On the Properties of a New Thiol Reagent:

6-Chloromercuri-2-nitrophenol *

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6-Chloromercuri-2-nitrophenol (CMNP) has been prepared from mercury(II) acetate and o-nitrophenol by an improved method.

CMNP was found to react rapidly and quantitatively above pH *ca.* 3.5 with all true and with some potential thiols investigated (giving MNP-thiols). The effect of reaction time, pH and NaCl concentration on this reaction has been studied.

A method is described for quantitative determination of thiols insoluble in toluene or in 1,2-dichloroethane. Concentrations down to the order of 10 μM can thus be determined.

CMNP was found to be a specific reagent for true and potential thiols at pH 4, the only exception found being the cyanide ion.

Mixtures of MNP-thiols have been separated by paper chromatography. R_F-values for some thiols of biochemical interest are given in 4 solvent systems.

A "ghost" spot on the chromatograms was identified as 2,2'-dihydroxy-3,3'-dinitro-diphenylmercury.

Organic mercury compounds, such as the now classical *p*-chloromercuri-benzoic acid [1], are among the reagents employed for thiols. As such they have the advantage of being relatively specific and fast reacting.

In this laboratory the study of a few mercury derivatives of nitrophenols was undertaken. These compounds have the advantage of being colored and their reaction with thiols can therefore be conveniently studied colorimetrically.

The substances investigated here were 4-acetoxymercuri-2-nitroresorcinol [2], 4-chloromercuri-2-nitrophenol [2], and 6-chloromercuri-2-nitrophenol [3]. Of these, 6-chloromercuri-2-nitrophenol was found to react rapidly and quantitatively with thiols above pH *ca.* 3.5. This reaction (Fig. 1) was studied in detail.

The results show that at pH 4, 6-chloromercuri-2-nitrophenol is a highly specific reagent for thiols.

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* Some preliminary investigations for this work were carried out at the Biochemical department of the Nobel Medical Institute, Stockholm, Sweden.
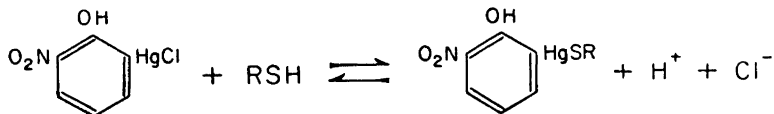
Fig. 1. The reaction between CMNP and thiols.

The reaction products of 6-chloromercuri-2-nitrophenol and various thiols have been separated by paper chromatography. The fact that these thiol derivatives are highly colored renders them especially suitable for chromatographic analysis.

The advantages of 6-chloromercuri-2-nitrophenol over some previously studied colored mercury-containing thiol reagents [4-6] (which all were azo compounds) are: a) the new reagent is easy to prepare in satisfactory yields; b) it can be used as a reagent in chromatography of thiols.

An improved method for the synthesis of 6-chloromercuri-2-nitrophenol is also reported.

MATERIALS AND METHODS

The thiols used in these investigations were from Mann Laboratories, Nutritional Biochemicals Corporation, and Eastman Company. *o*-Nitrophenol (m. p. 44—46°) was obtained from Eastman Company (samples from some other sources were less pure and gave lower yields of 6-chloromercuri-2-nitrophenol). Mercury (II) acetate was of analytical purity obtained from Merck Company. All solvents used in chromatography and elsewhere, as well as other chemicals, were of highest commercially obtainable purity. They were used without further purification.

Buffers used (unless otherwise stated):

pH 1.1— 6.6   0.1 M citrate
   7.2— 9.0   0.1 M tris(hydroxymethyl)aminomethane
   9.0—12   0.1 M glycine

Unless otherwise stated, all experiments were carried out at room temperature (25°C). All melting points (°C, Kofler method) are corrected. Spectrophotometric measurements were performed in 1 cm cuvettes with a Beckman Model DU spectrophotometer.

Abbreviations used:

CMNP = 6-chloromercuri-2-nitrophenol
MNP-thiol = S-(2-hydroxy-3-nitrophenyl mercuri)-thiol
EDTA = (ethylenedinitrilo) tetraacetic acid
ε_λ = molar extinction coefficient at λ mμ (1 cm cuvette)

Synthesis of 6-chloromercuri-2-nitrophenol.

The mercuration of nitrophenols with mercury (II) acetate generally proceeds easily, but the product obtained practically always seems to consist of a mixture of all theoretically likely monomercury derivatives plus considerable amounts of dimercuri derivatives [2,3,7].

