A COMPARATIVE STUDY
OF
THREE TOXIC LEGUME GLYCOPROTEINS

by

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CHAPTER 1

INTRODUCTION

The toxicity of certain natural compounds is an environmental hazard which has confronted mankind throughout his developmental history, through to the present day, and it is therefore not surprising that a fear of toxic plants and animals has become ingrained on the human psyche. Manifestations of the resulting interest in toxic organisms are to be found, historically, in the prominent position which various such organisms have occupied in the mythologies of many diverse cultures and, currently, in the world-wide research effort aimed at a study of toxic compounds.

In addition to the well-known and recognised toxic organisms, however, a new hazard is created by the demands placed upon the world's food resources by an ever increasing population. These demands have necessitated an investigation into new potential food sources, an effort having the concomitant danger of exposure to new toxic compounds. While in certain instances the effects of toxins encountered in this manner have proved to be dramatic (e.g. tetorodotoxin) in other cases the effect may not be immediately manifest as this may depend both upon the susceptibility of the test organism and on the level of toxin to which this organism is exposed, as much as upon the "intrinsic toxicity" of the compound.

Therefore, although at first sight there would appear to be no doubt as to what constitutes a toxin, the question of the level of exposure underscores a fundamental difficulty of definition. For example the recognised toxicity of polar bear liver (1) and also, it has been speculated, that of the South African red steenbras Petrus rupestris, is due simply to an unusually high level of vitamin A, this being normally an essential human nutrient. Conversely, foodstuffs commonly accepted as being innocuous may contain potent toxins, but in very small amounts. Examples are the toxic hallucinogen, myristicin, present in celery, parsley and nutmeg and the acetylenic alcohol "carototoxin" present in carrots and celery (2). Singleton and Kratzer (3), in their discussion of plant phenolic toxins, have consequently defined three arbitrary groups of toxic compound on the basis of the LD_{50} level. By their definition a compound is classed as slightly toxic if the LD_{50} lies within the range 5000 to 500 mg/kg, moderately toxic in the range 500 mg/kg to 50 mg/kg and highly toxic below 50 mg/kg.

The duration of exposure is also an important parameter in evaluating the toxicity of a compound. For example gossypol, a toxin from cottonseed, is only slightly toxic if administered in a single acute oral dose (LD_{50} 2400 - 3340 mg/kg). When administered in small amounts over a long period, however, the LD_{50} of a subsequent acute single dose drops to a much lower value (10 - 200 mg/kg). As a consequence animals fed toxic amounts of gossypol may appear normal for a few weeks to a year before suddenly succumbing (4). Effects such as this are of particular interest with regard to new foods for, traditionally, unless a toxic symptom is manifest within 24 hours of ingesting a food for the first time the food and the effect are unlikely to be considered as related.

Two more subtle dangers posed by certain toxins are of topical interest at the time of writing. The danger that apparently innocuous compounds could prove to be carcinogenic when administered for long periods has attracted a great deal of attention. However, while this danger is potentially very real, it is difficult to establish cause and effect relationships in such cases and this fact, doubtlessly coupled with the psychological considerations mentioned earlier, has led to the tendency to err on the safe side, as manifest by the banning of cyclamates on what may be considered meagre evidence. This approach has been partly influenced by disasters with the sedative drug, thalidomide, which have brought to light the danger that compounds which might be relatively innocuous to fully developed animals can cause irreparable damage to developing foetuses. The possible teratogenic activity of toxic compounds therefore deserves especial attention.

In the continuing world-wide search for more food, many authors have suggested greater exploitation of legume seeds, as these have a high protein content, comparable to that of animal-derived foods. However, legume seeds have long been recognised as containing factors having potent anti-physiological effects and which could be responsible for the relatively poor performance of raw legumes in maintaining the growth of young test animals. These factors include protease inhibitors (5, 6), haemagglutinins (7, 8, 9) and compounds which are toxic when injected intraperitoneally into rats and mice (10). Liener (11, 12) and Jaffé (13) have presented reviews of the literature concerning the protease inhibitors and haemagglutinins of plant foodstuffs but the factors with intraperitoneal toxicity, as such, have not received as much attention.

The intraperitoneal toxicity of crude legume extracts was first observed by Liener, who adopted the technique of intraperitoneal injection, as a means of establishing the effects of the soybean trypsin inhibitor, "without introducing the complicating factor of intestinal proteolysis" (10). By this means Liener was able to demonstrate that while a purified trypsin inhibitor did not elicit a toxic response when injected, a crude preparation of trypsin inhibitor was toxic.

This observation suggested the presence, in soybeans, of a toxic factor distinct from the trypsin inhibitor. This was later confirmed by Liener (14) when he observed that increasing purification of the toxic factor was attended by a corresponding decrease in trypsin inhibiting activity. Increasing toxic potency was, however, paralleled by increasing haemagglutinating activity. A similar close relationship between toxicity and haemagglutinating activity has also been reported by Jaffé and Gaede (15) who prepared two different haemagglutinins, both of which were intraperitoneally toxic, from the black bean (a cultivar of Phaseolus vulgaris).

As a result of these studies, haemagglutinating activity and toxicity came to be generally regarded as synonymous. However, evidence of the existence of additional, distinct, intraperitoneal toxins with little or no haemagglutinating activity has been presented by Stead, de Muelenaere and Quicke (16) for the Natal round yellow bean (another cultivar of *Phaseolus vulgaris*) and by Dennison, Stead and Quicke (17) for the jack bean (*Canavalia ensiformis*).

It is these intraperitoneal toxic factors with low haemagglutinating activity which constitute the subject of the study reported in this thesis. These specific factors were considered an appropriate subject for investigation as very little is known about this group of compounds. Furthermore, the likelihood that legumes will in future contribute an increasing proportion of the protein fraction of the human diet and of animal rations makes a study of the toxins in these legumes timeous.

The investigation reported in this thesis is a continuation of two earlier studies undertaken in this laboratory by the author (18) and by Stead (19), which were aimed respectively at the isolation of the toxic factors from the jackbean (*Canavalia ensiformis*) and the Natal round yellow bean (*Phaseolus vulgaris*).

The first part of the thesis concerns the preparation, by essentially established procedures, of toxic isolates from the jack bean, the soybean and the Natal round yellow bean (NRY-bean). In the preparation of these isolates attention was given to the comparative behaviour of the extracts from the three legumes.

The second section deals with an attempt to find a more efficient procedure for the isolation of the toxic factor from the jackbean which, if successful, might have had more general application. In particular, attention was focussed on the use of tanned gelatin as a medium for molecular exclusion chromatography. While the attempt failed, useful information was obtained concerning the properties of this chromatographic medium and these findings are included here.

The final section of this thesis deals with a comparative study of the toxic isolates from the NRY-bean, the jack bean and the soybean. This investigation was undertaken to ascertain whether or not the three preparations shared any common features, in the hope that this might give some insight into the origin of the toxic effect. It was found that while the toxins of the jack bean and the soybean have certain properties in common and may be placed in a single class, the toxin from the NRY-bean is markedly different.

CHAPTER 2

MATERIALS AND METHODS

2.1 Beans

A single batch of seeds of each species was used in these investigations. The jack beans (Canavalia ensiformis) were imported from Rhodesia while the Natal round yellow beans (Phaseolus vulgaris) and the soybeans (Glycine max - Geduld strain) were locally grown and were obtained from a local distributor. The seeds were stored in a cool dry environment but were not refrigerated.

The air-dried beans were milled in a Christy and Norris junior 5-inch laboratory mill (2.0 mm sieve). The meal was collected in a mutton-cloth bag which allowed a continual flow of air through the mill, thus avoiding blockage of the sieve and consequent overheating. Care was taken to avoid inhalation of the dust from jack beans as this gives rise to asthma-like symptoms.

2.2 Reagents and buffers

Unless otherwise stated all reagents were of analytical reagent grade.

All buffers were made up by dissolving a weight of the appropriate acid or base, sufficient to give the desired final molarity, in an amount of distilled water somewhat less than the intended final volume, and titrating to the desired pH with an appropriate base or acid, before diluting to the final volume. Ingredients in addition to the buffer salts were added before titration. In the case of buffers containing 8 M-urea, heat was applied to promote solvation of the urea but the temperature was maintained below 30°C to minimise the formation of cyanate.

Buffers used for molecular exclusion chromatography in the absence of 8 M-urea contained sodium azide (0.02% w/v) and buffers for ion exchange chromatography contained caprylic acid (20) to inhibit the growth of micro-organisms on the biodegradable chromatography media.

2.3 Intraperitoneal toxicity

Intraperitoneal toxicity was assayed by suspending the material under test in physiological saline and administering to young adult mice (20 - 22 g) by a single intraperitoneal injection of 1 ml of solution. The number of deaths occurring within 72 hours of injection were recorded. In those cases in which sufficient sample was available the LD_{50} was determined by the method of Litchfield and Wilcoxon (21).

2.4 Haemagglutinating activity

The assay used for the assessment of haemagglutinating activity has been the subject of two previous studies in this laboratory (19, 22). The procedure used by both of these authors was based upon that of Liener (23) which exploited the differential rate of settling of agglutinated and non-agglutinated erythrocytes. Liener estimated haemagglutinating activity by photometrically measuring the erythrocytes remaining in solution after a stated time interval. This principle was retained in the assay method finally adopted by Barlow (22), but Barlow introduced a new method of calculating the results. Barlow's method of assay and calculation were used in this study.

Barlow (22) and Stead (19) have brought to light many sources of variation in the assay technique viz. source of blood, trypsin preparation used to effect sensitisation of erythrocytes, age of sensitised erythrocytes, etc., so that meaningful comparisons can only be made between samples assayed concurrently using a common sensitised erythrocyte preparation.

2.4.1 Procedure

As it has been found in this laboratory that small changes can have a marked effect upon the assay results, the procedure used is described in detail.

