CHROMATOGRAPHY OF BLOOD-CLOTTING FACTORS AND SERUM PROTEINS ON COLUMNS OF DIATOMACEOUS EARTH*

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The purification of proteins still offers formidable difficulties; and this task is conspicuously troublesome in the study of blood coagulation. First, it is necessary to isolate a clotting factor from an overwhelming quantity of plasma proteins. Then, it is necessary to remove small traces of specific contaminants, because slight contamination can lead to an erroneous concept of blood coagulation. In these problems, and especially for the removal of trace contaminants, chromatography might be of unique assistance. For this purpose, diatomaceous earth has already shown certain desirable characteristics.

Preparations of diatomaceous earth have good flow rates, and have found much use as filter aids in the collection of protein precipitates and in the clarification of protein solutions (1). They have often been mixed with adsorbents to improve the flow rate of chromatographic columns. In many of these applications the adsorbent effect of the filter aid is negligible. However, it is now widely appreciated that the filter aids, themselves, can sometimes function as adsorbents (2).

Adsorption on diatomaceous earth, followed by elution of the desired material, has been used to purify bacterial penicillinase (3), alfalfa pectinesterase (4), orange pectinesterase (5–7), influenza virus (8, 9), Rous sarcoma virus (10–12), and melanized granules from mouse melanomas (13). Approximate distribution coefficients were estimated in the work on influenza virus and pneumonia virus of mice (9); and column chromatography was applied in the purification of Rous sarcoma virus (10) and melanized granules (13). Recently, Clauser and Li have investigated the chromatographic behavior of several proteins on columns of hyflo super-cel (14). They reported that growth hormone, bovine serum albumin, and methylated serum albumin were adsorbed at a pH below their isoelectric points, and were eluted in yields of 80 to 90 per cent when the pH was shifted to values above their isoelectric points.

In previous work on blood coagulation (15, 16) hyflo was used to adsorb a small portion of the protein from a solution of plasma globulins. After filtration on a Büch-

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ner funnel, the operations of washing and flowing elution were performed as if the hyflo cake were a flat chromatographic column. The eluate contained thrombokinase and a platelet-like thromboplastin.

In the present study, diatomaceous earth has been used to remove thrombokinase (or its precursor) from preparations of prothrombin. Prothrombin, itself, has been chromatographed on columns of conventional size and shape. Thrombin has also been chromatographed; and serum proteins have been separated into four major bands, with good recovery of protein. All operations have been performed within the pH range 5.0 to 8.1.

EXPERIMENTAL

Materials and Methods

Hyflo Super-Cel, Standard Super-Cel, and Filter-Cel.—Grades of diatomaceous earth supplied by Johns-Manville Corporation, New York. As quoted by the manufacturer's representative, (P. R. Deschere, personal communication January 4, 1955), determinations of specific surface area in square meters per gram have given the values: 1.9 for hyflo, 5.5 for standard super-cel and 22.8 for filter-cel.

E&D Filter Paper.—The Eaton-Dikeman Company, Mt. Holly Springs, Pennsylvania.

Buffers for Chromatography.—Reagents of analytical grade were used. One set of buffers was made with potassium dihydrogen phosphate and sodium hydroxide to give the molarities and pH specified. Those buffers containing acetate were made with potassium dihydrogen phosphate, sodium acetate, and sodium hydroxide. The pH 6.00, pH 7.59, and pH 8.01 buffers used for the chromatography of thrombin were made with potassium dihydrogen phosphate and potassium hydroxide.

Chromatographic Columns.—In all cases, the column was prepared by filtering a slurry of the adsorbent, with suction, on E&D no. 612 paper supported by a perforated plate. The column was washed until the effluent had a low optical density and was close to the pH desired for the experiment. In the earlier experiments, when the fractions were collected by hand, the column was stopped overnight by fitting a stopper in the top of the tube. The later experiments, performed with an automatic fraction collector, were run continuously, day and night. Wherever points were used in charting the columns, they were so placed that the point marking the first 20 ml. fraction would be at 20 ml., or at fraction 1, depending on the scale; *i.e.*, the points were at the ends of the fractions.

Optical Density.—Measured against distilled water at 280 mμ and corrected by subtracting the optical density of the particular buffer medium.