Hodgson [3] synthesized 6-acetoxymercuri-2-nitrophenol by fusing mercury (II) acetate with a large excess of *o*-nitrophenol. However, the yield is poor.

Contrary to Hodgson's statement [3] that 4-acetoxymercuri-2-nitrophenol is preferentially formed by boiling mercury(II) acetate and o-nitrophenol in solvent media, we found that when 1-butanol, 1-propanol or methanol were employed as solvents, the 6-acetoxymercuri derivative could be isolated from the reaction product in fairly good yields. However, employing high boiling solvents like 1-butanol caused large amounts of the dimercury derivative to form, and low boiling solvents like methanol gave poor yields. In boiling 1-propanol, however, the reaction proceeded rapidly and the yield of the 6-acetoxymercuri derivative was satisfactory. As has been pointed out by previous investigators [2,3] the 6- resp. 4-acetoxymercuri-2-nitrophenols are difficult to obtain in a pure form, mainly because the dimercuri derivative, which is formed simultaneously, shows somewhat similar solubility properties as the monomercuri derivatives. We found it advantageous to convert the resulting mixture of acetoxymercuri-2-nitrophenols into their chloride forms prior to purification. CMNP could then be preferentially extracted from the crude product with acetone. Another advantage of working with the chloride is that it has a conveniently located melting point (190°) and its purity could thus be checked easily. The acetate does not melt below 300°.

It should be pointed out, however, that there is no substantial difference between the chloride and the acetate in regard to their reactivity toward thiol groups.

Procedure of preparation. To a refluxed solution of 15.0 g (0.108 mole) of o-nitrophenol in 50 ml 1-propanol is added 34.5 g (0.108 mole) of mercury (II) acetate in small portions during one hour. The mixture is refluxed for another hour, poured onto 200 g of cracked ice and filtered when ice cold. The yellow precipitate is washed with 50 ml of distilled water and with 25 ml of 95 % ethanol. To convert the substances into their chloride forms they are vigorously stirred for 15 min with 100 ml of a saturated solution of sodium chloride in water. The suspension is then filtered and washed free of chloride ions with distilled water and dried at room temperature in the dark.

The dry, yellow substance obtained (31 − 34 g) is refluxed for 15 min with 200 ml of acetone and filtered. The undissolved residue is extracted for another 15 min with 100 ml of acetone and filtered. The pooled acetone extracts are evaporated to dryness in a stream of air at room temperature. 18 − 20 g (yield 45 − 50 %) of relatively pure CMNP (melting about 185°) is thus obtained. After two recrystallizations from 95 % ethanol it melts at 190°. (Upon storage the melting point tends to drop somewhat). Further recrystallizations do not change the melting point. (Hodgson [3] reports 185°.) The product was found to be fairly soluble in most organic solvents tested, especially in acetone, ethyl acetate and in chlorinated hydrocarbons. CMNP should be stored in a dark, dry and cool place.

Mercury determinations. 0.3 g of CMNP in 50 ml distilled water were mixed with 3 g of potassium iodide and with 1 g of iodine and carefully heated with occasional stirring until a clear solution resulted. Mercury was then determined as HgS in this solution according to Ref.[8] (Found: Hg 53.48, 53.55. Calc. for $C_6H_4ClHgNO_3$ (374.17): Hg 53.61).

Position of the mercury *. 0.15 g of CMNP (0.40 mmole), 0.10 g of iodine (0.40 mmole) and 0.5 g of potassium iodide in 10 ml of distilled water were carefully heated with occasional stirring until a clear solution resulted. The solution was cooled to room temperature, acidified with a few drops of 5 % hydrochloric acid and shaken with 10 ml of ethyl acetate. The ethyl acetate phase was separated and evaporated to dryness and the resulting crystals (0.10 g) were recrystallized once from 50 % ethanol. The golden yellow crystals obtained melted at 109° as reported for 6-iodo-2-nitrophenol [3,9]. No other mono-iodo-2-nitrophenol has a melting point close to the one found [10,12].

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* A similar technique has been used before [3,7] but no details were given.

It has been presumed that the iodine occupies the same position in the molecule as the mercury it replaces. According to the results of Henry and Sharp [13] this is not always true when halogens (e. g. bromine) react with a mercury atom attached to a benzene ring. These authors found, however, that iodine treatment gave the expected derivative in all cases studied.