For the purposes of this and related studies, a colony of pure-bred Californian rabbits was established. Blood was drawn from 2 to 3 of these rabbits (preferably litter mates) and collected into separate clean glass vessels containing anticoagulant solution (anhydrous sodium citrate, 2.0 g; 37% aqueous formaldehyde, 13.3 ml; 0.9% sodium chloride solution, 200 ml) at the level of 1 ml per 7.5 ml of blood. An equal volume of Alsever's solution (glucose, 20.5 g; sodium citrate, 8.0 g; sodium chloride, 4.2 g; citric acid, 0.55 g; distilled water to 1 litre) containing "Phenergan" preservative (Maybaker Pty. Ltd., Port Elizabeth) (300 mg/litre) was added and the mixture was centrifuged (225 x g, 20 min, 2°C). The supernatant was removed by suction and the cells resuspended in an equivalent volume of Alsever's solution and again centrifuged. This procedure was repeated and the cells were finally resuspended in an equal volume of Alsever's solution. At this stage the erythrocyte preparation derived from different rabbits could be pooled to give the stock erythrocyte suspension. The percentage of erythrocytes in the stock suspension was determined by centrifuging (225 x g, 20 min, 2°C) a measured sample (30 ml) in a calibrated centrifuge tube. The stock solution was stored at 4°C until required. Barlow (55) has recommended that the suspension be used as soon as possible, preferably within one day.

For each set of assays a standard trypsinated erythrocyte suspension (STES) was prepared. A volume of stock erythrocyte suspension, sufficient to give 150 ml of a 4% (v/v) suspension was centrifuged (225 x g, 20 min, 4°C), the supernatant removed and the packed cells resuspended in buffered saline pH 7.0 (NaCl, 8.0 g; K_2HPO_4, 1.21 g; KH_2PO_4, 0.34 g; distilled water to 1 litre) to 150 ml. A suspension (10% w/v) of crude trypsin (British Drug Houses, Poole, England) in 0.05 N - HCl (1.5 ml) was added and the mixture incubated at 37°C for 1 hour. Following incubation the cells were washed free of trypsin by alternate centrifugation and washing, with three 100 ml aliquots of physiological saline. The washed erythrocytes were suspended in physiological saline (approximately 100 ml) and the concentration was adjusted by the addition of further saline until a 1: 1 dilution of the suspension with physiological saline gave a reading of 0.5 ± 0.02 on the Eel colorimeter, using the wavelength of approximately 620 mμ selected by the red Ilford No. 608 filter. The resulting suspension served as the standard trypsinated erythrocyte suspension (STES).

Samples for assay were made up at a level of 2.5 mg/50 ml of saline. This solution (1 ml) was added to the first of nine test tubes (i.d. 10 mm), each containing physiological saline (1 ml). A series of dilutions ranging from 1/2 to 1/256 was prepared by transferring solution (1 ml) from the first tube into the second, mixing and transferring 1 ml into the next tube etc. STES (1 ml) was added to each tube in the series and the contents were well mixed. Three further tubes were prepared, a blank containing 2 ml saline and two tubes (E_{50} tubes) containing 1.5 ml saline and 0.5 ml STES. The E_{50} tubes were agitated and read in the colorimeter at zero time while the absorbances of the remaining tubes were read after 1 hour, as recommended by Barlow (22). During the interim period the tubes were maintained in a vertical position in a drilled wooden block holder.

Absorbance readings were obtained using an Eel colorimeter fitted with an Ilford No. 608 filter and a polythene tube holder having a 1 cm square window, 1 cm from the base to ensure that readings were taken from corresponding positions in each tube.

Each assay was carried out in duplicate.

2.4.2 Calculation of haemagglutinating activity

For the purposes of this study one haemagglutinating unit is defined as that weight of sample which causes 50% of the STES to sediment in one hour under the described conditions. The reciprocal of the weight (expressed in mg) containing one haemagglutinating unit therefore gives the HU/mg. Activity can conveniently be calculated by Barlow's equation (22).

$$x = A + \left(\frac{E_A - E_{50}}{E_A - E_B}\right)A \,, \qquad \ldots\ldots\ldots \qquad [2.1]$$

where x = wt of haemagglutinin required to sediment 50% of the STES;

A = wt of haemagglutinin in tube A (nearest tube having extinction greater than E_{50});

E_A = extinction of tube A;

E_B = extinction of tube B (nearest tube having extinction less than E_{50}) .

The reciprocal of x values gives HU/unit weight.

2.5 Assay for nitrogen

Samples were assayed for nitrogen by Kjeldahl digestion followed by Nesslerisation. Successful application of this method is dependent upon several factors and it was found that the method given below had to be followed closely if satisfactory results were to be obtained. The major considerations involved getting the amount of nitrogen in the assay aliquot within the correct range while maintaining the pH within the range (pH 11.5 to pH 12.3) in which the ammonia-mercuri iodide complex is soluble. Furthermore, the coloured complex is not formed instantaneously and eventually flocculates out of solution (24) so that the time after which the colour intensity is read must be accurately controlled.

A freeze-dried sample (± 10 mg) was weighed onto cigarette paper, and transferred to a micro-Kjeldahl digestion flask. Concentrated sulphuric acid (1 ml), one Kjeldahl tablet (Na_2SO_4, 1 g; Hg, equivalent of 0.1 g) and two glass beads were added and the mixture was digested for 3 hours. After cooling the digests were transferred quantitatively to 50 ml volumetric flasks and made up to volume with distilled water. An aliquot of this solution (3 ml) was transferred to a 100 ml volumetric flask, diluted to approximately 70 ml with distilled water and 1N NaOH (2 ml) was added. Nessler's reagent (5 ml) was added from a burette with constant stirring, the solution was immediately made up to volume with distilled water and thoroughly mixed. After exactly 30 min the absorbance at 470 nm was read in a Bausch and Lomb Spectronic 20 spectrocolorimeter.

A standard curve relating absorbance to nitrogen content was constructed for the range 0 to 200 µg N using a standard $(NH_4)_2SO_4$ solution (0.4717 g/L = 100 µg N/ml).

2.6 Assay for neutral sugars

The method of Dubois, Gilles, Hamilton, Rebers and Smith (25) was used to determine neutral sugar content. In carrying out this method all glassware was washed with conc. sulphuric acid, rinsed with distilled water and allowed to drip dry and all volumes were measured by means of burettes.

A sample (± 5 mg) of freeze-dried material was dissolved in distilled water and made up to 50 ml. Three aliquots (1 ml) of diluted sample containing between 0 and 200 µg of neutral carbohydrate were transferred to acid-washed test tubes and 5% (w/v) redistilled phenol (1 ml) was carefully added. Concentrated H_2SO_4 (5 ml) was added dropwise into the centre of each tube and the contents were well mixed. After cooling, the tubes were closed with polythene stoppers, inverted to ensure adequate mixing, and 30 mins after addition of the acid the absorbance at 485 nm was read in a Zeiss PMQ II spectrophotometer. A standard curve relating absorbance to sugar content was constructed for the range 0 - 200 µg of sugar using a standard mannose solution.

2.7 Assay for total phosphorus

Samples were assayed for total phosphorus by the method of Allen (26).

2.7.1 Reagents

Ammonium molybdate solution: ammonium molybdate, A.R. (8.3 g) dissolved in distilled water and made up to 100 ml.

Amidol reagent: amidol (2,4 diaminophenol hydrochloride) (2 g) and sodium bisulphite (40 g) dissolved in distilled water and made up to 200 ml. This reagent was kept in a black bottle.

Standard phosphate solution (1 mg P./ml). Oven dried KH_2PO_4 (1.0967 g) made up to 250 ml with distilled water. Suitable standard dilutions were made up from this stock solution.

Perchloric acid: 60% (v/v) solution.

Hydrogen peroxide: 30% (v/v) solution.

2.7.2 Procedure

Freeze-dried sample (5 mg), perchloric acid (2.2 ml) and two glass beads were heated together in a micro-Kjeldahl digestion flask until the solution had become colourless (± 2 h). If necessary, a few drops of hydrogen peroxide solution were added to complete combustion. After cooling, the contents of the digestion flask were transferred quantitatively to a 10 ml volumetric flask and made up to 10-ml with perchloric acid. A sample of this solution (2 ml) was transferred to a 50-ml volumetric flask, amidol solution (4 ml) and molybdate solution (2 ml) were added and the solution was made up to volume with distilled water. After 5 to 30 min the absorbance of the solution was read at 700 nm in a Bausch and Lomb Spectronic 20 spectrocolorimeter. A standard curve relating absorbance to phosphorus content was constructed for the range 0 to 0.1 mg phosphorus using the standard phosphate solution.

2.8 Amino acid analysis

Preparation of hydrolysates. Freeze-dried sample (1.5 mg) was accurately weighed into each of three acid cleaned pyrex glass test tubes (10 x 150 mm), the necks of which had been suitably constricted. "Analar" grade 6N - HCl (3 ml) was added, the contents of each tube were frozen in acetone/dry ice and strongly evacuated using a high vacuum pump system. The tubes were sealed under vacuum and the contents subjected to hydrolysis in an oven at 110°C for 24, 48 and 72 h respectively. The tubes were removed from the oven, cooled, carefully opened and an appropriate amount of mixed internal standard solution (usually 50 µl) (see below) was added and mixed thoroughly with the hydrolysate and washings from the tube cap. The hydrolysate was cleared by centrifugation and reduced to the point of dryness by evaporation under reduced pressure (water venturi pump) in a Buchi rotavaporator (water bath, 45°C; condenser, circulating tap water). Distilled water (± 5 ml) was added and the solution was again reduced to the point of dryness, before final suspension in pH 2.2 diluting buffer (2 ml) (20). Hydrolysates prepared in this way were stored at -15°C until analysed.

