Tricalcium Phosphate as an Adsorbent in the Chromatography of Proteins

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The recent spectacular success of the chromatographic method in solving many important problems in diverse fields of chemistry and biology has encouraged investigators in many laboratories to anticipate comparable success in the chromatography of proteins. However, in spite of the great interest in the problem and several encouraging preliminary experiments, a well developed method for general use has not yet appeared. The use of batch procedures involving consecutive adsorption and elution, particularly on aluminium hydroxide, bauxite, tricalcium phosphate and certain silicate minerals, is well known to enzyme chemists and some workers have also used chromatographic procedures (see especially Bamann & Myrbäck, 1941; Sumner & Somers, 1947 and Zechmeister, 1950).

On the basis of his observations on 'salting-out adsorption' on paper and silica gel, Tiselius (1948) suggested the possibility of protein chromatography using this method. Mitchel, Gordon & Haskins (1949) were able to separate considerable quantities of enzymes by salting-out adsorption on a paper 'chromatopile'. Franklin & Quastel (1949) have reported paper chromatography of proteins under conditions not involving salting out, although experiments by one of us (S.M.S.) indicate that such chromatograms may be artifacts resulting from the combined effects of the viscous resistance to flow offered by the protein spot and the irreversible adsorption of small quantities of protein in an immobile tail. Sober, Kegeles & Gutter (1949) performed a successful frontal analysis of egg white proteins using a cation-exchange resin.

The fact that chromatography of proteins has not yet had the brilliant success achieved, for example, with the amino-acids is probably in part due to the limitations imposed by having to work with systems that do not promote denaturation; this confines investigation to mild adsorbents and to aqueous media free of such detergents and organic solvents as would otherwise be chosen as promising eluting agents. It has been considered worth while in this Institute to extend the range of possible adsorbents by studying a variety of materials and to compare their behaviour critically. Shepard & Tiselius (1949) studied ex-

tensively the adsorption of several proteins on silica gel and conducted promising frontal analyses of mixtures, although attempts at elution or displacement were discouraging (private communication). The present paper reports similar studies with tricalcium phosphate, with reference to the effect of salt and of changes of pH on the elution analysis of phycoerythrin in phosphate buffers. Quantitative comparisons with some other adsorbents are also reported.

MATERIALS AND METHODS

Preparation of adsorbents. Hydrated tricalcium phosphate was prepared by the slow addition of conc. H_3PO_4 to a chilled solution of a calcium complex of sucrose, according to a method developed by MacIntire, Palmer & Marshall (1945). To 450 g. pure sucrose in 2 l. of water were added 75 g. CaO prepared by calcining 150 g. of precipitated $CaCO_3$ at 1000° for 3 hr. The suspension was agitated periodically for several hours until most had dissolved, and was clarified. Conc. H_3PO_4 (about 18 ml.) was added dropwise with continuous stirring over the course of an hour to 800 ml. of the calcium-sucrose solution, chilled to about 5° , until the pH dropped to 9.5; after this the stirring was continued for 4 hr. The resulting precipitate of tricalcium phosphate was thoroughly washed in a large centrifuge with many portions of distilled water. No special precautions were taken to exclude CO_2 . The product, a voluminous finely divided gelatinous material, was stored and used as a suspension in water (about 0.08 g./ml.).

Proteins. Phycoerythrin, the fluorescent red protein from the sea weed *Ceramium rubrum*, was prepared by extraction and fractional crystallization according to Kylin (1910). Bovine serum albumin, obtained through the courtesy of Dr K. O. Pedersen, had been prepared by $(NH_4)_2SO_4$ fractionation, and was reprecipitated before use.

