SYMPOSIUM ON CHARACTERIZATION OF PROTEINS

Introduction

In 1838 Mulder, at the suggestion of Berzelius, assigned the word "protein" to those complex organic nitrogenous substances found in all cells, animal or plant. The word protein was derived from the Greek word meaning primary or first. But it soon became apparent that proteins had no single primary function in the cell; rather they contributed in numerous ways to both the architecture and physiological state of the animal or plant. Proteins serve as structural elements in the various membranes and walls of the cell and as protective coatings in the hair, wool, and connective tissue of the animal. Proteins also function in both regulatory and defensive mechanisms. Proteins are enzymes, hormones, carriers of oxygen and essential elements, and antibodies. They are participants in energy transfer, that is, muscle contraction and photosynthesis, and in the transfer of genetic information.

Proteins also serve another function which is of major interest to this Division of Agricultural and Food Chemistry. Proteins, when consumed as food, provide essential nutrients for growth and maintenance. A listing of our basic food sources—milk, meat, fish, egg, and seeds, which include both cereals and oilseeds—shows that proteins are a significant factor in our dietary patterns. However, this selection cannot be delegated to a natural preference for the proper nutrients. It is rather the result of a highly subjective selection based on aroma, flavor, texture, and satiable characteristics which, in many instances, are due to the physical characteristics of the food proteins. As the role of the food industry shifts from one of food preparation to one of food formulation and fabrication, these physical functional characteristics will assume even greater importance.

Irrespective of the function which proteins serve, be it physiological, physical, or nutritional, the major objective of protein chemistry has been to understand the relationship between protein composition and structure, and protein functionality. It seemed to us particularly appropriate, therefore, that the new protein subdivision of the Agricultural and Food Division present a symposium on the characterization of proteins. An in-depth evaluation of all methods under development in this rapidly expanding field would be beyond the scope of this symposium. We have, however, attempted to highlight certain developing areas in the logical sequence of: (1) methods of separation; (2) methods of analysis of primary composition and structure; and (3) methods for evaluation of the secondary and tertiary structure of the protein molecule. By this approach we hoped to provide a timely overview of the analytical methodology of proteins.

We are deeply indebted to the contributors who so expertly covered their subjects by reviewing the literature and presenting results of their own research. With the excellent cooperation of the authors and the interest and exertion of Dr. Bates and his staff it has been possible to achieve early publication of the manuscripts. Through the cooperation of the American Chemical Society the articles presented here will be reproduced by the Society in book form in order to reach an ever larger audience. For this we are grateful.

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Columns for Large-Scale Gel Filtration on Porous Gels

Fractionation of Rape Seed Proteins and Insulin

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At the Institute of Biochemistry in Uppsala we have for several years been working on the technical problems encountered with the scaling up of gel filtration columns. The difficulties are especially noticeable when gels of high water regain are used in columns with diameters larger than about 20 cm. Two new columns for gel filtration on a preparative scale with porous gels are presented in this paper, the so-called compact and stacked sectioned column types, respectively. They are characterized by their unconventional dimensions, very short and with large diameters, *e.g.*, 15 × 45 cm. Several column sections are connected in series and the two types mentioned differ in the way these are connected to each other. The problem of sample application on large diameter columns is briefly discussed. An example of automation of a gel filtration experiment is given. The following applications are presented—the fractionation of rape seed proteins, serum proteins, and insulin.

Gel filtration is nowadays a well-established technique in chemical and biological laboratories all over the world. It is used both for analytical and preparative purposes for the separation and fractionation of low molecular weight substances, as well as high molecular weight material such as proteins, nucleic acids, polysaccharides, and even particles. Many thousands of publications have dealt with this technique in the last decade and the applications are numerous. A complete list of references is available from Pharmacia Fine Chemicals AB, Uppsala, Sweden, covering the use of their dextran gel Sephadex and their agarose gel Sepharose.

This paper is intended to provide examples from our own research of how gel filtration with Sephadex gels can be

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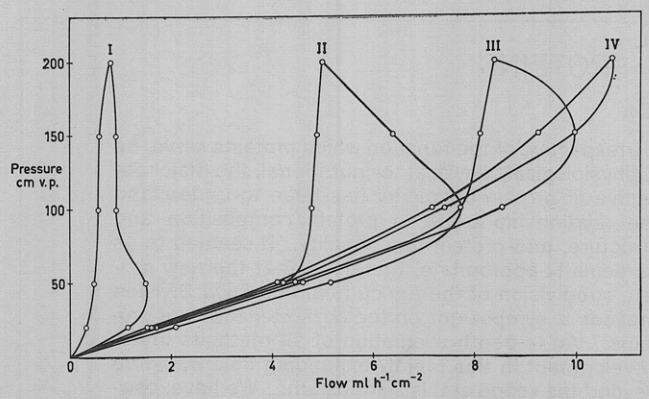

Figure 1. Flow rate as a function of the hydrostatic pressure (expressed as cm water column) for 30-cm diameter columns of varying heights, packed with Sephadex G-150. The columns are packed in the same way. The Roman numerals indicate the following bed heights: I = 52 cm; II = 23 cm; III = 13 cm; and IV = 8 cm

utilized both for guiding analytical experiments and for large-scale protein fractionations. Special emphasis will be on problems connected with the design of columns for large-scale work. The applications that will be presented are the isolation and fractionation of serum proteins, rape seed proteins, and insulin.

