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Organomercurial-polysaccharide, a Chromatographic Material for the Separation and Isolation of SH-Proteins

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A new chromatographic material for separation and isolation of SH-containing proteins has been synthesized. Its function is based upon the reversible reaction between SH-proteins and an organomercurial firmly anchored to crosslinked dextran. Proteins containing sulphydryl groups are in contrast to non-SH-proteins readily fixated to the material, and can be eluted in an active, unchanged state by means of small molecular thiols or other complexing agents with affinity for mercury.

The new material has been successfully applied to the separation of protein mixtures into an SH-fraction and a fraction containing no titrable SH-groups. Furthermore, it has been applied as a purification step in the isolation procedure of mercaptalbumin and of SH-enzymes as well as for concentrating dilute solutions of SH-enzymes. The material is particularly suited for chromatographic fractionation of individual SH-proteins utilizing a gradient and/or stepwise elution with mercury complexing agents.

Numerous methods are available for the separation, purification and isolation of proteins, *e.g.* ammonium sulphate-fractionation, separation according to the Cohn-system, adsorption- and ion-exchange chromatography, gel-filtration, electrophoresis *etc.*[1] The outcome of a separation according to these methods is governed by the character of the proteins considered as a whole (solubility, size, shape, amphoteric properties, number of ionizable groups, isoelectric point *etc.*). It should be emphasized that the presence or absence of specific reactive groups such as phenolic, thioether, or sulphydryl-groups, plays a minor role in these procedures.

In the present paper we report on a new chromatographic material "organomercurial-polysaccharide", by means of which it is possible to separate and isolate SH-proteins selectively from proteins not containing mercapto groups. Thus, in this procedure the presence of reactive sulphydryl groups is essential.

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Sulphydryl groups are undoubtedly the most reactive groups on a protein molecule. Their number may vary from zero to more than 100 SH-groups per mole, and the reactivity of the individual SH-group may vary considerably according to the steric arrangements *etc.* One of the characteristics of the mercapto group is its exceptionally high affinity for heavy metal ions, particularly for mercury. This property has previously been utilized for the isolation of mercaptalbumin, enolase, lactic dehydrogenase and a few other SH-proteins by way of crystalline Hg-complexes.[1] Some examples are also known where zinc, lead and cupric salts have been employed for the precipitation of certain SH-proteins.[1]

Our procedure is based upon the reversible reaction between SH-proteins and an organomercurial firmly anchored to crosslinked dextran. The attachment of an SH-protein to the column material and its elution in an unchanged state with a small molecular thiol or with certain other mercury-complexing agents, are in analogy with the inactivation of SH-enzymes with *p*-chloromercuribenzoate, and the subsequent reactivation with cysteine.

The first part of this report deals with the synthesis of organomercurial-polysaccharide, the second part deals with the chemical and chromatographic properties of the new material, and the third part demonstrates some of its applications.

SYNTHESIS OF ORGANOMERCURIAL-POLYSACCHARIDE

Principle. Highly cross-linked dextran, "Sephadex G-25" (I) is aminated with 2-aminoethyl hydrogensulphate (II) in the presence of alkali, and the resulting amino-Sephadex (III) thiolated with N-acetyl-homocysteine thiolactone (IV) in the presence of silver nitrate, yielding SH-Sephadex (V). This material on treatment with 3,6-bis-(acetatomercurimethyl)-dioxane (VI), a bifunctional mercurial synthesized from allyl alcohol and mercuric acetate or nitrate, yields the final product "organomercurial-polysaccharide" (VII) with approximately one "mercurial chain" per 30—40 glucose units. The reactions may be illustrated by the following scheme:

$$\underset{\text{I}}{R\cdot OH} + \underset{\text{II}}{HSO_3\cdot O\cdot CH_2\cdot CH_2\cdot NH_2}$$

$$\downarrow$$

$$R\cdot O\cdot CH_2\cdot CH_2\cdot NH_2 \quad \text{III}$$

$$\downarrow \quad \text{IV: } CH_2{<}^{S\,-\,C=O}_{CH_2-CH\cdot NHAc}$$

$$R\cdot O\cdot CH_2\cdot CH_2\cdot NHCO\cdot CH(\cdot NHAc)\cdot CH_2\cdot CH_2\cdot SH \quad \text{V}$$

