research during the past 20 years on sulfur–mercury interactions, the relationship between this chemical reaction and toxicological observations is yet to be explained.

Many studies on Hg-binding sites of proteins have utilized albumins as model systems. Binding of the mercuric ion (Hg^{2+}) to albumins has been demonstrated in vitro, and the reactive groups of the protein were the SH groups, as in mercaptalbumin (Hughes and Dintzis, 1964), or the COOH group, as in human serum albumin (Perkins, 1961).

The formation of methylmercuric cysteine in biological systems has been reported. A study of the accumulation of methylmercury has shown that ~95% of the muscle methylmercury was in the form of the methylmercury–cysteinyl complex (Westoo, 1966). After the injection of ^{203}Hg-labeled methylmercuric chloride ($CH_3{}^{203}HgCl$) in rats, methylmercury cysteine was found in the bile (Norseth and Clarkson, 1971).

It has been reported that, when methylmercuric chloride was administered to hens, the ovalbumin contained 97% of the egg white ^{203}Hg (Magat and Sell, 1979). The data presented in this paper deal with the chemical nature of

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the ^{203}Hg binding to the ovalbumin of the egg white after $CH_3{}^{203}HgCl$ was given to laying hens. The high affinity of organomercurials, in general, for SH and S–S groups enabled the separation of a ^{203}Hg-labeled amino acid residue of digested ovalbumin from nonmercurated compounds by chromatography on a disulfide-containing resin. A possible binding site of ^{203}Hg to ovalbumin is suggested.

EXPERIMENTAL SECTION

Apparatus. A γ counter (Model 1197, Tracor Analytic) with a thallium-activated NaI crystal was used for the measurement of ^{203}Hg radioactivity. The column chromatographic analysis setup included the following: a spectrophotometer (Beckman DU) equipped with a flow-through cell attachment (Model 203-A, Gilford), an automatic fraction collector (Model FC220-K, Gilson), a three-channel peristaltic pump (Model P-3, Pharmacia), and a recorder (Model 481, Pharmacia). The fractions were concentrated by a column eluate concentrator (Model CEC-1, Amicon) with a PM-10 membrane (Amicon). The amino acid composition was determined with a Durrum D-400 amino acid analyzer.

Reagents. $CH_3{}^{203}HgCl$ in 0.02 M Na_2CO_3, obtained from New England Nuclear, had a specific activity of 1.68 mCi/mg. Pronase (protease from *Streptomyces griseus*) and carboxymethylcellulose (7 mequiv/g) were both purchased from Sigma Chemical Co. Thiopropyl-Sepharose 6B (hydroxypropyl 2-pyridyl disulfide derivative) was a product of Pharmacia Fine Chemicals. 2,2′-Dipyridyl disulfide (98% purity) was obtained from Aldrich Chemical Co. The amino acid standards cysteine, cystine, and methionine (98% purity) were purchased from Nutritional Biochemicals, and homocysteine (90% purity) was obtained from Sigma Chemical Co.

Procedure. *Administration of $CH_3{}^{203}HgCl$ and Collection of Egg Whites.* Four White Leghorn hens were kept in individual cages and were maintained on a basal diet (Sell, 1977) for 1 week preceding administration of radioactive Hg. $CH_3{}^{203}HgCl$ (24 μCi) was given once per hen by injection into the breast muscle. The eggs were collected daily, and the egg whites were counted for radioactivity. The egg whites with the highest ^{203}Hg activity were used for fractionation of the egg white proteins.

Radioactivity Measurements. All ^{203}Hg activity measurements were performed on 1-mL samples of egg whites or column fractions by using the γ counter described in the preceding section.

Fractionation of Egg White Proteins. Column chromatography on carboxymethylcellulose, as described previously (Magat and Sell, 1979), was employed to isolate the major proteins of the egg white.