Depending on the type of separation, gel filtration procedures can roughly be divided into desalting and fractionation operations (Flodin, 1962). In desalting operations, the components to be separated have great differences in molecular weight, which makes it possible to use gels of low water regain such as Sephadex G-25 and G-50. These gels are very rigid (especially Sephadex G-25) and can be packed into large beds which allow very high flow rates. As an example, the columns manufactured by Pharmacia Fine Chemicals (Sephamatic GF 18-10) have a total volume of 2500 l., and, when packed with Sephadex G-25 coarse, have a capacity of about 1000 to 1500 l. of charge solution per hour.

In fractionation operations, on the other hand, and especially in the protein field, the gels most often used are of the high water regain type such as Sephadex G-100 and G-200. These porous gels are less dense than Sephadex G-25 and become easily, although reversibly, deformed by the hydrodynamic forces when certain critical column dimensions and flow rates are attained. This will result in clogging of the capillary system of the column. These critical column dimensions are of such a degree of magnitude that some sort of stabilization of the gel bed is necessary if the technique is to be transferred on to an industrial scale.

In this institute we have for some years been studying the possibilities of introducing some kind of gel bed stabilizers in large diameter ($\geqslant$ 30 cm) columns, packed with Sephadex G-150. The results of this investigation will be published elsewhere (Janson and Porath). A short summary of this developmental work will be given here, along with some applications where our columns and a similar column type which has been designed by Pharmacia Fine Chemicals AB have been used.

CONSTRUCTION OF LARGE DIAMETER COLUMNS FOR POROUS GELS

The Compact Sectioned Column Type. When determining the flow rates at different hydrostatic pressures as a function of the gel bed height for 30-cm wide columns packed with Sephadex G-150, the highest and most stable flow rates, and also the highest resolving power per unit bed length,

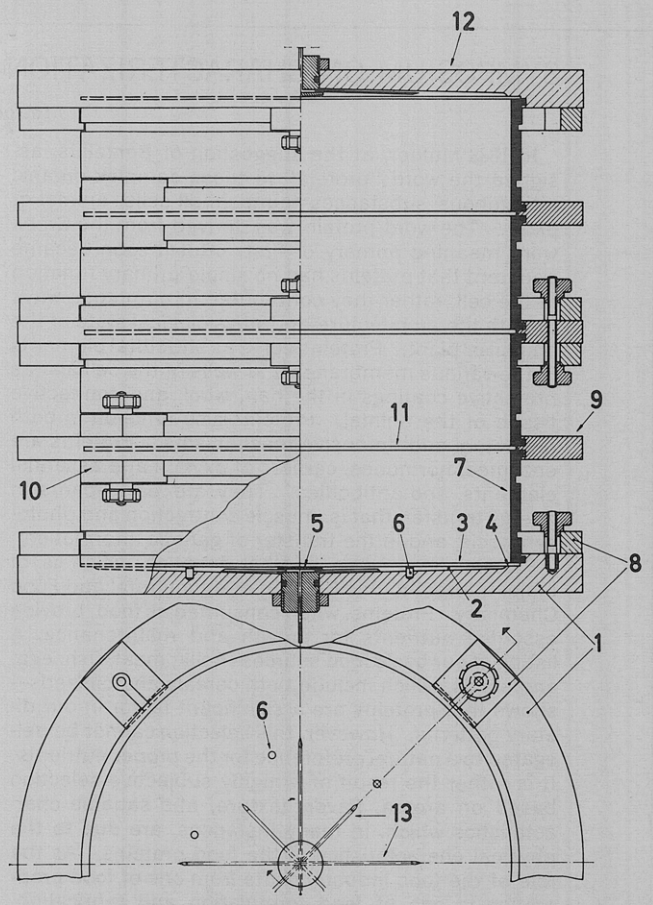

Figure 2. Partially sectioned drawing of the compact sectioned column type with internal sample distribution system. The column is entirely symmetric, the two endpieces are identical. (1, 12) End plates, made of Perspex; (2) Sample distribution chamber; (3) 1.5-mm thick sheet of porous plastic (Vyon No. 1); (4) Peripheral shelf (support for the Vyon sheet); (5) Center support and sample distributor; (6) Cylindrical Perspex supports for the Vyon sheet; (7) Column cylinder section (7.5-cm high and 30-cm diameter); (8, 10) Clamp made of polyvinylchloride; (9) Ring made of polyvinylchloride (notice the O-rings); (11) 3-mm thick rigid sheet of porous plastic Vyon No. 1; (13) Cannular tubing (sample distributors). The total volume, V_t, of this column is 21 l.

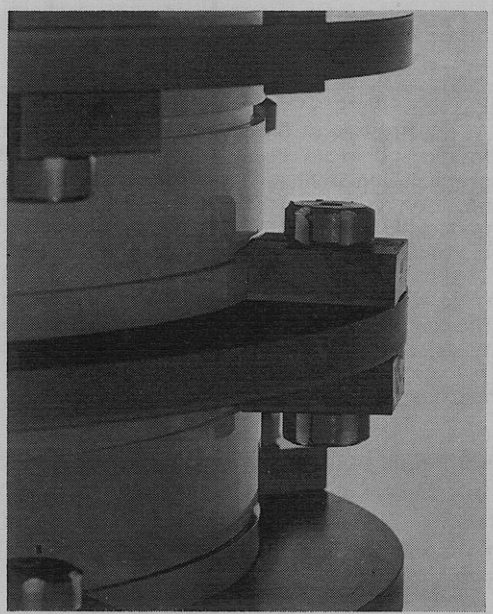

Figure 3. Detail of the compact sectioned column, showing the Perspex column sections and the sealing rings with clamps made of polyvinylchloride

were obtained with the shortest of the columns tested (Figure 1). On the basis of these results, a column type was designed which is built up of 7.5-cm high and 30-cm wide column sections (total volume 5.25 l.) made of Perspex cylinders separated by 3-mm thick rigid porous plastic sheets (Vyon No. 1, Porous Plastics Ltd., Dagenham Docks, Essex, England). The column sections are held together by rings and clamps of PVC (polyvinylchloride) which give an airtight seal with the aid of rubber O-rings. Figure 2 shows a drawing of such a column built up of four sections, and Figure 3 shows a detail of the column sections, with rings and clamps. The column endpieces, which are made of Perspex, are hollowed out at the inner surface to form a sample distributing chamber containing the sample distributing device made of stainless steel cannular tubing. The top and bottom sheets are also made of porous plastic (Vyon No. 1), 1.5-mm thick and supported by small Perspex supports evenly distributed over the bottom of the distributing chamber.