$$\downarrow \quad AcHg\cdot CH_2\cdot CH{<}^{O\cdot CH_2}_{CH_2\cdot O}{>}CH\cdot CH_2\cdot HgAc \quad \text{VI}$$

$$R\cdot O\cdot CH_2\cdot CH_2\cdot NHCO\cdot CH(\cdot NHAc)\cdot CH_2\cdot CH_2\cdot S\cdot Hg\cdot CH_2\cdot CH{<}^{O\cdot CH_2}_{CH_2\cdot O}{>}CH\cdot CH_2\cdot HgAc \quad \text{VII}$$

Choice of starting material. It was found that carbohydrates in general can be converted into mercurial compounds by the described route. Thus, cotton, powdered cellulose of fine and standard grade, DEAE-cellulose, Sephadex G-50 and Sephadex G-25, have all successfully been thiolated and converted into the respective mercurial forms. Of these materials, Sephadex G-25 (dextran with a high degree of crosslinking) was especially suitable, being inactive, insoluble, colourless and non-adsorbent for proteins in general. Furthermore, organomercurial-polysaccharide prepared from Sephadex G-25 is well suited for batchwise operation as well as for column chromatography.

Aminization of Sephadex G-25. Amino-Sephadex was prepared essentially according to the method of Reeves and Guthrie for aminization of cotton.[2] Sephadex G-25 of medium porosity (65 g) was rapidly stirred into a solution of sodium hydroxide (60 g) and 2-aminoethyl-hydrogensulphate (20 g) in water (156 ml). After a few minutes the swollen and apparently dry material was spread out on a glass plate and dried in an oven at 100 – 110° for about 4 – 5 h. The amino-Sephadex was then repeatedly washed with water until free from alkali. A degree of aminization represented by 0.3 – 0.5 % nitrogen was usually obtained by this method.

SH-Sephadex from amino-Sephadex. The thiolation was carried out according to the method of Benesch and Benesch.[3,4] To the swollen slurry of amino-Sephadex (65 g) was added a solution of N-acetylhomocysteine thiolactone (12 g) in water (800 ml). Under vigorous stirring at room temperature, 1 N $AgNO_3$ and 1 N NaOH were added incremently so as to maintain the pH at 7.5, until a total of 75.5 ml $AgNO_3$ had been used.

The pH was checked regularly during this process, and was not allowed to exceed pH 9 in order to avoid reduction of the silver ions by the reducing endgroups of the crosslinked dextran. The suspension was allowed to stand with frequent stirring at room temperature for a few hours. Removal of silver ions was accomplished with a concentrated solution of thiourea (140 g in the minimum amount of water) followed by 1 N HNO_3 (250 ml). The SH-Sephadex was washed with thiourea in dilute HNO_3 and with distilled water, and stored at 0 – 4° as a swollen suspension.

Vacuum dried thiolated Sephadex is a white powder which swells in water forming gel-grains with the same appearance and gel-filtration properties as ordinary Sephadex G-25. In contrast to thiols in general, the SH-groups of thiolated Sephadex are remarkably stable towards oxidation by air; thus, no oxygen uptake could be demonstrated in 8 h at 37°, pH 7.4. The nitroprusside test for SH-groups is strongly positive. The material possesses a very high affinity for heavy metals, and as a typical polythiol, it shows pronounced reducing properties. Unexpectedly, however, it cannot be titrated with iodine directly. The number of SH-groups are therefore best determined by treating a known amount of SH-Sephadex with excess of a neutral solution of tetrathionate and titrating the formed thiosulphate with standard iodine solution. SH-Sephadex G-25 prepared as described usually contained approximately 0.55 – 0.45 % SH or 1 SH per 30 – 40 glucose units.

3,6-Bis-(acetatomercurimethyl)-dioxane. This bifunctional mercurial may be prepared according to Edsall *et al.*[5] by heating a mixture of equivalent amounts of allyl alcohol and mercuric acetate to 60° and allow to stand. We found however, that the yield obtained in this way was very poor and that the formation and crystallization of the rather soluble organomercurial acetate required several days. We therefore adopted the method described by Chatt [5] whereby the more insoluble nitrate was obtained. This salt was subsequently converted by means of ion exchange on Dowex-1-acetate into 3,6-bis-(acetatomercurimethyl)-dioxane.