Chromatographic technique. Perspex columns (62 mm. long and 8 mm. diam.) were packed with a suspension containing 1 part by weight of tricalcium phosphate (dry weight basis) and 5 parts Super-Cel filter aid (Eimer and Amend, New York). The Super-Cel was first freed of foreign matter and larger particles by allowing a suspension to settle for a few seconds and then decanting the remaining more finely divided material for use. The column was packed by forcing the mixture as a fairly heavy suspension into the column from the top reservoir under a pressure of 3 kg./sq.cm. The column was thus tightly packed in a few minutes, after which the top was gently smoothed and packed with a spatula. The application of the spatula tended to squeeze out water, making the top layer very moist and fluid, and it was

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necessary to remove this moisture by intermittent gentle suction from below, taking care not to draw air into the column. The assembled column was washed with 1% (w/v) aqueous NaCl, and the sample of phycoerythrin (0.5 ml. of 0.2% (w/v) solution in saline) was pressed in and further washed with a few ml. saline. The eluting agent to be studied was then added to the reservoir and a few cm. Hg pressure applied as necessary to produce a flow rate of 2–3 ml./hr., corresponding to a linear velocity of the solution of about 50 mm./hr. After elution commenced, the volume of effluent solvent and the corresponding travel of the red band of protein were measured. The dead volume of the column was determined by frontal analysis of a solution of glucose (5%, w/v) under the assumption that the glucose was not adsorbed.

Adsorption isotherms. The adsorption of serum albumin on various materials was studied in batch experiments in which the solvent, adsorbent and protein with a total volume of 12 ml. were mixed, in the order given, in test tubes which were then tightly stoppered with the exclusion of bubbles and rotated end over end about 10 times/min. for several hours. The adsorbent was then removed by centrifugation and filtration or by two consecutive centrifugations, and the supernatant was analysed for protein by ultraviolet absorption at 276 m μ . The measured concentration was corrected by the use of blanks from which the protein was omitted, and the result subtracted from controls (having only protein and solvent, but no adsorbent) to give the amount of protein adsorbed.

RESULTS

The elution analysis of phycoerythrin

The effects of variations in pH, sodium chloride concentration and phosphate buffer concentration are presented in Table 1, which gives the velocity of the

protein as a fraction of that of the solution (R_F), and these data are graphically illustrated in Fig. 1. Phosphate buffer is an effective eluting agent for this system so that in concentrations above 0.04M-phosphate in 1% (w/v) saline there is rapid elution

Fig. 1. Iso-elution curves for phycoerythrin on tricalcium phosphate. The curves represent interpolated values for the relative motion of protein and solvent (R_F) as a function of salt concentration and phosphate concentration at pH 7.8 (see Table 1).

of the protein, while in the absence of phosphate there is no discernible motion of the band. The band moved with half the velocity of the solvent in 0.01M-phosphate in 1% saline. The effect of salt was more startling and more complicated. In the absence of salt there was very little retention of the protein,

Table 1. *The elution analysis of phycoerythrin in various solutions*

Sodium phosphate buffer concn. (M)	pH	NaCl concn. (%, w/v)	Elution velocity (R_F)	
			Protein	Phosphate
0.00	—	0	0.90	—
0.0025	7.8	0.1	0.10	—
0.0025	7.8	0.05	0.10	—
0.0025	7.8	0.002	0.83	—
0.006	5.2	1.0	0.10	—
0.005	7.8	0.5	0.10	0.43
0.005	7.8	0.25	0.26	—
0.005	7.8	0	0.78	—
0.007	6.7	1.0	0.34	—
0.008	7.8	0.5	0.37	—
0.01	7.8	3.0	0.30	—
0.01	7.8	1.0	0.38	0.60
0.01	7.8	0.25	0.56	—
0.0125	7.8	1.0	0.43	—
0.0125	5.2	1.0	0.52	—
0.0125	7.8	0	0.83	—
0.0125	5.2	0	0.78	—
0.0175	6.7	1.0	0.53	—
0.025	7.8	5.0	0.48	—
0.025	7.8	1.0	0.63	0.65
0.025	5.2	1.0	0.59	—
0.025	7.8	0	0.83	0.85
0.035	6.7	1.0	0.71	—
*	8.0	1.0	0.00	—

* 0.02M-sodium diethylbarbiturate buffer.

even at low phosphate levels. Experiments in distilled water were not always reproducible, presumably because of the failure to control the pH, but in some cases R_f 0.9 was obtained, whereas retention was very strong when even traces of salt were present. More reproducible results were obtained in 0.0025M-phosphate, where it was found that the addition of as little as 0.05% sodium chloride would cut the R_f from 0.85 to as little as 0.1.