After the developmental work which led to this column design was over (spring 1966), Joustra *et al.* (1967) published a paper in which similar ideas were put forward.

Figure 4 shows an elution diagram obtained when 500 ml of normal human serum is run on a compact sectioned column with four sections and with the bed dimensions of 30 × 30 cm, total volume 21 l., packed with Sephadex G-150.

The Stacked Sectioned Column Type. In an attempt to increase the diameter of the compact sectioned column from 30 to 45 cm, we found it very difficult to obtain the necessary airtight sealing between the column sections and the ring. This was due to irregularities in the large Perspex cylinders then available. In that situation it was natural to use an idea which came up during our discussions which produced the design of the compact sectioned column type. This was the possibility of coupling short columns in series to form a stacked sectioned type of column. There are many advantages with a column of this type over the compact one, even if the latter is less expensive in construction and less bulky. Thus it is possible to check the packing quality and separation capacity of each column section independent of the others, each section can be replaced within minutes if unsatisfactory by any means, and finally it is possible to disconnect and elute separately sections which contain, for example, slow migrating substances, thus making the cycle time for the process considerably shorter. Figure 5 shows a photograph of two stacks of sectioned columns made in our workshop.

The engineers at Pharmacia Fine Chemicals have, independently of us, arrived at a construction very similar to the one shown in Figure 5 (see Figure 15). The differences lie in the design of the endpieces where this company, as in all their smaller columns, prefers a combination of nylon fabric and plastic wire netting instead of porous plastic membranes, and in the sample and eluent distribution pattern where they find six inlets and outlets, arranged as in Figure 10, being an optimal solution.

The new sectioned column type of Pharmacia Fine Chemicals AB, which will appear on the market in spring 1971, will be molded in TPX (a polymerization product of methyl pentene), which is a superior material to Perspex in many respects, *e.g.*, it is autoclavable and is more resistant to solvents.

THE PROBLEM OF SAMPLE APPLICATION ON LARGE DIAMETER COLUMNS

A perfect initial sample zone and a perfectly homogeneous flow through the whole column cross section is of the greatest

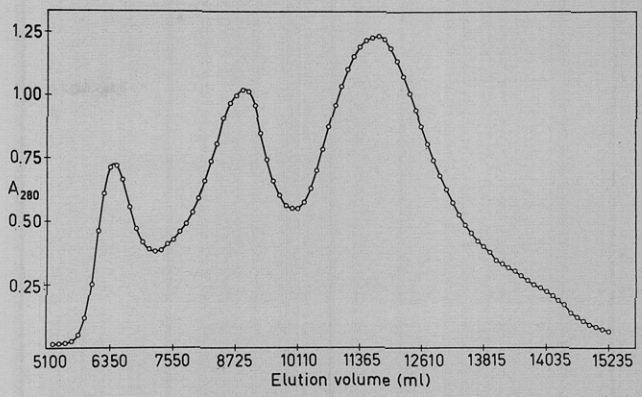

Figure 4. Elution diagram obtained when 500 ml of normal human serum is fractionated on the column described in Figure 2, packed with Sephadex G-150. Buffer: 0.05 *M* Tris-HCl, pH 8.0 + 0.5 *M* NaCl. Flow rate 250 ml/hr^{-1}

importance for the maximum utilization of the separation capacity of a gel bed. This is especially valid for short columns with large diameters. The depth of the sample distributing chamber, *i.e.*, the distance between the end plate and the porous plastic sheet, should be kept at a minimum to diminish dilution of narrow sample zones. Tailing at high sample densities is another serious problem. In our laboratory the following precaution is taken when applicable. The sample is applied through the bottom inlet of the column and is immediately followed by an appropriate volume of a solution of somewhat higher density than the sample, containing a low molecular weight "inert" substance such as sucrose or glycerol. One example of this procedure is presented in Figure 6. Being aware that this is merely a partial solution of the problem, although very effective when applicable, we have tried to find other more general solutions.

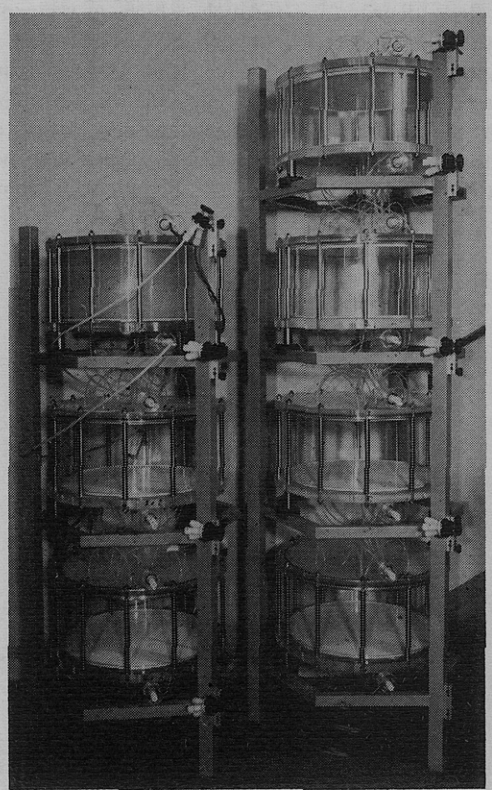

Figure 5. Two stacks of 45-cm diameter and 17.5-cm high column sections designed by the author and collaborators

