# PHOSPHOPROTEIN PHOSPHATASE FROM THE CHICK EMBRYO AND ITS ADSORPTION ON IONIC RESINS



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# Phosphoprotein Phosphatase from the Chick Embryo and its Adsorption on Ionic Resins

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Introduction.

While the high energy phosphate bond concept has been shown to be untrue in the literal sense (1), and the energy changes involved explained in terms of the thermodynamics of electrostatic energy changes (2), organic phosphorus compounds are involved in so many biological processes that any enzymatic reaction which promotes transfer of phosphorus is of interest. Embryonic tissue contains a large amount of phosphoproteins, which appear to act as storage proteins, being used up during growth. These include casein present in mammalian milk, and phosvitin present in hen's egg yolk.

Phosphoproteins differ from nucleoproteins in that they lack purine or pyrimidine bases and the phosphate group is esterified to an amino acid. Phosvitin has been isolated by Mecham and Olcott (3). It has a molecular weight of about twenty thousand and contains ten percent phosphorus, with one molecule of serine for each atom of phosphorus.

Foote and Kind (4,5) purified a phosphoprotein phosphatase from twelve day chick embryo by extracting the homogenate with near neutral saline buffer solution, and successive acetone and ammonium sulphate fractionation. They claimed an active, water soluble, freeze dried powder which was stable for several months when stored in vacuo at 0°C. The average recovery by this process was estimated at fifteen percent. They chose the embryo as the most suitable source of the enzyme, since the unferti-

lized egg showed no activity, and the yolk sack showed high acid phosphatase activity. The activity of the embryo increased steadily from four to twelve days.

Activity in the embryo was also found against p-nitro phenyl phosphatase and was believed due to another enzyme since this activity was heat stable. The ratio of phosphoprotein phosphatase to aromatic phosphatase activity ranged between two and four. Attempts to remove this contaminating enzyme were unsuccessful partly due to the variation of the ratio of the enzymes. The aromatic phosphatase was not active towards phosphoprotein of phosphopeptide.

Acid phosphatase also present in chick embryo was completely separated by acetone fractionation. This phosphatase was present in the discarded second acetone fraction, and was active to phosphoprotein and phosphopeptone but was heat stable. This was thought to be a beta-glycerol phosphatase.

The phosphoprotein phosphatase of chick embryo was distinguished from the other two phosphatases by its rapid thermal deactivation.

The purpose of phosphoproteins in normal adult tissue is not understood. Kennedy and Smith (6) have suggested a role as phosphotransferases alternately accepting and donating phosphorus. However, chick embryo phosphoprotein phosphatase was shown to be inactive towards the phosphorylated forms of likely acceptors: glycerol, serine, glucose and conversely not to add phosphorus to them (7).

The question of the point of attachment of the phosphate in phosphoprotiens has been the subject of debate. Since the isolation of O- phosphoserine from casein and vitellin by Lipmann (8) it has been generally held that protein phosphorus exists as a monoester of serine or some other hydroxyamino acid. In the case of phosvitin titration data indicated (3) an equal number of phosphorus and serine residues which supports a phosphoserine linkage. In addition Williams and Sanger (9) obtained evidence by paper electrophoresis of hydrolysates that phosvitin and casein contain peptides of formula $(Ser\ P)_2$, $(Ser\ P)_3$, $(Ser\ P)_6$, in which the serine residues were joined in a chain. The chain was attached also to glutamic acid or leucine residues.

In the case of casein Levene (10) isolated a phosphopeptone from a tryptic digest of casein. Lowndes (11) and others purified this and identified it as an octapeptide containing two phosphoseryl glutamic acid residues, one aspartic acid and one isoleucine residue. They assumed from chemical evidence that the end group was phosphoseryl glutamic acid.

The suggestion by Perlmann (12) of nitrogen phosphorus and other linkages in addition to the phosphoserine ester linkage in casein has been discredited by a variety of contrary evidence (13,14,15). Thus chick embryo phosphoprotein phosphatase was not found to have any activity against the nitrogen phosphorus bond as in phosphocreatine (7).

There is evidence that specific phosphoprotein phosphatases exist. Attempts to hydrolyse phosphorus from phosphoproteins such as casein by use of acid or alkaline phosphatases from mammalian tissues have generally resulted in failure. It is possible to explain the dephosphorylation of phosphoproteins as a two step process: an initial loosening or hydrolysis of the peptide chains followed by specific action on the phosphate ester. In the case of the chick embryo enzyme the phosphoprotein activity is heat sensitive, whereas proteolytic enzymes are known to be quite stable. Acid soluble nitrogen assayed by Kjeldahl before and after enzymatic cleavage resulted in no significant increase in acid soluble nitrogen (4). These results do not indicate preliminary proteolysis but that a specific phosphoprotein phosphatase is involved. In addition attempts to liberate phosphorus from organic phosphate substrates including serine phosphate by action of the chick embryo enzyme were not successful (7,16). Phosvitin, casein and the phosphopeptone isolated from casein by partial enzymatic hydrolysis were susceptible to the action of chick embryo enzyme.

