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A Chromatographic Method of Analysis for Thiols

BY C. A. PRICE* AND C. W. CAMPBELL

Horticulture Department, Purdue University, Lafayette, Indiana, U.S.A.

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Although there are a number of methods for the detection and estimation of thiol compounds (Patterson & Lazarow, 1954), no fully adequate chromatographic procedure has been hitherto available. The reduced forms of coenzyme A (Stadtman, 1952), cysteine (Toennies & Kolb, 1951), and glutathione (Miller & Rockland, 1952) have been detected on paper by means of thiol reactions, but the chief obstacle has been the ease of autoxidation of the thiol groups, leading to secondary spots and the excessive formation of 'comets' or tailing.

The specific problem of α -amino acid-thiol separations was first resolved by Hanes, Hird & Isherwood (1950) and expanded by Gutcho & Laufer (1954). In their methods the thiol group is masked with

* Present address: Research Laboratory of Biophysics, Department of Medicine, Harvard Medical School and Peter Bent Brigham Hospital, Boston, Mass., U.S.A.

N-ethylmaleimide (Friedman, Marrian & Simon-Reuss, 1949), a reaction that proceeds rapidly to completion in dilute aqueous solution over a wide pH range (Leopold & Price, in preparation). After chromatographic development of the *N*-ethylmaleimide-thiol conjugates, the compounds are detected with ninhydrin. A disadvantage of this method was that these conjugates were distinguished from other amino acids and peptides only by their R_f values. More recently (Benesch, Benesch, Gutcho & Laufer, 1956) a method has been found for detecting the conjugates of *N*-ethylmaleimide and any thiol. An alternative approach, suggested by Gutcho & Laufer (1954), is that the thiol-masking reagent contains a potential chromophore. Such a procedure is in fact the basis of a histochemical technique which has been developed successfully (Barnett & Seligman, 1954). The most promising substance of this sort, described recently

by Tsou, Barnett & Seligman (1955), is *N*-(4-hydroxy-1-naphthyl)isomaleimide. The isomaleimide moiety reacts rapidly and specifically with thiols (equation 1), whereupon the α -naphthol moiety may be combined with diazotized reagents to yield intensely coloured dyes (equation 2).

The method described below consists in the preparation of the *N*-(4-hydroxy-1-naphthyl)isomaleimide derivatives of thiols followed by chromatography and detection with tetraazotized di-*o*-anisidine.

A preliminary report of this work has appeared elsewhere (Price, 1956).

The following abbreviations are employed in this paper: cysteine, CySH; glutathione, GSH; naphthalene-1-acetic acid, NAA; oxidized glutathione, GSSG; *N*-(4-hydroxy-1-naphthyl)isomaleimide, HNI; tetraazotized di-*o*-anisidine, TDA.

METHODS

Materials

'Naphthanil diazo blue B' (Dupont), obtained from Fisher Scientific Company, St Louis, Mo., U.S.A., was used as a source of tetraazotized di-*o*-anisidine (TDA).

Cysteine (CySH), glutathione (GSH) and oxidized glutathione were products of Schwarz Laboratories; commercial preparations of homocysteine, djenkolic acid, *n*-butylthiol and thiomalic acid were gifts of Dr R. Henze. The sample of cysteinylglycine was a gift from Dr L. Laufer of the Schwarz Laboratories, Mount Vernon, N.Y., U.S.A. Thioctic acid (6:8-dimercaptooctanoic acid) was a gift of Dr L. Reed. Thiocoumarin was a gift from Dr R. Goodwin. Coenzyme A (approx. 75%) was a gift from the Pabst Laboratories, Milwaukee, Wis., U.S.A. It was reduced with potassium borohydride immediately before use (Jones, Black, Flynn & Lipmann, 1953).

Chromatographic solvents were all of the highest commercial grades available, but were not specially purified.

Krylon, a methacrylate solution, is manufactured by Krylon, Inc., Philadelphia 46, Pa., U.S.A.

