HYDROCARBON-COATED SEPHAROSES.
USE IN THE PURIFICATION OF GLYCOGEN PHOSPHORYLASE

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Summary: A homologous series of hydrocarbon-coated Sepharoses varying in the length of their alkyl side chains (Seph-$NH(CH_2)_nH$) was synthesized. These modified Sepharoses provide a versatile tool for the purification of proteins, since, by choosing the suitable member of the series, a desired protein can be extracted from a protein mixture. This is illustrated in the case of glycogen phosphorylase, which is not retained at all by methyl Sepharose (n=1), is retarded by propyl Sepharose (n=3), is adsorbed on butyl Sepharose (n=4) and can be eluted from the column by deforming buffers, and is so tightly adsorbed on hexyl Sepharose (n=6) that it could be eluted from the column only in the denatured form, by washing with 0.2 N CH_3COOH. On a preparative scale, a hundred-fold purification of phosphorylase could be achieved in one step, by passage of a crude muscle extract on a small butyl Sepharose column.

In view of the dramatic enzyme purifications achieved recently by the use of affinity chromatography (1,2), we attempted to coat Sepharose with glycogen and use it for affinity chromatography of glycogen phosphorylase and glycogen synthetase. This was performed (3) by activation of Sepharose with CNBr (4,5) and reaction with octamethylenediamine to obtain an ω-aminoalkyl Sepharose (2,6), then binding of CNBr-activated glycogen to it. The glycogen-coated Sepharose (3) indeed adsorbed phosphorylase b, and the enzyme could be eluted from the column by the use of deforming buffers such as imidazole-citrate (7) which were shown to have a marked, fully reversible effect on the structure of the enzyme. Sepharose 4B which was used as a carrier did not bind phosphorylase but, to our surprise, the control column of Sepharose which had reacted with octamethylene diamine also did bind phosphorylase b. On the other hand, if octamethylene

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diamine was replaced by tetramethylene diamine, no adsorption of phosphorylase occurred. This observation raised the possibility that the very length of the hydrocarbon chain might be involved in endowing the modified Sepharose with the capacity of adsorbing glycogen phosphorylase b.

MATERIALS AND METHODS

Enzymes. Glycogen phosphorylase b was prepared by the method of Fischer et al. (8) and recrystallized three times before use. The enzyme was freed from AMP by passage through a charcoal-cellulose column (9) and assayed by the method of Hedrick and Fischer (10). Enzyme concentrations were determined spectrophotometrically using an absorbance index $A_{280}^{1\%}$=13.1 (11). GAPD† (from Sigma) was assayed by the method of Velick (12) and its concentration was determined using an absorbance index $A_{280}^{1\%}$=10.0 (13).

Muscle Extract. Minced rabbit skeletal muscle was extracted with 2.5 liters of water per kg of meat. Extraction was allowed to proceed for 15 minutes at 22° and then the mixture was poured through cheesecloth and allowed to drain. After centrifugation for 40 min at 3000 g the supernatant was filtered through glasswool to obtain the crude extract used here for the isolation of phosphorylase.

Alkyl Sepharoses. Sepharose 4B (Pharmacia) was activated with CNBr at pH 11 and 22° by addition of 1 g CNBr to 10 g (wet weight) of Sepharose (4,5). The reaction was allowed to proceed for 8 minutes, maintaining the pH at 11 with 5N NaOH. Activation was terminated by filtration and washing of the gel with ice-cold de-ionized water. Meanwhile, a solution of the appropriate alkyl amine in 40% dioxane was prepared, and acidified to pH 9.0 with 6N HCl. The activated Sepharose was then suspended in cold 0.1 M $NaHCO_3$ and mixed with an equal volume of the alkyl amine solution. Dioxane was added to the slurry to a final concentration of 40% to keep the alkyl amine in solution. The reaction mixture now contained 4 moles of the alkyl amine per mole of CNBr used for the activation of

†Abbreviations: GAPD, D-glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12); Seph, Sepharose 4B.

the Sepharose. The alkyl amine was allowed to react with the activated Sepharose at 4° for 24 hours. Subsequently the hydrocarbon-coated Sepharose was washed with water, 0.1 M $NaHCO_3$, 0.05 M NaOH then water again. Under the microscope the coated beads appeared identical to unmodified Sepharose 4B.

Acrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and 2-mercaptoethanol was performed as described by Dudai et al. (14).