In the work of Foote and Kind it would appear that the yield of enzyme by the method used was low, the method itself might be expected to cause some denaturation, and that an-other phosphatase was present in the final product. It would be useful, therefore, to attempt to fractionate the enzyme by an alternative method, with the possibility that both the purity and yield might be increased. Since ion exchange adsorption was not used by Foote and Kind (4) this might be a good approach. Column chromato-

graphy of proteins is a relatively new technique of separating these labile substances and it appears to be of great potential value since all proteins are ionic to some extent and the technique is simple and inexpensive. Of the resins at present available there are three main kinds: dextran gels, synthetic polyvalent ions, and cellulose derivatives with ionic substituents. In order to obtain sharp bands and to avoid clogging the ion exchange column with excess material it is necessary in any chromatographic separation of proteins to reduce the volume of the material to be chromatographed to a few millilitres before applying it to the column. The ionic resins being strongly adsorbing may be used to effect some initial concentration of the protein, provided the adsorption and elution can be carried out without appreciable denaturation or loss of the protein. In practice this is difficult to avoid in a batch process since in this case the adsorption is usually strong and the closer the interaction between the protein and the resin the more likely the stripping off of any protective water atmosphere, and the rupture of delicate configurations. From this point of view it would appear that the best prospect of success lay with the substituted cellulose resins, which one might expect to be more gentle in action. The dextran gels which depend on a non-ionic mechanism might be the most useful for fine separations on columns.

Gel filtration like many of the techniques of modern protein chemistry originated in the laboratories of A. Tiselius, the main authors of its development being

Porath and Flodin (17,18,19). In highly cross linked dextran gels, proteins because of their large size do not penetrate the gel particle, but inorganic ions and amino acids being smaller do penetrate the gel particles. This results in possibilities of separation of proteins from inorganic salts, and of separation of proteins according to size. The major effect is molecular sieving, and the chromatographic behaviour is not very sensitive to pH change. However, some cation exchange also occurs as basic substances are retarded.

Ion exchange celluloses are made by attaching substituent groups with basic or acidic properties to the cellulose molecule, giving anionic or cationic resins. Peterson and Sober (20, 21) first described these materials and their preparations have been made commercially available both by Whatman using conventional cellulose polymer, and by Sephadex using dextran resins as the polymer.

The cellulose matrix is hydrophilic as opposed to the hydrophobic nature of most synthetic resins. As a result of this hydrophilic nature the cellulose exchangers have high water binding capacity and, therefore, are characterised by a high exchange capacity. Even moderate substitution with exchangeable groups, about 0.5 - 1.0 milliequivalent per gram, gives sufficient capacity. If the substitution is too high they form gel like columns with poor flow rates. The fibrous nature of the exchanger results in the majority of functional groups being close to the surface, readily available, therefore, to exchange with large molecules which could not normally penetrate the heavily cross-linked structure of synthetic resin exchangers.

Of the synthetic resins available the literature supports the use particularly of IRC-50, a polycarboxylic acid resin in the sodium form, and a cation exchanger (22). This resin has been described as being useful for neutral and basic proteins due to the weak ionisation of its acid groups (23, 24).

Dowex-2 an anion exchanger of low capacity derived from a quaternary ammonium base on a polystyrene skeleton, has been reported as having a small affinity for haemoglobin and, therefore, useful for separating proteins which are adsorbed by it, from haemoglobin (25).

Best described as a mineral resin, calcium phosphate has a long and proven record as an adsorbent for proteins. It is most suitable as an adsorbent in the form of hydroxyl apatite. Study of its possibility in column form has been made by Tiselius and others (26). Separation of proteins on this resin is best effected by gradient elution.

The polyvalent nature of protein molecules seems to be responsible for much of their characteristic behaviour during ion exchange and adsorption chromatography. The linkages may be ionic, Van der Waals, or hydrogen bonding, and the exact behaviour depends on the relative importance of these forces and the extent of the water envelope around the protein molecule. In the case of the hydroxyl apatite resins Tiselius explains the behaviour as being dependent on the necessity of breaking simultaneously all the multiple linkages of the polyvalent protein which can be done by increasing the ionic strength to a point where it

masks the charge of the protein. At the ionic strength at which this occurs the change in combined polyelectrolyte force for small change in ionic strength will be very sharp. The Rf value of a column for particular ionic strength is then either zero or unity. The resulting power of a column cannot then be increased by lengthening the column, but only by using a more gradual change in composition of the eluant.

While this theory applies well to columns of tri-calcium phosphate, it does not account for the occurrence of the reversible distributions as in the case of IRC-50 resin where the Rf can vary from 0.7 to 1.0. This behaviour is best explained in terms of a reduction of the effective polyvalency of the protein by burying of cationic sites in the interior of the protein, and by reduction of the effective charge by presence of high ionic strength buffer.