Furthermore, 4-chloromercuri-2-nitrophenol as prepared by Hodgson [3], has a different melting point (205°) from the one found for CMNP. It seems therefore safe to presume that the mercury in CMNP occupies the 6 position.

The acetone insoluble fraction from the synthesis was treated in a similar manner and after two recrystallizations from 50 % ethanol the product melted at 96°, possibly indicating it to be 4,6-diiodo-2-nitrophenol (m. p. 98°) [11]. However, 5-iodo-2-nitrophenol has a similar melting point (96°) [12]. Mercury determinations performed on the acetone insoluble product were inconsistent, but indicated the presence of two mercury atoms per molecule of o-nitrophenol.

The acetone insoluble fraction was then treated with potassium bromide and bromine in an analogous manner to the one described above. After two recrystallizations from concentrated acetic acid the brominated compound melted at 119°, close to that reported for 4,6-dibromo-2-nitrophenol (120—121°) [13].

Thus the acetone insoluble fraction mainly consists of 4,6-di(chloromercuri)- 2-nitrophenol.

Absorption spectra of CMNP. Fig. 2 shows the absorption spectra of CMNP at pH 1.6 and at pH 9.1.

For quantitative measurements the peak at 420 mμ shown only in basic solutions was found convenient to use. From Fig. 3 it is seen that the pH-optimum for absorption at this wavelength is between pH 9—10 ($\varepsilon_{420} = (5.28 \pm 0.02) \times 10^3$ at pH 9.5).
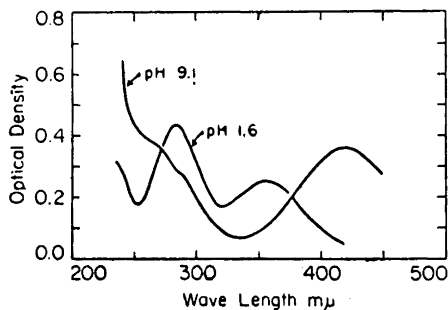

*Fig. 2.* Absorption spectra of CMNP (66.8 μM) at pH 1.6 and at pH 9.1.

In this pH-range the absorption follows Beer's law.

The absorption spectra of MNP-thiols were found to be almost identical with those presented in Fig. 2, provided the thiol itself does not absorb in this region. MNP-cysteine was found to have the same molar extinction coefficient as CMNP at pH 9.5 (420 mμ). That of MNP-glutathione is practically the same ($\varepsilon_{425} = (5.22 \pm 0.03) \times 10^3$) at this pH, but the absorption maximum was shifted somewhat towards longer wavelengths (425 mμ).

The color at 420 mμ (pH 9.5) was found to be stable within reasonable time limits (Table 1).

Table 1. Color stability of MNP-cysteine (85.8 μM) at pH 9.5.

Time minutes	Optical density at 420 mμ
0	0.452
60	0.453
270	0.452
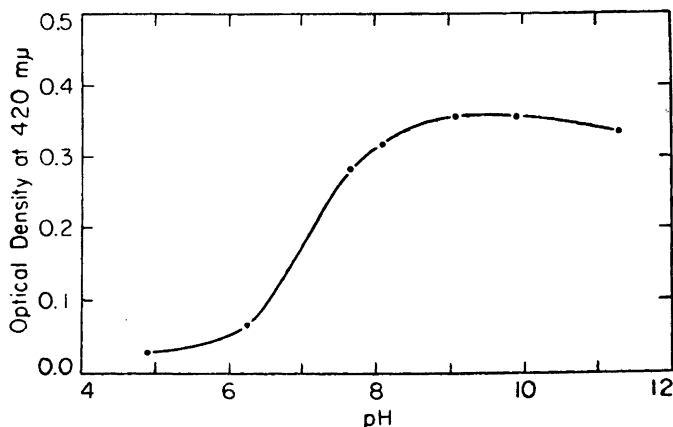
Fig. 3. Effect of pH on absorption peak at 420 mμ. Concentration of CMNP 66.8 μM.
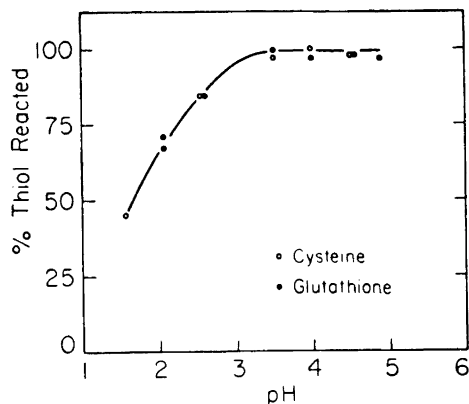
THE REACTION BETWEEN CMNP AND THIOLS

When a thiol dissolved in a buffer of about pH 4 is shaken with a solution of CMNP in an organic solvent, *e.g.* toluene, a yellow color or precipitate generally appears in the water phase. The reaction taking place is described in Fig. 1.