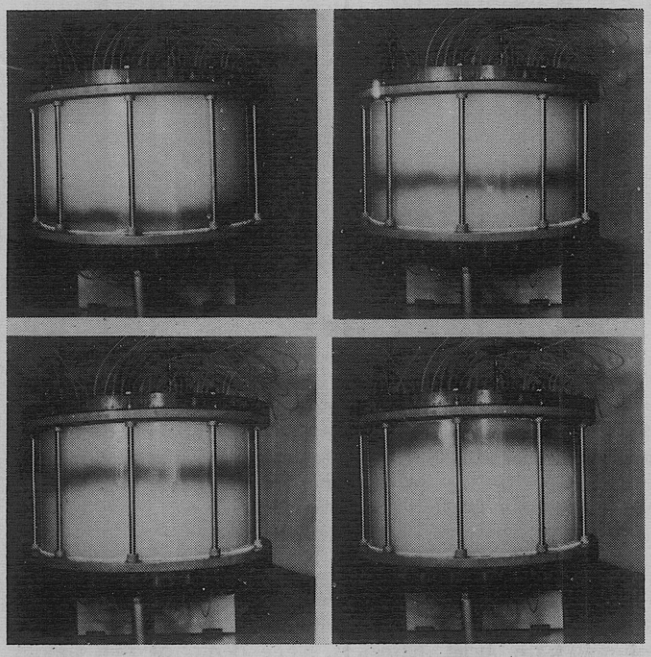

Figure 6. The photographs show the almost perfectly homogeneous flow through a 45-cm diameter and 27.5-cm high experimental column equipped with 61 inlets and outlets. The test zone consists of 1 l. of hemolyzed serum. The column is packed with Sephadex G-25 fine. The linear upward flow rate is 12 cm/hr^{-1} (18 l./hr^{-1}). Buffer: 0.05 *M* Tris-HCl, pH 7.5. In order to avoid considerable tailing due to the high density of the sample, this was immediately followed by 0.5 l. of 15% sucrose solution before the eluting buffer was applied

Multiple inlets in the column end plates, an arrangement which will be discussed below, made it possible to use another technique for breaking stationary areas of high density during the sample application procedure. In connection with Figure 8 the technique will be described in more detail. The principle is that every second inlet is connected to two external flow distributors through which a reciprocating flow may be obtained with the aid of two solenoid valves controlled by a time pulse relay. The sample is applied through all of the inlets, and for an appropriate period of time thereafter the buffer flow is allowed to reciprocate as described above at a somewhat higher flow rate than what is used in the run. The technique has been used with success

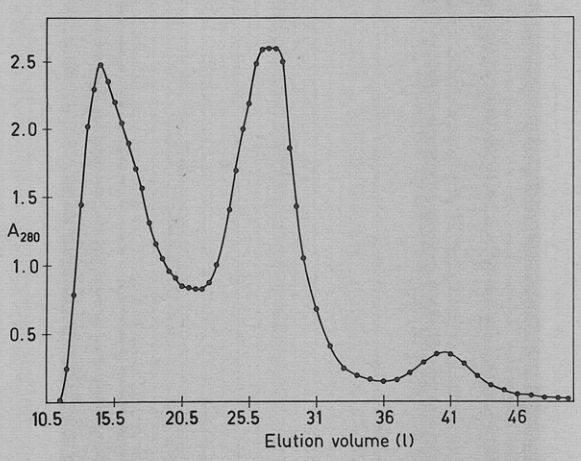

Figure 7. Elution diagram from an experiment where 1.7 l. containing a 6.5% solution of a crude insulin preparation (10% insulin) is fractionated on the column shown in Figure 5, packed with Sephadex G-50 fine. Flow rate: 4.8 l./hr^{-1}. Buffer: 0.01 *M* HCl. The insulin is recovered from the rear fractions of the middle peak

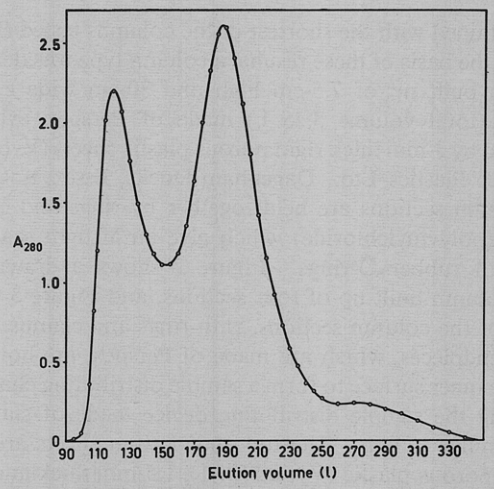

Figure 8. Elution diagram from an experiment with our largest (300-l.) column. Sample: 10 l. of a 5.8% solution of a crude insulin preparation similar to that in Figure 6. The column is packed with Sephadex G-50 coarse. Flow rate: 22.5 l./hr^{-1}. Buffer: 0.01 *M* HCl. The insulin is recovered from the rear fractions of the middle peak

in connection with sample application in our 300-l. column (Figure 8).