In some cases the forces involved in the interaction between adsorbent and resin may be non-ionic. Clearly in the case of gel filtration this is so, but also in the case of a resin where both the resin and protein ionic groups have been suppressed, separations of this kind may occur. The polycarboxylic resin IRC-50 being a weak acid is not fully ionised till about pH 8 or 9. In the case of neutral proteins, therefore, at a neutral pH, the force involved must be largely non-ionic, hydrogen bonding and Van der Waals, and separations which would not be possible otherwise are effected (23, 24).

Since the successful use of any resin as an adsorbent depends on the recovery of enzyme on completion of the adsorption elution cycle, an estimation of the efficiency of this process for any resin is a necessary first step in its use. Hirs, Moore and Stein (22) have studied distribution coefficients for a number of resins including IRC-50 with respect to Pancreatic Ribonuclease. The distribution coefficient they defined as the ratio to the concentration of protein per millilitre of resin to that per millilitre of solution. This coefficient could be related to the hold-up volume. If the distribution coefficient was within the range 2-5 then the enzyme emerged from the column after 2-5 hold up volumes. They emphasise, however, that a favourable distribution coefficient between resin and solution is not sufficient unless it is a true reversible distribution, and that in nearly all cases reequilibrium with fresh solvent showed that protein was not released.

The present study is an account of a systematic determination of adsorption and elution coefficients of the chick embryo phosphoprotein phosphatase with respect to the ion exchange resins described above as being suitable for protein adsorption.

Experimental.

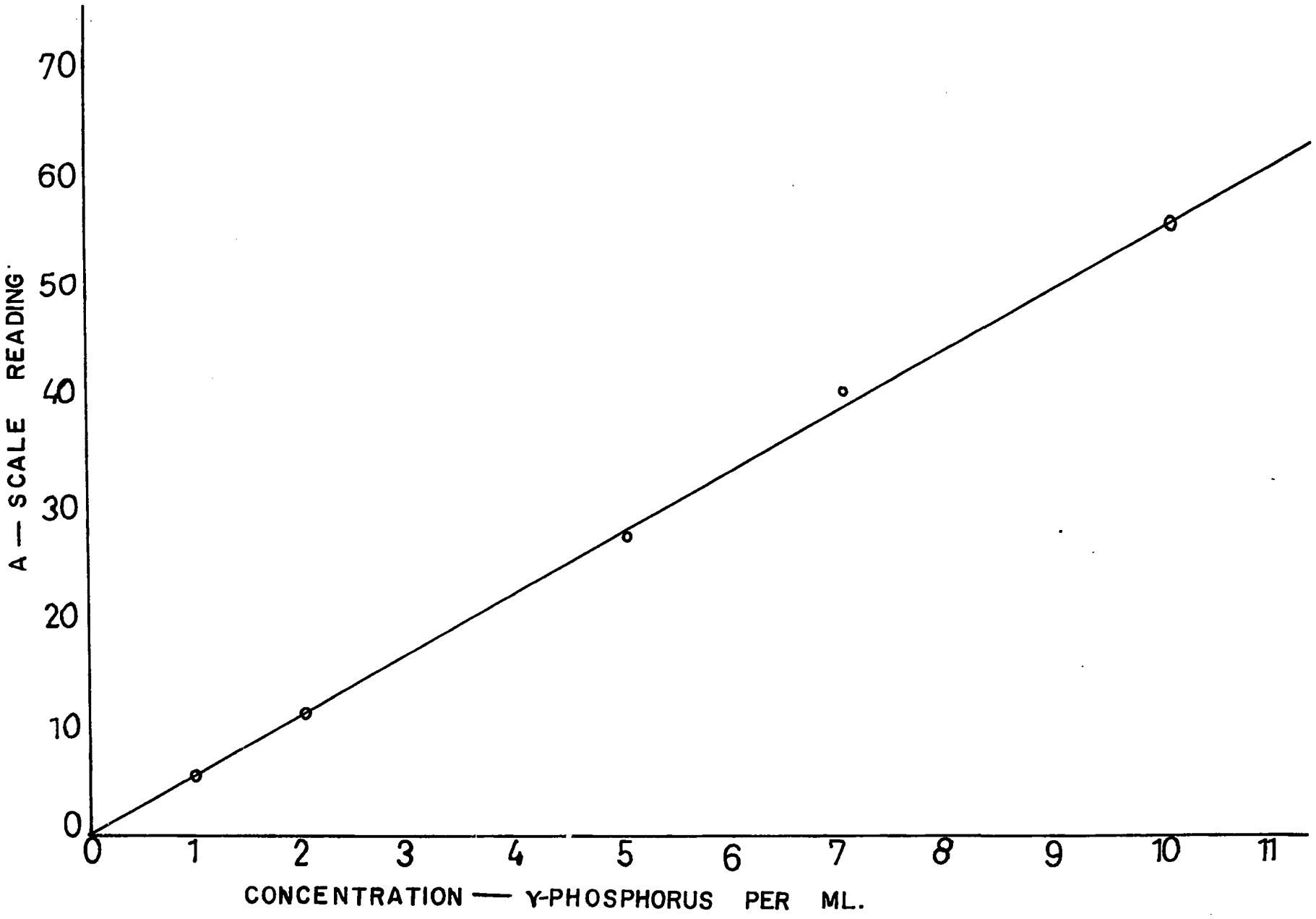
Phosvitin was prepared by magnesium sulphate precipitation from the yolk of hen's egg (27), the preparation being carried out in the cold room at 4°C with the use of a preparative centrifuge (at a speed of 15,000 rpm, force 30,000 g). After dialysing against citrate buffer at pH 4.0 to remove magnesium ions, followed by dialysis against distilled water, the protein was freeze dried. The protein was analysed after freeze drying for nitrogen and phosphorus. Nitrogen by microkjeldahl, phosphorus by ammonium molybdate stannous chloride colorimetric method, preceded by digestion of the protein by seventy per cent perchloric acid (28). Analysis showed 12.5 % nitrogen and 9.33 % phosphorus. The same protein preparation was used throughout the investigation. The protein was stored in a stoppered glass vessel and kept in the refrigerator.