Internal standards. The use of an internal standard, which is usually an uncommon amino acid, is essential for the estimation of errors due to (i) mechanical losses in the steps following acid hydrolysis; (ii) manipulative errors in sample application; and (iii) possible changes in colour development. Allowance for these errors is required if the absolute percentage of each amino acid, and also the total amino acid contribution to the sample nitrogen, is to be calculated. Furthermore, in any given analysis, the acidic and neutral amino acids and the basic amino acids are analysed by two independent runs and hence, unless an internal standard is included in each run, there is a possibility of error bias between the two sets of results. In this study, norleucine (27) and 2,4 diaminobutyric acid (28) were used as standards for the long and the short column systems respectively. The two standards were made up in the form of a mixed solution containing oven-dried norleucine (26.2 mg) and 2,4 diaminobutyric acid dihydrochloride (38.0 mg), dissolved and made up to 100 ml with pH 2.2 diluting buffer, the resulting solution containing 2 μ mole/ml of each standard. Mixed standard solution was added to the samples as described above, the volume added being selected so that the peaks due to the standards were of the same order as the peaks due to the sample amino acids. The internal standard solution was distributed into 2 ml portions which were stored at -15°C until required.

<u>Analysis procedure</u>. Hydrolysed samples (1 ml) were analysed for amino acid content using Spackman's accelerated system (29, 30) on a Beckman 120B amino acid analyser. A summary of the analysis conditions is presented in Table 1.

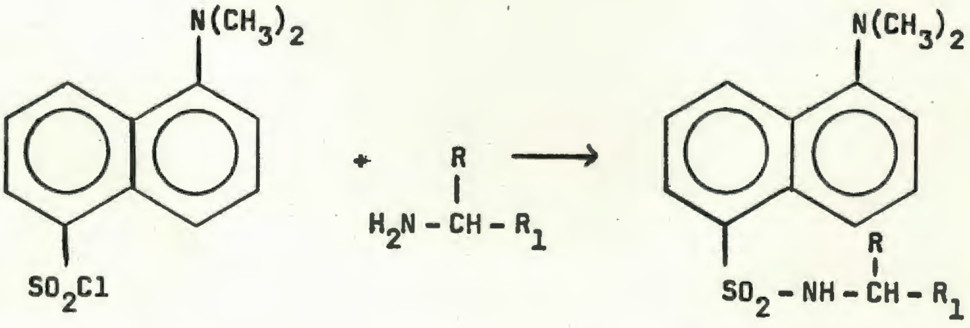
TABLE 1 .../

TABLE 1 Operating conditions of Beckman 120B amino acid analyser

Condition	Acidics/neutrals	Basics
Resin type	PA 28	PA 35
Packed resin dimensions	0.9 × 50 cm	0.9 × 11.5 cm
Buffer flow rate	66.7 ml/hr	66.7 ml/hr
Ninhydrin flow rate	33.3 ml/hr	33.3 ml/hr
Buffer back pressure	300 psi	100 psi
Sample	0.75 mg } in 1 ml	0.75 mg } in 1 ml
Internal standard	25 µl } in 1 ml	25 µl } in 1 ml
Buffers and times (times at inlet)	pH 3.23 (0.2N) Na-citrate 0 – 85 min pH 4.25 (0.2N) Na-citrate	pH 5.28 (0.35N) 0 – 120 min
Temp.	85 – 200 min/55°C	55°C

2.9 Determination of N-terminal groups

The reagent currently favoured for N-terminal group determinations is 1-dimethylamino-naphthalene-5-sulphonyl chloride (DANS-Cl) (31), which reacts with amino terminal groups as follows:



DANS-Cl

Dansyl derivative

The so-called dansyl derivatives are stable to acid hydrolysis so that after reaction of the N-terminal and ε-amino groups, the peptide can be acid hydrolysed and the derivatives identified by thin layer chromatography or electrophoresis

An advantage of the DANS-Cl reagent is that the dansyl derivatives exhibit an intense yellow fluorescence under UV-irradiation so that very small amounts (0.1 - 0.001 mμM) can be detected. This is an important consideration as both the DANS-Cl reagent and the previously used 2,4 dinitrofluorobenzene (32) are only appreciably soluble in organic solvents whereas the protein reactant on the other hand is only appreciably soluble in aqueous solutions. A low yield of coloured derivatives is therefore to be expected so that a high colour value is an advantage.

2.9.1 Procedure

The method described by Gray (31) was used. About 1 mg of protein was dissolved in A.R. 8 M-urea (0.5 ml) containing 0.5 M - $NaHCO_3$. Dansyl chloride solution (0.5 ml), containing 20 mg dansyl chloride per ml of acetone, was carefully added with constant mixing and the mixture was left to react overnight at room temperature. Excess reagent, urea etc. was removed by dialysis against running distilled water, the dialysed solution freeze-dried and subsequently hydrolysed for 18 h at 110°C with 6N - HCl (0.5 ml). The hydrolysate was dried in vacuo over NaOH pellets and resuspended in glacial acetic acid (50 μl).

Identification of the N-terminal derivatives was attempted by chromatography on silica gel thin-layer plates (E. Merck AG, Darmstadt, Germany), following the methods of Deyl and Rosmus (33). Standard dansyl derivatives were obtained from the Seikagaku Kogyo Co. Ltd., 2 - 9 Nihombashi-honcho, chuo-ku, Tokyo, Japan.

2.10 Column chromatography

2.10.1 Molecular exclusion chromatography

Since its introduction by Porath and Flodin (34) in 1959, molecular exclusion chromatography has become one of the most popular methods used for the separation of macromolecules. A major advantage of the method is that the gels used are chemically relatively inert so that interaction with the solute is usually minimal. With regard to protein characterisation, this has the advantage that changes in experimental conditions have little, if any, effect upon the interaction of the sample with the gel matrix and, consequently, can be interpreted in terms of the effect on the sample alone.

The first choice for characterisation of an unknown system, or for use with dissociating media, is Sephadex G-100 as this combines good flow properties with a separating range (4,000 to 150,000 daltons) encompassing a majority of proteins and protein sub-units. For the separation of molecules too large to be accomodated by Sephadex G-100, the author prefers the use of macroreticular agarose gels as these have a greater mechanical strength than microreticular gels (e.g. Sephadex) of an equivalent pore size.

Preparation of gels. Dry Sephadex resin (Pharmacia Fine Chemicals, Uppsala, Sweden), as supplied, was allowed to sink into buffer solution and was set aside to swell for at least 3 days. Fine particles were removed by sedimenting the resin through a column of buffer in a measuring cylinder and decanting the supernatant. The sedimented material was collected as a smoothly-flowing slurry which was evacuated to remove trapped air and packed under gravity into the chromatography column, essentially as described by Determann (35).

Sepharose resins (Pharmacia Fine Chemicals, Uppsala, Sweden) are supplied as a wet slurry which was diluted with buffer and packed into the column as above.

Estimation of molecular weights and diffusion coefficients from molecular exclusion data. Molecular weights were estimated by means of a standard curve relating K_{ar} to log MW, as suggested by Fischer (36), using data obtained by chromatography of a number of standard proteins (Fig. 35 and Appendix 1).

Similarly diffusion coefficients (D) were estimated by means of a standard curve relating $1/D$ to K_{av}, as suggested by Andrews (37), using both data obtained by chromatography of standard proteins and the values of D for these proteins as tabulated by Andrews.

2.10.2 Chromatography on tanned gelatin

As there is little published information concerning tanned gelatin, some of the considerations involved in the use of this material in chromatography will be considered here in some detail.

Selection of gelatin. The major consideration in the selection of the gelatin is to obtain a product giving the greatest gel strength. In their original publication, Polson and Katz (38) suggested the use of a Difco gelatin which required fractionation by polyethylene glycol precipitation to obtain a high molecular weight fraction giving a rigid gel. A limitation of this procedure is that it is difficult to ensure complete removal of the polyethylene glycol after the precipitation step. Obviously, if the polyethylene glycol were not reproducibly removed, it would not be possible to subsequently reproduce a gel made up to a given nominal concentration.

This problem can be by-passed if a gelatin of defined strength is used as the starting material, as in this case fractionation would not be necessary. In this study, therefore, a high strength acid-process pigskin gelatin marketed by Messrs Canada Packers Ltd., 60 Paton Rd., Toronto 4, Canada, was used.

Formation of particles. Primary considerations concerning the particles of a chromatographic medium are the surface area to volume ratio, the packing density, the operating back-pressure and the cost. The greatest possible surface area to volume ratio for optimal sample equilibration must be balanced against a practicable operating back-pressure. Furthermore, in the case of molecular exclusion chromatography, in which separation is limited to elution volumes between V_0 and V_{max}, it follows that columns with the greatest packing density (i.e. the lowest value of V_0) will effect the best separation.

Particles formed by fragmentation have a high surface area to volume ratio and are easily prepared. However, for a given particle size the back pressure and the void volume are higher than for spheres of the same size. Spheres, on the other hand have a minimal surface area to volume ratio and therefore spheres of a smaller size are required for the same separating efficiency. In the case of both types of particle, however, the particle size distribution has the greatest influence on the void volume and the back pressure, the latter being at a minimum when the particle size distribution is at a minimum. In the case of spheres, at least, the void volume is within limits not dependent upon the actual particle size.

In the present study, the ether spray method of Polson and Katz (38) and the organic solvent method of Hjerten (39), for the formation of spherical particles were investigated. Both of these methods were found to present difficulties in standardisation of the conditions, particularly between different gel concentrations. For this reason the more simple, straight-forward and reproducible method of fragmentation in a Waring blender was finally used for the formation of granular particles. These particles were found to give satisfactory back pressures without further classification into sizes although the packing density was somewhat low compared to Sephadex resins.

Chromium and formalin tanning. Chromium and/or formalin tanning insolubilises gelatin gel, reduces the nett charge and at the same time increases the gel rigidity by crosslinking adjacent polypeptide chains. Chromium tanning involves carboxylic acid and amino side chain groups, reaction occurring with the non-ionised carboxylic acid group so that maximal reaction occurs at a low pH. The resulting product is thought to involve a combination of covalent, co-ordinate and hydrogen bonding. Formalin, on the other hand, reacts with amino groups in the uncharged form and can bring about cross-linking by reaction with two amino groups on different polypeptide chains. The requirement for the unionised form of the amino group means that reaction is maximal at high pH.

