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Poly Vinyl(*N*-phenylenemaleimide). A New Selective Binding Agent for Thiols*

E. G. Richards, D. L. Snow, and C. W. F. McClare

ABSTRACT: The preparation of poly vinyl(*N*-phenylenemaleimide) from polyaminostyrene and maleic anhydride is described. This material was found to react rapidly and irreversibly with sulfhydryl compounds but not with nucleotides, soluble ribonucleic acid, and other nonsulfhydryl compounds.

It is frequently desirable to remove thiols from solutions. Although an organomercurial polysaccharide has been described (Eldjarn and Jellum, 1963) and used to fractionate proteins into SH proteins and non-SH proteins, it suffers from the disadvantage that several substances such as chelating agents interfere with its mode of action. Furthermore the preparation of the material is somewhat complicated. We have therefore developed an alternative material involving the use of a maleimide derivate of polyaminostyrene. The purpose of this paper is to describe the preparation and some properties of this material.

Experimental Section

Materials. High molecular weight polyaminostyrene in the form of a 400 mesh powder was obtained from Kodak Ltd., London, technical grade maleic anhydride was obtained from Hopkins and Williams Ltd., London, *N*-ethylmaleimide, L-cysteine, and glutathione from B.D.H. Ltd., London, thioglycolic acid, thiosalicylic acid, and β-mercaptoethanol from Koch-Light Laboratories Ltd., London, nucleotides from Sigma Chemical Co., St. Louis, and yeast and *Escherichia coli* soluble RNA from General Biochemicals Inc., New York. All other reagents were Analar grade, supplied by B.D.H. Ltd.

Analytical Methods. Sulfhydryl groups in solution were estimated by a method similar to that described by Roberts and Rouser (1958) and Alexander (1958). Aliquots of the solution to be analyzed were mixed with aliquots of 2 mM *N*-ethylmaleimide in phosphate buffer, pH 7, and allowed to stand for 20 min. The absorbance at 302 mμ was measured using a Zeiss PMQ II spectrophotometer, and the change of absorbance with respect to a control was used to calculate the thiol content. A value of 0.61×10^3 (Gregory, 1955) for the change in absorptivity was assumed. Suspensions of resin were filtered using a 0.22 μ millipore filter.

Electrometric titrations were carried out with the aid of an E.I.L. Model 23A direct reading pH meter in conjunction with a GM 23 micro glass electrode supplied by the same firm.

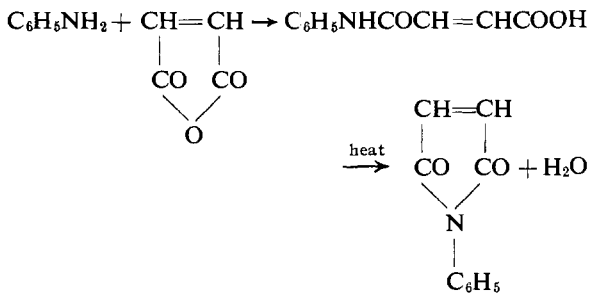
Preparation of Poly Vinyl(N-phenylenemaleimide) (PVM[1]). The preparation of this material was based on the well-known synthesis of *N*-phenylmaleimide (Cava *et al.*, 1961) from aniline and maleic anhydride according to the reaction shown in Scheme I.

The polyaminostyrene was washed with 1 N HCl to remove soluble material and allowed to stand overnight with 1 N NaOH. The resin in the unprotonated form was washed extensively with distilled water until the filtrate was neutral and then dried *in vacuo* over P_2O_5.

Maleic anhydride (80 g) was placed in a 500-ml flask fitted with a reflux condenser surmounted by a $CaCl_2$ drying tube. The flask was gently heated on a sand bath

* From the M. R. C. Biophysics Research Unit and Department of Biophysics, King's College, Drury Lane, London, W. C. 2, England. *Received October 25, 1965.*

[1] Abbreviations used in this work: PVM, poly vinyl (*N*-phenylenemaleimide); NEM, *N*-ethylmaleimide.



SCHEME I

till the anhydride melted (57°) and the temperature reached 100°. Washed polyaminostyrene (10 g) was then added to the melt and the temperature was increased to 140°. At this temperature an exothermic reaction took place with the evolution of water vapor. The temperature was increased to 170° and kept at this value for 30 min. The mixture was then cooled and extracted with hot acetone. The crude product was further extracted with boiling acetone in a soxhlet apparatus until the washings gave no residue on evaporation. Finally the light-brown powdered product was dried *in vacuo* over P_2O_5. The final yield was 13.2 g.