The activity of the enzyme was measured by dissolving eight milligrams of phosvitin in five millilitres of distilled water, mixing with ten millilitres of enzyme extract whose pH had been adjusted to pH 6.0 by dialysis, and incubating at 35°C for one or one and a half hours. The phosphorus released was compared with that of a control sample for the native enzyme homogenate, acting upon the phosphoprotein under the same conditions.

The phosphorus released from the protein by enzyme action was analysed by the ammonium molybdate stannous chloride method. The protein present was first removed by

the addition of 20% trichloroacetic acid, and centrifugation. The details of this procedure were as follows. After incubation one millilitre of the sample was withdrawn from each reaction vessel and transferred to a 15 ml centrifuge tube containing five millilitres of water and one millilitre of 20% trichloroacetic acid. The sediment was separated by centrifuging. Five millilitres of this clear supernatant were then added to a fifty millilitre flask containing ten millilitres of distilled water. To this flask were then added five millilitres of ammonium molybdate reagent followed by one millilitre of stannous chloride solution. Colorimetric estimation of phosphorus was then made and a blank in absence of phosvitin used to correct this figure. A graph showing relation of a phosphorus concentration to absorption is shown in the figure.

The chick embryo extract was prepared from twelve day chick embryo, obtained from hen's egg by incubation at 105°F, by homogenizing with a rotating razor blade stirrer. Saline solution (10 % NaCl) was added to the homogenate to bring the per cent sodium chloride to 1.0 %. The homogenate was cleared in an international centrifuge, model HT, at 15,000 rpm for 10-15 mins. The clear supernatant was dialysed against 0.01 M sodium chloride solution for 3-4 days in the cold room at 4°C. This reduced the phosphorus content to an acceptable level. The phosphorus content in the undialysed homogenate was very high and prevented measurement of its activity. In the case of the adsorption on hydroxyl apatite the resin contained high


70
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CONCENTRATION — γ-PHOSPHORUS PER ML.

phosphorus so that the extract was dialysed after adsorption, and the homogenate dialysed for only fifteen hours before hand.

The conditions for adsorption on the resins were standardised as follows. Twenty millilitres homogenate, adjusted by dialysis for one hour to the ionic strength and pH required, were mixed with a known weight of resin, which had been equilibrated against the same buffer. After five minutes at 4°C, the suspension was centrifuged, and the sediment stirred with 20 ml. eluant for five minutes. The supernatant, eluate, and untreated homogenate were tested for activity as described above and the ratio of the activity of the supernatant or eluate to the homogenate expressed as a coefficient in decimal fraction. These results are tabulated for each resin. In the case of the cellulose exchangers the amount of resin taken per adsorption was eight grams, for the synthetic resins Dowex-2 and IRC-50 the amount was 40 grams, and in the case of hydroxyl apatite the sediment from 50 ml. slurry, containing an estimated 0.05 moles of calcium ion. Adsorption was generally effected at neutral pH and low ionic strength (ca, I 0.05) and elution at higher ionic strength, generally 0.5 M sodium acetate plus 0.5 M sodium chloride. In some cases a second elution was undertaken. In cases where elution was carried out at a pH other than 6.0, the pH of the eluate was brought to pH 6.0 by dialysis against sodium acetate buffer.

In the case of hydroxyl apatite the buffers used for adsorption and elution were 0.001 M ph 6.8, pH6, pH 7 and pH8, and 0.5 M pH 7.6, pH6, pH7 and pH8 sodium phosphate buffer respectively.

In addition the method of adsorption used was to expose the homogenate to fresh resin three successive times and combine the sediments obtained for elution in one stage. Thus the adsorption coefficient may be artificially high compared with the results for other resins.

The pretreatment of the resin consisted of washing with water and obtaining equilibrium between the buffer and resin at the desired pH, by stirring the resin with buffer. In the case of IRC-50 which is known to adsorb large amounts of alkali slowly, the resin was stirred by magnetic stirrer while titrating by automatic titrator overnight, it was then stirred with buffer as for the other resins.

In the case of Sephadex ion exchangers, ten grams of the resin was swollen in water for one hour, the fines removed by centrifuging, and the gel washed alternately with 0.5 N sodium hydroxide, water and 0.5 N hydrochloric acid, and finally equilibrated with buffer. The C-50 grade was used. All the resins were obtained commercially except for the hydroxylapatite resin which was prepared by the method of Tiselius, Hjerten and Levin (26).