Enzymic Digestion of Ovalbumin. The ovalbumin fractions obtained from the egg white protein fractionation were concentrated by using a column eluate concentrator. Then, the ovalbumin was diluted to a concentration of 0.5% with 0.1 M Tris-HCl, pH 8. The method of enzymic digestion of ovalbumin was that of Yamashina and Mikuno (1962). Pronase, in the ratio of 50:1 (ovalbumin:enzyme), and 0.1 M calcium acetate were added. A few drops of toluene were placed on top of the reaction mixture to prevent bacterial growth. Incubation was at 37 °C for 2 days.

Separation of ^{203}Hg-Labeled Amino Acid from Digested Ovalbumin. Thiopropyl-Sepharose 6B contains a disulfide with 2-thiopyridyl protecting groups (2-pyridyl-S-S-propyl-Sepharose or 2-Py-S-S-Sepharose). Characteristics and use of this gel are described elsewhere (Pharmacia Fine Chemicals Brochure, 1977). The gel was prepared as follows. Eight grams of freeze-dried thiopropyl-Sepharose 6B were allowed to swell in 0.1 M Tris-HCl, pH 7.5, containing 0.3 M KCl and 1 mM EDTA (Carlsson and Svenson, 1974) at room temperature. The gel was washed with several volumes of buffer (200 mL/g of freeze-dried powder) and was then suspended in a 5-fold volume of buffer. The pH of the digested ovalbumin was adjusted to 7.5, and the digest was added to the Sepharose suspension. Nitrogen was bubbled through the mixture for ~30 min to purge air from the sample The mixture was stirred gently overnight at room temperature to facilitate the coupling reaction.

Covalent chromatography consisted of the elution of unbound materials from the Sepharose, followed by removal of the bound material by a reducing agent. The coupling mixture was poured into a column (2 × 7 cm) and eluted with 0.1 M Tris-HCl, pH 7.5 (containing 0.3 M KCl and 1 mM EDTA). Absorbance of the eluate was monitored at 280 nm. The flow rate was maintained at 12 mL/h and fractions were collected every 10 min. All fractions were counted for ^{203}Hg activity and were tested with a modified ninhydrin reagent according to the photometric method of Moore and Stein (1954). Elution was continued until A_{280nm} was back to the base-line value, and the eluates were ninhydrin negative and had no detectable radioactivity. The fractions corresponding to the protein peak were pooled for analysis. The gel was washed with 1.0 M NaCl and then with 0.2 M NH_4Ac, pH 8.6 (Egorov et al., 1975). The removal of any covalently attached compound from the Sepharose column was done by elution with 0.02 M mercaptoethanol solution in 0.2 M NH_4Ac, pH 8.6. Measurement of absorbance of the eluates at 280 nm was continued. Fractions of the previously covalently bound material were collected and were measured for radioactivity and characterized as described in the preceding section. Radioactive and (or) ninhydrin-positive fractions were pooled for further identification.

The gel was regenerated after each use by reacting with 2,2′-dipyridyl disulfide, 1.5 M in 0.1 M Tris-HCl containing 0.3 M KCl and 1 mM EDTA, as described by Brocklehurst et al. (1973).

Characterization of the Column Chromatography Fractions. (1) Paper Chromatographic Analysis. The individual fractions obtained during chromatography were applied on paper (Whatman No. 1) and developed in the buffer system butanol–pyridine–water (1:1:1) (Nigam and McConnell, 1969; Shrift and Virupaksha, 1965). After development, the papers were sprayed with 0.2% ninhydrin in 95% ethanol. Spots produced by the samples were compared with those of the standards (cysteine and methionine) and were cut out of the paper chromatogram for measurement of ^{203}Hg activity. The pooled fractions from both the Tris-HCl and mercaptoethanol elutions also were analyzed.

(2) Amino Acid Analysis. The pooled fractions containing material that had been attached previously to the Sepharose and had ^{203}Hg activity were analyzed for amino acid composition. All samples obtained from four different ovalbumin fractions representing different egg whites were tested. These samples were first hydrolyzed with constant-boiling HCl containing 0.1% phenol at 110 °C for 22 h. The hydrolysates were then oxidized with 70% formic acid and 30% H_2O_2 according to the procedure described by Moore (1963). The products were analyzed for amino acid composition by a Durrum D-400 amino acid analyzer.