A small layer depth of the sample distributing chamber, however, precludes the use of an internal sample distribution system of the type described above, as this will seriously disturb the formation of a homogeneous initial sample zone. For that reason we have chosen the alternative of external sample and eluent distribution for columns with diameters of 45 cm and larger. This means that the in- and out-flow of the column are evenly distributed over the end plates in such a way that all parts of the gel surface are covered with the sample within the shortest possible period of time. We found, as could be expected, that the best results were obtained with the largest number of inlets and outlets tested, in this case 61, using a hexagonal distribution pattern. Figure 5 shows a series of photographs of a test zone passing through such a column with a diameter of 45 cm and the length of 27.5 cm. Figure 7 shows the elution diagram obtained when a crude preparation of insulin is fractionated on the same column, packed with Sephadex G-50. The excellent properties of this column encouraged us to build a still larger one, now with an inner diameter of 87 cm and 51-cm high. The total volume is 300 l. As no Perspex cylinder with this large diameter was available on the market, we made one ourselves by bending Perspex plates in an oven and glueing the segments together to form the column cylinder. Figure 8 shows the elution pattern obtained when a sample of a crude insulin preparation, similar to that shown in Figure 6, is chromatographed on this column packed with Sephadex G-50 coarse. Figure 9 shows a schematic drawing of the 300-l. column and its auxiliary equipment.

Even if the best results were obtained with a large number of inlets and outlets on these two mentioned special columns, we are anxious to stress that as few as six or seven, distributed as shown in Figure 10, have given quite satisfactory results in experiments with 45-cm diameter columns.

LARGE-SCALE FRACTIONATIONS ON THE STACKED SECTIONED COLUMN TYPE

Isolation and Fractionation of Rape Seed Proteins. Rape seed, *Brassica napus* var. oleifera, a major oilseed in many countries, *e.g.*, Canada and Sweden, contains besides

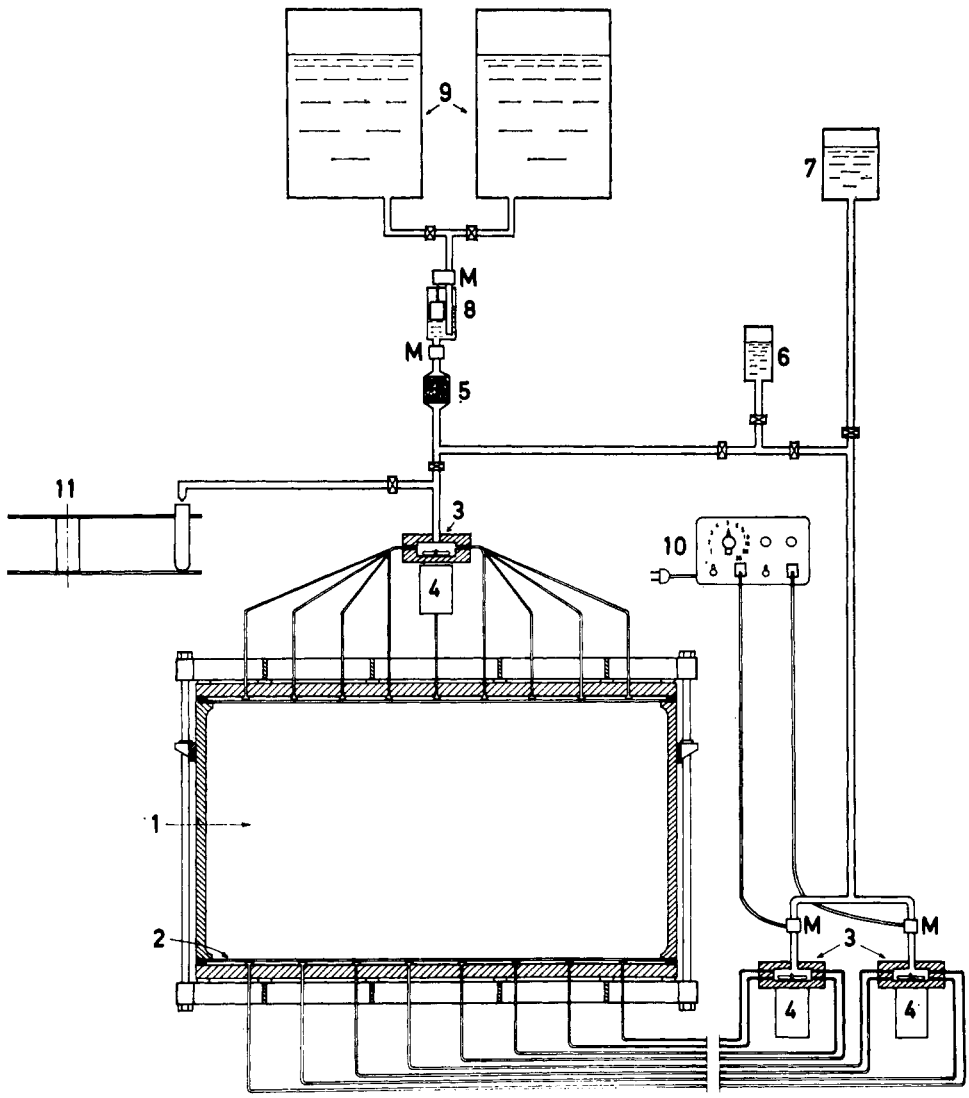

Figure 9. Schematic drawing of the set-up used in connection with large-scale gel filtration experiments on the 300-l. column. (1) Gel bed (87 × 51 cm); (2) Porous plastic sheet (Vyon No. 1, 3 mm); (3) External sample and eluent distributors containing PTFE-coated stirrer magnets; (4) Electric magneto stirrers; (5) Glass wool filter cartridge; (6) Sample container; (7) Buffer container to be used for obtaining maximum hydrostatic pressure during pulse breaking of stationary high-density zones in the sample distribution chamber; (8) Level control; (9) 700-l. vessels made of polythene for the storage of eluent buffers; (10) Time pulse relay, which regulates the two solenoid valves (M) controlling the flow through the two sample and eluent distributors. Immediately after the sample solution has been applied, the time pulse relay is switched on and the flow will alternate between the two distributors which are connected to every second of the 61 inlets. The pulse time can be varied between 1 sec and 25 sec in ten steps. The result is a very efficient breaking of possible stationary zones of high density in the thin layer between the porous plastic sheet and the column end plate; (11) Fraction collector containing 60 10-l. polythene bottles (not of the type shown on the drawing)