The main characteristics of the resin which was used are described below.

The Substituted Celluloses. The preparation of these exchange resins is described by Peterson and Sober (20). They are made available commercially by Whatman based on Whatman cellulose, and by Sephadex based on Dextran gels. Sephadex ion exchangers consist of cross linked dextran chains, with functional groups attached to the surface and inside the particles. They swell rapidly in water

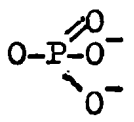
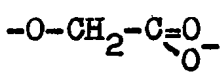
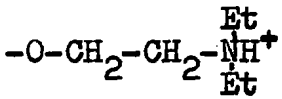
and show low non-specific adsorption. The C25 grade is tightly crosslinked and large molecules of molecular weight greater than ten thousand are adsorbed only on the surface. The C50 grade is more porous and therefore suitable for high molecular weight molecules. Sephadex ion exchangers swell when the ionic strength is lowered and shrink when it is raised.

Carboxymethyl cellulose. A weak cation exchanger. It's active group is a monofunctional acetic acid group with pK between pH 4 and 5. It functions most readily on the alkaline side of its pH. It is based on cotton cellulose.

Phosphorylated Cellulose. A strong cation exchanger. It has a high nominal exchange capacity. Its active group is bifunctional phosphate with pK at pH 2 and 7. Between pH 2 and 7 it is therefore monofunctional, and above pH 7 bifunctional. It is based on cotton cellulose.

Diethyl amino ethyl cellulose. A weak anion exchanger, The active group is a tertiary amine base, a monofunctional group with pK in the region of pH 7.5 to 9.5. It will not function in strongly alkaline solutions. According to Sober, Gutter, Wyckoff, and Peterson (21), the effect of pH on its activity is two fold. Reducing the pH from the alkaline side suppresses ionisation of the carboxyl groups of the protein but increases the ionisation of the tertiary amine groups of the resin.

Epichlorohydrin Triethanolamine. A weak anion exchanger of low capacity. The exact formulation of the reactive substituents is uncertain. It is a weakly basic derivative with tertiary amino groups functioning as exchange sites. It is useful particularly for nucleic acids.

| Resin | Type Exchange | Substituent | Reagent in preparation |
|---|---|---|---|
| Phosphorylated Cellulose | Strong cation exchange |  | NaOH, $POCl_3$, Cellulose, final product pH6 |
| Carboxymethyl cellulose | Weak cation |  | NaOH, Cotton Cellulose, Chloroacetic acid. |
| Diethyl amino ethyl cellulose (DEAE) | Weak anion |  | NaOH, Solka Floc cellulose, a wood cellulose, 2 chloro-triethylamine |
| Epichlorohydrin Triethanolamine (ECTEOLA) | Weak anion | Formulation uncertain | Triethanolamine Epichlorohydrin. |

Dowex-2(25). An anion exchanger of low capacity. The active group is a quaternary ammonium base and the polymer base is polystyrene.

IRC-50 (XE64). A weak cation exchanger (22). The active groups are carboxylic groups of a polycarboxylic acid resin. The ionisation of these are suppressed below pH 3 and full exchange capacity is not reached till pH 8 or 9. Adsorption of neutral or basic proteins is greatest at neutral pH since at this pH ionic groups are suppressed for both the resin and protein. It is particularly used for separating neutral or basic proteins for this reason, since the non-ionic forces can be used to differentiate them. In this connection it is of interest that haemoglobin which has an isoelectric point between pH 4.5 and 5.6 is not adsorbed below pH 5.8. The elution volume for this protein is very dependent on ionic strength. Below I 0.22 it increases exponentially, and for elution in a workable volume a higher ionic strength must be used.

The resin requires considerable quantity of alkali to adjust the pH and reaches equilibrium slowly (22).

Hydroxyl apatite (26). An anion exchanger. Some phosphoric acid is lost by the lattice in the formation of hydroxyl apatite from brushite which is hydrated calcium phosphate. The active substituents must therefore, be excess charge of the calcium ions of the hydrated calcium phosphate lattice. In the case of this resin it appears that the protein is bound by multiple linkages between the protein as polyelectrolyte and the resin as polyvalent resin. These can

be broken by elution with polyvalent anion such as phosphate, at a suitable ionic strength. The ionic strength is quite critical and elution occurs at the buffer front. (Hence gradient elution is the most successful means of chromatography. The resolving power of the column being determined not by its length but by the rate of change of concentration of the buffer used in the gradient elution). Due to the nature of the resin, changes in resin composition might be expected at extremes of pH, which is therefore restricted to near neutrality.

| Resin | Type Exchange | Substituent | Preparation |
|---|---|---|---|
| Dowex-2 | Anion | Quaternary ammonium base | - |
| IRC-50(XE64) Polycarboxylic acid | Cation | Polycarboxylic acid (Na) | - |
| Hydroxyl-apatite | Anion | Xs $Ca^{++}$ ions in lattice | Treat brushite pptt. with alkali. $CaHPO_4\ 2H_2O \rightarrow Ca_5(PO_4)_3OH + H_3PO_4$ |

## Results.

The results of the adsorption-desorption studies are recorded in tabular form. With the exception of the hydroxyl apatite resin adsorption was made from a low ionic strength sodium acetate buffer, and eluted by 0.5 M sodium acetate buffer with 0.5 M sodium chloride added. In the case of hydroxyl apatite, adsorption was made from 0.001 M sodium phosphate buffer and eluted with 0.5 M sodium phosphate buffer at different pH's.