RESULTS AND DISCUSSION

A series of alkyl-Sepharoses, which differ in the length of their hydrocarbon side chains, was synthesized (Fig. 1). Mixtures of GAPD and phosphorylase b were passed through short columns of the above modified Sepharoses. It was found (Fig. 2) that GAPD is not adsorbed or retarded by any of these columns (Seph-C_1 to Seph-C_6). However, while Seph-C_1 did not retain phosphorylase b, Seph-C_2 retarded the enzyme and higher alkyl Sepharoses (Seph-C_4 to Seph-C_6) adsorbed it. Elution of phosphorylase b from the columns on which it was adsorbed was not possible by merely increasing the ionic strength with NaCl (up to 0.5 M) but using a deforming buffer (composed of 0.4 M imidazole and 0.05 M 2-mercaptoethanol, adjusted to pH 7.0 with citric acid (7)) the enzyme could be eluted from Seph-C_4, though not from Seph-C_6. The binding of phosphorylase b to Seph-C_6 is apparently very tight, since the enzyme could not be eluted even when the pH of the deforming buffer was lowered to 5.8. Recovery of the enzyme from Seph-C_6 was possible only in the denatured form, by washing the column with 0.2 M CH_3COOH.

The above results suggest that the length of the hydrocarbon "arms" of the Sepharose beads has a marked effect on the capacity of the column to bind phosphorylase b, passing from no retention, through retardation, to reversible binding up to a very tight binding, as the hydrocarbon chain is gradually lengthened. The question was raised whether this is true only for phosphorylase. Out of several proteins tested (lysozyme, bovine serum albumin, bovine γ-globulin and GAPD) only phosphorylase b was retained by Seph-C_4 (Figs. 2 and 3).

Abbreviation	Structure
Seph-C_1	-NH-CH_3
Seph-C_2	-NH-CH_2-CH_3
Seph-C_3	-NH-CH_2-CH_2-CH_3
Seph-C_4	-NH-CH_2-CH_2-CH_2-CH_3
Seph-C_5	-NH-CH_2-CH_2-CH_2-CH_2-CH_3
Seph-C_6	-NH-CH_2-CH_2-CH_2-CH_2-CH_2-CH_3
Seph-C_8	-NH-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-CH_3

Fig. 1. Structure of alkyl-Sepharoses used in this work.

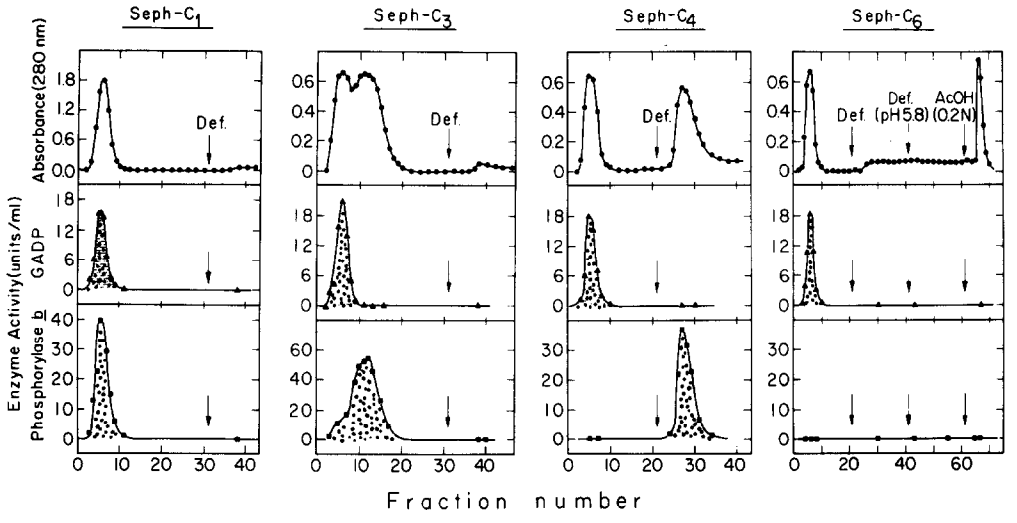

Fig. 2. Preferential adsorption of phosphorylase b on hydrocarbon-coated Sepharoses varying in the length of their alkyl chains. The following samples were applied on the columns (8 x 0.9 cm) Seph-C_1, 0.75 ml containing 5.0 mg GAPD and 5.1 mg phosphorylase b; Seph-C_3, 0.9 ml containing 4.0 mg GAPD and 4.2 mg phosphorylase b; Seph-C_4, 1.0 ml containing 4.9 mg GAPD and 4.1 mg phosphorylase b; Seph-C_6, 1.0 ml containing 4.9 mg GAPD and 4.1 mg phosphorylase b. The columns were equilibrated at 22° with a buffer composed of sodium β-glycerophosphate (50 mM), 2-mercaptoethanol (50 mM) and EDTA (1 mM), pH 7.0. Fractions of 1.6 ml were collected and their absorption at 280 nm (—●—) as well as their GAPD activity (—▲—) and phosphorylase b activity (—■—) were monitored. Non-adsorbed protein was washed off with the buffer mentioned above and then elution with a deforming buffer (0.4 M imidazole, 0.05 M 2-mercaptoethanol adjusted to pH 7.0 with citric acid) was initiated (arrow). In the case of Seph-C_6, attempts were made to elute phosphorylase using also a more acidic deformer (pH 5.8) and subsequently - 0.2 N CH_3COOH.