40–45% edible oil about 25% protein. The nutritional value of this protein, as judged from its amino acid pattern where an excess of lysine and sulfur-containing amino acids is noticeable, seems to be very favorable (Liedén, 1965). Defatted rape seed meal is, however, an inferior food because of the presence of certain low molecular weight antinutritional substances, such as glucosinolates. In the presence of water, these glucosinolates are enzymatically split to yield isothiocyanates. These compounds, and especially one rearrangement product, L-5-vinyl oxazolidinethione (goitrin), are potent goitrogenic substances. Many attempts have been made to solve the problem of removing the glucosinolates so as to be able to utilize the valuable protein for nutritional purposes. Maybe the best approach hitherto is that of Tape *et al.* (1970). They make use of a simple water-extraction procedure on coarsely crushed seeds. Their de-

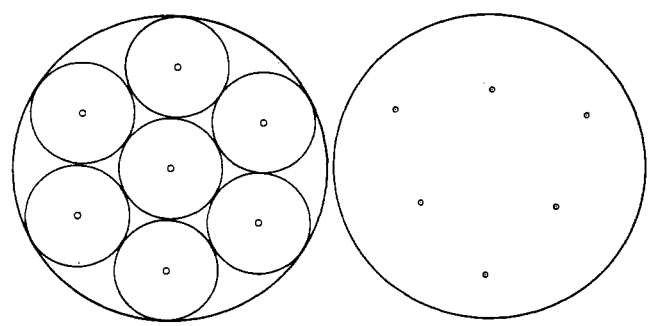

Figure 10. Inlet and outlet distribution patterns used on the stacked column sections. The double triangular pattern is found on the prototypes of Pharmacia Fine Chemicals (six inlets and outlets). The hexagonal pattern (seven inlets and outlets) is used on the columns designed by the author and collaborators

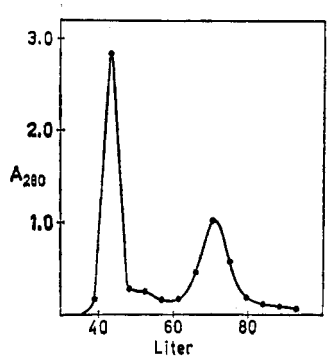

Figure 11. The elution profile obtained when a neutral extract of 250 g of defatted rape seed meal is fractionated on a column battery consisting of six 45-cm diameter and 15-cm high sections, packed with Sephadex G-50 fine. Sample volume: 2.7 l. Flow rate: 9 l./hr^{-1}. Buffer: Sodium phosphate, 0.1 M, pH 7.0. NaN_3, 0.02%, is used as a preservative. Fraction volume: 4.5 l. The column is of the stacked sectional type with an external sample distribution system. The low molecular weight material has been eluted separately between the second and third sections of the column battery

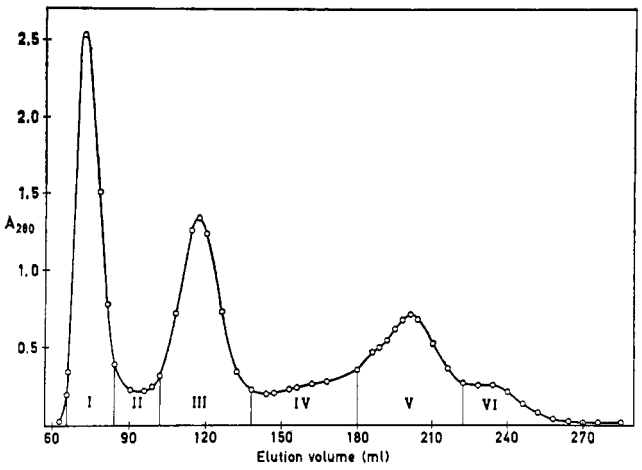

Figure 13. Analytical gel filtration of a neutral extract of defatted rape seed meal. The extract was previously desalted on Sephadex G-25. Column: Two columns 2 × 41 cm coupled in series, total volume 257 ml, packed with Sephadex G-200. Sample volume: 4.5 ml (5% protein). Flow rate: 9 ml/hr^{-1}. Buffer: Tris-HCl, 0.05 M, pH 8.6. The high optical density of the void peak is mainly due to turbidity

fatted product is virtually glucosinolate-free and contains 55–60% protein. However, their procedure involves a loss of about 20% of the total protein during the water-extraction step, and furthermore it does not seem possible to increase the protein content of their final product further.

In cooperation with S-Å. Liedén, a nutritional biochemist at our institute, we are investigating the potentialities of Sephadex chromatography for removing the antinutritional factors from aqueous extracts of defatted rape seed meal. The separation problem as such is one of the most favorable possible applications of the gel filtration technique: the separation of high molecular weight material (proteins) from low molecular weight substances (glucosinolates). It makes it possible to use gels like Sephadex G-25 coarse, suitable for the largest possible industrial scale columns (such as Sephamatic units), and to apply the largest possible charge volumes, requirements which naturally are essential for a process intended to produce proteins for consumption.