In the case of some resins notably IRC-50, but also CM-Sephadex, adsorption was accompanied by visible denaturation with the appearance of a white sediment. It is known that the former requires large amounts of alkali to neutralise it, but even when neutralisation was effected by an automatic titrator with overnight stirring, followed by equilibration with the buffer, denaturation still occurred.

TABLE I

| Adsorbent | Type | Adsorbing solution I | pH | Activity (% dephosphorylation in 1 or 1½ hrs). control | Activity (% dephosphorylation in 1 or 1½ hrs). supernatant | Activity (% dephosphorylation in 1 or 1½ hrs). Eluate I | Activity (% dephosphorylation in 1 or 1½ hrs). Eluate II | Coefficient = Assay/Control supernatant | Coefficient = Assay/Control adsorption | Coefficient = Assay/Control Eluate I | Coefficient = Assay/Control Eluate II |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Dowox-2 | Anion | 0.02. | 6.0 | 15.2 | 1.3 | 2.7 | - | 0.09 | 0.91 | 0.18 | - |
| | | 0.05 | 6.0 | 15.2 | 4.5 | 0.8 | - | 0.30 | 0.70 | 0.05 | - |
| | | 0.02 | 7.0 | 15.2 | 6.0 | 3.0 | - | 0.40 | 0.60 | 0.20 | - |
| | | 0.05 | 7.0 | 15.2 | 9.6 | 0.0 | - | 0.63 | 0.37 | 0.00 | - |

## TABLE II

| Adsorbent | Type | Adsorbing solution I | | Activity (% dephosphorylation in 1 or 1½ hours). control | | | | Coefficient = Assay / Control | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | supernatant | Eluate I | Eluate II | supernatant | adsorption | Eluate I | Eluate II |
| IRC-50 | Cation | 0.02 | 6.0 | 11.04 | 0.90 | 0.0 | 0.80 | 0.08 | 0.92 | 0.0 | 0.07 |
| | | 0.05 | 6.0 | 11.04 | 1.64 | 0.15 | 0.16 | 0.15 | 0.85 | 0.01 | 0.01 |
| | | 0.02 | 7.0 | 11.04 | 0.0 | 0.0 | 0.75 | 0.0 | 1.00 | 0.0 | 0.07 |
| | | 0.05 | 7.0 | 11.04 | 0.0 | 1.50 | 0.75 | 0.0 | 1.00 | 0.14 | 0.07 |

TABLE III

| Adsorbent | Type | Adsorbing solution I | | Activity (% dephosphorylation in 1 or 1½ hrs.) Control | Supernatant | Eluate I | Eluate II | Coefficient = Assay/Control Supernatant | Adsorption | Eluate I | Eluate II |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Cellulose DEAE (Whatman DEI Floc) | Weak anion | 0.02 | 6.0 | 11.04 | 2.54 | 2.24 | 0.75 | 0.23 | 0.77 | 0.20 | 0.07 |
| | | 0.05 | 6.0 | 11.04 | 1.94 | 1.88 | 0.45 | 0.18 | 0.82 | 0.17 | 0.04 |
| | | 0.02 | 7.0 | 9.69 | 3.73 | 1.34 | 0.90 | 0.39 | 0.61 | 0.14 | 0.09 |
| | | 0.05 | 7.0 | 9.69 | 4.33 | 0.93 | 1.19 | 0.45 | 0.55 | 0.10 | 0.12 |

TABLE IV

| Adsorbent | Type | Adsorbing solution I | pH | Activity (% dephosphorylation in 1 or 1½ hrs.) Control | Supernatant | Eluate I | Eluate II | Coefficient = Assay/Control Supernatant | Adsorption | Eluate I | Eluate II |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Cellulose DEAE (DEAE Sephadex) | Weak anion | 0.05 | 6.0 | 13.42 | 0.15 | 1.64 | 0.45 | 0.01 | 0.99 | 0.12 | 0.03 |
| | | 0.10 | 6.0 | 13.42 | 1.19 | 1.49 | 0.48 | 0.09 | 0.91 | 0.11 | 0.04 |
| | | 0.05 | 7.0 | 10.44 | 0.90 | 2.39 | 0.70 | 0.09 | 0.91 | 0.23 | 0.07 |
| | | 0.10 | 7.0 | 10.44 | 2.09 | 1.94 | 0.90 | 0.20 | 0.80 | 0.19 | 0.09 |
| | | 0.05 | 8.0 | 10.51 | 1.28 | 1.19 | 0.75 | 0.12 | 0.88 | 0.11 | 0.07 |
| | | 0.10 | 8.0 | 10.51 | 0.96 | 0.90 | 0.45 | 0.09 | 0.91 | 0.09 | 0.04 |