Preparation of Methylmercuric Cysteine in Vitro and Covalent Chromatography on 2-Py-S-S-Sepharose. This procedure was employed to determine whether methyl-

Table I. Proportion of the ^{203}Hg Activity of Egg White Present in Ovalbumin

	^{203}Hg, dpm		
egg	egg white[a]	ovalbumin[b]	% recovery
A	436 713	418 664	95.9
B	488 986	471 917	96.5
C	423 435	372 917	88.1
D	385 971	380 722	98.6

[a] Radioactivity of egg whites before fractionation by chromatography on carboxymethylcellulose. [b] Total radioactivity of ovalbumin fraction after carboxymethylcellulose chromatography.

mercuric cysteine prepared by direct reaction of CH_3-$^{203}HgCl$ with cysteine would result in a compound exhibiting characteristics similar to those of the material isolated from digested ovalbumin. Five trials were run. For each trial, cysteine was dissolved in 0.1 M NaAc, pH 6, to a final concentration of 0.02 M (Dale et al., 1975). A measured volume of $CH_3{}^{203}HgCl$ (equivalent to 1.8–2.0 μmol) of known radioactivity (Table III) was added and the mixture allowed to react in a 50 °C water bath with agitation (Dale et al., 1975). After cooling to room temperature, the mixture was added to the prepared Sepharose suspension and chromatographed as described earlier for digested ovalbumin. All fractions eluted from the column during chromatography were measured for radioactivity and were analyzed by paper chromatography and were compared with the radioactive Sepharose-bound material isolated from ovalbumin.

Preparation of Mercuric Cysteine in Vitro. The same procedure was followed as that described for the preparation of methylmercuric cysteine, except $HgCl_2$ solution (0.04 M final concentration) was substituted for CH_3-$^{203}HgCl$. The reaction mixture was filtered to remove precipitates that formed during the reaction, and the filtrate was analyzed by paper chromatography as previously described for the material isolated from ovalbumin. The R_f was compared with those of the thiopropyl-Sepharose-bound material obtained from ovalbumin and the standards, cysteine, homocysteine, and methionine.

RESULTS AND DISCUSSION

The ^{203}Hg activities of egg whites were greatest 3–4 days after administration of the $CH_3{}^{203}HgCl$ dose to hens. Therefore, egg white proteins of these eggs were fractionated, and ovalbumin fractions were isolated. The radioactivities of the egg whites and the corresponding ovalbumins are shown in Table I. Between 88 and 99% of the ^{203}Hg in the egg whites was present in the ovalbumin. The latter contained 58% of the total egg white protein, as determined previously (Magat and Sell, 1979).

To determine the binding site of ^{203}Hg to the ovalbumin molecule, it was necessary to digest the ovalbumin and thus obtain ^{203}Hg-labeled amino acid(s) or peptides, which could be separated from the other digestion products. Enzymic digestion with Pronase was employed instead of acid hydrolysis to reduce the potential loss of ^{203}Hg. Pronase, a protease from *S. griseus*, has been used successfully for the digestion of ovalbumin (Bogdanov et al., 1964; Narita and Ishii, 1962; Yamashina and Mikuno, 1962).

After digestion, the hydrolysate was blended with 2-Py-S-S-Sepharose for the coupling reaction. Subsequent column chromatography was used to separate compounds with thiol and (or) Hg groups from other substances in the digest. Unbound materials were eluted from the Sepharose with Tris-HCl buffer as shown by the 280-nm peak obtained (Figure 1) as well as by the analysis of the fractions