Mercuric nitrate (16.5 g) was dissolved in the minimum amount of nitric acid containing 40 g conc. HNO_3/l, and heated to 50°. Allyl alcohol (3.5 ml) was added and the mixture was allowed to stand for 30 min. The precipitate of white crystals was washed with ice-cold water, dissolved in the minimum quantity of 0.5 N NaOH and reprecipitated by acidification with nitric acid. A 1 % solution of the recrystallized organomercurial nitrate in the minimum amount of NaOH was finally passed through a column of "Dowex-1-acetate" and the eluate adjusted to pH 7.6 with phosphate buffer (final concentration 0.01 M). The solution was stable when stored in the dark.

In order to facilitate the characterization-studies on organomercurial-polysaccharide, 3,6-bis-(acetatomercurimethyl)-dioxane containing radioactive mercury has also been

synthesized. The ^{203}Hg isotope, purchased as mercuric acetate, was mixed with carrier mercuric nitrate and incorporated as above, but in reduced scale quantities.

Organomercurial-polysaccharide. Excess of a cold 1 % solution of 3,6-bis-(acetatomercurimethyl)-dioxane in 0.01 M phosphate buffer pH 7.6 was poured into a cold suspension of swollen SH-Sephadex and stirred for about 15 min at 0°. In order to avoid crosslinking through the bifunctional mercurial, excess of the latter was essential. This was checked by adding a few drops of the supernatant to a potassium iodide solution. Turbidity or a white precipitate of the insoluble organomercurial iodide should be obtained. The organomercurial-polysaccharide was then repeatedly washed with distilled water. The product was stored at 0—4° suspended in distilled water.

CHARACTERIZATION OF ORGANOMERCURIAL-POLYSACCHARIDE

Chemical properties. Dry organomercurial-polysaccharide is a white powder which swells in water forming gel-grains similar to ordinary Sephadex, and with the gel-filtrations properties preserved. Evenly packed columns giving good flowrates (up to 15 ml per min for a 20 × 1 ½ cm column) are easily prepared. The material shows the chemical reactions of organomercurials such as exceptionally high affinity for SH-groups and consequently for SH-proteins, ability to bind anions like iodide, bromide or other substances forming insoluble mercurial salts, and also high affinity for a number of complexing agents like EDTA, Tris, glycine *etc.* (Table 1).

The 3,6-bis-(acetatomercurimethyl)-dioxane is firmly linked to the SH-Sephadex via a stable, covalent S—Hg bond. The only substances found capable of breaking this bond, thus liberating the bifunctional mercurial and regenerating the SH-Sephadex, were smallmolecular thiols like cysteine, cysteamine, 2-mercaptoethanol and although less efficient, reduced glutathione. The material is unaffected by low pH. Strongly alkaline solutions, however, (pH above 11) slowly removes the mercurial.

The organomercurial-polysaccharide is stable towards oxidation by air, thus no O_2-uptake could be measured at 37°, pH 7.7 during 8 h. In order to avoid bacterial growth we have always stored the product in the cold.

Provided crosslinking of adjacent SH-groups through the bifunctional mercurial has been avoided, the number of free acetatomercury groups should equal the number of free SH-groups in the original SH-Sephadex. This was verified as follows:

Organomercurial-polysaccharide prepared from SH-Sephadex containing a known amount of SH-groups, was titrated with potassium iodide solution containing trace amounts of radioactive iodide (^{131}I). When every free acetatomercury group had combined with one iodide ion, the supernatant became radioactive. The results obtained in this way, proved that about 97 % of the theoretical acetatomercury groups were present in the free state, confirming that only a negligible amount of crosslinks had been introduced.