As both chromium and formalin tanning involve amino groups it is to be expected that the order of tanning will have an effect on the final product and this is confirmed by the results of Polson, Fawcett and Katz (40). As the chromium-formalin tanned product was found by these authors to be isoelectric at pH 7.0, this material was chosen for use in this study.

An interesting point concerning the tanning processes is that as amino groups are involved in the tanning reactions, buffers containing amino groups, for example glycinate and tris, should not be used during the tanning processes as these might react preferentially. Conversely, however, these buffers are desirable for subsequent chromatography as they can scavenge unreacted cross-linking groups and thus prevent reaction with the sample protein.

Procedure for preparation of gels (see also reference 41). Unfractionated acid process pigskin gelatin (Gelrite type A, 275 Bloom Canada Packers Ltd., 60 Paton Road, Toronto 4, Canada) was made up at the required concentration in distilled water and allowed to set in a glass container kept in an ice/water mixture. The gel was cut into small cubes (approximately 0.5 cm) and tanned overnight at 4°C in a solution of Kromex Chrome tanning salt (Chrome Chemicals (Pty.) Ltd., Merebank, Durban) (Kromex 80 g, sodium chloride 8.5 g, dissolved by heating and made up to one litre with distilled water). The tanned cubes were fragmented briefly (± 1 min) in a Waring blender into granules which were packed into a column (4 x 45 cm) and washed with 0.14 M NaCl until the effluent was free of chromium. The 0.14 M NaCl was then replaced by formalin tanning solution (37% formaldehyde solution, 1 part; 0.14 M NaCl, 1 part; pH adjusted to 9.0 with NaOH) which was run through the column until formalin could be detected in the effluent. At this stage the gelatin was extruded from the column, suspended in formalin tanning solution, adjusted to pH 9.0 with 5N NaOH and maintained at this pH for 2 h by the addition of further NaOH, prior to being set aside for 24 h at 4°C. After this period the granules were repacked into the column, washed free of formalin with 0.14 M NaCl and equilibrated with buffer (the formalin should not be washed out directly with the buffer if this contains an amino group as these two components react and precipitate out of solution). The granules were then extruded from the column, homogenised for 3 to 5 min in a Waring blender to yield finer fragments and finally, after temperature equilibration, were packed into a jacketed analytical column (1.75 x 125 cm). After packing the column was equilibrated with at least two column volumes of buffer before use.

The various washing steps described above were performed in columns, thus taking advantage of the cascade principle and ensuring maximal washing efficiency in a minimal time and with the expenditure of a minimum volume of washing solution.

2.10.3 Ion exchange chromatography

Preparation of resin. A new fibrous, fines reduced, anion exchange resin (Whatman DE 23), manufactured by W. and R. Balston Ltd., Maidstone, Kent, was used. The resin was precycled before use by a procedure described by the manufacturers in their laboratory manual (42). Pre-cycling ensures that the resin is fully swollen and that the charged groups are consequently fully accessible.

After pre-cycling the "fines" were removed by allowing the resin to settle twice through a column of distilled water in a 2 l measuring cylinder, the supernatant being discarded each time.

At this stage the resin was in a form convenient for a preliminary equilibration with phosphate buffer. The resin was washed with buffer on a Buchner funnel after which it was suspended in buffer and adjusted to pH 7.6 by titration with approximately 1N - KH_2PO_4. This was followed by further washing with buffer on the filter before the resin was suspended in buffer and packed into the column.

2.10.4 Operation of columns

Before use columns were usually equilibrated with at least two column volumes of buffer. Ion exchange columns, however, were equilibrated with a larger volume (± 5 column volumes).

Ascending or descending elution, as considered appropriate, was carried out either by gravity flow or by the use of suitable pumps. The column effluent was monitored either at 253.7 nm in a Uvicord absorptiometer or at one or more selected wavelengths in a DB spectrophotometer, equipped with a flow-through cell. Further details are included in the relevant sections on experimental procedure.

In the case of molecular exclusion columns where elution volumes were measured, the effluent was collected in a fraction collector equipped with a volume measuring device. The parameters V_t and V_o were determined by chromatography of tryptophan (1 mg) and blue dextran (5 mg) dissolved in 1 ml of buffer solution.

2.10.5 Concentrating and desalting of fractions

Unless stated otherwise the following procedure was used for the concentration and desalting of samples from column chromatography.

Selected pooled fractions were concentrated by ultrafiltration (also known as pressure dialysis or reverse osmosis) in a 50 ml Amicon "Diaflo" ultrafiltration cell, using a UM-2 membrane, with a nominal retention cut-off of 1,000 MW, operating under a nitrogen pressure of 50 p.s.i. The major advantage of the Amicon system lies in the unique membrane which, in the words of the manufacturer, "consists of a very thin (0.1 to 1.5 micron) layer of extremely fine pore texture (2 to 100 Å) upon a much thicker (50 to 250 micron) layer of open-celled, microporous sponge." As a consequence of this structure, rejection occurs at the membrane surface resulting in the high through-put and non-clogging characteristics.

An advantage of the ultrafiltration system is that only the macromolecules are concentrated so that there is no increase in the ionic strength of the medium, which could have adverse effects upon the sample protein.

For desalting in the ultrafilter the sample was reduced to a suitable minimum volume by application of nitrogen pressure. The pressure was released, distilled H_2O was added to give a suitable volume and pressure was again applied. This procedure was repeated until the ultrafiltrate gave no reaction for chloride when tested with ammoniacal $AgNO_3$.

The minimum volume to which the sample was reduced and the volume to which it was increased by adding distilled water were estimated empirically, taking into account the estimated amount of material in the sample. However, as desalting by ultrafiltration is analogous to the classical analytical technique of washing precipitates on a filter, the same expression (24) was assumed to apply, viz:-

$$x_n = x_o\left(\frac{u}{u + v}\right)^n \qquad \ldots\ldots\ldots \quad [2.2]$$

where n = number of "washings";

x_o = conc. of salts before desalting;

x_n = conc. of salts after n "washings";

u = the volume to which the sample was reduced after each "washing";

v = volume of dist. H_2O added for each washing.

Consequently the factors u and v were maintained at a minimum in order to achieve desalting in the shortest time possible, i.e. for a given total volume used for washing, n was kept as large as possible.

Ultrafilter-desalting could not be used in the case of proteins which are not soluble in distilled water, e.g. concanavalin A.

2.11 Immunological techniques

2.11.1 Preparation of antisera

Littermate pure-bred Californian rabbits were used for the preparation of antisera to minimise differences in response between different animals. Antisera were developed in adult rabbits using an initial intramuscular injection (1 mg antigen in 1 ml physiological saline) followed by a further five intravenous injections (2 mg antigen in 1 ml physiological saline) at 3-day intervals. Three days after the last injection, blood (± 25 ml/rabbit) was collected in clean, dry Erlenmeyer flasks and set aside for 2 hours at room temperature and subsequently overnight at 4°C. The antiserum was decanted and stored at -15°C until required.

2.11.2 Immuno-electrophoresis

Immuno-electrophoresis, a two-dimensional analytical system involving electrophoretic separation in one direction and subsequent immunodiffusion at right angles to this, has become an accepted and powerful means of characterising protein isolates, particularly with regard to the detection of heterogeneity. The method was developed for the analysis of serum proteins, which are potent antigens, and this remains the most successful area of application. In the case of weaker antigens, such as the legume proteins described by Stead (19) and also those used here, less intense precipitin bands are obtained but the method can, nevertheless, be used.

Experimentally, conditions selected represent a compromise between optimal conditions for electrophoresis and optimal conditions for immuno-precipitation. Fortunately, the low ionic strength required for minimal heat generation during the electrophoresis stage is compatible with the low salt concentration required for maximal immuno-precipitate development (43). The antibody-antigen reaction is also pH dependent, the optimum pH being 7.0 while no immunoprecipitate is formed below pH 4.5 or above pH 10.0 (44). A balance must therefore be struck between the optimum pH for electrophoretic separation, the optimum for immunoprecipitation and the pH stability limits of the antigen. In this study pH 7.0 was selected due to the latter two considerations.

A further consideration is that for the detection of all of the antigens in a given sample it is necessary to arrange for each antigen to be present in the gel at a concentration equivalent to the corresponding antibody. As the antibody concentrations cannot be known in advance it is necessary to do replicate immuno-electrophoretic runs covering a range of antigen concentrations (19).

Materials. Buffer: 0.1 µ Tris-HCl, pH 7.0 (c.f. Bloemendal (102)). Tris (12.99 g) was dissolved in distilled water (± 950 ml), titrated to pH 7.0 with approx. 5N - HCl and made up to 1 litre.

1% Agarose gel: Agarose (0.3 g) (Miles-Seravac (Pty) Ltd., Packer Avenue, Epping Industria, Cape Town) was heated in tris-HCl buffer, pH 7.0 (30 ml) until dissolved and 1% merthiolate (0.6 ml) was added.

Stain: Ponceau S (0.5 g) (BDH Chemicals Ltd., Poole, England), trichloracetic acid (7.5 g) and sulphosalicylic acid (7.5 g) were dissolved in distilled water and made up to one litre.

Procedure. Clean microscope slides (25 x 75 mm) were fitted into a holder (Shandon Instruments, Ltd.) and covered with molten agarose to a depth of approximately 2 mm. When set, sample wells were cut into the gel using a die made for this purpose. The slide holder was placed on a cooled plate in the electrophoresis tank and filter paper wicks impregnated with buffer were connected from the buffer compartments to the slides. Sample (40 mg/ml) was placed into the first of four sample wells, using a micro-syringe, and serial 1 : 1 dilutions of the sample with buffer were placed into the remaining three wells. A potential (100 V D.C.) was applied across the gels for 1 hour, cooling during this period being effected by passage of a coolant through the labyrinth of the cooled plate. The slide holder was removed from the electrophoresis tank and troughs were cut into the gel, again with a purpose-made die. The troughs were filled with antiserum containing merthiolate preservative (± 1 drop of 1% solution/ml) and the gels were set aside for three or more days at 37°C in an environment saturated with water vapour. After development of the bands, unprecipitated protein was washed out of the gels with large volumes of saline, changed frequently over a 48 hour period. Each gel was covered with a layer of moistened filter paper (Whatman No. 1) cut to the size of the slide and pin-prick holes were made in the paper corresponding to the positions of the trough and wells. The gels were subsequently dried at 37°C, stained in Ponceau S for 5 to 10 minutes, decolourised in 5% acetic acid and finally again dried. During the first drying step excess salt is absorbed by the filter paper.