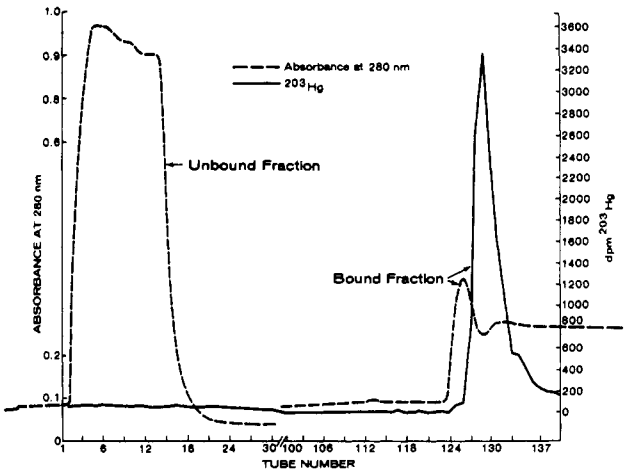

Figure 1. Covalent chromatography of enzyme-digested ovalbumin on 2-Py-S-S-Sepharose; tube no. 1–99 = unbound fractions, and tube no. 100–140 = bound fractions. The column measured 2 × 7 cm and the fraction size was 2.0 mL. Eluting buffers were as follows: Tris-HCl, pH 7.5, containing 0.3 M KCl and 1 mM EDTA (tube no. 1–99); 0.02 M mercaptoethanol in 0.2 M NH_4Ac, pH 8.6 (tube no. 100–140). The flow rate was maintained at 12 mL/h.

Table II. Covalent Attachment of ^{203}Hg-Labeled, Enzyme-Digested Ovalbumin to 2-Py-S-S-Sepharose

	^{203}Hg, dpm		
egg	ovalbumin[a]	bound fraction[b]	% binding
A	92 751	92 006	99.2
B	92 905	68 024	73.2
C	118 361	106 601	90.1
D	75 657	63 071	83.4

[a] Radioactivity of ovalbumin before enzymatic digestion. [b] Total radioactivity of pooled fraction bound to 2-Py-S-S-Sepharose.

with ninhydrin reagent. Measurement of the radioactivities of all fractions showed no appreciable amount of ^{203}Hg activity associated with the unbound fractions (Figure 1 and Table II). During the detachment of bound materials with mercaptoethanol solution, a small peak appeared (Figure 1). This may have been due to thiopyridone released during the disulfide exchange reaction with 2-Py-S-S-Sepharose. Thiopyridone absorbs at both 280 and 343 nm. The bound fractions removed by mercaptoethanol contained most of the radioactivity, amounting to 73–99% of the total ^{203}Hg activity applied (Figure 1 and Table II).

Paper chromatographic analysis of the unbound fractions showed several ninhydrin-positive components (Figure 2), but the fractions previously bound to Sepharose produced only two spots on paper, one of which was identical with that of the cysteine standard (Figure 2). The darker of the two spots exhibited a greater mobility than the cysteine standard but less than that of methionine. Identical results were obtained with Sepharose-bound fractions from four different ovalbumins. Radioactivity measurements of the paper chromatograms corresponding to the spots and the origin have shown that the ^{203}Hg activity was concentrated in the spot exhibiting the higher R_f value while the origin contained an average of ~22% of the total radioactivity.

Further identification of the ^{203}Hg-labeled, Sepharose-bound fractions was done by amino acid analysis. After acid hydrolysis and oxidation of the samples, cysteic acid was the only amino acid derivative that appeared (Figure 3a,b). Because cysteine and cystine are both oxidized to