Prothrombin Assay.—Essentially as described (16–19), but with some modifications. By this procedure, before or after the modifications, about 1900 units of prothrombin have been found in 1 ml. oxalated plasma. The usual activator system: thrombokinase, cephalin, calcium, and dilute bovine serum, will be discussed in the accompanying paper (20). The results have not been corrected by comparison with a stable standard, and hence are significant mainly in comparison with other tests run at the same time. The unit is much smaller than the NIH unit.

Thrombin Assay for Chromatography of Thrombin.—Essentially as previously described, but converted to standard NIH units. To do this, a working standard of glycerinized thrombin was prepared (19). This, in turn, was assigned a value in terms of NIH units by direct comparison with a dry standard thrombin received from the National Institutes of Health through the courtesy of Dr. W. G. Workman. The column fractions were tested in direct comparison with the working standard.

Removal of Thrombokinase from Prothrombin

For these experiments, plasma globulins were fractionated as described previously (15, 16). The major prothrombin fraction, eluted from barium sulfate by 0.3 M phosphate, pH 6.6, will be designated the "first prothrombin eluate." The thrombokinase or prothrombokinase present in various fractions will be designated "thrombokinase" throughout this paper, without any attempt to decide whether the thrombokinase was in the precursor form at a given stage of the operations.

The "first prothrombin eluate" has regularly contained a small, but troublesome, portion of the total thrombokinase. In the past, most of this contaminant was removed by stirring with filter-cel (16). However, the loss of prothrombin was great; and the removal of thrombokinase was not complete. In search of a better method, the absorbent effects of hyflo super-cel, standard super-cel, and filter-cel were studied further.

Each adsorbent was mixed with the first prothrombin eluate in the proportions indicated in Fig. 1. After 15 minutes at room temperature, the adsorbents were removed by centrifugation. The optical densities of the supernates were determined; and after dialysis, assays were performed for prothrombin. Susceptibility to activation in the presence of cephalin and calcium was taken as a rough measure of thrombokinase.

Hyflo, with the smallest surface area per gram was the least effective adsorbent. Filter-cel, with the largest surface area per gram, was the most effective adsorbent. This type of correlation has been observed in previous studies on celite filter aids (8, 9). On a percentage basis, the amount of prothrombin adsorbed tended to be somewhat less than the amount of protein; whereas the amount of thrombokinase removed was considerably greater.

It was especially interesting that the first prothrombin eluates were more stable after they had been treated with adsorbents, as exemplified in Fig. 2.

Samples which had been treated with different amounts of filter-cel were dialyzed against cold 0.9 per cent saline for 4 hours and against cold veronal-buffered saline, pH 7.4, for 22 hours. Assays for prothrombin were performed at the end of the dialysis and again after 5 more days at 5°C. With the smaller quantities of filter-cel the initial loss of prothrombin was more than counterbalanced by the greater stability of the remaining prothrombin, as shown in Fig. 2. A trace of thrombin was detected in the untreated control immediately after dialysis; but no thrombin was found in the treated samples.

The quantitative adsorbent behavior of standard super-cel has varied somewhat

from one batch to another, but not enough to make it act like hyflo on one hand or like filter-cel on the other. Passage through a cake of standard super-cel proved to be effective in removing thrombokinase without prohibitive loss of prothrombin.

A slurry composed of 140 gm. standard super-cel and 1 liter 0.3 M phosphate, pH 6.6, was filtered on E&D no. 612 paper on a Büchner funnel 13 cm. in internal diameter. It was washed with 3 liters of the same phosphate buffer. As 700 ml. of "first prothrombin eluate," followed by 1400 ml. of the phosphate buffer, were passed