The sample of *N*-(4-hydroxy-1-naphthyl)isomaleimide (HNI) principally used was a generous gift from Dr A. Seligman and was part of the original batch described by Tsou *et al.* (1955). Dr L. Laufer, of the Schwarz Laboratories, also kindly supplied two samples and one lot was prepared in this laboratory.

Reactions and purification of HNI. The original sample of HNI dissolved in ethanol and chromatographed in butanol-water contained at least two components (Fig. 1). When increasing amounts of thiol were made to react with the reagent (for conditions, see below) and the products chromatographed, the more rapidly moving component was observed to disappear in proportion to the appearance of a new spot. The slower-moving component did not change in intensity (this is not evident from the tracing) and was concluded, therefore, to be a contaminant.

Except in the solvent systems rich in butanol, the presence of the contaminant was not a serious disadvantage, since both contaminant and reagent moved close to the solvent front. Where the contaminant might have obscured other

components, a purified HNI reagent was employed. This was prepared by streaking a large quantity of HNI on a sheet of Whatman no. 20 filter paper, chromatographing in *n*-butanol-water and eluting the faster component with ethanol. The material obtained no longer gave the 'base reaction' (see below), but was free of contaminant and still reacted with thiols (Fig. 1).

In several preparations of HNI, the reagent contained widely varying quantities of contaminant. Further work is therefore required to standardize the chemical preparation. Moreover, to the extent that the structural formula (Tsou *et al.* 1955) was deduced from the properties of an impure sample, some reservations must attach to the assigned structure.

One property of HNI observed by Tsou *et al.* (1955) was the formation in aqueous alkaline solution of an intense transient red colour. This 'base reaction' is irreversible and corresponds to the loss of an absorption maximum at 430 m μ . In our experiments upon prolonged exposure to water at acid or neutral pH there was also a loss in the potential for the 'base reaction' and the 430 m μ absorption, but altered HNI none the less reacted with thiols as before.

The combination between HNI and thiols was greatly accelerated at increasing pH. With GSH, for example, at pH 8 the reaction was complete within a few seconds; at pH 2, on the other hand, the reaction was only half-completed in 45 min. This dependence of the reaction rate on high pH is in contrast to that of *N*-ethylmaleimide: with the latter reagent the rates are high at all pH values tested, but are accelerated under the more acid conditions (Leopold & Price, in preparation).

The detection reaction between HNI and TDA also proceeds rapidly only under alkaline conditions. Since

Fig. 1. Reaction of cysteine and HNI. Samples (5 μ l.) of 0.1% (w/v) HNI with several concentrations of cysteine (also 5 μ l.) chromatographed in solvent 7, ascending. Shown at right: the product of purified HNI (HNI-1) with cysteine is identical with that of unpurified reagent.

hydroxide salts of diazo compounds are known to be readily decomposed in water, it is essential to store the TDA in a weakly acidic solution.

Reagents and solutions required. 'Stock HNI': 0.2% (w/v) of HNI in 95% (v/v) ethanol; it is stable indefinitely at -10° .

'Standard HNI': 0.02% (w/v) of HNI in 50% (v/v) ethanol; stable for at least 1 month at -10° .

TDA: 1 g. of 'naphthanil diazo blue B' was stirred for several minutes with 100 ml. of 0.01N-HCl in absolute methanol, gravity-filtered through Whatman no. 4 paper, and the filtrate diluted to 1 l. with acetone. The small precipitate that formed overnight was discarded. The initially pale-orange solution slowly decomposed and turned red at -10° , more rapidly at room temperature.

m-NaHCO₃ and 2N-HCl.

Solvent systems for chromatography: solvent 1, 95% (v/v) ethanol-formamide-N acetic acid (75:10:15, by vol.); solvent 2, propan-2-ol-N acetic acid (80:20, v/v); solvent 3, propan-2-ol-N acetic acid (85:15, v/v); solvent 4, propan-2-ol-n-butanol-N acetic acid (75:10:15, by vol.); solvent 5, propan-2-ol-n-butanol-N acetic acid (45:40:15, by vol.); solvent 6, propan-2-ol-n-butanol-formamide (75:10:15, by vol.); solvent 7, water-saturated n-butanol.