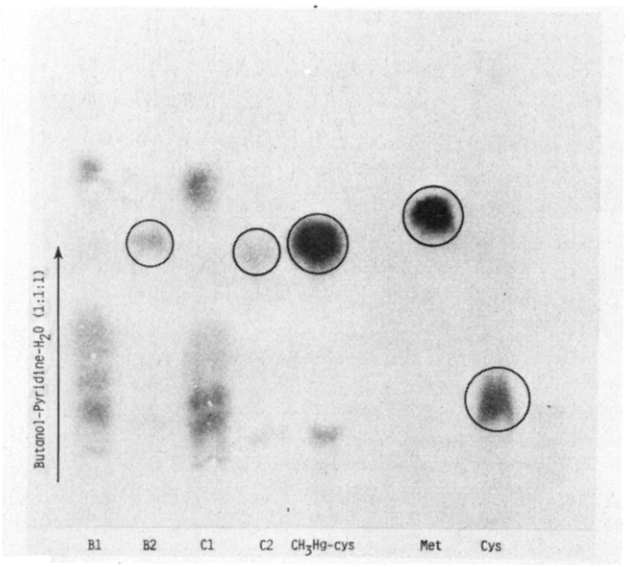

Figure 2. Paper chromatography of fractions from covalent chromatography of digested albumins. Paper was stained with 0.2% ninhydrin in 95% ethanol. B_1 and C_1 are unbound peak fractions eluted with Tris-HCl. B_2 and C_2 are bound peak fractions eluted with mercaptoethanol. CH_3Hg-cys is the bound fraction from the in vitro preparation of methylmercuric cysteine. Standards are cysteine (Cys) and methionine (Met). Buffer system: butanol–pyridine–H_2O (1:1:1). Important chromatographic spots have been circled so that their positions are clearly shown.

cysteic acid, analysis was also performed on the hydrolyzed, but unoxidized, sample. This showed the presence of a compound that eluted at 0.73 h (Figure 3c). In comparison, cysteine (not shown herein) eluted at 0.64 h, and cystine eluted at 0.82 h (Figure 3b). The elution times for cysteine and homocysteine, as determined in a separate analysis, were 0.64 and 0.99 h, respectively. The material eluting at 0.73 h (Figure 3c) probably was a mercury–amino acid complex, either methylmercuric cysteine or mercuric cysteine.

The presence of the mercury–cysteine complex, either as methylmercuric cysteine or as mercuric cysteine in the ^{203}Hg-labeled, Sepharose-bound fractions was, therefore, indicated by amino acid analysis and paper chromatography. The binding of Hg^{2+} to cysteine could have involved the SH group of the amino acid, in which case the Hg^{2+} would be lost during performic acid oxidation before amino acid analysis. This hypothesis is supported by the finding that amino acid analysis of the hydrolyzed and oxidized samples from ovalbumin produced only cysteic acid.

Because the form of Hg administered to the hens was CH_3HgCl and because the C–Hg bond is known to be relatively stable, the mercury–amino acid complex present in the fraction isolated from ovalbumin would likely contain the CH_3Hg group bound to the amino acid (methylmercuric cysteine). On the other hand, the presence of the compound, mercuric cysteine, would be possible if a cleavage of the C–Hg bond did occur either during the incorporation of the Hg into the amino acid molecule in the in vivo synthesis of ovalbumin or during the coupling reaction with 2-Py-S-S-Sepharose of the digested ovalbumin in the present experiment. Cleavage of the C–Hg bond has been demonstrated to occur to some extent in blood as well as in the cerebrum, liver, and kidney of rats fed methyl (^{203}Hg) chloride (Garcia et al., 1974a,b).

Both methylmercuric cysteine and mercuric cysteine were prepared in vitro as described earlier. Cysteine was