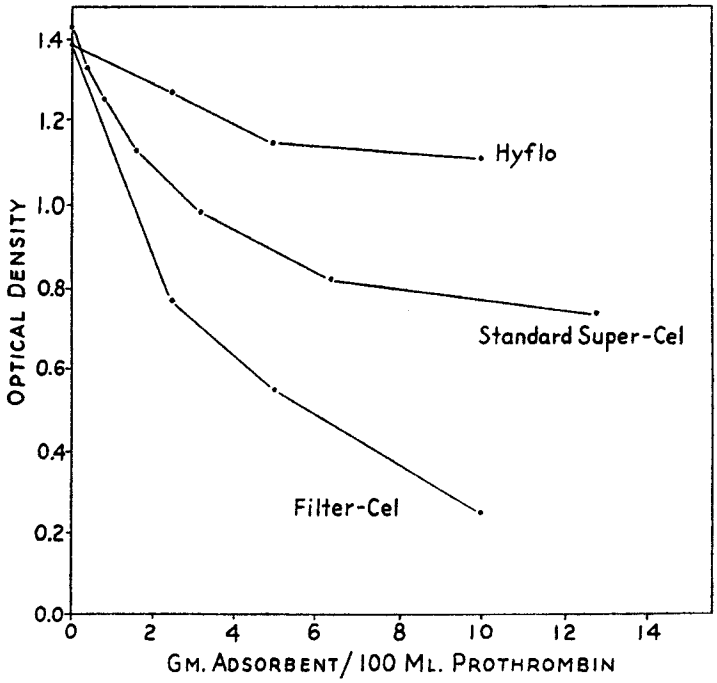

FIG. 1. Batch adsorptions with partially purified prothrombin.

through the cake at an even rate, eleven effluent fractions were collected in 164 minutes at room temperature.

Inspection of Fig. 3 reveals that the fifth fraction had the highest concentration of prothrombin and the highest specific activity. This fraction had about as much prothrombin per milliliter as the original "first prothrombin eluate." The total recovery of prothrombin activity in the eleven fractions was 62 per cent. As evident in Table I, thrombokinase was readily demonstrable in sample 0, (the original "first prothrombin eluate"), which rapidly produced thrombin in the presence of cephalin and calcium. Thrombokinase was barely detectable in fractions 7 to 11; and it was not found in fractions 4 to 6, which contained most of the effluent prothrombin. That such fractions contain very little, if any, thrombokinase has been verified by other tests, some of which will be presented in the accompanying paper (20).

A trace of thrombin was found in dialyzed sample 0; but none was found in dialyzed samples of fractions 4 to 8, which contained almost all the effluent prothrombin.

Numerous experiments have shown that treatment with diatomaceous earth tends to reduce three independently measured variables: (1) contam-

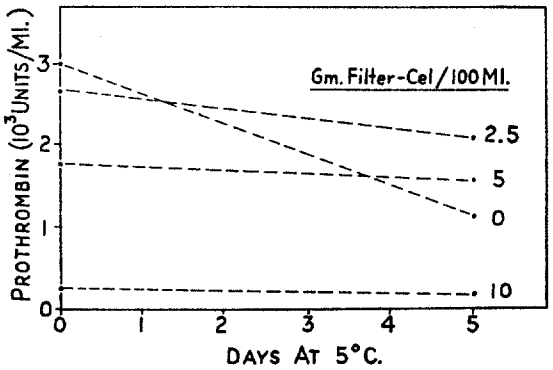

FIG. 2. Greater stability of prothrombin after treatment with filter-cel.

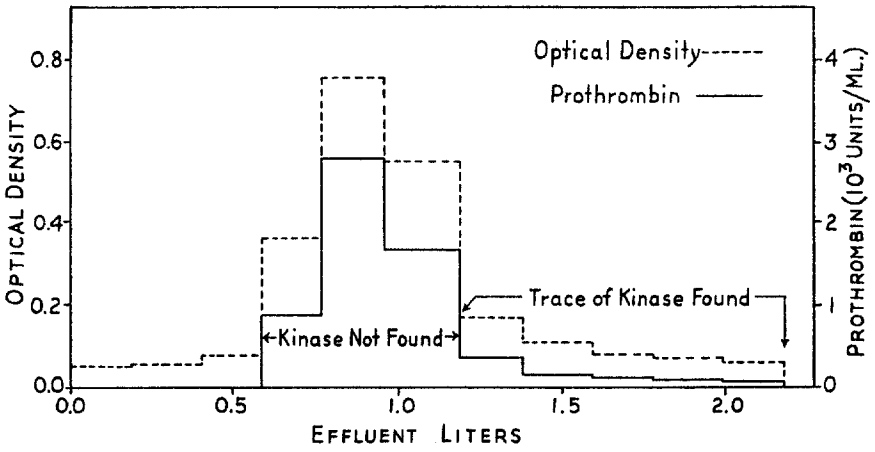

FIG. 3. Removal of thrombokinase from prothrombin by standard super-cel.

ination with thrombokinase, (2) "spontaneous" production of thrombin, and (3) loss of prothrombin upon aging. This association of effects would be expected. However, it is doubtful that thrombokinase is the only factor responsible for loss of prothrombin upon aging.