Effect of pH. Cysteine and glutathione dissolved in buffers of varying pH were mechanically shaken with a solution of CMNP in toluene and the decrease in optical density at 355 mμ (the absorption at this wavelength follows Beer's law) in the toluene layer was followed. Simultaneously, blanks containing only the corresponding buffers and reagent in toluene were shaken at the same rate. The difference in optical density between the two toluene layers after completed reaction was taken as a measure of the amount of reacted thiol. From the molar extinction coefficient for CMNP in toluene ($\varepsilon_{355} = (3.91 \pm 0.02) \times 10^3$) it was thus possible to calculate the amount of reacted thiol.

As is seen from Fig. 4 both glutathione and cysteine react quantitatively (97—100 %) under the conditions employed. The difference in structure between glutathione and cysteine apparently does not have a significant influence on the pH dependence of the reaction.

Above pH 5 the reagent becomes very water soluble (Table 2) and the reaction becomes difficult to follow with the described technique. However,

Fig. 4. Effect of pH on the reaction between cysteine, glutathione and CMNP. 6 ml 0.3 mM solutions of thiols in buffers of varying pH (blanks: 6 ml of corresponding buffer) were shaken for 1 minute at the rate of 300 cycles/min with 6 ml 0.03 % CMNP in toluene. For calculation of data see text.

it can be shown by paper chromatography that the thiols investigated also react with the reagent in the pH range 5—12.

Table 2. Distribution of CMNP between buffers and 1,2-dichloroethane. Data were obtained by shaking 0.5 % solutions of CMNP in 1,2-dichloroethane for 10 min at the rate of 300 cycles/minute with an equal volume of buffer of indicated pH. The concentration of CMNP in the water phase was calculated from the optical density at 420 mμ of the water phase after adjustment to pH 9.5. It should be noted that the distribution of CMNP between the phases is much affected by the organic solvent used.

pH	Concentration of CMNP in water phase (μM)
1.56	20
2.59	20
3.52	19
4.00	24
4.90	259

Effect of shaking time. As is shown in Fig. 5 the reaction between gluta-thione, cysteine and the reagent is completed within one minute under the experimental conditions used. Prolonged shaking increases the optical density in the toluene layer (*i.e.* decreases the difference in readings between blank and sample) thus showing a slight decomposition of the reaction product. This decomposition could be almost completely prevented by the addition of excess cyanide (see Fig. 5), but not by EDTA in similar concentrations.

It thus appears likely that cyanide does not act mainly by binding traces of heavy metals present (which catalyze the autooxidation of thiols) but by forming a cyanide complex with the MNP-thiol. This view is supported by the fact that cyanide, but not EDTA, reacts with CMNP itself under identical
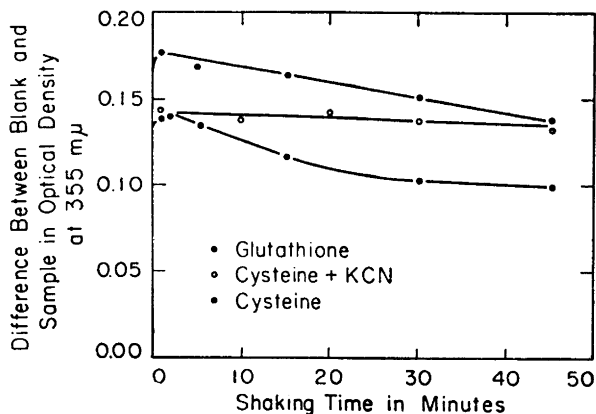
Fig. 5. Effect of shaking time on the reaction between cysteine, glutathione and CMNP. 6 ml thiol of pH 4.0 (blanks: 6 ml buffer of pH 4.0) were shaken at the rate of 300 cycles/min with 6 ml of 0.03 % CMNP in toluene. Optical density of 0.5 ml toluene phase + 3.0 ml of toluene was followed. Concentrations: Cysteine 0.25 mM; glutathione 0.32 mM. KCN in cysteine solution and in corresponding blank: 1.7 mM.

conditions. A possible degradation product is 2,2'-dihydroxy-3,3'-dinitro-diphenylmercury (see later paragraphs).