Slides prepared in this way were stable for an indefinite period and could be easily stored.

2.11.3 Determination of diffusion coefficients by immuno-precipitation

Polson (46) has described a method for the determination of diffusion coefficients from immuno-precipitation data. During immunodiffusion (double diffusion) on gel plates or in gel columns the point of first appearance of a precipitin band, when the antibody and antigen are present in approximately optimal proportions, is described by the equation

$$\log \frac{C_{go}}{C_{bo}} = \log \frac{C_g}{C_b} + \frac{x_g}{\sqrt{\pi D_g t}} - \frac{x_b}{\sqrt{\pi D_b t}} \quad \ldots\ldots \quad [2.3]$$

where C_{go} and C_{bo} = original concentrations of antigen and antibody respectively;

C_g and C_b = concentrations of antigen and antibody at optimal proportions;

x_g and x_b = distances of precipitin band from antigen and antibody origins respectively;

D_g and D_b = diffusion coefficients of antigen and antibody respectively;

t = diffusion time;

$x_g + x_b$ = H (distance between antigen and antibody origins).

When the antigen and antibody are actually present in optimal proportions for reaction, then

$$\frac{C_{go}}{C_{bo}} = \frac{C_g}{C_b} \ .$$

Hence, from eqn [2.3]

$$\frac{x_g}{\sqrt{\pi D_g t}} = \frac{x_b}{\sqrt{\pi D_b t}}$$

and

$$\frac{x_g^2}{x_b^2} = \frac{D_g}{D_b} \qquad \ldots\ldots\ldots \ [2.4]$$

Therefore, at optimal proportions the position of the precipitin band is independent of time and depends only upon the ratio of the diffusion coefficients of the antigen and antibody. Since the majority of immunoglobulins are of the I_gG type (45) the diffusion coefficient of the antibody may be assumed to be 4.6×10^{-7} cm^2 sec^{-1} and the diffusion coefficient of the antigen may be calculated from accurate measurements of x_g and x_b at optimal proportions.

Although the method can be applied to double-diffusion systems on gel plates, Polson (46) has described an apparatus which takes advantage of the lower sample concentrations required for double diffusion in one dimension. The apparatus, as used in this study, consisted of three perspex bars (12 x 20 x 200 mm) and a thinner section (12 x 7 x 200 mm) which served as a lid. Holes (0.4 cm diameter) were drilled through the lid and upper two bars and into, but not through, the lower bar. The bars were machined and polished so that a watertight seal was obtained when the interface between the bars was lightly greased.

To carry out a determination, the bars were lightly greased and placed into juxtaposition, excess grease being removed from the holes with cotton wool. The holes were brought into apposition and antiserum, containing merthiolate preservative (1 drop of 1% soln/ml), was introduced into the compartments in the lower bar. This bar was displaced slightly, sealing off the lower compartments and molten 0.5% agarose, in buffered physiological saline, (pH 7.0; NaCl, 8.0 g; K_2HPO_4, 1.21 g; KH_2PO_4, 0.34 g and merthiolate, 0.1 g dissolved and made up to 1 litre with distilled water), cooled to just above the gelling point, was introduced into the compartments in the second bar which in turn was displaced relative to the top bar. Serial dilutions of antigen in buffered saline were introduced into the compartments in the top bar which was sealed by displacement of the lid. Finally the compartments in the three bars, but not the lid, were brought into apposition and the apparatus was set aside for several weeks at room temperature for the precipitin bands to develop.

When the bands had developed sufficiently, the parameters x_g and x_b were measured in the columns in which the bandwidths were at a minimum.

Polson (46) has described a refinement to allow for the contingency that the point of optimal precipitation lies intermediate between two selected concentrations. He has suggested plotting the precipitate bandwidth against the antigen concentration, the minimum bandwidth point, corresponding to the point of maximal precipitation, being used to read off the values of x_g and x_b from a plot of antigen concentration vs apparent x_g. This method is difficult to use when more than one antigen is present, however, as the precipitin bands overlap when the concentrations are not optimal.

2.11.4 Reaction with concanavalin A

Concanavalin A, the phytohaemagglutinin of the jack bean (Canavalia ensiformis) has the unique property of reacting with polysaccharides having a non-reducing terminal glucose, mannose, or fructose residue (47). In most cases, interaction of concanavalin A with the polysaccharide leads to the formation of a precipitate, so that in several respects the reaction is analogous to an antibody-antigen precipitin reaction. The development of precipitin bands during gel double-diffusion of concanavalin A against a sample glycoprotein is therefore an indication of the presence of terminal non-reducing glucose, mannose or fructose residues in the glycoprotein and furthermore, it was realised that the position of the band could be used to calculate the diffusion coefficient of the glycoprotein by the method of Polson (46), since the diffusion coefficient of concanavalin A is known (48).

2.12 Discontinuous gel electrophoresis

The discontinuous gel electrophoresis method developed by Ornstein (49) and Davis (50) consists of two phases. In the first phase application of a potential across the gel, maintained in a vertical attitude, causes a sharp interface to form between the so-called leading ion (an ion of high mobility) and the trailing ion (an ion of relatively lower mobility) in accordance with the regulating function of Kohlrausch (51). The interface moves downwards through a gel of large pore size (the stacking gel), which stabilises the system against convection, and protein molecules of a mobility intermediate between the leading and trailing ions are focussed into extremely thin layers at the interface. In the second phase, the interface reaches a gel of smaller pore size (the running gel) at which point a change in pH increases the mobility of the trailing ion so that the interface overtakes the protein bands, leaving these to separate according to charge in a uniform voltage gradient. An additional factor affecting the separation is the frictional resistance imposed by the gel matrix, this being proportional to molecular size.

It follows from the above that disc gel electrophoresis is possible only at certain pH values at which suitable leading and trailing ions are available (52).

2.12.1 Materials

Anionic system (49).

Stock solutions:-

A: Tris (36.6 g), TEMED (N : N : N' : N' Tetra-methyl-1 : 2-diamino-ethane) (0.23 ml) adjusted to pH 8.9 with 1N - HCl and made up to 100 ml.

B: Tris (5.98 g), TEMED (0.46 ml) adjusted to pH 6.7 with 1N - HCl and made up to 100 ml.

C: Cyanogum 41 (Xg) made up to 50 ml with distilled water. (X = 2 x nominal percentage of final running gel).

D: Cyanogum 41 (12.0 g) made up to 100 ml with distilled water.

E: Riboflavin (4 mg) made up to 100 ml with distilled water.

F: Sucrose (40 g) made up to 100 ml with distilled water.

Stock buffer (pH 8.3) : Tris (6.0 g) and glycine (28.8 g) made up to 1 litre with distilled water.

Working solutions:-

Running gel soln 1: 1 part A
2 parts C
1 part distilled water.

Running gel soln 2: Ammonium persulphate (0.14 g) made up to 100 ml with distilled water.

Stacking gel soln : 1 part B
2 parts D
1 part E
4 parts F.

N.B. The stacking gel solution was kept in a vessel wrapped with foil to exclude light.

Working buffer : Stock buffer diluted 1 : 10 with distilled water.

Anionic system in 8 M-urea.

Stock solutions:-

A: Tris (14 g), urea (48.05 g) and TEMED (0.23 ml) dissolved in distilled water, titrated to pH 8.9 with 1N - HCl and made up to 100 ml.

B: Tris (5.98 g), urea (48.05 g) and TEMED (0.46 ml) dissolved in distilled water, titrated to pH 6.7 with HCl and made up to 100 ml.

C: Cyanogum 41 (Xg) and urea (24.03 g) made up to 50 ml with distilled water. (X = 2 x nominal percentage of final running gel).

D: Cyanogum 41 (12.0 g) and urea (48.05 g) made up to 100 ml with distilled water.

E: Riboflavin (4 mg) and urea (48.05 g) made up to 100 ml with distilled water.

F: Urea (48.05 g) made up to 100 ml with distilled water.

Stock buffer (pH 8.3):- The stock buffer did not contain urea and was prepared as before.

Working solutions:-

Running gel soln 1: 1 part A
2 parts C
1 part 8 M-urea (F).

Running gel soln 2: Ammonium persulphate (0.14 g) and urea (48.05 g) made up to 100 ml with distilled water.

Stacking gel soln : 1 part B
2 parts D
1 part E
4 parts F.

N.B. The stacking gel solution was kept in a vessel wrapped with aluminium foil to exclude light.

Working buffer : Stock buffer diluted 1 : 10 with distilled water.

<u>Cationic system (53)</u>.

Stock solutions:-

A: 1N - KOH (48 ml) and TEMED (4.0 ml) titrated to pH 4.5 with glacial acetic acid and made up to 100 ml.

B: 1N - KOH (48 ml) and TEMED (0.46 ml) titrated to pH 6.0 with glacial acetic acid and made up to 100 ml.

C: Cyanogum 41 (Xg) made up to 100 ml with distilled water. (X = 2 x nominal percentage of final running gel).

D: Cyanogum 41 (10 g) made up to 100 ml with distilled water.

E: Riboflavin (4.0 mg) made up to 100 ml with distilled water.

Working solutions:-

Running gel soln 1: 1 part A
2 parts C
1 part distilled water.

Running gel soln 2: Ammonium persulphate (0.28 g/100 ml).

Stacking gel soln : 1 part B
2 parts D
1 part E
4 parts distilled water.

Working buffer : β-alanine (31.2 g) and glacial acetic acid (8.0 ml) made up to 1 litre with distilled water.

Cationic system in 8 M-urea.