Chromatography of Prothrombin

Since prothrombin preparations proved to be relatively stable once they had been passed through a cake of standard super-cel, it was feasible to per-

form experiments of longer duration. For this purpose a prothrombin concentrate was prepared and stored frozen in 10 ml. portions. Of several modifications introduced into the preparative procedure, the most pertinent were passage through cakes of standard super-cel, precipitation with ammonium sulfate at 0.6 saturation, and resolution in 0.1 M acetate. The optical density of the concentrate was 3.43 and the pH, 5.26.

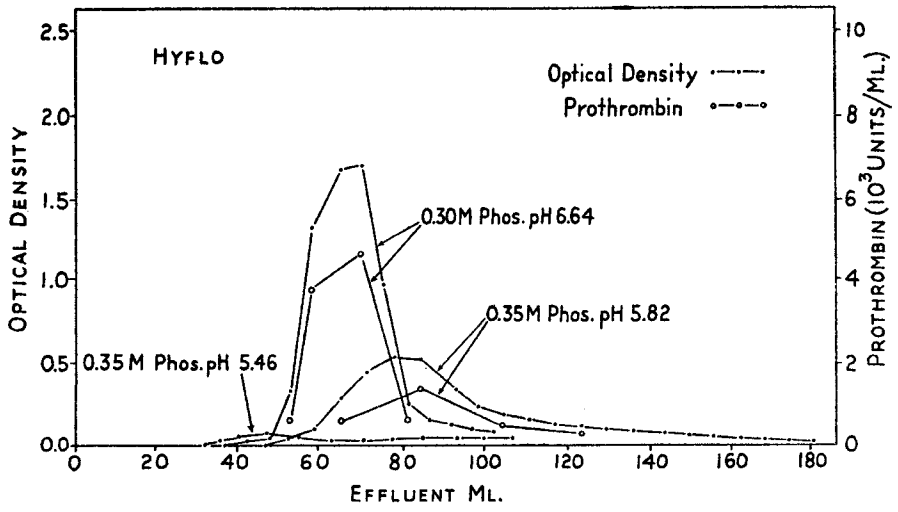

FIG. 4 *a*

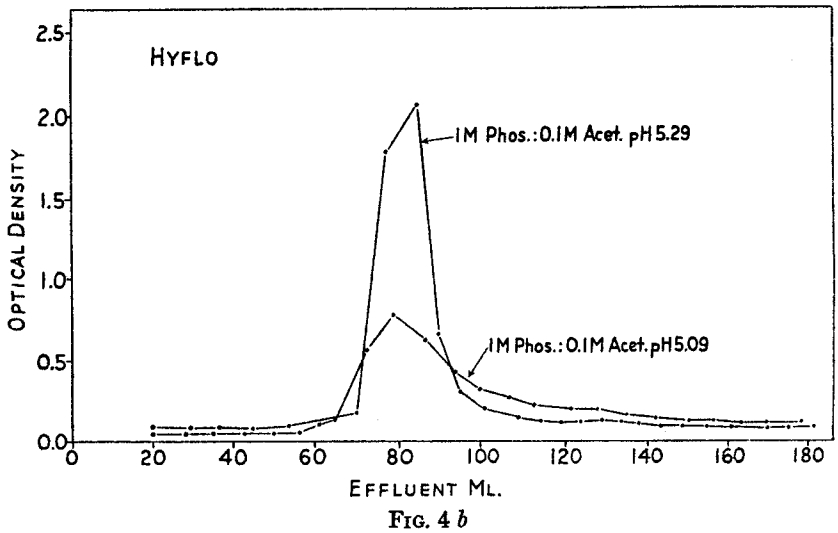

FIG. 4 *b*

FIGS. 4 *a*, *b*, and *c*. Column chromatography of prothrombin at 3.5 ± 0.5°C.