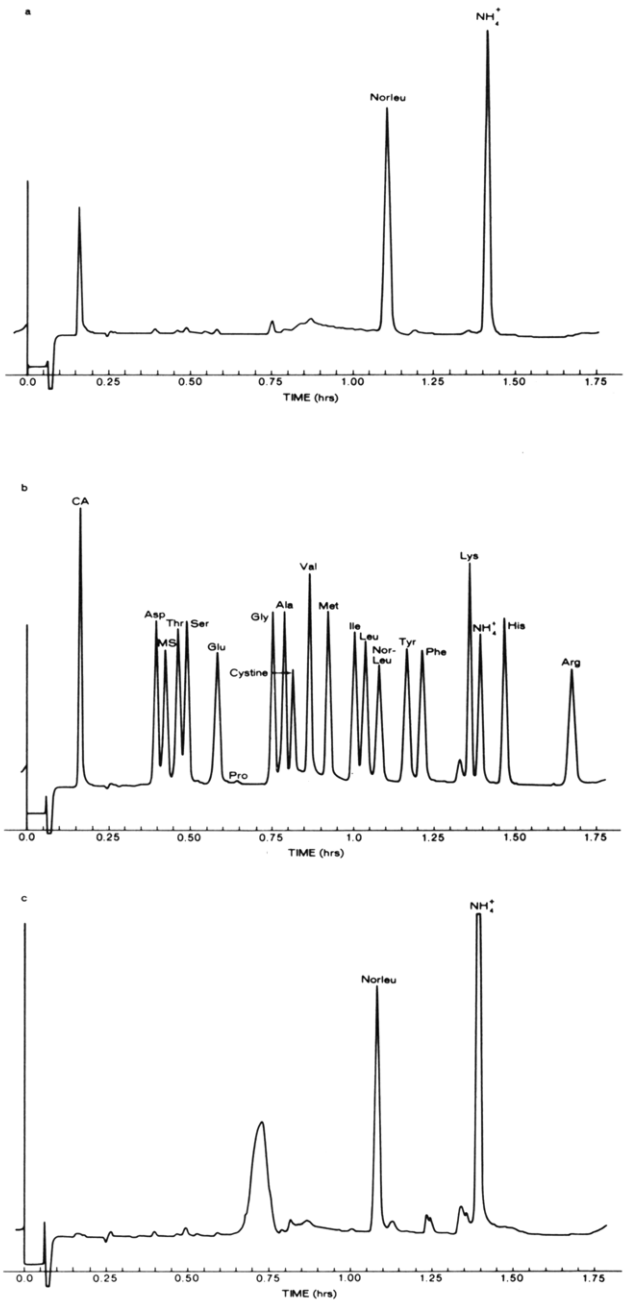

Figure 3. Amino acid analysis patterns. Part a shows the Sepharose-bound fraction from enzymically digested ovalbumin, after acid hydrolysis and oxidation. Part b represents the elution pattern of the amino acid standards. Part c shows the Sepharose-bound fraction from enzymically digested ovalbumin, after acid hydrolysis (standard = norleucine).

reacted with $CH_3{}^{203}HgCl$ and chromatographed on 2-Py-S-S-Sepharose as was performed with the ovalbumin hydrolysates. The Sepharose-bound fraction produced a single peak corresponding to that produced by the bound fraction from ovalbumin (Figure 1). The column fractions were then chromatographed on paper. It was shown that the Sepharose-bound fractions contained only ~15% of the applied radioactivity (Table III) and produced a pattern on paper chromatography similar to those of the Sepharose-bound fractions from egg ovalbumins (Figure 4). The main constituent seemed to be unreacted cysteine. The other component had a mobility greater than that of cysteine and seemed identical with the ovalbumin component suggested to be the mercury–cysteine complex (Figures 2 and 4).

Table III. Covalent Attachment of Mercurated Cysteine to 2-Py-S-S-Sepharose

trial	^{203}Hg, dpm		
	$CH_3{}^{203}HgCl$[a]	unbound fractions[b]	bound fractions[c]
A	1 146 205	856 831	148 874
B	1 118 848	885 158	110 293
C	1 008 020	846 903	45 395
D	1 146 205	990 779	127 344
E	1 146 205	942 878	86 113

[a] Radioactivity of $CH_3{}^{203}HgCl$ added to cysteine. [b] Total radioactivity of pooled Tris-HCl fractions. [c] Total radioactivity of pooled mercaptoethanol fractions.