Procedure

Preparation of marker solutions. To portions (1 ml.) of 'stock HNI' were added 0.6 ml. of water, 0.2 ml. of 10^{-2} M thiol in water and 0.1 ml. of m-NaHCO₃. The solution was stirred and at the end of 1 min. the 'base reaction' was stopped with 0.1 ml. of 2N-HCl. (If an excess of thiol were present, the red colour of the 'base reaction' product bleached within 1 sec. This was avoided as it led to multiple spot formation; cf. Fig. 1.) Marker solutions thus prepared were mM with respect to thiol and about 3 mM in excess of HNI. When stored at -10° , no deterioration was observed over many weeks.

Extraction of free thiols from plant tissues as conjugates with HNI. Samples of about 200 mg. fresh wt. of tissue were placed in conical glass homogenizers of 3 ml. working capacity; 1 ml. of 'standard HNI' was added, followed by 0.1 ml. of m-NaHCO₃. Grinding was completed in 1 min. and the reaction stopped with 0.1 ml. of 2N-HCl. The contents of the homogenizer were transferred to a 5 ml. calibrated and ground-glass-stoppered centrifuge tube with

the aid of sufficient 50% (v/v) ethanol to bring the volume to 2 ml. If further treatment were necessary to coagulate proteins, the tubes were placed in a boiling-water bath for 5 min. before final adjustment of volume. The tubes were then centrifuged at 1500 g for 10–20 min. and the supernatants collected.

Chromatography. Whatman filter paper no. 20 was used almost exclusively for chromatography, although no. 5 was perhaps equally satisfactory. With faster-running grades the spots were not sufficiently compact. In general, circumstances that led to a rate of movement of the solvent exceeding 20 cm./24 hr. produced elongate spots.

On a sheet of paper ($18\frac{1}{2}$ in. $\times$ $22\frac{1}{2}$ in.) a series of line segments were drawn 1 cm. long, 2 cm. apart and 12 cm. from the long edge of the paper. For routine screening of solvents, 5 μ m-moles (5 μ l. of mM marker solutions) gave spots of convenient size. The samples were applied along the line segments either with an Agla micrometer syringe (Burroughs Wellcome Inc., Tuckahoe, N.Y., U.S.A.) or with calibrated capillary micropipettes, prepared according to the directions of Mr R. Hems (personal communication). The paper was dried with a stream of cool or warm, but not hot, air from a hair dryer. (Decomposition of the HNI-thiol conjugates occurred at elevated temperatures.)

With plant-tissue extracts (10%, w/v) about 20 μ l. was usually found to provide a detectable spot corresponding to glutathione.

The chromatograms were allowed to develop in the usual manner for 24–48 hr., by which time the front had descended 20–40 cm. beyond the starting lines. Pre-equilibration of the paper with solvent vapours was not found to be advantageous. The chromatogram was then dried in air. To detect the thiol-HNI conjugates, the papers were exposed briefly to NH₃ vapour, dipped into the TDA solution, and hung in a vertical position with the starting line upwards. Within a few seconds blue or red spots appeared.

The pigments formed were stable for months, but spraying with a preservative (Krylon) was sometimes employed.

RESULTS

Results with marker solutions. Cysteine and glutathione, the most common naturally occurring thiols, were readily separated with a number of solvent systems containing ethanol or propan-2-ol

Table 1. R_f values of N-(4-hydroxy-1-naphthyl)isomaleimide (HNI) derivatives of various thiols

All samples were applied on Whatman no. 20 paper in 5 μ m-mole quantities; except for solvent 6, descending development was used throughout. For details of solvent systems see Methods.