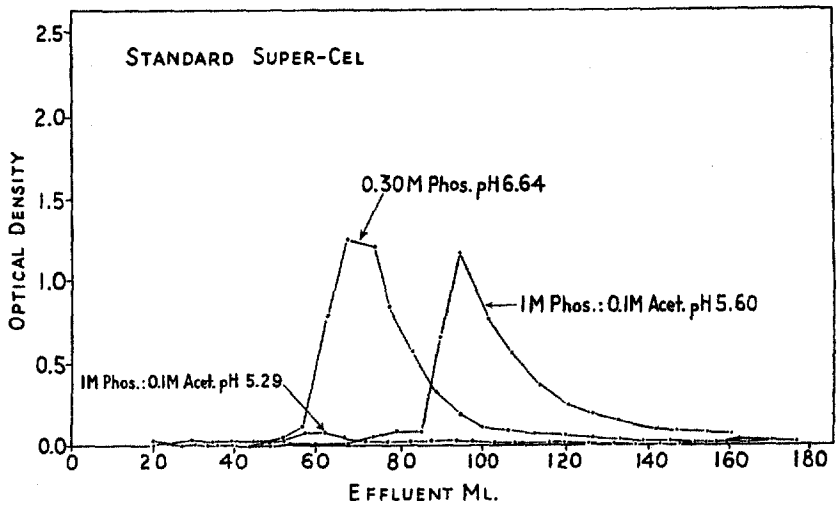

FIG. 4 *c*

TABLE I

Insusceptibility of Prothrombin to Activation by Cephalin and Calcium as Shown by Effluent Fractions from a Cake of Standard Super-Cel

Mixtures				Period of incubation			
0.3 ml.	0.5 ml.	0.1 ml.	0.1 ml.	10 min.	20 min.	60 min.	28 hrs.
Buffer	Sample 0	Cephalin	$CaCl_2$	21 sec.	15 sec.	13 sec.	10 sec.
"	Fraction 4	"	"	N.c. 27 hrs.	N.c. 27 hrs.	N.c. 27 hrs.	N.c. 1 hr.
"	" 5	"	"	N.c. 27 "	N.c. 27 "	N.c. 27 "	N.c. 1 "
"	" 6	"	"	N.c. 27 "	N.c. 27 "	N.c. 27 "	N.c. 1 "
"	" 7	"	"	N.c. 27 "	N.c. 27 "	N.c. 27 "	1050 sec.
"	" 8	"	"	N.c. 27 "	N.c. 27 "	1-27 "	720 "
"	" 9	"	"	1-27 "	1-27 "	1-27 "	660 "
"	" 10	"	"	1-27 "	1-27 "	1-27 "	600 "
"	" 11	"	"	1-27 "	1-27 "	1-27 "	720 "

Buffer, veronal-buffered saline, pH 7.4. Cephalin, 0.1 per cent suspension in buffer. $CaCl_2$, 0.025 M Ca, in buffer. Sample 0, original material, not passed through cake. Room temperature, 24°C.

At the specified times, 0.1 ml. of mixture was added to 0.3 ml. oxalated fibrinogen. The resulting clotting times are given in the body of the table. N.c. 27 hrs. = no clot in 27 hours.

Exploratory runs at room temperature revealed that the protein could be adsorbed on columns of hyflo, and eluted by phosphate buffers. When the column was operated in a cold room at 3.5 ± 0.5°C., somewhat less protein was eluted by a given solution. Some of the results are shown in Figs. 4 *a*, *b*, and *c*.

In each case the column was made with 24.5 gm. diatomaceous earth. In every

case but one, the column was 21 mm. in diameter and about 170 mm. high. Flow rates of the different columns varied from 1.4 to 7.0 ml. per hour. Each column was used for a freshly thawed 10 ml. portion of the same batch of prothrombin.