In the procedure we have worked out rape seeds are milled twice in a rolling mill, extracted extensively with hexane at room temperature, and then the meal is dried in a vacuum evaporator. In the aqueous extraction of the proteins from the defatted rape seed meal, we have tried to attain conditions which fulfill the following three requirements: maximum yield of protein in solution; the nutritional value of the proteins maintained intact; and no spontaneous precipitation of proteins from solution. The milieu we have found that meets these three demands best is an aqueous solution of ammonia at pH 10.5, containing about 1% NaCl. After filtering and centrifuging in a basket centrifuge and a sep-

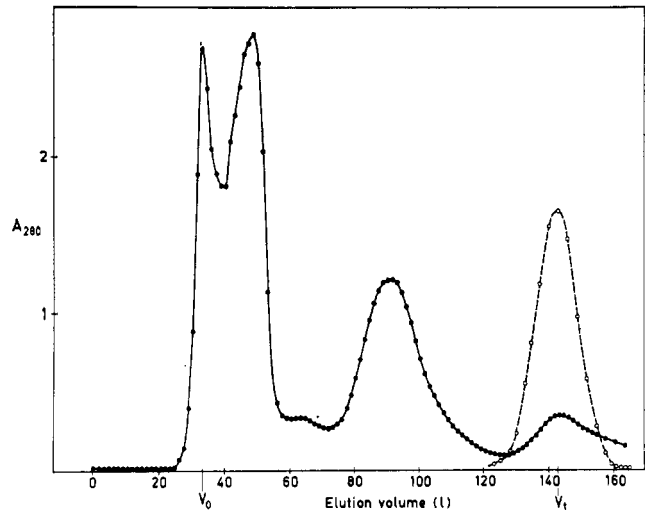

Figure 12. The elution pattern obtained when 150 g of rape seed proteins are fractionated on Sephadex G-200 packed in the same type of column battery as used for the experiment described in Figure 11. Sample volume: 3.8 l. Flow rate: 6 l./hr^{-1}. Buffer: Aqueous ammonia at pH 10.5. The sharp void peak is partly due to turbidity. The broad peak at the elution volume 90 l. contains at least four main components. They are all basic proteins, $I_p > 10$, and with molecular weights around 15–20,000. The total number of proteins in rape seed, as analyzed by isoelectric focusing in polyacrylamide gel, is around 40–50. The broken peak indicates salts still present in the not-fully equilibrated original sample solution

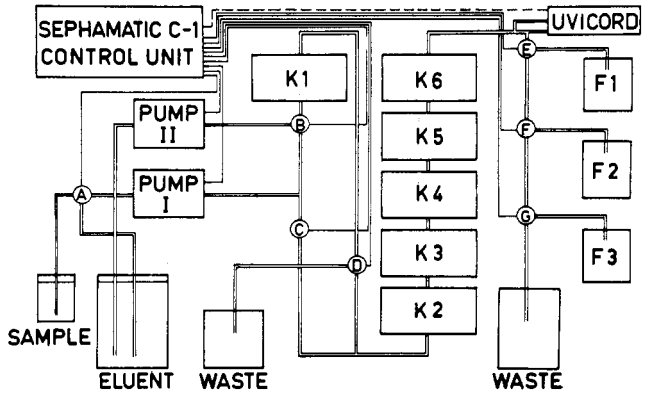

Figure 14. Flow scheme of an automatic process for the detoxification and/or fractionation of rape seed protein extracts. K1–K6 indicate the gel filtration column sections, numbered in the direction of flow. A–G indicate the solenoid valves. F1–F3 indicate the vessels for collecting of fractions. In order to be able to elute column number K1 separately, without disturbing the elution of the proteins through K2–K6, the solenoid valves B, C, and D are coupled in parallel together with pump number II. Their function is regulated by one extra self-repeating timer

arator, respectively, the dark brown extract is subjected to gel filtration on Sephadex G-25 or G-50 equilibrated with an aqueous solution of ammonia at pH 10.5. The fast-moving protein fraction is concentrated by vacuum evaporation and finally lyophilized to yield a light colored powder, easily soluble in water and with an agreeable taste.

On Sephadex G-50, with appropriate loading, it is possible to combine this detoxification step with a partial fractionation of the proteins. This is conceivable because of the fact that about 20% of the proteins in rape seeds are of relatively low molecular weight (15–20,000) as compared with the bulk of the proteins whose average is about 150,000. With the new stacked sectioned column type this can be utilized very efficiently if one elutes the strongly retarded colored substances and other low molecular weight material from the first one or two sections, while the proteins still are being separated on the other columns in the stack. This therefore diminishes the cycle time for the whole procedure. Figure 11 shows the elution profile obtained when fractionating a neutral extract of defatted rape seed meal on a column battery containing six sections, 45 cm in diameter and 15-cm high (prototype of Pharmacia Fine Chemicals), packed with Sephadex G-50 fine, and with a total volume of 135 l. This pattern is almost identical with those obtained on analytical columns under comparable conditions.

As mentioned by way of introduction, the most important advantage of the sectioned column type is the possibility of utilizing the porous Sephadex gels for protein fractionation on a large scale. Figure 12 shows the elution diagram obtained when 150 g of rape seed proteins are fractionated on Sephadex G-200 packed in the same type of column battery as was used in the experiment described in Figure 11. Figure 13 shows for comparison the result obtained when a similar extract is fractionated on an analytical column under somewhat more favorable conditions.

