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REFERENCES

1 M. COHN AND A. TORRIANI, *J. Immunol.*, 69 (1952) 471.
2 M. COHN AND A. TORRIANI, *Biochim. Biophys. Acta*, 10 (1953) 280.
3 H. A. SOBER, F. J. GUTTER, M. M. WYCKOFF AND E. PETERSON, *J. Am. Chem. Soc.*, 78 (1956) 756.
4 M. LAMBORG AND N. O. KAPLAN, *Biochim. Biophys. Acta*, 38 (1960) 272.
5 N. O. KAPLAN AND F. E. STOLZENBACH, in COLOWICK AND KAPLAN, *Methods in Enzymology*, Vol. III, Academic Press Inc., New York, 1957, p. 902.
6 M. LAMBORG, F. E. STOLZENBACH AND N. O. KAPLAN, *J. Biol. Chem.*, 231 (1958) 685.
7 O. WARBURG AND W. CHRISTIAN, *Biochem. Z.*, 310 (1941) 384.
8 S. SPIEGELMAN, H. O. HALVORSON AND R. BEN-ISHAI, in MCELROY AND GLASS, *Amino Acid Metabolism*, The Johns Hopkins Press, Baltimore, 1955, p. 144.
9 J. MONOD, G. COHEN-BAZIRE AND M. COHN, *Biochim. Biophys. Acta*, 7 (1951) 565.
10 J. MONOD AND M. COHN, *Advances in Enzymol.*, 13 (1952) 67.
11 F. C. NEIDHARDT AND B. MAGASANIK, *Biochim. Biophys. Acta*, 21 (1956) 324.
12 J. S. GOTS AND E. G. GOLLUB, *J. Bacteriol.*, 72 (1956) 858.
13 H. HALVORSON, *Antibiotics & Chemotherapy*, 4 (1954) 948.
14 E. F. GALE AND J. P. FOLKES, *Biochem. J.*, 53 (1953) 493.
15 J. BRACHET, in CHARGAFF AND DAVIDSON, *The Nucleic Acids*, Vol. II, Academic Press Inc., New York, 1955, p. 476.
16 E. BOREK AND A. RYAN, *J. Bacteriol.*, 75 (1958) 72.
17 F. C. NEIDHARDT AND F. GROS, *Biochim. Biophys. Acta*, 25 (1957) 513.
18 A. B. PARDEE, K. PAIGEN AND H. S. PRESTIDGE, *Biochim. Biophys. Acta*, 23 (1957) 162.

Biochim. Biophys. Acta, 38 (1960) 284–293

A CHLOROMERCURIBENZOATE RESIN FOR THE SELECTIVE BINDING OF NONPROTEIN SULFHYDRYL COMPOUNDS

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SUMMARY

An insoluble reagent formed by the binding of sodium *p*-chloromercuribenzoate to Dowex-2 resin has been used for the selective removal of thiols from solutions or from tissue homogenates by reverse dialysis. It was possible to liberate the bound thiol from the reagent by exchange with other sulfhydryl compounds.

INTRODUCTION

Many enzyme systems require the presence of sulfhydryl groups for their activation, and often incorporate a sulfhydryl reservoir such as glutathione. Presumably,

Abbreviations: *p*CMB, *p*-chloromercuribenzoate; EDTA, tetrasodium ethylenediamine tetraacetate.

glutathione maintains the enzyme thiol radicals in reduced form. It is at times desirable to remove or inactivate this reservoir in order to study the properties of the unprotected constituents of the system. While it is possible to dialyse low molecular weight thiols from protein preparations, other substances (not all of them known or replaceable) may be removed as well. Therefore, a method for the selective removal of thiols from biological systems seems desirable.