In basic solutions, however, MNP-thiols seem to be much less subject to decomposition, which can be shown by paper chromatography.

Reversibility of the reaction. By shaking a suspension of a MNP-thiol, such as MNP-glutathione, with an acid of about pH 1 the MNP-thiol is completely decomposed and all the color of the original solution can be taken up in *e.g.*, 1,2-dichloroethane. Analysis of the yellow dissociation product by paper chromatography after treatment of MNP-thiols with 0.1 M hydrochloric acid showed it to consist of CMNP contaminated with a small amount of 2,2'-dihydroxy-3,3'-dinitro-diphenylmercury (see later paragraphs about this compound).

Quantitative measurements. The results of the above experiments suggested that CMNP can be used as a quantitative reagent for thiols. Fig. 6 shows the results of the quantitative determination of cysteine.

The reaction was carried out by shaking cysteine solutions of pH 4.0 with a reagent solution in toluene, and the amount of reacted thiol was determined by measuring the decrease in optical density at 355 mμ in the toluene layer. In each instance 98—100 % reaction was obtained as calculated from ε_{355} for CMNP in toluene. The addition of cyanide to the reaction mixture gave little, if any, improvement when the shaking time was kept short. Furthermore, large excess of cyanide gave low readings.

When very small amounts of thiol were present higher accuracy was achieved by measuring the optical density of the water phase after completed shaking with CMNP. In such cases it is convenient to use a solvent for the reagent which has a density higher than 1 (*e.g.* 1,2-dichloroethane) so that a sample of the water layer can be directly pipetted off after completed reaction.
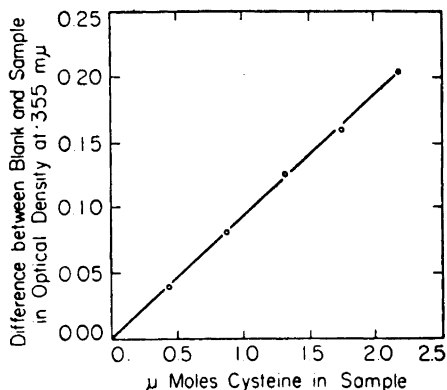
Fig. 6. Determination of cysteine with CMNP. 6 ml of cysteine solution of pH 4.0 (blanks: 6 ml buffer of pH 4.0) were shaken with 6 ml of 0.03 % CMNP in toluene for 1 minute at the rate of 300 cycles/min. The optical density of 0.5 ml toluene phase + 3.0 ml of toluene was determined.

The sample is then adjusted to pH 9.5 by the addition of a buffer of a suitable strength and the optical density at about 420 mμ is determined (the exact wavelength varies somewhat with the thiol used).

This method is generally not applicable when solutions of a high thiol concentration are used, as a precipitate then tends to form in the water-phase.

Care should be taken to avoid very high ionic strengths in the solutions to be measured as this decreases the completeness of the reaction (Fig. 7).
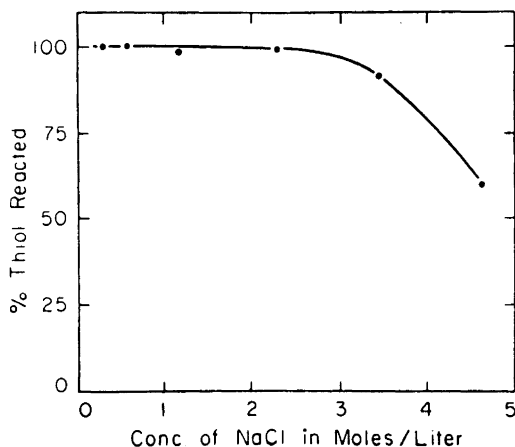
Fig. 7. Effect of NaCl concentration on the reaction between cysteine and CMNP. 0.3 mM solutions of cysteine in buffer containing various amounts of NaCl (final pH adjusted to 4.2 — 3.8; blanks contained the same buffer — NaCl mixtures) were shaken for 1 min at the rate of 300 cycles/min with equal volumes of 0.03 % CMNP in toluene. The reaction was followed by the decrease in optical density in the toluene phase. The amount of reacted cysteine was calculated as in Fig. 4.