Component	R_f value in				
	Solvent 1	Solvent 4	Solvent 5	Solvent 6 (ascending)	Solvent 7
HNI	0.98	0.93	0.90	0.98	0.92
HNI contaminant	0.98	0.84	0.77	0.94	0.69
n-Butylthiol	0.98	0.95	0.90	0.98	0.92
Coenzyme A	0.42	0.04	0.02	—	0.01
Cysteine	0.78	0.56	0.46	0.4	0.28
Cysteinylglycine	0.75	0.42	0.32	—	0.18
Glutathione	0.57	0.22	0.12	0.06	0.02
Homocysteine	0.84	0.57	0.46	—	0.31
Thioglycollate	0.95	0.74	0.71	0.28, 0.12	0.56
Thiomalate	0.90	0.63	0.62	—	0.29
Thioctic acid	0.98	0.90	0.90	0.6	0.86

with 10–20 % (v/v) of water or when butanol was incorporated up to 15 % (v/v) of the organic phase (Table 1). Such a system was also suitable for homocysteine, cysteinylglycine and coenzyme A. The non-amino acid thiols of low molecular weight move near the reagent in these solvent systems. For thioglycolic and thiomalic acids, solvent 5 and solvent 7 were more satisfactory. In these systems, however, glutathione sometimes became obscured, probably by interaction with carbonate.

The less polar thiol, thioctic acid, could be separated with solvent 6, but the resolution was not satisfactory. *n*-Butylthiol has thus far eluded separation from the reagent; it does, however, react as indicated by the rapid bleaching of the 'base-reaction' colour in the presence of excess of *n*-butylthiol.

As was reported by Tsou *et al.* (1955), HNI does not react with disulphides or thioethers. This was confirmed for cystine, djenkolic acid, thiourea and thiocoumarin.

The marker solutions of coenzyme A and cysteinylglycine gave multiple spots. The sample of coenzyme A included glutathione, cysteine, a trace of cysteinylglycine and three unidentified thiols running with R_F values of 0.27, 0.66 and 0.70 in solvent 4. (We are advised that current batches of Pabst coenzyme A are free of glutathione.) Cysteinylglycine marker solution also contained cysteine.

Sensitivity. Although 5 μ m-mole quantities were convenient for exploratory studies, the dye formed by the reaction of HNI and TDA was sufficiently

intense to be detected after development of the chromatogram down to 0.1 μ m-mole. An experiment was performed in which decreasing quantities of marker solutions of cysteine and glutathione (30–0.1 μ m-moles) in solvent 3 were applied on two halves of a filter-paper sheet. After development one half was dipped in TDA and the other in 0.25 % ninhydrin (Gutcho & Laufer, 1954). In the first case all loci containing α -naphthol were stained including, of course, the reagent. It was apparent that, with the exception of cysteine, all levels of thiol could be detected with TDA; the lowest quantities of HNI–TDA were best perceived under ultraviolet light (366 m μ .) as bright-red fluorescent spots. With ninhydrin the sensitivity was three- to ten-fold less. Benesch *et al.* (1956) detected glutathione down to 33 μ m-moles, a sensitivity 300-fold less than the present method.

Applications to plant material. Inasmuch as this laboratory has been occupied with problems of plant growth, the applications of thiol chromatography have been for the most part restricted to studies with plant material. One analysis is presented here solely for the purpose of illustration.

Week-old etiolated pea stems were cut into 10 mm. subapical sections; 200-mg. lots were placed in Petri dishes containing 15 ml. of water or auxin solution 50 μ M NAA and harvested at 4 and 24 hr. Extracts of the HNI derivatives of the free thiols were prepared as described above. The results are shown in Fig. 2.

Glutathione was found in amounts of the order of 0.1–0.3 μ mole/g. fresh weight. Cysteine, although obscured by a light-pink substance present in the 'no-HNI' controls, was thought to be present at perhaps one-fifth of this amount. No other thiols were detected. Whereas differences between treatments with and without auxin could not be detected, a steady decline of thiols in both lots was evident over the 24 hr. growth period.

A more detailed report of the applications of this method for thiol analysis will be reported elsewhere (Price & Leopold, in preparation).