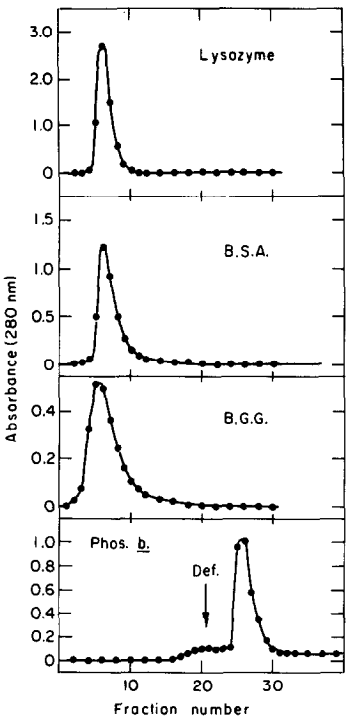

Fig. 3.

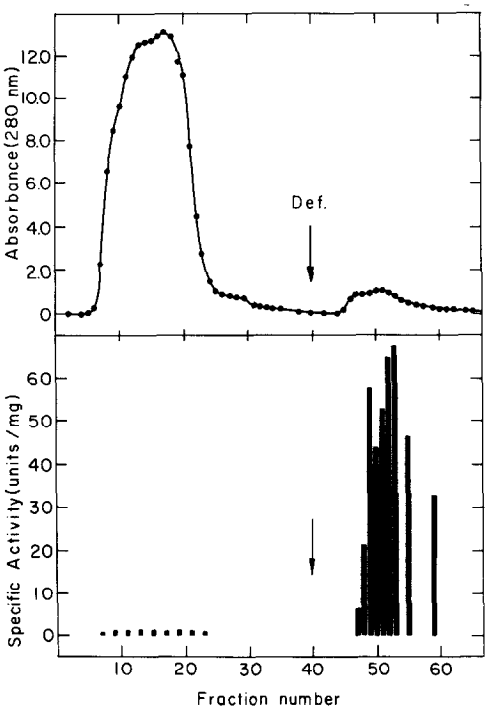

Fig. 4.

Fig. 3. Comparison of various proteins in their capacity to bind to Seph-C_4. Four Seph-C_4 columns (8 x 0.9 cm) were equilibrated at 22° with the glycerophosphate buffer described in the legend to Fig. 2. The following samples were applied on the columns: lysozyme-4.0 mg in 0.7 ml; bovine serum albumin (B.S.A.) - 10 mg in 0.4 ml; bovine γ-globulin (B.G.G.) - 3.3 mg in 0.7 ml; phosphorylase b (phos. b) - 4.2 mg in 0.6 ml. The columns were washed, eluted and monitored as described in the legend to Fig. 2.

Fig. 4. Preparative scale purification of phosphorylase from muscle extract on Seph-C_4. Thirty ml of the extract (see Materials and Methods) with phosphorylase activity of 0.7 units/mg were applied on a Seph-C_4 column (16 x 1.1 cm) equilibrated at 22° with the glycerophosphate buffer described in the legend to Fig. 2. Fractions of 2.6 ml were collected and their absorption at 280 nm (—●—) as well as their phosphorylase activity (black bars) was monitored. Non-adsorbed protein was washed off with the glycerophosphate buffer and then elution with the deforming buffer (cf. Fig. 2) was initiated (arrow). The phosphorylase specific activity was measured after dialysis of the fractions against 100 volumes of the glycerophosphate buffer.

This observation prompted us to attempt the purification of phosphorylase from crude muscle extract, using this column. As seen in Fig. 4, Seph-C_4 proved to be very efficient in fishing out phosphorylase from the extract. Over 95% of the proteins (based on absorption at 280 nm) are excluded from the column. These