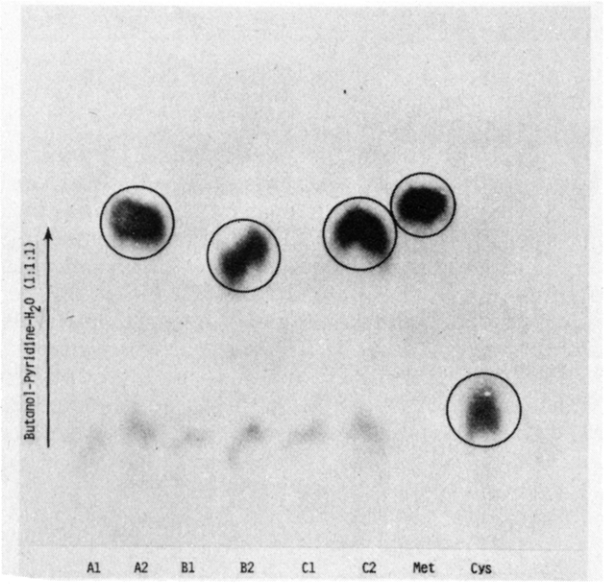

Figure 4. Paper chromatography of fractions from covalent chromatography of methylmercuric cysteine on 2-Py-S-S-Sepharose. A_1, B_1, and C_1 are unbound peak fractions eluted with Tris-HCl. A_2, B_2, and C_2 are bound peak fractions eluted with mercaptoethanol. Standards are cysteine (Cys) and methionine (Met). Buffer system: butanol–pyridine–H_2O (1:1:1).

Mercuric cysteine was prepared in vitro by using $HgCl_2$ as previously described. On paper chromatography, the ninhydrin-positive spots produced (Figure 5) showed the presence of unreacted cysteine as well as two other components with mobility greater than that of cysteine and with R_f's close in value to homocysteine and less than those obtained with methylmercuric cysteine (Figure 4) and the egg ovalbumin fraction (Figure 5, A_2, and Figure 2).

These results demonstrate that the methylmercury present in ovalbumin is bound primarily to the amino acid cysteine. The formation of mercuric cysteine or methylmercuric–cysteine complexes has been reported for other biological materials such as muscle (Westoo, 1966) and bile (Norseth and Clarkson, 1971). The observation that methylmercury in ovalbumin is bound in the same manner as in other biological materials raises the question "Why should methylmercury bind preferentially to ovalbumin (Magat and Sell, 1979)"? The total number of S atoms per unit of ovalbumin is not greatly different from that of other proteins of egg white (Gilbert, 1971). Also, the presence of a relatively high concentration of methionine in ovalbumin is not a satisfactory explanation for the preferential binding because the research reported here showed that the methylmercury occurred in the form of methylmercuric cysteine. Additional research will be required to obtain definitive information about this facet of methylmercury metabolism.

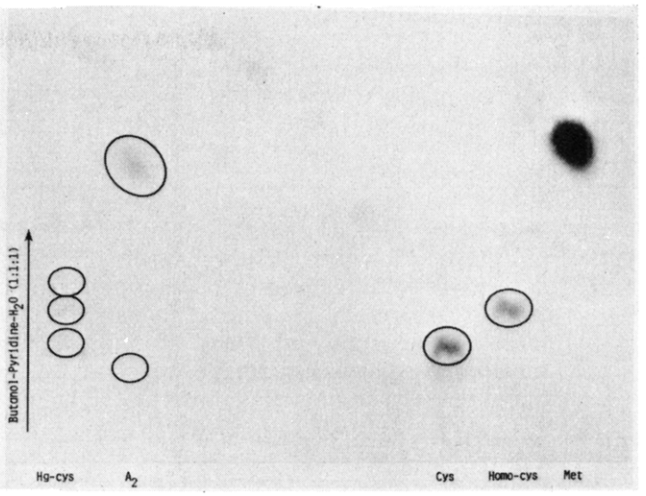

Figure 5. Paper chromatography of mercuric cysteine prepared in vitro. Hg-cys is mercuric cysteine; A_2 is the bound fraction from digested ovalbumin. Standards are cysteine (Cys), homocysteine (Homo-cys), and methionine (Met). Buffer system: butanol–pyridine–H_2O (1:1:1).

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