AUTOMATION OF THE CHROMATOGRAPHIC PROCEDURE

Thus it is feasible to produce sufficient quantities of at least three different groups of proteins to be able to perform long-term nutritional and toxicity tests on rats. To accomplish this, several runs still have to be carried out. To save manpower we have worked out an automatic process based on the Sephamatic C-1 Control Unit manufactured by Pharmacia Fine Chemicals AB. The Sephamatic C-1 contains four sequentially connected timers which regulate the functioning of five solenoid valves and two pumps. The chromatographic operations which may be controlled are sample application, elution, and the collecting of fractions. Three fractions and one waste may be collected either by time regulation or on a detector (*e.g.*, uv-monitor or refractometer) basis. The chromatographic cycle may be repeated automatically. The manufacturer is willing to discuss modifications to meet with the special requirements of the customers. We have ourselves made modifications which make it possible to elute automatically the slow migrating material out of the first column in the series without disturbing the elution of the protein through the others. Figure 14 shows the flow scheme for this process. By merely adjusting the timers, the scheme is applicable for fractionations on Sephadex G-50 as well. Figure 15 shows a photograph of the set-up used for automatic gel filtration with Sephamatic C-1 on the stacked sectioned column type.

PURIFICATION OF INSULIN

Insulin is an example of a substance which has been prepared on an industrial scale for more than 30 years by means of classical biochemical separation methods such as extractions, precipitations, and crystallizations. In cooperation with Vitrum AB, Stockholm, Sweden, we have been investigating the possibilities of introducing modern column chromatographic separation methods in the industrial production of insulin. Vitrum suggested that one of their intermediate preparations would be a suitable starting material for our investigation.

At the beginning of this project we were convinced that a combination of at least two entirely different separation methods was necessary to solve this superficially complicated purification problem. And it was not long before we arrived at a procedure which, on a laboratory scale, yielded a virtually pure insulin as analyzed by polyacrylamide gel electrophoresis

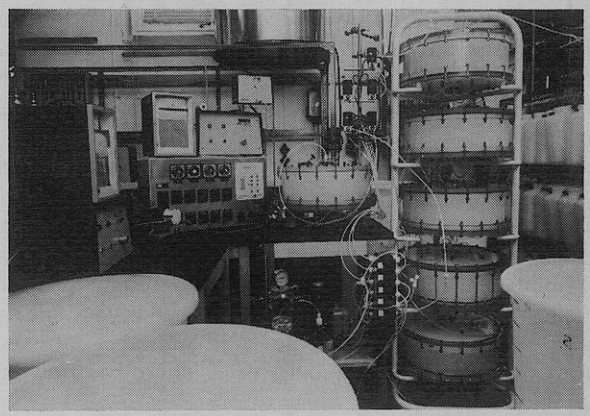

Figure 15. Equipment for automatic gel filtration on a technical scale, arranged according to the flow scheme shown in Figure 14. The set-up shown is adapted to the simultaneous detoxification and protein fractionation of rape seed meal extracts, but otherwise programed it has been used for the fractionation and purification of insulin. The column battery is a prototype manufactured by Pharmacia Fine Chemicals AB, Uppsala, Sweden

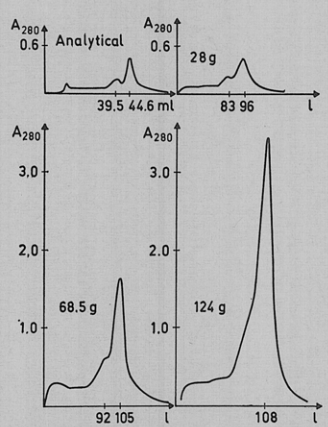

Figure 16. Column elution patterns obtained when very similar preparations of insulin (for the analytical and 28-g runs, identical) are fractionated on Sephadex G-50 fine in 0.01 *M* HCl under the following conditions: *Analytical*: 1-ml 2.8% sample on a 1-cm × 97-cm column (total volume 76 ml). Flow rate: 8.5 ml/hr^{-1}. Fraction volume: 1.04 ml. *28-g*: 1000-ml 2.8% sample on a battery of six 45 × 15-cm columns coupled in a series (Figure 15). Total volume: 135 l. Flow rate: 5 l./hr^{-1}. Fraction volume: 1.25 l. *68.5-g*: 1700-ml 4% sample. Fraction volume: 2.5 l. Other conditions as for the 28-g run. *124-g*: 2060-ml 6% sample. Flow rate: 8.5 l./hr^{-1}. Fraction volume: 2.4 l. Other conditions as for the 28-g run. The insulin is recovered from the main peak in an electrophoretically pure form, as analyzed by polyacrylamide gel electrophoresis and in a good yield

(Hjertén, 1963). This procedure was based upon a combination of gel filtration of Sephadex G-50 and ion exchange chromatography on a cation exchanger. However, we did not succeed in using this procedure on a preparative scale. The reason is that even if the gel filtration step could be scaled up easily, this was not the case with the ion exchange chromatography step. The cause of the difficulties which arose during the attempts to scale up the ion exchange chromatography step is the difference between adsorption capacity and solubility. Under conditions where insulin is readily soluble, the capacity of the ion exchanger is low and *vice versa*. We considered the use of urea and other substances in order to increase the solubility of insulin to be of no technical interest.

However, all these problems disappeared when the new high-resolving stacked sectioned column type was available for application of the insulin preparations of Vitrum AB. One passage of a solution of this insulin preparation in 0.01 *M* HCl through six 45-cm wide and 15-cm high column sections, prototypes, designed and manufactured by Pharmacia Fine Chemicals, packed with Sephadex G-50 fine, equilibrated in the same solvent, gave a good yield of an insulin which was pure as analyzed in polyacrylamide gel electrophoresis. This process is now automated and in use in the industrial production of insulin at Vitrum AB, Sweden. Figure 16 shows elution patterns obtained when fractionating insulin preparations under different conditions on Sephadex G-50 fine. Figure 15 shows a photograph of a set-up which has been used for the automatic fractionation of insulin.

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