In Fig. 1 the relationship between the effects of salt and phosphate is illustrated by plotting lines of equal protein elution velocity, expressed as R_f , on a graph of salt concentration against phosphate concentration. The graph is drawn only for concentrations below 1% saline, where most of the effect occurs. Somewhat surprisingly, there was no effect of pH variations between 5.2 and 7.8 within the experimental errors. Even pH 8.0 sodium diethylbarbiturate buffer would not give elution in the absence of phosphate ions. The possibility that pH effects exist below 0.01M-phosphate was not studied.

The phycoerythrin band was usually very regular, indicating that the packing was uniform, although a pronounced upward convexity of the front was apparent on the extruded and sectioned adsorbent column. Under conditions where the retention was slight ($R_f > 0.6$), the band was very narrow and well defined, but invariably some material of a slightly darker red colour was left at the top of the column. In addition, there was a faint trace of colour uniformly distributed over the column, presumably as a result of slight irreversible tailing. With higher retention there was a progressively more diffuse tail behind the sharp front and more material left near the starting point in a diffuse band so that chromatograms with R_f less than 0.3 appeared almost continuously coloured with phycoerythrin up to the sharp leading edge, this edge being used in the measurements. When stronger phosphate was sent through such a chromatogram it would pick up almost all of the trailing material as a second sharp band which would eventually catch up and merge with the leading band. In a series of exploratory experiments, a mixture of phycoerythrin and the blue phycocyan were readily separated, with the red band leading.

In interpreting the results and planning new experiments it is important to know the position of the phosphate front relative to the protein in order to decide whether the phenomenon is essentially displacement or elution. This was checked at four typical concentrations, using ammonium molybdate as a test reagent for the appearance of the phosphate front in the effluent, and the corresponding values of the velocity of the front relative to the solvent are reported in Table 1. In 0.01M-phosphate in 1% saline, where the elution of the protein proceeded at

an intermediate rate and showed considerable tailing for several mm. behind the front, the phosphate front was well ahead of the phycoerythrin, indicating that the phenomenon is true elution. On the other hand, under conditions where the protein moves rapidly in a narrow band, the phenomenon appears to be essentially displacement, with the protein band lying within the concentration gradient of the phosphate front, which may account for its extreme sharpness. This was checked at a phosphate concentration of 0.025M in 1% saline, in which case the presence of phosphate in the effluent was first detected three drops before the appearance of the phycoerythrin. The other values reported for the mobility of the phosphate front, as measured in the absence of protein, similarly indicate that the phenomenon may be either elution or displacement, depending on whether the protein retention is small or great.

Preliminary experiments with serum albumin gave elution in a sharp band. However, the serum albumin was more strongly retained and required 0.03M-phosphate for moderately fast elution. In an attempted chromatographic analysis of partially purified D-amino-acid oxidase, done in collaboration with Dr W. Frisell, the elution was accomplished with successive portions of phosphate buffer at progressively increasing concentrations. The activity was eluted in two sharp bands, one carried along in 0.005M- and the second in 0.02M-phosphate. The suggestion is made that the second band might have been material left as a tail behind the first band and subsequently picked up by the more concentrated phosphate. The total recovery of enzyme activity was 61%. When the experiment was repeated using only 0.025M-phosphate, the recovery was again 61%, with the activity appearing in a single band.