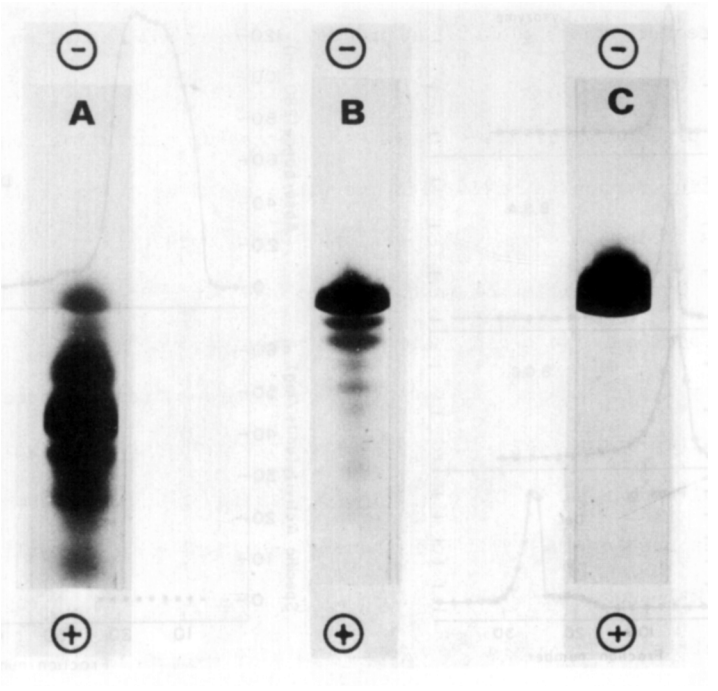

Fig. 5. Acrylamide gel electrophoresis of (A) crude muscle extract (50 γ protein); (B) phosphorylase obtained from the crude extract after chromatography on Seph-C_4 (30 γ protein); (C) three times crystallized phosphorylase b (24 γ protein). The electrophoresis (5% gels) was performed in the presence of sodium dodecyl sulfate (0.1%) and 2-mercaptoethanol (0.01 M), pH 7.2.

fractions exhibit essentially no phosphorylase activity as measured in the presence of AMP. Upon elution with the deforming buffer, a small amount of protein emerges from the column, with very high phosphorylase activity. The specific activity of the eluted enzyme (after dialyzing out the deforming buffer) is up to 68 units/mg, which is very close to that reported for the pure, crystalline enzyme (80 units/mg (10)). It should be noted, however, that the specific activity is not constant throughout the eluted fractions, suggesting the presence of some impurity. As seen in Fig. 5, the enzyme isolated on Seph-C_4 is not as pure as the three times crystallized phosphorylase b prepared in the classical method (8). However, since the crude extract had a phosphorylase activity of 0.7 units/mg and the specific activity of the enzyme purified on Seph-C_4 was up

to 68 units/mg, we have achieved here roughly a hundred-fold purification in one step. Moreover, the yield of the process was over 95%, based on activity measurements.

The present paper illustrates the impressive resolution that can be achieved by coating Sepharose beads with hydrocarbon chains. The availability of a homologous series of such Sepharoses provides a versatile tool for protein purification. Other families of such hydrocarbon-coated Sepharoses which have on their hydrocarbon side chain another functional group, e.g. an ω-amine, behave differently. For example, in the alkyl-Sepharose series a hydrocarbon side chain 4-carbon-atoms long sufficed to retain phosphorylase b, while in the ω-amino alkyl-Sepharose series a hydrocarbon chain of 6 carbon atoms was needed to retain phosphorylase b. In a forthcoming publication (3) it will be shown that ω-amino alkyl-Sepharose with 4-carbon side chains excluded glycogen phosphorylase but efficiently retained glycogen synthetase, and it was therefore possible to use this column for the separation of these two enzymes and for the purification of the synthetase (3). This last example suggests that the isolation of glycogen phosphorylase on Seph-C_4 is not a unique case. There may well be quite a few proteins which can preferentially be adsorbed by an appropriate member in the various series of hydrocarbon-coated Sepharoses. In view of the fact that all the alkyl Sepharoses used throughout this work were similar in structure except for the length of the hydrocarbon side chain, and since a 4-carbon side chain does not bear any special resemblance to the substrates of phosphorylase or to one of its known effectors, it seems plausible to assume that the retention of phosphorylase on Seph-C_4 occurs through interactions between the hydrocarbon side chains and hydrophobic pockets in the enzyme, for example between its protomers. This assumption could account for the gradation in the binding to the various alkyl-Sepharoses and for the fact that deforming agents, which bring about conformational changes in the enzyme and dissociation into protomers (7,15) bring about elution of the adsorbed enzyme from the column.

The results presented here have to be taken into consideration in the

design and use of columns for affinity chromatography. It was previously shown that interposing a hydrocarbon extension ("arm") between a ligand and the Sepharose core has often a dramatic effect on the binding capacity of an affinity chromatography column (1,2,6,16,17). Moreover, the length of the extension was found to be important. This effect was attributed to a relief of steric restrictions imposed by the matrix backbone and to an increased flexibility and mobility of the ligand when it protrudes farther into the solvent (6). The observations reported in this manuscript do not disprove these suggestions. However, they raise the possibility that hydrophobic interactions may themselves contribute significantly to the tightness of binding, through a mechanism which does not involve the specific recognition of the ligand *per se*. Furthermore, there may be a considerable number of "free arms" on the beads which are not connected to a ligand. These may bind the desired protein but also other proteins with hydrophobic pockets, reducing the specificity of the binding step.

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