With both methods a thiol concentration of 0.02 mM (3 μg cysteine/ml) gives a change in optical density of the order of 0.1.

Quantitative determination by the above methods is obviously limited to those thiols, which are not soluble in the organic solvents used.

Specificity of the reaction. As is seen from Table 3 CMNP reacts almost exclusively with true or potential thiols at pH 4, the only exception found being the cyanide ion. Thioethers, thiolactones, disulfides, sulfonic acids and sulfinic acids do not seem to react. At higher pH's, however, some substances were found to react with the reagent. *E.g.*, EDTA reacts at pH 7.

Table 3. Reactivity of compounds towards CMNP. Data were obtained by dissolving the compounds to be tested (1 — 2 mg/ml) in 1 M citrate buffer of pH 4.0 and shaking the solutions for one minute with a solution of CMNP (0.2 %) in 1,2-dichloroethane. The appearence of a yellow color or precipitate in the water phase indicated positive reaction. Reactivity values were obtained from quantitative determinations of 0.3 mM solutions of the compounds as described above (1 min shaking time).

Compounds that react with CMNP	Reactivity %		Nonreactive compounds
2-Aminoethanethiol	103		Benzenesulfinic acid
Cysteine	100		DL-(+)-*allo*-Cystathionine
Ergothioneine			L-Cystin
Glutathione	100		6,8-Dithio-*n*-octanoic acid
Homocysteine	100		DL-Homocysteine thiolactone
Hydrogen sulfide			DL-Methionine
Mercaptoacetic acid			Sodium bisulfite
2-Mercaptoethanol *			Taurine
L-2-Mercaptohistidine	100		Adenosine-5'-triphosphate
6-Mercaptopurine			L-Alanine
Mercaptosuccinic acid			L-Arginine
6-Methylthiouracil			DL-Aspartic acid
Potassium cyanide			Betaine
Sodium thiosulfate	100		Choline
2-Thiobarbituric acid	63;	77[a]	Creatinine
2-Thiocytosine	72;	69[a]	Disodium hydrogenphosphate
2-Thiouracilcarboxylic acid			EDTA
Thiourea	100		Glycine
			L-Histidine
			Potassium cyanate
			L-Proline
* = color of organic phase deepens			DL-Serine
[a] = 2 minutes shaking time			tris(hydroxymethyl)aminomethane
			DL-Tryptophane
			L-Tyrosine

PAPER CHROMATOGRAPHY OF MNP-THIOLS

Success in applying paper chromatography as a separatory means of MNP-thiols fundamentally depends on the stability of these compounds. As mentioned above MNP-thiols are not quite stable at slightly acid pH's. Fortunately, the MNP-thiols investigated were found to be more stable in alkaline solution. In fact, solutions of most investigated MNP-thiols in 0.1 M sodium hydroxide or in 1 % piperidine did not remarkably change their chromatographic properties after storage for a few days, even if the solutions were kept at room temperature.

Paper chromatography was thus attempted with the main emphasis on basic solvent systems in combination with treated and untreated varieties of paper. Out of some 300 combinations of solvents and varieties of papers tested, the best results were obtained when MNP-thiols were chromatographed on paper, which previously had been impregnated with sodium carbonate. A wide variety of solvent systems were found to be useful in combination with this paper.

"*Ghost*" *spots.* When chromatographing MNP-thiols in the manner just described, a small yellow red "ghost" spot was practically always observed accompanying the main MNP-thiol spot. This "ghost" spot had the same R_F-value independent of the MNP-thiol from which it was derived. Its position on the chromatgrams was different from that of CMNP and from MNP-H_2S. It was not identical with *o*-nitrophenol. The "ghost" spot did not contain sulfur, as judged by the cyanide-nitroprussiate reaction. Nor was it ninhydrin positive when derived from amino group containing MNP-thiols.

The size of the "ghost" spot relative to that of the parent MNP-thiol spot varied with the solvent used for the development of the chromatogram. For instance, exceptionally large "ghost" spots were obtained when using solvent systems rich in pyridine. Also, some MNP-thiols, especially MNP-cysteine, gave relatively more "ghost" spot than others. The addition of cyanide to the solvent systems did not significantly decrease the amount of "ghost" spot.