DAVISON AND HOFMANN have shown[1] that the addition of N-ethyl maleimide to rat adrenal tissue will arrest steroidogenesis; however, this procedure blocks the thiol groups of proteins, enzymes and glutathione indiscriminately, and therefore the results are difficult to interpret. In the course of studies on the relation of glutathione to adrenal cortical function[2], we encountered the same problem. To achieve a selective removal of soluble sulfhydryl compounds, we sought to develop an insoluble sulfhydrylbinding agent which could be used for reverse dialysis. A preliminary description of this reagent has been published[3]; subsequently we have become aware of a prior note by MILES, STADTMAN AND KIELLEY[4], describing a mercurial bakelite polymer which was prepared for much the same reasons.

EXPERIMENTAL AND RESULTS

Preparation of the reagent

Dowex-2 X 7.5 resin, 25–50 mesh (lot No. 2050, obtained from the Dow Chemical Company), was purified in the chloride form in 100-g lots by stirring mechanically for 10-min intervals with successive 200-ml portions of the following solutions: 4 % NaOH (3 changes); distilled water (4 changes); 4 % HCl (2 changes), distilled water (3 changes); 4 % HCl (2 changes); distilled water (10 changes). The prepared resin was stored in chloride-free distilled water, which had a pH of 6.8. A weighed quantity of wet resin was placed in a small Erlenmeyer flask surmounted by a filter funnel. Into another flask was weighed a quantity of sodium *p*-chloromercuribenzoate (*p*CMB) in about 10 % excess of the milliequivalents absorbable by the resin (in this case 3.1 $\pm$ 0.3/dry gram). The *p*CMB crystals were shaken with water for about 5 min, allowed to settle, and the supernatant solution poured through the filter onto the resin, which was then stirred magnetically for a minute or two. The aqueous supernatant from the resin was then poured back through a filter into the flask with the *p*CMB crystals and the process repeated over and over. It is most important to avoid contaminating the resin with *p*CMB crystals. Maximum uptake of *p*CMB was determined by testing the supernatant solution from the resin for dissolved *p*CMB. 0.5 ml of the solution was added to 0.5 ml of a glutathione standard (9.3 mg/100 ml of 2 % aqueous sulfosalicylic acid) and the resulting SH concentration measured amperometrically[5]. When there was no further uptake of *p*CMB, the resin was washed 10 times with distilled water. About 90 to 92 % of the theoretical amount of *p*CMB absorbable was bound to the resin.

Properties of resin–pCMB

Storage of resin–*p*CMB in distilled water at 4° for periods up to 60 days, storage for several days at room temperature, or drying of the resin had no effect on its sulfhydryl-binding properties so long as it was kept in the dark.

The uptake of cations was tested by adding approximately 2500 counts/min

^{43}KCl in water, to a small portion of resin–*p*CMB. After standing for a few minutes, the supernatant solution was decanted and the resin washed repeatedly with water. The combined washings contained 93.8 % of the added radioactivity, indicating little cation retention.

Properties of the resin–pCMB–glutathione complex

1. *The effect of chelating agents*: Resin–*p*CMB reacted with glutathione in aqueous or dilute sulfosalicylic acid solutions in a manner characteristic of *p*CMB alone. The influence of chelating agents was of interest and was investigated in two ways. In one group of experiments, glutathione or [^{35}S]glutathione was dissolved in water or in 10 % aqueous sulfosalicylic acid to which had been added 50 mg tetrasodium ethylenediamine tetraacetate (EDTA)/100 ml solution. This reagent was shaken mechanically with small portions of the resin–*p*CMB for 10 min and the supernatant solution examined by liquid scintillation counting[2], by amperometric titration of sulfhydryl groups, or by both methods. The removal of glutathione was essentially quantitative. In other experiments, the resin–*p*CMB was first shaken with a solution of EDTA, and the glutathione or [^{35}S]glutathione added subsequently; the same result was observed.