All stock solutions were made up as above but with the additional inclusion of urea (48.05 g/100 ml). Working solutions were made up as above except that 8 M-urea was used in place of distilled water. No urea was included in the working buffer which was made up as above.

2.12.2 Apparatus

Two types of apparatus were used, a Buchler polyanalyst model 1004 and an apparatus developed from a design originated in this laboratory by Williams (54). The Buchler apparatus was based upon the design of Davis (50), employing cylindrical gels, but the lower electrode compartment consisted of a double glass-walled jacket, cooled by circulating water ($4^{0}C$), and platinum electrodes were used. Williams' slab gel apparatus was developed as a means of obtaining gels suitable for scanning in the equipment available in this laboratory. At the time at which this apparatus was constructed and used, no comparable equipment was commercially available.

Williams' apparatus was constructed of a perspex tube (12.0 cm i.d.) with a wall thickness of 6.0 mm. The tube was cut in two, forming two electrode compartments which were floored with 6.0 mm perspex discs, sealed with 3.0 mm O-rings in recessed channels. Two parallel slots (0.6 x 7.2 cm) were cut into the floor of the upper reservoir to accomodate the two cells. Cooling was effected by means of latex painted copper coils attached to the lid of the upper compartment and the floor of the lower. The platinum wire electrodes were fixed parallel to and equidistant from the ends of the cells in the two compartments and were attached next to the cooling coils. The glass (1.0 mm) cells enclosed a rectangular volume (10.0 cm x 5.2 cm x 2.0 mm), into which the gels were cast, and were fitted with perspex collars 9.7 cm x 3.0 cm x 5.0 cm fixed at right angles to the cell and 1.5 cm from one end. The component parts of the cell were cemented together with clear epoxy adhesive.

In the course of this study some modifications were made to Williams' apparatus. Difficulty was experienced in removing gels from the cells at the end of an electrophoresis run; in many cases the gel was broken and the material lost. In consequence, a demountable cell was constructed (Fig. 1). This cell was essentially the same as that described above but one side was constructed of two sheets of glass 8.0 cm x 7.0 cm x 1 mm and 2.0 cm x 7.0 cm x 1.0 mm. The smaller sheet formed an integral part of the cell and was cemented in place at one end of the cell and surrounded by the perspex collar which was now placed 8 mm from the end. The larger sheet was loose fitting and was held in position as described below.

A further problem concerned the latex-coated copper cooling coils. The coating was found to be unreliable as an electrical insulator so that leakage of current occurred through the coolant solution and on occasion the coolant vessel became charged to a dangerously high potential. Several alternative coating materials, epoxy marine lacquer, generator winding lacquer etc. were tried without success. Consequently cooling of the upper electrode compartment was abandoned, as in the case of the Buchler apparatus, and the double glass walled container of the Buchler apparatus was used as the lower electrode compartment (Fig. 2).

2.12.3 Procedure

Buchler polyanalyst apparatus. The cylindrical glass cells (0.5 cm i.d. x 7.5 cm) used with this apparatus were thoroughly cleaned by immersion in diluted Photoflo solution (Kodak Ltd., London) and allowed to dry. The cells were placed in a holder, maintaining them in a vertical position, and the ends were sealed with rubber caps. Sucrose solution (60% w/v) was carefully layered to a depth of ± 1 cm in each tube followed by 1.5 cm of stacking gel solution and finally ± 2.0 ml of distilled water. Care was taken to ensure that smooth interfaces were formed between the layers. The stacking gel was photopolymerised

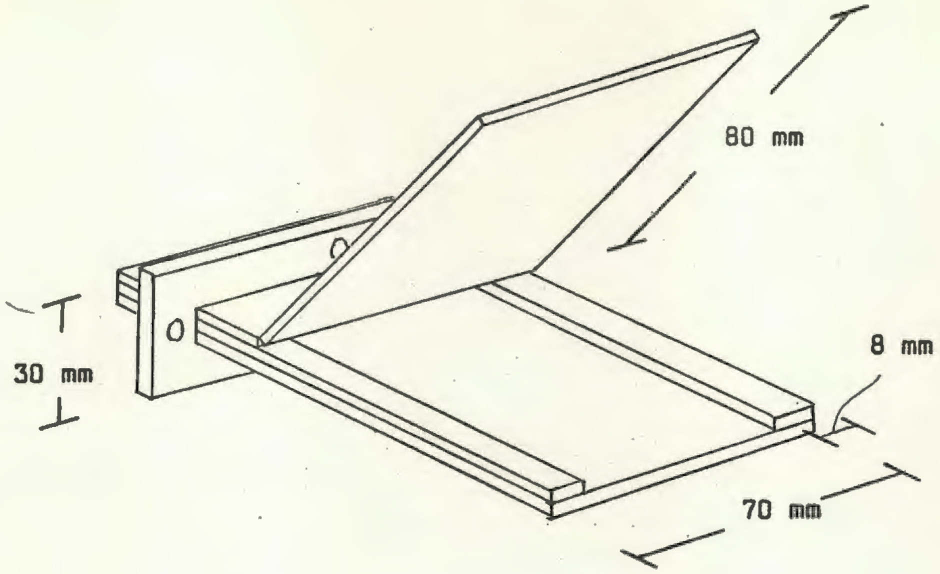


FIGURE 1 Demountable cell for slab-gel electrophoresis

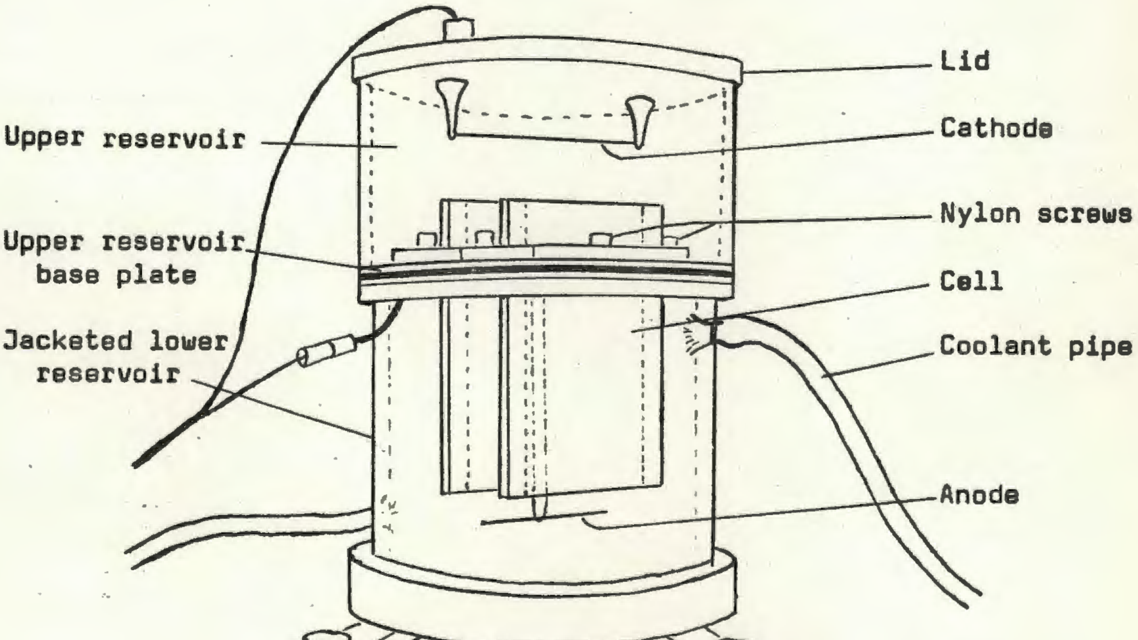


FIGURE 2 Apparatus for slab gel electrophoresis

by exposure to light emitted by two daylight fluorescent lamps at a distance of 20 - 30 cm for 20 min. The water layer was removed and the cells rinsed with a small amount of a freshly prepared mixture of running gel solutions 1 and 2 (1 : 1 v/v) and finally filled with the same solution. Air was excluded by sealing each tube with a small square of polyethylene film or parafilm.

At this stage the lower electrode compartment was filled with buffer and circulation of the coolant ($1^{\circ}C$) was commenced.

After polymerisation of the running gel (± 40 min) the 60% sucrose solution was rinsed out with buffer and the tubes placed into position in rubber grommets in the floor of the upper electrode chamber. A drop of buffer was suspended from the end of each gel and the two halves of the apparatus were brought together, submerging the gels into the lower electrode buffer. The upper electrode vessel was filled with buffer and 1.0 ml of bromophenol blue (0.001%) was added as a marker for observing the migration of the leading ion/trailing ion interface. Samples were layered onto the top of the stacking gels using a microsyringe and a potential was applied, giving a constant current of 2 mA per tube until the samples had penetrated the stacking gels after which the current was increased to 5 mA/tube. The potential was applied until the marker dye had reached a position 1 cm from the end of the gel at which stage the potential was discontinued and the gels were removed from the tubes by rimming with a jet of water from a hypodermic syringe.

The gels were stained by overnight immersion in amido black solution (0.1% in 7.0% acetic acid) and destained in 7.0% acetic acid by longitudinal electrophoresis in tubes of a slightly larger diameter than the gels, having one end constricted to support the gel. Air bubbles were obviated by inserting the gel into the tube immersed in 7% acetic acid. A constant current of 15 mA per tube was sufficient to complete destaining in 30 - 40 mins. Alternatively, the gels were stained for glyco-protein as described in section 2.11.4.

No equipment was available for the scanning of the cylindrical gels and these were recorded photographically.

Slab gel apparatus. The glass cells of the apparatus were thoroughly cleaned but prolonged immersion in Photoflo solution was avoided as this was found to weaken the epoxy adhesive bonds. The free glass sheets were hinged onto the cells with a strip of 'Sleek' plastic adhesive strapping (Smith and Nephew (Pty) Ltd., Pinetown, Natal), 12 mm wide, and sealed against the edges of the cell with a minimum adequate amount of rubber adhesive. Care was taken to ensure that no rubber adhesive entered into the gel space as this material inhibits gelling. Finally, the bottom of the cell was sealed with Sleek strapping and rubber adhesive. The assembled cells were screwed into place on the floor of the upper electrode compartment, a water-tight seal being ensured by the use of grease-free rubber gaskets.