Preparation of N-Phenylmaleimide. Since the method employed for the preparation of PVM was more violent than that described by Cava *et al.* (1961), a model experiment was carried out. Aniline (13.9 g) was added dropwise with stirring to 64 g of molten maleic anhydride in an open beaker at 70°. A transitory deep yellow color was followed by precipitation of maleanilic acid. On heating the mixture to 140° an exothermic reaction took place, water was evolved, and the solid dissolved. The temperature was raised to 170° for 30 min. On cooling, the orange solution became semisolid. Excess maleic anhydride was removed by boiling with 300 ml of water, cooling, and filtering three times. The crude residue contained an impurity, possibly polymeric, the concentration of which increased with the reaction temperature. It was recrystallized from ethanol–water to give yellow needles melting at 86° in 48% yield. After recrystallization from cyclohexane the melting point was 88° (89°). The product reacted with SH groups as expected and gave the characteristic deep red solution with ethanolic KOH.

Characterization of Polyaminostyrene and Reaction Product. The equivalent weight of the polyaminostyrene with respect to basic groups was determined by stirring weighed samples of the resin with 5-ml aliquots of 5 N HCl for 24 hr. After filtration, aliquots of the filtrate were titrated with standard alkali. The equivalent weight was estimated to be 170 ± 5. This figure was in satisfactory agreement with the equivalent weight with respect to nitrogen, as estimated from Kjeldahl nitrogen determination of samples of the digested resin, of 177 ± 5. This agreement suggests that all the nitrogen is present in the form of titratable amino groups. The mean of these two estimations is 174 ± 5, and it is calculated that each styrene residue bears on the average 0.66 amino group.

The number of millimoles of *N*-substituted maleimide per gram of product was estimated from Kjeldahl nitrogen determinations and by treatment of weighed samples with 0.1 N alkali followed by filtration and back titration with standard acid carried out electrometrically. In the ensuing calculations it was assumed that the increase in weight of the product was solely due to the formation of *N*-substituted maleimide groups and that the equivalent weight of the polyaminostyrene was 174 ± 5. In the latter reaction, the imide ring is opened to yield the maleamic acid (Gregory, 1955) which takes up 1 equiv of base. Although there is the possibility of complete hydrolysis to give free maleic acid in the supernatant, there was no material which titrated at the first p*K* (6.5) of maleic acid. Similarly, if *N*-phenylmaleimide is boiled with 1 N NaOH, it is rapidly hydrolyzed to the maleamic acid, but liberation of free aniline cannot be detected.

The results of the two methods of estimation were in satisfactory agreement: 2.56 ± 0.28 mmoles/g from the Kjeldahl determination and 2.25 ± 0.04 mmoles/g from the alkali treatment. The weighted mean of these figures is 2.25 ± 0.04, which corresponds to reaction with 56% of the amino nitrogen of the polyaminostyrene. This agreement also indicates that the major product is indeed the *N*-phenylmaleimide derivative, though the possibility that some polymerized maleic anhydride (or other by-product) is trapped on the column cannot be entirely eliminated. Treatment with 5 N HCl led to complete hydrolysis of both *N*-phenylmaleimide and of PVM to free maleic acid and the amine.

Treatment of the PVM resin with 0.1 M phosphate buffer, pH 7.0, for 12 hr followed by filtration and spectrophotometric examination of the supernatant showed negligible absorbance in the 260–300 mμ band, which suggests that the resin is effectively insoluble at this pH.

Preliminary investigations showed that a 3 × 1 cm column of the PVM effectively bound up to 0.25 g (3 mmoles) of β-mercaptoethanol in 2% solution in 0.1 M phosphate buffer, pH 7.0. Thus after reaction no purple color with alkaline sodium nitroprusside could be detected in the eluent. After extensive washing the resin gave a strongly positive Lassaigne test for sulfur; an unreacted sample of the PVM gave a negative reaction.

Reaction of PVM with SH Compounds. *N*-Substituted maleimides were originally reported to react specifically with SH compounds (Marrian, 1949) by addition across the double bond. Although the specificity is now thought to be incomplete (Smyth *et al.*, 1960), it is sufficiently great for the reagent still to be useful. The addition reaction and the hydrolysis of the imide ring to the maleamic acid are both base catalyzed, but at pH 7 the rate of the former reaction is considerably greater than that of the latter (Gregory, 1955). Accordingly the reaction of the PVM resin with a variety of SH compounds and other reagents was investigated in 0.1 M phosphate buffer, pH 7.0.