The effect of pH: Under the influence of varying pH, dissociation of the resin-*p*CMB glutathione complex could occur either at the resin-carboxyl bond, the mercury-carbon bond, or at the mercury-mercaptide bond. These possibilities were investigated in the following experiments. 1.0 g resin–*p*CMB was treated at neutral pH with an excess (3.257 mmoles) of [^{35}S]glutathione. The resin–*p*CMB–[^{35}S]glutathione complex was washed repeatedly with 10-ml portions of water until the 25th wash contained less than 2 % of the initial radioactivity. The washings were combined and counted, and $2.08 \cdot 10^6$ counts/min (1.06 g GSH + G^{35}SH/g resin) were found to have been incorporated in the glutathione-saturated resin–*p*CMB. Solutions at integral pH's from 1 to 10 were prepared by the addition of HCl or NaOH to 0.1 *M* aqueous acetic acid. To 10-ml aliquots of these solutions, 100-mg portions of the resin–*p*CMB–[^{35}S]glutathione were added and stirred magnetically for 5 min. The supernatant solution was filtered off; two aliquots of 0.3 ml were used for measurement of radioactivity by liquid scintillation counting[2] and the remainder was reserved for the determination of mercury in duplicate by the ignition technique of FEIGL[6]. The results of two such experiments are summarized in Table I. Disruption of the

TABLE I

DISSOCIATION OF RESIN–*p*CMB–GLUTATHIONE COMPLEX AT VARIOUS pH VALUES

pH	*Liberation of* ^{35}S, %	*Liberation of mercury*	
		μmoles	%
1	8.3	0.01–0.02	0.01–0.03
2	11.6	0.02–0.05	0.05–0.06
3	3.5	0.5	0.63
4	3.0		
5	3.4	0.6	0.79
6	4.3		
7	5.9	0.8	0.95
8	5.2	2.5	3.2
9	6.0	5	6.3
10	2.2	$<$ 0.01	—

resin-carboxyl bond (or conceivably the mercury-carbon bonds), with the liberation of organic mercury, was detectable over the entire pH range although only microgram quantities appeared at the extreme ends of the pH scale. Maximum appearance of mercury in the aqueous phase occurred at pH 9, with 6.3 % of the mercury dissociating from the resin. The mercury-mercaptide bond behaved quite differently, showing maximum dissociation at pH 2 and 9, with a minimum at pH 3.0.

Exchange of carboxyl groups: Acetate was used as a model compound to measure carboxyl exchange at the resin-carboxyl bond. 2.0 ml of an aqueous 0.1 *M* solution of sodium [1-^{14}C]acetate was shaken with 24.6 mg resin–*p*CMB for 30 min. No removal of radioactivity from the supernatant solution was observed; therefore, the exchange of acetate-carboxyl for *p*CMB-carboxyl in the resin–*p*CMB complex appeared to be negligible. Additional evidence was secured by shaking an aqueous solution of 66.4 mg *p*CMB-glutathionate with 17.9 mg resin–*p*CMB [^{35}S]glutathionate. After 0.5 h of shaking, only 1 % of the [^{35}S]glutathione appeared in the supernatant solution and washings, indicating that little exchange occurred at the carboxyl bond under these conditions.

Stability of the mercury-mercaptide bond in the presence of thiols: 25 mg resin–*p*CMB saturated with 120,000 counts/min [^{35}S]glutathione was shaken for 30 min with 2 ml water containing 15 mg cysteine hydrochloride. 17.5 % of the [^{35}S]glutathione appeared in the washings. With 50 mg of glutathione instead of the cysteine, only 5.6 % of the [^{35}S]glutathione was washed off. However, when 1-g batches of resin–*p*CMB saturated with $4.0 \cdot 10^6$ counts/min of [^{35}S]glutathione were shaken with a solution of 1 g glutathione in 18.5 ml water adjusted to pH 6.4 with bicarbonate, from 41.2 to 53.7 % of the [^{35}S]glutathione appeared in the aqueous phase. As mentioned in previous section, *p*CMB–glutathione did not displace significant amounts of [^{35}S]glutathione from the resin.