It was found, however, that if the MNP-thiols were prepared at 0° and chromatographed with the solvent systems listed in Table 4, relatively smaller amounts of "ghost" spot was present on the finished chromatograms than when they were prepared at 25°. It is thus clear that much of the compound responsible for the "ghost" spot is formed during the preparation of MNP-thiols, when this takes place at room temperature.

Furthermore, some solvent systems decompose MNP-thiols during chromatography more easily than others.

A substance with the same chromatographic and spectrophotometric properties as the "ghost" spot is formed when MNP-thiols are heated in neutral solutions (*e.g.* when attempts are made to recrystallize MNP-thiols from water or ethanol). A convenient way to obtain this substance in quantity is to boil an ethanol soluble MNP-thiol * (1 g) in 95 % ethanol (50 ml) for 30 min and to recrystallize the crystals appearing upon cooling from the same solvent. The decomposition product thus obtained is chromatographically pure.

Preliminary investigations of the compound, which does not melt below 300°, showed that it contains mercury. Upon treatment with iodine + iodide as described in earlier paragraphs, the compound is slowly converted into 6-iodo-2-nitrophenol and mercury(II) iodide. The substance did not react with thiols when the CMNP procedure was applied, suggesting that the mercury is bound by nonionizable bonds.

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* *E.g.*, MNP-2-mercaptoethanol.

Using the molar extinction coefficient for CMNP at pH 9.5 (which seemed justified as an approximation method since all MNP-thiols investigated show very similar molar extinction coefficients) a molecular weight of 257 was obtained. This value, however, does not account for both the mercury and for the nitrophenol residue present. The presence of two nitrophenol residues in the substance must then be presumed. This would approximately double the molar extinction coefficient and give a molecular weight of the compound of the order of 500, which only allows for one mercury atom per molecule of compound.

Elementary analysis of the compound, finally, was in agreement with these findings. (Found: C 29.67; H 1.96; Hg 42.23. Calc. for $C_{12}H_8HgN_2O_6$ (476.81); C 30.23; H 1.69; Hg 42.07)

Thus the isolated compound is identical with 2,2'-dihydroxy-3,3'-dinitro-diphenylmercury, and the decomposition of MNP-thiols by heat *etc.* is most likely represented by the scheme in Fig. 8.
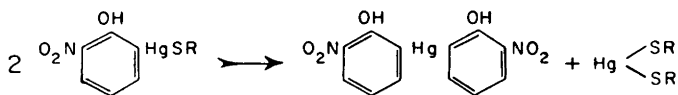

Fig. 8. Probable scheeme for the formation of ''ghost'' spots.

Preparation of MNP-thiols. Good yields of MNP-thiols were generally achieved by the following procedure carried out at 0°. 0.1 g thiol is dissolved in 5 — 10 ml of 1 M citrate buffer of pH 4 and an equivalent amount of CMNP in ethylacetate is added under vigorous stirring. A yellow precipitate appears immediately. If changed, pH is adjusted back to 4 with 0.1 M sodium hydroxide. The precipitate is filtered off and washed with 50 ml of water and with 10 ml of ethylacetate (ethylacetate soluble MNP-thiols are preferably washed with a small amount of ethanol). The substances can be further purified by dissolving them in the minimum amount of 0.1 M sodium hydroxide and reprecipitating them at pH 4 by the addition of 10 % acetic acid. After washing with water and with ethylacetate (ethanol) the MNP-thiols are dried at room temperature in the dark.

The MNP-thiols thus prepared are stable when stored in a dark, dry and cool place.

To prepare reference solutions for paper chromatography of thiols not soluble in organic solvents it is generally sufficient to shake the thiol, dissolved in buffer of pH 4 at 0°, with an excess of CMNP in 1,2-dichloroethane at 0°. After centrifugation a sample of the water phase (preferably containing some of the precipitate formed) is pipetted off and 0.1 M sodium hydroxide or 1 % piperidine is added dropwise until the solution turns yellow red (pH 9 — 10).

Chromatographic procedure. Whatman No. 1 papers were used exclusively. Prior to chromatography the papers were impregnated with a solution of 1 % $Na_2CO_3 \cdot H_2O$ in water by dipping the papers into the solution or by spraying them with the sodium carbonate solution until the papers were completely saturated. The papers were dried at room temperature.

For best results freshly impregnated papers should be used. The dried papers were spotted with MNP-thiols dissolved in 1 % piperidine or in 0.1 M sodium hydroxide.