As shown in Fig. 4 *a*, the prothrombin titer tended to parallel the optical density; and no gross fractionation was achieved. This was to be expected, for the preparation had already been fractionated by standard super-cel. In general, the results illustrated, together with others, showed that elution

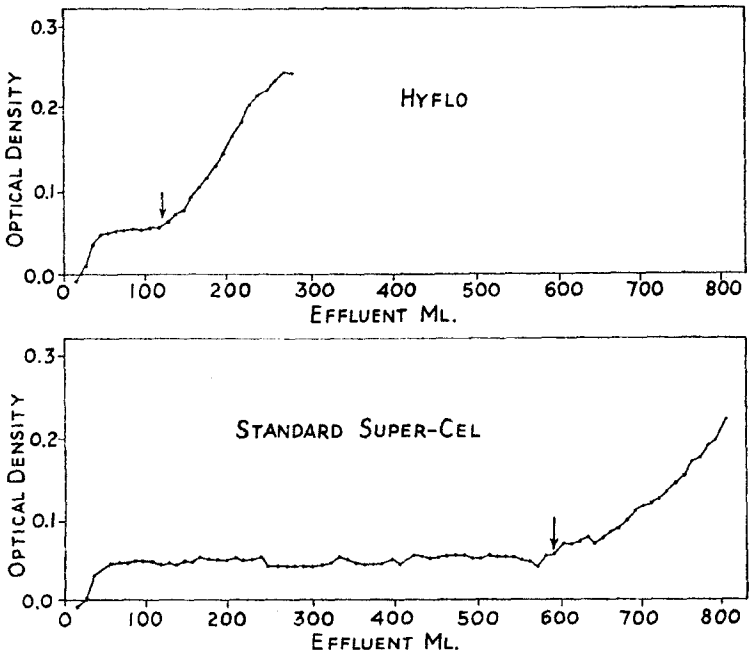

FIG. 5. Parallel breakthrough experiments with dilute prothrombin.

was greater at higher pH, within the range of 5.0 to 6.6. It was greater at higher molarity of phosphate, within the range 0.1 to 1.0. Within limits, it was possible to select the desired pH of operation and then to raise the phosphate concentration until the desired degree of elution occurred. A given eluent was more effective with hyflo than with standard super-cel.

The difference between standard super-cel and hyflo was further evident in breakthrough experiments performed simultaneously at room temperature.

Dilute prothrombin, with optical density about 0.35, phosphate concentration about 0.28 M, and pH 5.11, was introduced at the rate of 20 ml. per hour into columns made with 10 gm. adsorbent. The effluent fractions are plotted in Fig. 5.

As seen in Fig. 5, a small amount of light-absorbing material began to

emerge from the column directly after the hold-up volume. Each curve then described a plateau until a second, bigger, rise began. With respect to the protein in the second rise, the capacity of standard super-cel was almost six times that of hyflo.

By an extension of the foregoing procedure, prothrombin has been adsorbed from dilute solution, and eluted at four times the original concentration.

Chromatography of Thrombin

Chromatographic results with thrombin have been similar to those with prothrombin. However, in the case of thrombin, the material put into the

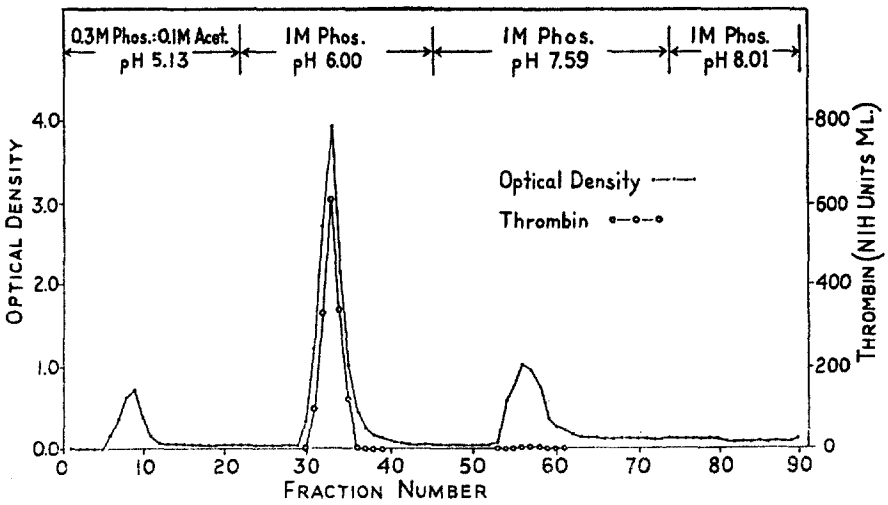

FIG. 6. Chromatography of thrombin on standard super-cel at 3.5 ± 0.5°C.

column had not been subjected to quite so much pretreatment with diatomaceous earth; and more impurities were left for gross separation by the column.