The upper electrode compartment was supported with the cells in a vertical position and a freshly-prepared mixture of running gel solutions 1 and 2 was introduced to a depth of approximately 70 mm in each cell. Air bubbles, which tended to adhere to the Sleek strapping, were dislodged with a pasteur pipette and distilled water was carefully layered over the running gel solution to a depth of 10 - 20 mm. After polymerisation of the running gel solution (approximately 45 min) the water layer was decanted, the cells rinsed with stacking gel solution, and stacking gel solution was layered to a depth of 15 mm followed by an equivalent layer of distilled water. Photopolymerisation of the stacking gel was initiated by two daylight fluorescent lamps, placed at a distance of 15 to 20 cm and was complete after approximately 15 min.

The Sleek strapping sealing the bottom of each cell was cut away with a sharp scalpel and the cells were tested for leaks by filling the upper electrode compartment with buffer. The two halves of the apparatus were brought together, immersing the cells into the lower electrode buffer, previously chilled to ± 1°C by circulation of coolant through the compartment jacket. The sample applicators were placed into position in each cell, 1 ml of bromophenol blue (0.001%) was added as a marker and the samples were introduced into the slots of the sample applicators using a microsyringe. A potential giving a current of 25 mA was applied until the leading ion/trailing ion boundary, made visible by the bromophenol blue, had migrated ± 7.5 mm into the stacking gel. At this point the potential was switched off, the sample applicators removed from the cells and the potential reinstated to give a constant current of 60 mA. This current was maintained until the leading ion/trailing ion boundary had migrated to a position approximately 1 - 1.5 cm from the lower edge of the gel. The current was switched off, the cells removed from the apparatus and opened to permit easy removel of the gel. The gels were notched on one side, close to the lower end, to facilitate subsequent orientation, removed from the cell and immersed in amido black stain (0.1% in 7% acetic acid). Staining was carried out overnight and subsequent destaining in 7% acetic acid was brought about by transverse electrophoresis at a potential of 12 V D.C. and was completed within 10 mins.

The gels were sliced longitudinally, supported on clean microscope slides and scanned in a Zeiss model 3 photometric scanner. The slit-width of this instrument had been reduced to one third of the normal size and the rate of movement of the carriage was reduced by mechanical coupling to the chart-paper drive of a Metrohm lab-recorder.

2.12.4 Staining for glycoprotein (55)

Fuchsin-sulphite stain. Potassium metabisulphite (16 g) and c. HCl (21.0 ml) were dissolved in distilled water and made up to 2 litres. Basic fuchsin (8 g) was added and the mixture stirred gently for 2 h. A small amount of activated charcoal was added and the solution was filtered after 10 min. This solution was stored in a brown bottle in the refrigerator.

Procedure. Polyacrylamide gels were immersed in trichloracetic acid solution (12% w/v) for 30 min, rinsed with distilled water, immersed in periodic acid solution (1% in 3% acetic acid solution) for 50 min and washed overnight in a large excess of distilled water. The gels were immersed in Fuchsin-sulphite stain in the dark for 50 min and subsequently washed three times with freshly-prepared 0.5% metabisulphite solution for approximately 10 min/wash. Finally, excess stain was removed by washing in distilled water and the gels were stored in 7% acetic acid solution.

2.13 Gel electrophoresis in Sodium dodecyl sulphate (SDS)

SDS-gel electrophoresis, which was introduced by Shapiro, Vinuela and Maizel (56) is becoming an increasingly popular method for the detection of heterogeneity and for the estimation of protein molecular weights. The method is dependent in the first instance upon the interaction of the protein molecule with SDS. This is a detergent having an ionised hydrophilic head and an aliphatic, hydrophobic tail and it is thought (56) that the protein, in some manner, organises the SDS into a micellar structure, presumably with the hydrophobic parts of the SDS molecules buried in the hydrophobic interior of the protein and with the charged groups arrayed upon the surface.

In the case of most proteins, approximately 1.4 mg of SDS is bound per milligram of protein (57) and at this level the negative charge due to the SDS is sufficient to mask the charge on the protein and all proteins consequently have an anodic migration (58). As the intrinsic charge differences between proteins is masked by the SDS, separation of proteins is due solely to differences in size and hence the method can be used to determine molecular sizes.

Two considerations of practical importance are relevant to SDS-gel electrophoresis. Firstly, the lack of charge differences between proteins in SDS means that a stacking phase will not be as advantageous as in conventional disc gel electrophoresis. For this reason the stacking phase is usually omitted. However, a stacking system has been devised and used by Neville (59) for the preliminary concentration of dilute samples in SDS. Secondly, as SDS has been demonstrated to cause irreversible changes in the structure of certain proteins (60), SDS-gel electrophoresis must be regarded as essentially an analytical method which at this stage appears to have little promise for preparative applications.

2.13.1 Reagents

Stock solutions:-

1·12 g } in 2·8 ml
\+ ·04 g }

A: Acrylamide, recrystallised from chloroform, (40 g), Bis (N' : N' - methylene-bis-acrylamide) (1.5 g) made up to 100 ml with distilled water.

·2g/ml

B: Sodium dodecyl sulphate, recrystallised from 96% ethanol at 9°C, (20 g) made up to 100 g with distilled water.

C: Ammonium persulphate (1.5 g) made up to 100 ml with distilled water.

D: TEMED (N : N : N' : N' - tetra-methyl-1 : 2-diamino-ethane) (0.5 g) made up to 100 ml with distilled water.

Stock buffer: Tris (48.456 g), anhydrous sodium acetate (16.408 g), disodium EDTA (7.445 g) dissolved in approx. 950 ml of distilled water, titrated to pH 7.4 with acetic acid and made up to 1 litre.

Working solutions:-

Gel soln:	1.4 parts A
	0.5 parts B
	1.5 parts C
	0.5 parts D
	1.0 parts stock buffer
	5.6 parts distilled H_2O.
Buffer :	Stock buffer (100 ml), solution B (50 ml) made up to 1 litre with distilled water.

2.13.2 Procedure

The procedure used was essentially that of Fairbanks, Steck and Wallach (61), except that the reducing agent dithiothreitol (DTT) was omitted from the sample solutions. The experimental procedure was similar to that used in disc gel electrophoresis although in this case only a single gel, as opposed to a running and a stacking gel, was used.

2.14 Iso-electric focussing (62)

Protein molecules are ampholytes, bearing a pH-dependent nett charge which is positive at pH values lower than the pI and negative at pH values higher than the pI. The point at which the protein bears no nett charge, the so-called iso-electric point, is a characteristic parameter and is usually different for different proteins.

If a protein is distributed throughout a solution in a pH gradient, then upon application of an appropriate electrical potential, molecules in the low pH zone will migrate through zones of increasing pH towards the cathode while those in the high pH zone will conversely migrate through zones of decreasing pH towards the anode. Each molecule will migrate through the pH gradient until it reaches the pH value at which it has no nett charge at which point migration ceases. After a sufficient time, therefore, the respective molecules will, in consequence, be "focussed" at the iso-electric point. In this way a mixture of proteins can be separated, as each will focus at a characteristic iso-electric point, and furthermore the iso-electric points can be readily determined.

In practice, three difficulties must be overcome; a stable, uniform pH gradient must be established and maintained, the system must be stabilised against disturbances due to convection and a system for the measurement of the pH gradient must be devised. The major recent advance, which has transformed the principle into a practicable and widely used technique, has been the development of the material known as "Ampholine" (LKB-Produkter AB, Stockholm, Sweden), consisting of a mixture of polyamino-polycarboxylic acid ampholytes which, when exposed to an electrical potential under suitable conditions, generate and maintain a stable linear pH gradient. Stabilisation against convection disturbances is usually achieved, in preparative systems, by conducting the experiment in a sucrose gradient, in which case the pH gradient can readily be measured by eluting the solution at the conclusion of an experiment (62). In analytical systems a polyacrylamide gel is often used as an anti-convection medium (63) and in this case the pH gradient is measured either by segmenting and eluting the gel (64) or by the use of standard dyes (65) which are focussed at characteristic points.

2.14.1 Reagents

A: Riboflavin (0.001 g) made up to 50 ml with distilled water.

B: Cyanogum 41 (0.5 g), TEMED (0.005 ml), Ampholine (0.5 ml, pH 3 - 10) and solution A (5 ml) made up to 10 ml with distilled water. This solution was stored in a foil-wrapped container. For iso-electric focussing in 8 M-urea this solution additionally contained 4.8 g urea.

C: Sucrose (0.4 g) made up to 10 ml with distilled water.

D: Trichloracetic acid (120 g) made up to 1 litre with distilled water.

Anode electrode solution: Phosphoric acid (50 ml) diluted to 1 litre with distilled water.

Cathode electrode solution: Ethylenediamine (50 ml) diluted to 1 litre with distilled water.

2.14.2 Procedure

The Buchler polyanalyst model 1004 disc gel apparatus described in section 2.12.2 was also used here for gel iso-electric focussing. The cylindrical glass cells (75 mm x 5 mm i.d.) were placed in a holder, maintaining them in an upright position and the lower ends were sealed with rubber caps. Solution C was layered to a depth of ± 1.0 cm, sample solution (approximately 50 µl) containing ± 0.6 mg of protein was added to solution B (3 ml), well mixed, and the resulting solution was layered over solution C to within ± 1 cm of the top of the cell. Distilled water was layered over this solution, filling the cell, and photopolymerisation of gel solution B was brought about by exposure to two daylight fluorescent lamps placed at a distance of 15 to 20 cm. When polymerisation was complete (± 15 min) the sucrose solution was rinsed out and the cells were placed in position through the grommets in the upper electrode vessel. The upper electrode compartment was filled with anode electrode solution (5% phosphoric acid) and the lower compartment with cathode electrode solution, chilled to ± 1°C. The upper compartment was brought into position and air bubbles were removed from below the gels using a bent-tipped pasteur pipette.