Properties as material for protein chromatography. Data will be presented showing the general characteristics of organomercurial-polysaccharide when applied as a material for column chromatography of SH proteins. Hemoglobin (M.W. = 66 500) which contains 8 SH per mole of which 4 SH seem to be particularly reactive,[7,8] was for the sake of simplicity chosen as a representative SH-protein for these studies. Although the results express the behaviour of

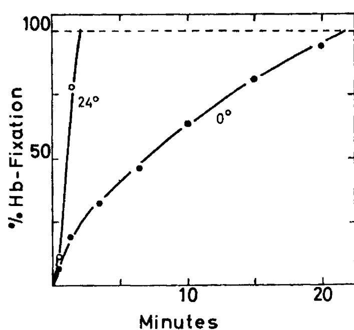

Fig. 1. Effect of temperature on the rate of attachment of hemoglobin to organomercurial-polysaccharide. 2 ml of the column material suspension, 10 ml 0.01 M phosphate buffer (pH 7.4) and 8 mg hemoglobin were used. The amount of hemoglobin present in the supernatant was spectrophotometrically determined at intervals.

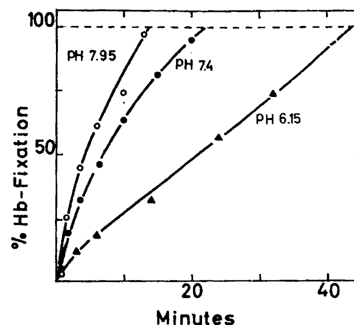

Fig. 2. Effect of pH on the rate of attachment of hemoglobin to organomercurial-polysaccharide. Experimental conditions as in Fig. 1, except that phosphate buffers of different pH were used, and that the temperature was kept constant at 0°.

hemoglobin towards organomercurial-polysaccharide, we have found that these informations can more or less be extended to many other SH-proteins. The data may therefore serve as useful guides for experiments with SH-proteins in general.

The conditions for fixation and elution of hemoglobin were established by experiments carried out batchwise in centrifuge tubes.

Fig. 1 shows that the rate of attachment of hemoglobin to the organomercurial-polysaccharide is faster at higher temperatures. The reaction proceeds more readily at pH 7.95 than at pH 6.15 (Fig. 2) indicating that the degree of ionization of the SH-groups is an important factor.

Fig. 3 shows the effect of pH on the elution of hemoglobin from the organomercurial-polysaccharide. The protein is seen to remain firmly attached between pH 5.5 and 11. Lowering of pH from 5.5 to 5.3 is seen to bring about a drastic change from 100 % fixation to 100 % elution. This steepness of the

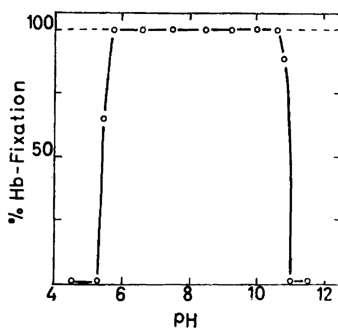

Fig. 3. Effect of pH on the elution of hemoglobin from organomercurial-polysaccharide. Batchwise treatment with 0.05 M buffers at 0°. Hemoglobin in the supernatant was measured. Phthalate buffer was used between pH 4 and pH 6, phosphate buffer between pH 6 and pH 8 and bicarbonate buffer above pH 8.

pH-elution curve is noteworthy since this fact most likely can be utilized to separate different SH-proteins with different pH-elution characteristics, by a gradient or stepwise alteration of the pH.

The effect of a number of different compounds on the elution of hemoglobin has been studied. Since radioactive organomercurial-polysaccharide was employed in these experiments it has been possible to classify the substances as seen in Table 1.

Table 1. Effect of various compounds on the elution of hemoglobin from organomercurial-polysaccharide. ^{203}Hg-labelled organomercurial-polysaccharide with hemoglobin attached to it was treated with 0.05 M solutions of the different substances in 0.01 M phosphate buffer of pH 7.5 at 0°. Radioactivity as well as hemoglobin concentration was determined in the supernatant.