TABLE V

| Adsorbent | Type | Adsorbing solution | | Activity (% dephosphorylation in 1 or 1½ hours). | | | | Coefficient = Assay / Control | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | I | | Control | Supernatant | Eluate I | Eluate II | Supernatant | Adsorption | Eluate I | Eluate II |
| Carboxy methyl cellulose (Whatman CM1) | Weak cation | 0.02 | 6.0 | 10.74 | 0.0 | 0.75 | 0.90 | 0.0 | 1.00 | 0.07 | 0.08 |
| | | 0.05 | 6.0 | 10.74 | 0.0 | 1.19 | 0.30 | 0.0 | 1.00 | 0.11 | 0.03 |
| | | 0.02 | 7.0 | 10.59 | 1.81 | 2.24 | 1.49 | 0.17 | 0.83 | 0.21 | 0.14 |
| | | 0.05 | 7.0 | 10.59 | 2.98 | 2.98 | 0.96 | 0.28 | 0.72 | 0.28 | 0.09 |

TABLE VI

| Adsorbent | Type | Adsorbing solution | | Activity (% dephosphorylation in 1 or 1½ hours). | | | Coefficient = Assay / Control | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | I | pH | control | supernatant | Eluate I | Eluate II | supernatant | adsorption | Eluate I | Eluate II |
| Carboxy methyl cellulose (CM Sephadex) | Weak cation | 0.05 | 6.0 | 10.44 | 0.60 | 0.06 | 0.30 | 0.06 | 0,94 | 0.01 | 0.03 |
| | | 0.10 | 6.0 | 10.44 | 0.36 | 0.09 | 0.03 | 0.04 | 0.96 | 0.01 | 0.00 |
| | | 0.05 | 7.0 | 11.63 | 0.45 | 0.60 | 1.04 | 0.04 | 0.96 | 0.05 | 0.09 |
| | | 0.10 | 7.0 | 11.63 | 0.09 | 0.30 | 0.45 | 0.01 | 0.99 | 0.03 | 0.04 |

TABLE VII

| Adsorbent | Type | Adsorbing solution | | Activity (% dephosphorylation in 1 or 1½ hours). | | | | Coefficient = Assay / Control | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | I | pH | Control | Supernatant | Eluate I | Eluate II | Supernatant | Adsorption | Eluate I | Eluate II |
| Ecteola | - | 0.02 | 6.0 | 16.3 | 6.0 | 0.8 | - | 0.37 | 0.63 | 0.05 | - |
| | | 0.05 | 6.0 | 16.3 | 3.7 | 0.0 | - | 0.23 | 0.77 | 0.00 | - |
| | | 0.02 | 7.0 | 16.3 | 4.9 | 0.0 | - | 0.30 | 0.70 | 0.00 | - |
| | | 0.05 | 7.0 | 16.3 | 4.3 | 0.9 | - | 0.26 | 0.74 | 0.06 | - |

TABLE VIII

| Adsorbent | Type | Adsorbing solution I | Adsorbing solution II | Activity (% dephosphorylation in 1 or 1½ hours). Control | Activity (% dephosphorylation in 1 or 1½ hours). Supernatant | Activity (% dephosphorylation in 1 or 1½ hours). Eluate I | Activity (% dephosphorylation in 1 or 1½ hours). Eluate II | Coefficient = Assay/Control. Supernatant | Coefficient = Assay/Control. Adsorption | Coefficient = Assay/Control. Eluate I | Coefficient = Assay/Control. Eluate II |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Cellulose Phosphate (Whatman) | Strong cation | 0.02 | 6.0 | 15.81 | 5.97 | 0.45 | - | 0.38 | 0.62 | 0.03 | - |
| | | 0.05 | 6.0 | 15.81 | 6.50 | 0.30 | - | 0.41 | 0.59 | 0.02 | - |
| | | 0.02 | 7.0 | 14.73 | 6.40 | 0.25 | - | 0.41 | 0.59 | 0.02 | - |
| | | 0.05 | 7.0 | 14.73 | 5.85 | 0.15 | - | 0.37 | 0.63 | 0.01 | - |

TABLE IX

| Adsorbent | Type | Adsorbing solution I | | Activity (% dephosphorylation in 1 or 1 ½ hours). Control | Coefficient = Assay/Control: Supernatant | Eluate I | Eluate II | Eluate III | Supernatant | Adsorption | Eluate I | Eluate II | Eluate III |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Hydroxyl apatite | Anion | 0.001M $Na_2HPO_4$ | pH 6.8 | 10.5 | 4.1 | 5.3 | - | - | 0.39 | 0.61 | 0.51 | - | - |
| | | | 6.8 | 11.5 | 3.7 | 5.1 | 1.0 | 0.5 | 0.32 | 0.68 | 0.44 | 0.09 | 0.04 |
| | | | 6.0 | 18.7 | 5.0 | 1.0 | - | - | 0.27 | 0.73 | 0.05 | - | - |
| | | | 7.0 | 18.7 | 3.0 | 4.0 | - | - | 0.16 | 0.84 | 0.21 | - | - |
| | | | 8.0 | 18.7 | 6.0 | 2.0 | - | - | 0.32 | 0.68 | 0.11 | - | - |

Discussion.