Comparison with other adsorbents

A number of isotherms were determined for the adsorption of bovine serum albumin on various materials. These isotherms were curved near the origin and tended to level off to a saturation value when the protein concentration in the supernatant reached about 0.05%. Since these studies are incomplete, only the approximate adsorption value at this supernatant concentration will be reported here for purposes of comparison. Paper (Munktell 3-Grycksbos Pappersfabriks AB, Grycksbo, Sweden) at pH 5.0 or 5.5 gave salting-out adsorption amounting to 0.5 mg. protein/g. paper in 1.5M-ammonium sulphate, 1.5 mg./g. in 2.0M, and 4.0 mg./g. in 2.5M. A Brockmann-type aluminium oxide buffered at pH 5.0 with 0.1M-sodium acetate buffer gave an optimum adsorption in 0.5M-ammonium sulphate of about 10 mg./g., and about half this adsorption in 1.0M-ammonium sulphate or in its complete ab-

sence. A sample of silica gel prepared by Dr R. L. M. Synge for chromatographic use gave an adsorption of 110 mg./g. at pH 5.0 without the addition of ammonium sulphate, and a commercial anhydrous silicic acid gave comparable results.

The hydrated tricalcium phosphate used in the chromatographic experiments reported here was studied only in the absence of ammonium sulphate, and adsorbed serum albumin from a 0.005M-sodium acetate buffer (pH 5.0) to the extent of about 310 mg./g., calculated on the dry weight basis. Addition of 0.05M-sodium phosphate buffer (pH 6.3) reduced the adsorption to about one-fifth, and gave a nearly linear isotherm as far as the curve was studied (up to 0.1% (w/v) of protein in the supernatant solution). The adsorption was reduced to 130 mg./g. by 0.03M-phosphate buffer at pH 6.3. A sample of tricalcium phosphate precipitated from trisodium phosphate by the addition of calcium chloride had similar adsorption characteristics, but a commercial anhydrous material showed no adsorption at all. Visually estimated experiments with the adsorption and subsequent phosphate elution of phycoerythrin on gamma aluminium hydroxide (Willstätter) indicated that its behaviour was similar to that of tricalcium phosphate.

DISCUSSION

On the basis of these preliminary experiments, precipitated tricalcium phosphate seems to be a promising material for protein chromatography, being readily prepared in a reproducible form with desirable physical characteristics, and showing reversible adsorption for many proteins. Certainly it is worthy of further investigation. The effectiveness of phosphate buffers as eluting agents probably reflects the tendency of the calcium phosphate crystals to attract ions of the same kind from the solution, thereby decreasing their affinity for protein, and it is to be noted that the phosphate solutions show considerable retention on the column. The more surprising effect of small salt concentrations in promoting the adsorption of phycoerythrin cannot be so readily explained, and a theoretical discussion seems out of order until more data are available. It is to be noted that salt also considerably increases the retention of the phosphate buffer, but that the effect

on the protein predominates. The absence of a marked pH effect is in sharp contrast with the behaviour of silica gel.

A possible difficulty with the system is the tendency for the protein to tail behind the front when the elution is slow. This tailing, which seems to occur particularly when the phosphate front is ahead of the protein, is probably a consequence of the curving of the adsorption isotherm, and it is difficult to predict whether it will prove an obstacle in the analysis of mixtures. The elimination of tailing in higher phosphate concentrations appears to be the result of almost complete displacement of the protein which is then carried along in the phosphate front, and suggests the possible advantage of eluting with a progressively increasing phosphate concentration. The material left at the starting point is probably a mixture of impurities and denatured protein already present in the sample used. Whether the process of adsorption analysis itself causes any denaturation is a question which should be thoroughly investigated. The experiments with D-amino-acid oxidase had that point as one objective (as any denaturation should cause a loss in enzymic activity) and established that the tendency toward denaturation was certainly not great. However, considerably more work will be necessary to prove that the danger of denaturation can be ignored.

SUMMARY

1. Hydrated tricalcium phosphate as an adsorbent for proteins has been investigated with respect to the effects of phosphate buffer, pH and sodium chloride on the chromatography of phycoerythrin.

2. Phosphate buffer is an effective displacing agent. In low concentrations it gives elution analysis of phycoerythrin with considerable tailing, and in higher concentrations it gives displacement analysis in sharp bands. Variations in pH have little effect.

3. Sodium chloride in low concentrations promotes to a great extent the adsorption of phycoerythrin, and to a lesser extent the adsorption of phosphate buffer.

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