Compounds without effect.	Compounds which elute the hemoglobin, but not the organomercurial.	Compounds which elute the hemoglobin as well as the organomercurial.
Phosphate	Tris-buffer	Cysteine
(up to 4 M without effect)	EDTA	(10^{-4} M is sufficient).
Carbonate	Thiocyanate	
Bicarbonate	Thiouracil	Cysteamine
Phthalate	Ammonium-salts	2-Mercaptoethanol
Acetate	Glycin	Reduced glutathione
Citrate	Diethyldithiocarbamate	
Chloride	Iodide	Strong bases, pH > 11
Nitrate	Bromide	
Sulphate		

From the table it can be inferred that the protein S—Hg bonds are more easy to split than the bonding between the SH-Sephadex and the organomercurial, since only thiols are able to break the latter, whereas complexing agents like EDTA and Tris as well as other ions with affinity for Hg, rupture the former link. Consequently these substances must be absent in experiments where the purpose is to retain SH-proteins on the organomercurial column. On the other hand, these buffers may be used as eluting agents. The elution with complexing agents is analogous with the well known fact that EDTA may actually overcome the *p*-chloromercuribenzoate inhibition of certain SH-enzymes.[9]

An indication that the hemoglobin actually was bound to the organomercurial-polysaccharide through the SH-groups was the fact that an increase in the ionic strength up to 4 M phosphate buffer failed to elute the hemoglobin, whereas it immediately was liberated by very small amounts of thiols (*e.g.* 10^{-4} M cysteine).

A standard procedure adopted in connection with the use of organomercurial-polysaccharide, involves attachment of the SH-protein to the column material, elution with cysteine, and removal of excess cysteine as well as cysteineorganomercurial-complex by gel-filtration on ordinary Sephadex G-25. When

hemoglobin was subjected to this procedure, the protein always was recovered without any signs of denaturation or changes as judged by the following criteria:

1. The absorbtion spectra between 2000 and 8000 Å were identical before and after the experiment (Fig. 4).

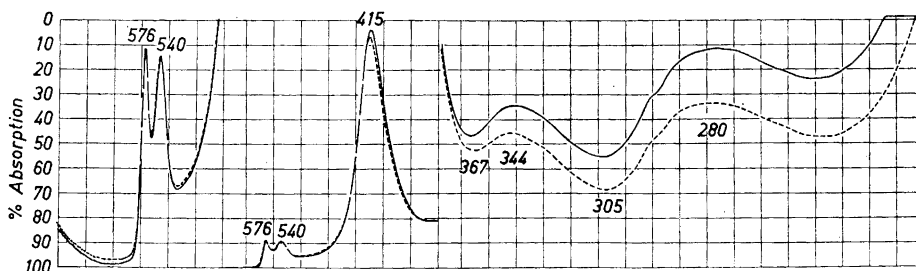

Fig. 4. Absorption spectra of hemoglobin before and after elution from organomercurial-polysaccharide.

2. The sedimentation properties in the Spinco-analytical ultracentrifuge were unchanged.

3. The number of reactive readily titrable SH-groups of the hemoglobin remained essentially unchanged (4.33 SH/mole before and 4.19 SH/mole after the experiment). The SH-titer was determined by amperometric silver titration,[10,11] and the hemoglobin concentration calculated from the absorbancy at 540 mμ.

APPLICATIONS OF ORGANOMERCURIAL-POLYSACCHARIDE

We have found that organomercurial-polysaccharide can be used for a variety of purposes such as the quantitative separation of SH-proteins from non-SH-proteins, and the isolation of SH-proteins from dilute solutions. Probably the most important application is to the fractionation of individual SH-proteins by chromatography on organomercurial-polysaccharide. These applications of the new column-material will be demonstrated by appropriate examples utilizing human serum albumin and several well known SH-enzymes.

Quantitative separation of proteins into an SH- and a non SH-fraction

This application can be clearly demonstrated with human serumalbumin. This protein consists of approximately two-thirds of mercaptalbumin with 1 SH per mole. The remaining fraction either contains no SH-group or a less reactive, non-accessible one.[10] Mercaptalbumin which was discovered by Hughes may be isolated by precipitation with mercuric chloride,[12] or with certain organomercurials.[5] Human albumin can also be divided into mercaptalbumin and non-SH-albumin by passage through the organomercurial-polysaccharide

column. The non-SH-fraction, in contrast to the mercaptalbumin, is not retained by the column.

Experimental procedure. A method has been developed which may be applied to the separation of most protein mixtures into an SH- and a non-SH-fraction. This procedure will therefore be described in general terms.

If the protein mixture to be separated contains small molecular thiols, EDTA, Tris, ammonium salts or other interferring complexing agents, (Table 1) these substances have to be removed, *e.g.* by gel-filtration on a column of Sephadex G-25 coarse grade, equilibrated with 0.01 M phosphate buffer pH 7.6.