With all tissue preparations a 'no-HNI' control was essential, for a number of naturally occurring substances formed coloured products with TDA. This was particularly true of inflorescences and may have been due to the abundance of phenolic compounds related to anthocyanins. Ultraviolet light provides a further means for distinguishing HNI derivatives of thiols from other chromophores; only in the presence of HNI were any red-fluorescing materials observed.

SUMMARY

1. A method is described for the paper chromatography of thiols. The thiol is first treated with *N*-(4-hydroxy-1-naphthyl)isomaleimide and after

Fig. 2. Chromatography of thiols extracted from growing pea-stem sections. Extracts were prepared as described in text from tissue floating on water or 50 μ M NAA solution and harvested at various times. Extracts were chromatographed in solvent 2.

chromatographic development detected with tetra-azotized di-*o*-anisidine.

2. The technique is applicable to a variety of thiols, but is not yet suitable for alkyl thiols.

3. Quantities of thiols as low as 0.3 μ m-mole were detected, and under optimum conditions 0.1 μ m-mole was visualized under ultraviolet light. The sensitivity of the method is therefore superior to the *N*-ethylmaleimide detection of thiols or the ninhydrin detection of 2-amino acids.

4. A sample application of the method is presented.

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The Synthesis of some Peptides Containing Glutamic Acid

BY D. A. ROWLANDS AND G. T. YOUNG
The Dyson Perrins Laboratory, University of Oxford

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For use in experiments concerning the mechanism of the assimilation of glutamic acid by *Staphylococcus aureus* (Rowlands, Gale, Folkes & Marrian, 1957), a number of peptides containing glutamic acid have been prepared, of which L-valyl-L-glutamic acid, L-valyl- α -L-glutamyl-L-valine, L-leucyl- α -L-glutamyl-L-leucine and α -L-glutamyl- α -L-glutamyl-L-glutamic acid are new. α -L-Glutamyl-L-alanine has been prepared by an alternative route.

For the synthesis of L-valyl- α -L-glutamyl-L-valine, benzyloxycarbonyl-L-valine was converted into the mixed anhydride, with ethyl chloroformate in toluene solution, and this solution was allowed to react with an aqueous solution of the sodium salt of α -L-glutamyl-L-valine; the coupling product crystallized immediately, and hydrogenation gave the tripeptide. An analogous procedure succeeded in the preparation of L-leucyl- α -L-glutamyl-L-leucine. For the synthesis of α -L-glutamyl- α -L-glutamyl-L-glutamic acid, benzyloxycarbonyl-L-glutamic anhydride was allowed to react with diethyl methyl α -L-glutamyl-L-glutamate; the isomeric products were separated by fractional extraction with aqueous sodium carbonate, and the α -isomer was hydrolysed and then hydrogenated to the tripeptide. The synthesis of L-valyl-L-glutamic acid requires no comment. α -L-Glutamyl-L-alanine was prepared in

the normal fashion from benzyloxycarbonyl-L-glutamic anhydride (e.g. Le Quesne & Young, 1950a); its synthesis by a different route has since been reported by Sachs & Brand (1953).

The preparation of the γ -glutamyl peptides used in the work reported in the succeeding paper has been described previously (Le Quesne & Young, 1950b; Rowlands & Young, 1952), but recently Sachs & Brand (1954) have reported that the intermediate benzyloxycarbonyl- γ -L-glutamyl azide may isomerize during coupling, to yield both α and γ products. We have therefore examined our original samples by paper chromatography with high concentrations of peptide and a mixture of *n*-butanol-acetic acid-water, as the mobile phase, and by comparison with samples to which known quantities of the α -isomer have been added we have estimated the approximate amount of the latter. The γ -L-glutamyl-glycine, -L-valine, -L-leucine, -L-tyrosine and -glycylglycine showed only traces (less than 0.2%) or none of the α -isomer. The γ -L-glutamyl-L-alanine was estimated to contain less than 0.5% and γ -L-glutamyl-L-glutamic acid less than 2% of the α -isomer, but the sample of γ -L-glutamyl-L-aspartic acid contained considerably more. In the synthesis of γ -L-glutamyl-L-glutamic acid only one of the intermediates after coupling