All chromatograms were developed by the ascending technique and were run for 2 — 4 h in the dark. Longer runs are preferably carried out in a cold room at 0 — 4°, in order to decrease the amount of decomposition of MNP-thiols during chromatography. MNP-thiols appear as yellow spots. Amounts down to the order of 5 mμmoles/cm^3 are easily detected (Fig. 9).

Table 4. Approximate R_F-values of MNP-thiols chromatographed 3—4 h at room temperature on Whatman No. 1 paper impregnated with 1 % $Na_2CO_3 \cdot H_2O$.

Solvents (figures indicate volumes):
1. 95 % ethanol : water, 4:1
2. methanol : N,N-dimethylformamide : water, 3:1:1
3. 95 % ethanol : benzene : water, 4:1:1
4. 95 % ethanol : N,N-dimethylformamide : water, 3:1:1

Substance	Solvent No.			
	1.	2.	3.	4.
CLMNP	0.21	0.12	0.35	0.22
MNP-2-aminoethanethiol	0.55	0.53	0.74	0.63
MNP-cysteine	0.28	0.33	0.45	0.39
MNP-ergothioneine	0.51	0.53	0.75	0.65
MNP-glutathione	0.07	0.13	0.10	0.09
MNP-homocysteine	0.23	0.32	0.35	0.28
MNP-mercaptoacetic acid	0.41	0.47	0.61	0.58
MNP-2-mercaptoethanol	0.85	0.79	0.97	0.90
MNP-2-mercaptohistidine	0.21	0.29	0.41	0.33
2,2′-dihydroxy-3,3′-dinitro-diphenylmercury	0.41	0.51	0.69	0.67
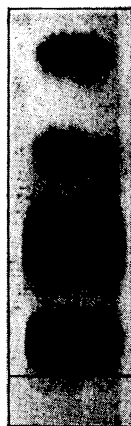
Fig. 9. Paper chromatogram of MNP-thiols (5—10 µg/spot) developed at room temperature (2 h) on Whatman No. 1 paper impregnated with 1 % $Na_2CO_3 \cdot H_2O$. Solvent: 95 % ethanol: N,N-dimethylformamide:water = 3:1:1. The MNP-thiols used were prepared at room temperature to demonstrate the formation of 2,2′-dihydroxy-3,3′-dinitro-diphenylmercury. (The chromatogram was photographed with the aid of a blue filter.)

The solvent systems listed in Table 4 were found to give minimum amounts of "ghost" spots.

Spray reagents. In addition to the R_F-values the following spray reagents were found to be useful aides in the identification of MNP-thiols on the chromatograms. The color reactions described were all obtained on sodium carbonate impregnated paper.

1) All MNP-thiols (and CMNP) give a brown color when sprayed with *p*-nitrobenzenediazonium fluoborate (0.5 % in 50 % ethanol). Thus weak spots can be made to show up more clearly.

2) To confirm the presence of sulfur in the MNP-thiol spots the cyanide-nitroprussiate reagent [14] can be used. Of the MNP-thiols listed in Table 4 all gave an immediate redbrown spot, except MNP-ergothioneine and MNP-2-mercaptohistidine, which do not react.

3) MNP-thiols containing amino groups give brown spots when sprayed with ninhydrine (0.5 % in ethanol). The colors are best developed at room temperature. Depending on the amount of substance present positive response is obtained after 1—12 h.

4) MNP-ergothioneine and MNP-2-mercaptohistidine give grey blue spots immediately when sprayed with N,2,6-trichloro-*p*-benzoquinone imine (0.2 % in 50 % ethanol).

Effect of light on MNP-thiols. Exposure of MNP-thiol spotted chromatograms to ultraviolet radiation prior to chromatography invariably showed a deposit of a brownish colored decomposition product at the starting line on the finished chromatogram (solvent: ethanol:water, 4:1). The blank spots, which had been covered during the UV-exposure, showed no trace of this decomposition product. Even UV lamps with a low wattage (9 watts) when used at a distance of about 10 cm from the paper caused considerable decomposition during one hour.

It was noted on the finished chromatograms that the size of the "ghost" spot relative to the size of the MNP-thiol spot was not significantly changed after UV exposure. Thus the decomposition of MNP-thiols by ultraviolet light is qualitatively different from the decomposition noted by *e.g.* heat, which is represented by Fig. 8.

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