A concentrated thrombin subfraction was prepared essentially as previously described (16), except that the final dialysis was performed against 0.3 M phosphate: 0.1 M acetate, pH 5.11. Its optical density was 27.75. A column, 19 mm. in diameter and approximately 260 mm. high, was prepared with 25 gm. standard super-cel, washed, and equilibrated with the buffer used for dialysis of the thrombin. After 5.0 ml. concentrated thrombin was introduced into the column, it was followed by the buffers indicated on Fig. 6. The fluid was run at 3.4 ml. per hour. Fractions of about 5.2 ml. were collected by an automatic fraction collector.

As illustrated in Fig. 6, the protein emerged in three distinct bands; and the thrombin activity was associated with the middle band. Close inspection

reveals that the ratio of thrombin to optical density varied within the middle band. Thus, the chromatographic analysis revealed that not even the middle band represented pure thrombin. Fraction 34 had the highest specific activity, 160 NIH units of thrombin per unit of optical density. This represented a purification of 2.46 times over the original concentrate, which had a specific activity of 65.

As estimated from the volumes and optical densities of the fractions, 89 per cent of the protein was recovered in fractions 1 to 90. Fractions 31 to 35 accounted for 83 per cent of the thrombin put into the column.

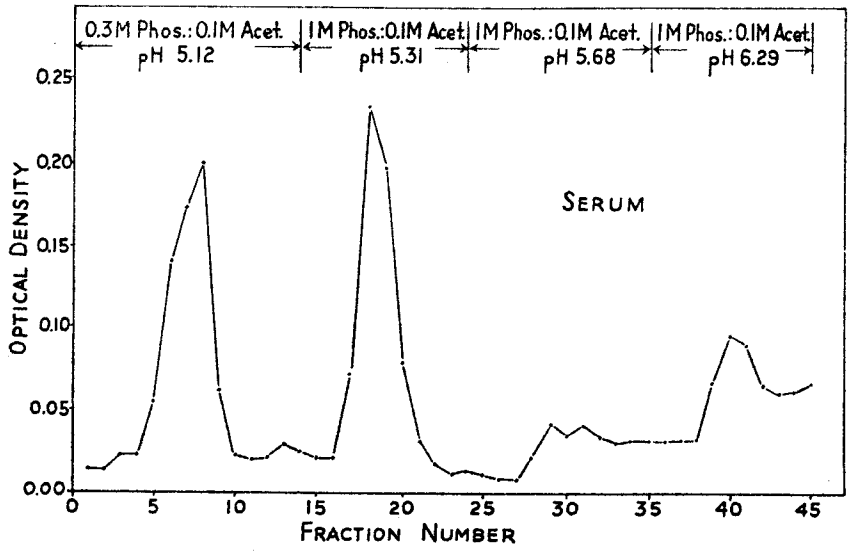

FIG. 7. Chromatography of serum proteins on standard super-cel at 3.5 ± 0.5°C.

Chromatography of Serum Proteins

The foregoing results have shown that chromatography on diatomaceous earth can be of considerable help in the purification of certain clotting factors. The experiment charted in Fig. 7 shows that conditions can be adjusted to permit good recovery of total serum protein.

Bovine serum was dialyzed against cold 0.3 M phosphate: 0.1 M acetate, pH 5.15. Its optical density was 67.8. A column, 19 mm. in diameter and approximately 260 mm. high, was prepared with 25 gm. standard super-cel, washed, and equilibrated with the first buffer of Fig. 7. After 0.5 ml. dialyzed serum was introduced into the column, it was followed by the buffers indicated on Fig. 7. The flow rate was 5.4 ml. per hour. Fractions, averaging 13.8 ml. in volume, were collected by an automatic drop-counting fraction collector.

Four major bands of serum protein are seen in Fig. 7. As estimated from the volumes and optical densities of the fractions, the forty-five fractions accounted for 94 per cent of the serum protein put into the column. The first band, comprising about 32 per cent of the protein, came through the column in the original solvent.