A column of organomercurial-polysaccharide 15 × 1 $\frac{1}{2}$ cm is packed and thoroughly washed with 0.01 M phosphate buffer, pH 7.6, until no turbidity is produced in the eluate upon addition of iodide. About 5—10 ml of the protein solution, is applied and drained into the column. The flow is then shut off and the proteins are allowed to stand in contact with the column material for 15—30 min at 0 to 4°. A 0.01 M phosphate buffer pH 7.6 is then passed through the column, and fractions of 5 ml are collected at a flowrate of 3 to 15 ml/min. Fractions 2 to 5 will contain the proteins not retained by the column, whereas the following fractions usually are free from protein. Fractions 2 to 5 are combined and excess cysteine added to ensure that sulphydryl groups, if present, are free and not blocked by liberated trace amounts of organomercurial. Excess cysteine is removed by gel-filtration on Sephadex G-25, equilibrated with 0.9 % NaCl. The pure protein solution thus obtained usually gives very little or no response in the amperometric silver titration,[10,11] and therefore comprises the "non-SH-fraction".

The other proteins which remain firmly attached to the organomercurial-polysaccharide column, may be eluted with cysteine (0.05 M in 0.01 M phosphate buffer pH 7.4). Excess cysteine as well as the cysteineorganomercurial-complex is completely removed by gelfiltration on Sephadex G-25 equilibrated with 0.9 % NaCl. The collected proteins always contain titrable SH-groups and may therefore be termed the "SH-fraction". All steps in the described procedure is carried out at 0—4°.

Regeneration of the column material. The organomercurial-polysaccharide column material can readily be regenerated after use. This is preferentially done in a batchwise procedure, but can also be carried out on the column. A 0.05 M cysteine solution of pH 7.4 is passed through the column thus regenerating the SH-Sephadex. After washing with distilled water, the 1 % solution of 3,6-bis-(acetatomercurimethyl)-dioxane is applied as previously described, and the organomercurial-polysaccharide column thus is regenerated.

Results and discussion. The above standard procedure was applied to chrystalline human serum albumin * which originally contained 0.58 SH/mole. This protein was split into one fraction without titrable SH-groups and another fraction which in three separate experiments was found to contain 1.02, 1.06 and 1.00 SH per mole, respectively. The molecular weight was taken as 69 000 and the protein concentration calculated from the absorbancy at 276 mμ ($E^{1\%}_{1\,cm} = 5.50$).

* Kindly donated by AB Kabi, Stockholm.

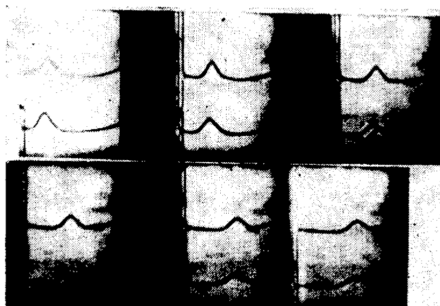

Fig. 5. Sedimentation diagram of original albumin and mercaptalbumin eluted from organomercurial-polysaccharide.

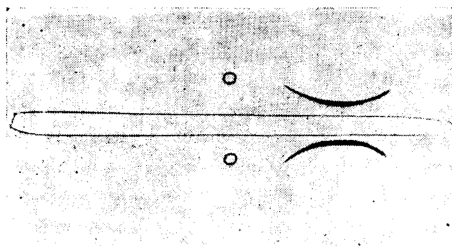

Fig. 6. Immuno-electrophoresis of original albumin and mercaptalbumin eluted from organomercurial-polysaccharide.

Fig. 5 shows that the sedimentation property of the mercaptalbumin in the ultra-sentrifuge was identical with that of the original albumin. Fig. 6 demonstrates that the immuno-electrophoretic behaviour was unchanged before and after the experiment. Judged by these criteria, separation of human albumin was achieved without denaturation.