Samples (10 mg) of the PVM were stirred magnetically with 5-ml aliquots of 10 mM thioglycolic acid for various

TABLE I: Binding of Various Compounds by 10 mg of PVM Resin from 5 ml of Solution in 20 min.[a]

Compd	Mol wt	Concn of Solution (mM)	Binding (mmoles/g of resin)
β-Mercaptoethanol	78	10	1.9
Thioglycolic acid	92	10	1.8
L-Cysteine	121	10	1.6
Thiosalicylic acid	154	10	*ca.* 0.5
Glutathione	307	10	1.9
Salicylic acid	138	10	0.05
DL-Lysine	146	10	0.03
2′(3′)-Adenylic acid	347	2	0.01
2′(3′)-Guanylic acid	363	2	0.01
2′(3′)-Cytidylic acid	323	2	0.01
2′(3′)-Uridylic acid	324	2	0.01
Yeast soluble RNA (25,000)		*ca.* 0.1 mM in P	2×10^{-4} in P
E. coli soluble RNA (25,000)		c.0.1 mM in P	2×10^{-4} in P

[a] The solutions contained 0.1 M phosphate buffer, pH 7.0.

times. After reaction, the solutions were filtered and assayed for sulfhydryl groups. The results were calculated as millimoles of thioglycolic acid bound per gram of resin and are shown in Figure 1. It is seen that the bulk of the reaction has taken place after 20 min but that some binding takes place very slowly after this for several hours. Similar results were obtained for L-cysteine and for β-mercaptoethanol. Even after 2-hr reaction the maximum uptake is less (65%) than the maximum expected (2.25 mmoles/g). It is probable that maleimide residues near the surface of the resin particles react rapidly but those buried beneath the surface more slowly. A similar effect was observed in the direct electrometric titration of the polyaminostyrene with acid.

The reaction with a variety of other sulfhydryl compounds was investigated by treating 10-mg samples of the PVM resin with 5-ml aliquots of 10 mM solution for 20 min, filtering, and assaying for SH groups. The reaction with several ultraviolet absorbing compounds was investigated similarly, but using 2 mM solutions of the reactant and assaying spectrophotometrically after appropriate dilution. The reaction with lysine in 10 mM solution was investigated using a ninhydrin assay as described for ammonia by Jacobs (1964).

The results of these experiments are shown in Table I. It is clear that only SH compounds react appreciably with the PVM. The binding of the other compounds was in no case significant. It has been shown previously (Roberts and Rouser, 1958) that *N*-ethylmaleimide does not react with the four common nucleotides, adenosine, guanosine, uridine, and cytidine monophosphates. The results in Table I confirm that they do not react with PVM either, and thus, as expected, the latter does not react with soluble RNA.

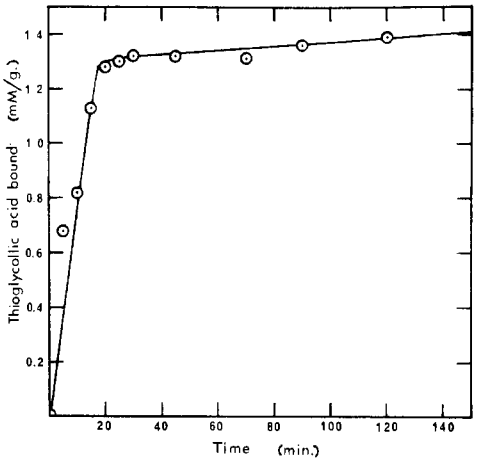


FIGURE 1: Plot of time *vs.* mass of thioglycolic acid adsorbed from 5 ml of 10 mM solution by 10 mg of PVM. The ordinate scale is given in units of millimoles of thioglycolic acid bound per gram of PVM.

Conclusions and Discussion

Poly vinyl(*N*-phenylenemaleimide), which is easily prepared, appears to act rapidly with a high degree of specificity with a variety of sulfhydryl compounds. It may thus be used to remove the latter from solution either by batch processing or by means of a column of the resin.

In particular, nucleotides and RNA are not bound to the material. It is highly probable that there are no other reactive groups on the material besides the maleimide moiety, in contradistinction to the organomercurial polysaccharide described by Eldjarn and Jellum (1963). Thus the styrene residues are inert to most compounds of biochemical interest and the unreacted amino groups are uncharged except at low pH (the acid pK of aniline is 4.7), so that no ion exchange is expected or found at pH 7. On the other hand, the reaction is irreversible and the SH compounds may not be recovered easily, though, by treatment with strong acid, the complex of thiol and maleic acid may be removed from the resin.

The capacity of the column is of the order of 1.8 mmoles/g for a variety of thiols, including glutathione, with a molecular weight of 307. It is probable that the capacity for macromolecules such as proteins or nucleic acid derivatives would be lower on account of steric effects.

Carbon *et al.* (1965) and Lipsett (1965) have reported the presence of thiopyrimidines in *E. coli* s-RNA. Examination of yeast s-RNA failed to detect any absorption band at 335 mμ even in solutions of which $A_{260} \simeq 100$. Therefore, presumably 4-thiouracil is absent from the latter material. Thiouracil might be expected to react with maleimide derivatives in a similar fashion to thiosalicylic acid but neither yeast nor *E. coli* s-RNA is bound to PVM. In the case of *E. coli* s-RNA this perhaps is because the thiouracil moiety is involved in secondary structure as suggested by Lipsett (1965). It is possible however that thiouracil-containing RNA molecules may be bound under denaturing conditions; this matter is presently under investigation in this laboratory.

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