DISCUSSION

The columns of diatomaceous earth, as herein described, have given a fair degree of resolution and good recovery, in terms of both protein and clotting activity. Moreover, the chromatography of serum proteins has offered some hope of more general applicability. The removal of thrombokinase from prothrombin has been most useful.

Other procedures, approaching those of chromatography more or less closely, have been applied to the blood coagulation problem. Owren (21) has employed controlled, repeated Seitz filtration to free plasma from prothrombin without removing factor V. A later modification of the Seitz procedure was reported to remove proconvertin, and to leave the prothrombin in the plasma (22).

Work reported from this laboratory in 1949 (15) and 1951 (16) showed that proteins which had been adsorbed on barium sulfate could be harvested in two portions by percolating two successive eluents through the barium sulfate-hyflo cake. The first portion, eluted by 0.3 M phosphate, pH 6.6, contained most of the prothrombin. The portion subsequently eluted by 0.6 M phosphate, pH 7.9, was a good source of thrombokinase. In 1952-53, Duckert, Koller, and Matter (23) described a similar procedure. In their method, barium sulfate was stirred with plasma, washed, and made into a column together with half its weight of hyflo. First, prothrombin was eluted by 0.14 M citrate, pH 5.8; then factor VII was eluted by 0.14 M citrate, pH 7.8.

It is evident from the present results that the hyflo may well contribute to the effect of barium sulfate–hyflo mixtures. To a large extent, the effects of the two adsorbents were separated by the method described in 1949-51. In this, an entirely separate adsorption with a large amount of hyflo was performed first. This removed an appreciable amount of protein readily adsorbable on hyflo, before the barium sulfate adsorption was begun. However, during the preliminary attempts to remove the last trace of thrombokinase from prothrombin, it was observed that the purification achieved by one particular barium sulfate–hyflo column was due almost entirely to the hyflo. This was revealed by repeating the experiment with a column which contained the same amount of hyflo, but no barium sulfate.

Recently, Tager (24) has used hyflo-amphojel columns to purify CRF (coagulase-reacting factor) from Seitz-filtered plasma. Previously, Tager (25) had reported that CRF was not adsorbed on Berkefeld filters, but was

taken up by aluminum hydroxide. (Berkefeld filters are made of diatomaceous earth; and amphojel is a pharmaceutical preparation of alumina gel.)

The present study has followed previous ones in emphasizing that small changes in conditions can make appreciable differences in the adsorption and elution of proteins. Consequently, it is hazardous to identify clotting factors merely by their apparent adsorptive behavior in different laboratories.

In 1938, Seegers, Brinkhous, Smith, and Warner (26) reported that their prothrombin was activated promptly by lung extract, but very slowly by cephalin. In the present study the best prothrombin fractions failed to produce thrombin when incubated with calcium and cephalin for 28 hours.

SUMMARY

1. In batch adsorptions with prothrombin solutions, hyflo was the weakest adsorbent, standard super-cel intermediate, and filter-cel strongest. Of these three grades of diatomaceous earth, hyflo has the smallest surface area per gram and filter-cel the largest. In parallel breakthrough experiments, a column of standard super-cel had a capacity almost six times that of a hyflo column.

2. After partial removal of impurities by diatomaceous earth, prothrombin preparations contained less thrombokinase, were more stable, and displayed less tendency to form thrombin "spontaneously." Thrombokinase (or its precursor) was removed from a preparation of prothrombin by passage through a filter cake of standard super-cel. The specific activity of the prothrombin was increased; and 62 per cent of the activity was recovered.

3. Prothrombin was adsorbed from an ammonium sulfate solution at pH 5.26 by columns of hyflo or standard super-cel. When eluted by phosphate solutions, the protein moved down the columns more readily at higher pH and higher concentration of phosphate salts, within the pH range 5.0 to 6.6, and within the phosphate range 0.1 to 1.0 M.

4. Thrombin was adsorbed on a column of standard super-cel at pH 5.11. As successive eluents passed through the column, the thrombin emerged between two bands of impurities. The specific activity of the thrombin was raised; and 83 per cent of the activity was recovered.

5. With a column of standard super-cel, and with a series of eluents within the pH range 5.1 to 6.3, total serum proteins were separated into four major bands. About 94 per cent of the protein was recovered.

Dr. Harold G. Cassidy made a number of helpful suggestions several years ago, and again during the preparation of this manuscript.

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