Elution of mercaptalbumin from the organomercurial-polysaccharide column was in contrast to hemoglobin also brought about by increasing the concentration of phosphate buffer. Thus 0.2 M phosphate buffer pH 7.4 was found sufficient for complete removal. This difference in behaviour may be explained by the fact that only 1 SH per mole albumin is available for bond formation with the column, whereas the more firmly attached hemoglobin may be linked to the mercurial-polysaccharide through as many as 8 SH-groups. Batchwise experiments confirmed that also the rate of attachment to organomercurial-polysaccharide was considerably slower with albumin than with hemoglobin. In fact the batchwise reaction proceeded so slowly with albumin, that we found it essential to apply column technique in all experiments performed with this protein.

To ensure that only SH-albumin is being fixated to the column, it is important to keep the phosphate buffer concentration at a certain level. Thus, if the organomercurial-polysaccharide column is equilibrated with distilled water only, and an aqueous solution of albumin applied, non-SH-albumin in addition to mercaptalbumin will be retained, probably due to a non-specific adsorption. Under such conditions, the eluate was found only to contain from 0.7 to 0.9 SH per mole.

Although human albumin has been studied particularly detailed, we have also separated other protein mixtures into an SH and a non-SH-fraction. Thus human serum was readily split into two such fractions. A crude solution of urease, originally containing 15.6 SH per mole, was also divided into two fractions, one containing 0 SH per mole and another fraction containing active enzyme titrating 25.2 SH per mole.

Purification of crude enzyme extracts

The degree of enzyme purification is usually expressed in terms of specific activity, *i.e.* enzyme activity per mg of protein. When applying organomercurial-polysaccharide chromatography to the purification of SH-containing enzymes, it is likely that inactivation sometimes may occur, in analogy with the fact that certain enzymes will be irreversibly inactivated by *p*-chloromercuribenzoate.

In the experiments to be described we have used two well known SH-enzymes which may be obtained in a pure, crystalline state, *viz.* aldolase and glyceraldehyde phosphate dehydrogenase. Glyceraldehyde phosphate dehydrogenase (GAPDH) from rabbit muscle was prepared as described by Velick [13] and recrystallized five times. The enzyme was assayed according to Velick.[13] Crystalline aldolase was obtained from Boehringer & Soehne and assayed according to Warburg and Christian.[14]

In order to establish the amount of denaturation occurring during a separation on organomercurial-polysaccharide, the pure crystalline enzymes were subjected to the standard procedure for fractionation into an SH- and a non-SH-fraction, and the specific activity of the enzymes determined before and after the experiment. The result as seen in Table 2 shows that no significant

Table 2. Specific activity of GAPDH and aldolase before and after elution from organomercurial-polysaccharide. The increment in optical density per minute at 340 mμ and at 22°, divided by the enzyme-concentration (extinction read at 278 mμ), was taken as the specific activity.

SH-enzyme	Specific activity (arbitrary units)
Original crystalline aldolase	30.5
Aldolase eluted from the column	30.4
Original crystalline GAPDH	20.7
GAPDH eluted from the column	20.2

denaturation occurred on the column, since the specific activity of GAPDH as well as aldolase remained essentially unchanged.

The use of organomercurial-polysaccharide as a purification step was demonstrated as follows. Rat muscle homogenate was centrifuged at 25 000 $\times$ g for 10 min and the supernatant applied to an organomercurial-polysaccharide column at 0°. After 10 min the column was washed with a mixture of 0.2 M ammonium sulphate and 0.01 M phosphate buffer, pH 7.6. About two thirds of the proteins originally present in the muscle extract were eluted by this solution, but aldolase activity did not appear. When a cysteine solution was applied, the remaining proteins including the aldolase were recovered. The specific activity of the eluted aldolase had increased three times, from originally 7.5 to finally 22.7 (Units as defined in Table 2).

The isolation of SH-proteins from a dilute solution

The isolation of a given protein from a dilute solution is a step frequently encountered in protein chemistry. By means of organomercurial-polysaccharide, it is, as can be demonstrated with glyceraldehyde phosphate dehydrogenase, possible to concentrate dilute solutions of SH-enzymes. 500 ml of a very dilute enzyme solution in 0.01 M phosphate buffer, pH 7.6, and with known activity per ml was continually passed through a $14 \times 1\frac{1}{2}$ cm organomercurial-polysaccharide column at $0-4°$ at a rate of 3 ml/min. At this rate complete fixation occurred. However, if the flow rate was increased to 3.8 ml/min 7.5 % of the original enzymic activity could be detected in the effluent, whereas at a flow rate of 6.5 ml/min 25 % of the original activity was found in the effluent. After passage of the total enzyme solution through the column at the maximum safe flow rate of 3 ml/min the attached enzyme was eluted with cysteine and the activity determined. The GAPDH activity pr ml was found to be increased with a factor of 20 by this treatment, and the recovery was more than 90 %.