Removal of tissue thiols by reverse dialysis

Deproteinized tissue extracts: 10 g of fresh rat liver was homogenized in a micro Waring blendor in cold 10 % sulfosalicylic acid, made up to 100 ml volume, and filtered after standing for 1 h. 8 g of resin–*p*CMB were enclosed in a bag of 1 cm wide Visking dialysis casing, with the air displaced by about 15 ml of the liver filtrate. The bag was placed in a 2.5 cm diameter glass cylinder which was partially filled with 45 ml of filtrate, stoppered, and oscillated mechanically 30 times/min. At hourly intervals, 1.0-ml samples of filtrate were removed from the cylinder and the sulfhydryl concentration measured amperometrically. (The stability of resin–*p*CMB in the presence of sulfosalicylic acid was demonstrated in the model experiments described above.) By 4 h, there had been a 40 % removal of sulfhydryl, and by 6 h a total of 93 %.

Tissue incubations. In these studies both rat-liver homogenate and cow adrenal whole-cell homogenate in KREBS-RINGER-phosphate were studied using a standardized technique for tissue incubation. The dialysis bags consisted of 4 cm lengths of 1 cm wide Visking casing. 0.1 g of resin–*p*CMB was placed in the bags and air displaced by adding filtrate from a sample of boiled homogenate. 1 ml of homogenate or brei, 114,000 counts/min of [^{35}S]glutathione and a resin bag were placed in a 15 × 60 mm vial closed with a plastic-lined screw cap. A number of these vials were inserted end to end within lengths of 20 mm diameter glass tubing which were maintained at 37°

and rotated at 13 rev./min. Vials were removed at intervals, aliquots of the homogenate precipitated with sulfosalicylic acid and 0.3-ml portions of the supernatant solution taken for liquid scintillation counting. The results of experiments with adrenal whole-cell homogenates are shown in Table II. Several experiments with liver homogenates were comparable.

TABLE II

REMOVAL OF THIOLS FROM ADRENAL HOMOGENATES

Reaction time	*No. of Expts.*	*$G^{35}SH$ removed, %*
0	2	0
30	4	24.0
60	5	33.0
90	20	62.2

DISCUSSION

The studies indicate that the combination of sodium *p*CMB with Dowex-2 forms an insoluble and relatively stable reagent, which can be used successfully for the removal of low molecular weight sulfhydryl compounds from solutions or tissue suspensions by reverse dialysis. In experiments to be reported elsewhere, it has been found that removal of a sufficient fraction of sulfhydryl from adrenal whole-cell homogenates results in cessation of steroidogenesis, but that this effect can be prevented if the homogenate is protected with added glutathione. This is evidence of the selective action of the resin–*p*CMB reagent for sulfhydryl groups and implies that exchange of tissue constituents with *p*CMB on the resin did not occur measurably in this system. Whether this would obtain in systems with very high concentrations of anions such as phosphate, sulfate, nitrate, etc. or whether these ions would cause release of the *p*CMB by the resin would have to be determined in the individual systems. In biologic as contrasted to purely chemical systems, return of biologic activity by restoration of the sulfhydryl compounds must be demonstrated before the selective action of the reagent can be accepted. The relative ease with which bound glutathione can be removed from the resin–*p*CMB reagent by exchange with added thiol (particularly if a volatile thiol or H_2S can be added instead of cysteine) suggests that the reagent might be of value for concentrating or isolating thiols in circumstances where classical procedures such as formation of copper or cadmium mercaptides are not feasible.

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REFERENCES

[1] C. DAVISON AND F. G. HOFMANN, *Endocrinology*, 54 (1954) 654.
[2] J. W. GOLDZIEHER, P. K. BESCH AND M. E. VELEZ, *J. Biol. Chem.*, 231 (1958) 445.
[3] P. K. BESCH, J. W. GOLDZIEHER AND S. MCCORMACK, *Science*, 126 (1957) 650.
[4] H. T. MILES, E. R. STADTMAN AND W. W. KIELLEY, *J. Am. Chem. Soc.*, 76 (1954) 4041.
[5] J. W. GOLDZIEHER, W. B. RAWLS AND M. A. GOLDZIEHER, *J. Biol. Chem.*, 203 (1953) 519.
[6] F. FEIGL, *Spot tests*, Vol. II (Organic Applications), Elsevier Publishing Company, Houston, N.Y., 1954.