The chick embryo phosphoprotein phosphatase was shown by Foote and Kind to be extremely unstable, at body temperature. Eighty three per cent of the activity of an extract was lost in twelve hours. Moreover, they reported large losses in activity when fractionation of the untreated extract by ammonium sulphate was attempted, this being almost the mildest method of fractionation which could be used. This evidence of instability is in agreement with results obtained herein. While adsorption on the resins was always good and in some cases quantitative recovery by elution was in most cases very poor. This can be explained in terms of denaturation on adsorption. The results with carboxymethyl cellulose resin, prepared by Whatman, were relatively good. At I 0.05 pH 7.0 the recovery from the resin was thirty-nine per cent. This resin might therefore be used for chromatographic purposes.

There appeared to be no significant correlation between the efficiency of a resin in adsorbing protein, and thecationic or anionic nature of the resin. Since proteins are normally amphoteric, and the measurements were all made at near neutrality to avoid denaturing the enzyme, this is perhaps not unexpected.

In the case of hydroxyl apatite the redovery from the resin was as high as eighty three per cent, and the efficiency of the adsorption elution process fifty one per cent. The hydroxyl apatite resin might therefore be used to effect an initial concentration and possibly

some purification by a chromatographic process, and the concentrated fraction obtained from the front of the elutant boundary, used as a starting sample on other resins or gels. In view of the lability of the enzyme more might be expected of the Dextran gels as column materials than of the ion exchange resins examined here.

Summary.

The adsorption of chick embryo phosphoprotein phosphatase on a number of ion exchange resins known to be suitable for protein chromatography has been systematically studied. In the case of the hydroxyl apatite resin the enzyme was efficiently adsorbed and desorbed without great loss. This resin could be used to effect initial concentration of the enzyme prior to column chromatography.

## REFERENCES


1. Hill, T. L. and Morales, M. F., J. Am. Chem. Soc., 73, 1656 (1951).
2. George, P. and Rutman, R. J., Prog. in Bioph. and Biophys. Chem. 10, 1 (1960).
3. Mecham, D. K. and Olcott, H. S., J. Am. Chem. Soc., 71, 3670 (1949).
4. Foote, M. W. and Kind, C. A., Archiv. Biochem. Biophys., 46, 254 (1953).
5. Foote, M. W., Thesis, University of Connecticut, 1954.
6. Kennedy, E. P. and Smith, S. W., J. Biol. Chem., 207, 153 (1954).
7. Küttner, R., Thesis, University of Connecticut, 1959.
8. Lipmann, F. A. and Levene, P. A., J. Biol. Chem. 98, 109 (1932).
9. Williams, J. and Sanger, F., Biochem. Biophys. Acta, 33, 294 (1959).
10. Levene, P. A. and Hill, D. W., J. Biol. Chem., 101, 711 (1933).
11. Lowndes, J., Macara, T. J. R. and Plimmer, R. H. A., Biochem. J., 35, 315 (1941).
12. Perlmann, G., Phosphorus Metab., Baltimore, 2, (1952).
13. Hofmann, T., Biochem. J., 69, 135 (1958).
14. Peterson, R. F., Nauman, L. W. and McMeekin, T. L., J. Am. Chem. Soc., 80, 95 (1958).
15. Anderson, L. and Kelley, J. J., J. Am. Chem. Soc., 81, 2275 (1959).

16. Koehn, P. V., Thesis, University of Connecticut, (1963).

17. Porath, J., and Flodin, P., Nature, 183, 1657. (1959).

18. Flodin, P., J. Chromatog., 5, 103 (1961).

19. Porath, J., Biochem. Biophys. Acta, 39, 193 (1960).

20. Peterson, E. A. and Sober, H. A., J. Am. Chem. Soc., 78, 751 (1956).

21. Sober, H. A., Gutter, F. J., Wyckoff, M. M. and Peterson, E. A., J. Am. Chem. Soc., 78, 756 (1956).

22. Hirs, C. H. W., Moore, S. and Stein, W. H., J. Biol. Chem., 200, 493 (1953).

23. Boardman, N. K. and Partridge, S. M., Nature, 171, 208 (1953).

24. Boardman, N. K. and Partridge, S. M., Biochem. J. 59, 543 (1955).

25. Boman, H. G. and Westlund, L. E., Archiv. Biochem. Biophys., 64, 217 (1956).

26. Tiselius, A., Hjerten, S. and Levin, O., Archiv. Biochem. Biophys., 65, 132 (1956).

27. Joubert, F. J. and Cook, W. H., Can. J. Biochem. and Physiol., 36,389 (1958).

28. McKenzie, H. A., Australian J. Chem., 7, 55 (1954).