Fractionation of SH-proteins by chromatography on organomercurial-polysaccharide

During the studies on organomercurial-polysaccharide it soon became evident that different SH-proteins showed different affinity for the column material. This can easily be explained by the fact that the number of available,

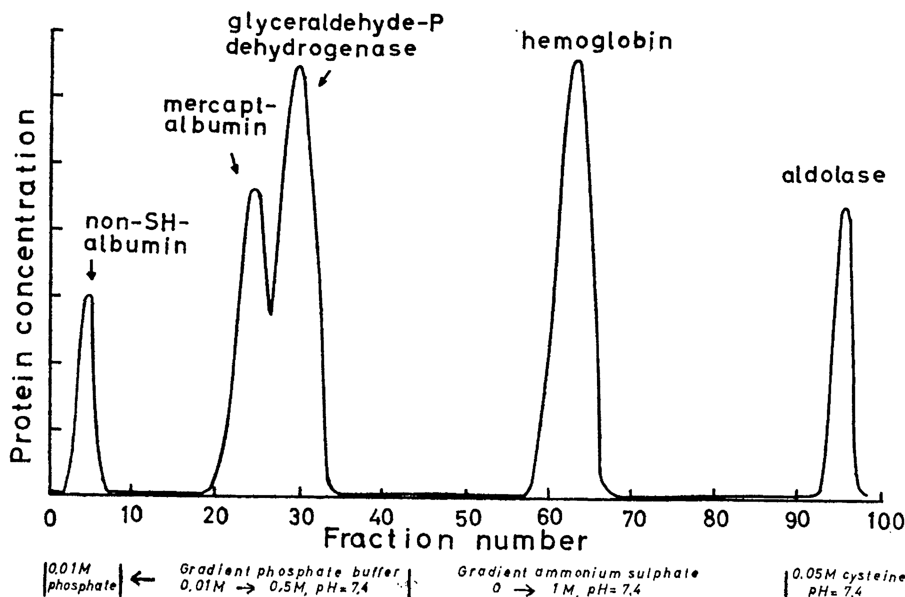

Fig. 7. Fractionation of a mixture of human albumin, hemoglobin, muscle glyceraldehyde phosphate dehydrogenase, and aldolase on a $20 \times 1\frac{1}{2}$ cm organomercurial-polysaccharide column at 0°.

reactive SH-groups may very greatly from protein to protein. Conventional gradient or stepwise elution with change in pH, ion composition and/or in ionic strength, therefore ought to bring about separation of at least some SH-proteins. This we have confirmed. Thus, mercaptalbumin is eluted ahead of glyceraldehyde phosphate dehydrogenase by a gradient phosphate buffer.

What opens up a large number of possibilities for separation of individual SH-proteins on organomercurial-polysaccharide is the fact that in addition a number of complexing agents (Table 1) may be used for elution. Most likely all these agents possess different affinity for the mercury of the column material and therefore demonstrate different ability to displace or elute the attached SH-proteins.

One of the many combinations of gradient and stepwise elution with or without complexing agents, have been used to separate a mixture of human albumin, glyceraldehyde phosphate dehydrogenase, hemoglobin and aldolase. Fig. 7 shows the result.

The results indicate that in addition to the actual number of SH-groups, the size or molecular weight of the protein molecule also plays an important role, for its elution from the column. As usual, the non-SH-fraction is not retained by the column at all, the mercaptalbumin with 1 SH per M.W. 69 000 is eluted ahead of GAPDH with 11 SH per M.W. 120 000. Hemoglobin with 8 SH per M.W. 66 500, is more firmly bound than the former, and finally aldolase with 29 SH per M.W. 147 000 has extremely high affinity for the column material and is neither eluted by phosphate buffer nor by 1 M ammonium sulphate. Cysteine, however, immediately elutes also this protein.

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