Electrofocussing was performed at a potential of 150 V D.C., applied for 2 h, after which the ampholytes were washed from the gel with several changes of 12% trichloroacetic acid solution. As the gels were symmetrical, orientation was effected by pushing a pin into one end of the gel before this was removed from the cell. The ampholytes, which stain as proteins, were removed from the gel with several changes of 12% trichloroacetic acid. The gels were stained with amido black (1% in 7% acetic acid) and destained electrophoretically as in the case of disc gel electrophoresis (2.12.3). It was noticed that the gels used for iso-electric focussing suffered large changes in volume during the washing, staining and destaining operations.

The pH gradient was measured by segmenting a gel, run concurrently with the sample gel, eluting the segments with distilled water and measuring the pH of the eluted solutions as described by Catsimpoolas (64).

2.15 Analytical ultracentrifugation

The analytical ultracentrifuge has become accepted as a virtually standard tool for the measurement of the physical parameters of biological macromolecules. Of these parameters the two of greatest interest, for characterisation purposes, are the molecular weight and the sedimentation coefficient.

The methodology used in the determination of sedimentation coefficients additionally provides a convenient and quick method of assessing the homogeneity of a given preparation. A typical determination requires only one hour of experimental work, and a few milligrams of sample. Nevertheless, analytical ultracentrifugation has drawbacks as a means of detecting heterogeneities. In particular, the Schlieren optical system imposes a limitation such that the instrument has only coarse selectivity, approximately on a par with molecular exclusion chromatography, and is therefore unable to detect other than gross heterogeneity.

Molecular weights can be estimated from sedimentation coefficients, provided the diffusion coefficients of the sample molecules are known, by means of the Svedberg equation:

$$M = \frac{RTs}{D(1 - \bar{V}\rho)} \qquad \ldots\ldots\ldots \quad [2.5]$$

where
R = universal gas constant;
T = absolute temperature;
s = sedimentation coefficient;
D = diffusion coefficient;
$\bar{V}$ = partial specific volume of solute;
ρ = density of medium.

This is the method of choice for the estimation of the molecular weights of the components of a mixture but where it has been established that the material under study is largely homogeneous, sedimentation equilibrium analysis is preferred as this is a primary measurement with less potential for error.

In sedimentation equilibrium analysis, a speed of rotation is used so that sedimentation of the molecule is balanced by diffusion. At equilibrium, if the sample is homogeneous, a linear relationship exists between log C (where C is the protein concentration) and r^2 (where r is the radius of rotation) and the molecular weight can be determined from the slope of this line, and the equation

$$M = \frac{2RT}{(1 - \bar{v}\rho)\omega^2} \cdot \frac{2.303\ (d \log c)}{d(r^2)} \quad \ldots\ldots \quad [2.6]$$

where ω = angular velocity in radians per second.
Other symbols are as in equation [2.5].

If the sample is heterogeneous, a curvilinear relationship is obtained between log C and r^2 and the slope of the line is not constant. It follows that for the estimation of molecular weights by sedimentation equilibrium analysis, the purity of the protein must be established beforehand so that the practicability of the analysis can be assessed.

In all ultracentrifugal analysis it is necessary to transform the results obtained into results under standard conditions, thus facilitating comparisons with other published work, and it is also desirable to perform the analyses at various concentrations and to extrapolate the results to infinite dilution.

2.15.1 Procedure

A Beckman model E analytical ultracentrifuge was used for ultracentrifugal analysis, following essentially the procedures described by Chervenka (66). Relevant experimental details are presented with each figure.

Sedimentation coefficients were estimated from the photographic plates recording the position of the sedimenting boundary at automatically measured time intervals. The distance from the reference hole line to the peak maximum was measured in each case with a magnifying profile projector and calculation of the sedimentation coefficients from the resulting data was effected by means of an IBM 1130 electronic computer using the programme listed in Appendix , which was kindly provided by Mr J. Conradie of the Natal Institute for Immunology. The computer was also used to fit a least squares linear regression to the values of $S_{20,w}$ obtained at different sample concentrations, thus facilitating an extrapolation to $S^{o}_{20,w}$.

Molecular weights were estimated by conventional sedimentation equilibrium analysis, using the six sector cell of Yphantis (67). Three different sample concentrations were analysed at two different speeds of rotation. At equilibrium the concentration distribution across the sectors was measured at 280 nm using the UV absorption scanner. Baselines were determined at the conclusion of a run by overspeeding to clear the upper portion of the sector. The distribution of sample across each sector was measured manually from the recorder trace and the data was tabulated as optical density versus radius of rotation. Calculation of the apparent molecular weight for each sample concentration and rate of revolution was effected from the tabulated data by means of the IBM 1130 computer, using the programme listed in Appendix 3.

The reciprocals of the values obtained for the apparent molecular weights were plotted against the sample concentration and an extrapolation was made to determine the apparent molecular weight at infinite dilution.

2.16 Spectral analysis

Absorption spectra, which have long been recognised as a valuable means of characterising certain molecules, were measured on a Beckman DB spectrophotometer, using 10 mm quartz cells.

A more powerful method of structural analysis is provided by the differential absorption of left and right circularly polarised light by optically active compounds. The resultant circular dichroism (CD) spectrum provides valuable information concerning the secondary and tertiary structural organisation of the sample molecule. Although such spectra are often difficult to interpret in terms of absolute structure they are particularly valuable in detecting changes in structure brought about by changed conditions. The methodology and interpretation of CD spectra have recently been reviewed by Tinoco and Cantor (68).

For the measurement of CD spectra a Jasco J-20 spectropolarimeter was used. Freeze-dried and desalted samples were dissolved in buffer (see below) at a concentration of 1 mg/ml. CD spectra were measured, in the wavelength range 350 nm to 250 nm, using a 10 mm quartz cell and for wavelengths shorter than 250 nm a 1 mm quartz cell was used.

Baselines were measured on samples of buffer only and the difference $(h_1 - h_2)$ between the recorder traces obtained from the protein sample and from the buffer were measured manually at 2 nm intervals. Values of $[\theta]$ were calculated from the values of $(h_1 - h_2)$ taking into account the instrument sensitivity setting (usually $0.002^\circ\ cm^{-1}$), the mean residue weight (assumed to be 110 daltons), the light path length and the sample concentration.

Buffers used in the measurement of CD spectra were; 0.01 M Tris-HCl, pH 7.6 containing 0.15 M NaCl, 0.01 M acetate, pH 4.0 containing 0.15 M NaCl and 0.01 M glycinate, pH 9.5 containing 0.15 M NaCl. In each case spectra were measured using these buffers with or without 8 M-urea.

2.17 Histological examination of intoxicated tissues

Moribund intoxicated animals were sacrificed and in each case the liver, kidney, spleen and brain were immediately removed and fixed overnight in 10% formalin (37% formalin (10 ml) made up to 100 ml with distilled water). The respective organs were embedded, sectioned and stained with hematoxylin-eosin stain, using the techniques described by Humason (69). The sections were examined in the microscope and recorded photographically.

CHAPTER 3

PREPARATION OF TOXIC PROTEIN ISOLATES

Following up the demonstration by de Muelenaere (70) that the extraction procedure of Lyman and Lepkovsky (71), for the preparation of crude trypsin inhibitor from soybeans, yielded toxic extracts when applied to the jack bean (*Canavalia ensiformis*) and the NRY-bean (*Phaseolus vulgaris*), the present author (18) and Stead (19), respectively, used this extraction procedure as a basis for the isolation of toxins from these sources.

In his study on the NRY toxin Stead (19) employed pH fractionation, $(NH_4)_2SO_4$ precipitation, organic solvent precipitation and ion-exchange on DEAE-cellulose to achieve group separation, but this procedure was somewhat specific for the NRY-bean toxin. An alternative procedure (I) was subsequently developed by the author (18) for the preparation of a jack bean isolate and it was hoped that the conditions selected would provide a more widely applicable method, suitable for use in a comparative study. The latter procedure was further investigated in the present study, using the jack bean as a model system, and was consequently slightly modified. The modified method will be referred to as procedure IA.

An attempt was made to apply the revised procedure to the soybean and the NRY-bean but it was found to be unsuitable in the case of the NRY-bean. Stead's method was therefore again adopted for the preparation of a toxic isolate from this bean.

It should be emphasised that, while the toxic isolates described in this chapter represent purified toxins, no claim is made regarding their ultimate homogeneity. The purification procedures were pursued only to the point where the respective isolates were considered sufficiently pure to permit a meaningful comparison of their characteristics.

3.1 Preparation of crude extracts

3.1.1 Investigation of extraction procedure I as applied to jack beans

For the extraction of jack beans, the method developed by the author (17, 18) was initially used. This procedure, which in this thesis is designated extraction procedure I is presented in Fig. 3. The overall extraction procedure has been discussed in a previous thesis (18) and hence will be considered here only with regard to detailed aspects to which further attention was given.

Effect of pH. A modification introduced in this study was that after exposure to pH 4.2, the extract was adjusted back to pH 7.6 instead of pH 7.0 in the belief that this might bring the protein into an ionic form requiring minimal equilibration against the pH 7.6 chromatography buffers subsequently used. However, the change to pH 7.6 was attended by a marked increase in the amount of flocculent precipitate formed, although no equivalent precipitate had been observed in pH 7.6 buffers containing 0.15 M-NaCl. It is assumed that the presence of salt promotes solubilisation of this material.

The pH 7.6 precipitate (which might involve phytate salts as these are sparingly soluble at this pH) was found to be toxic (Table 2) but chromatography of this material, after it had been resolubilised, revealed a complex mixture with several species with $A_{260} > A_{280}$ (Fig. 4). The elution position of this material was different to that of the toxin under study. By contrast, the elution profile on tanned gelatin of the soluble material prepared at pH 7.6 (CJ-7.6) was indistinguishable from that prepared at pH 7.0 (CJ-7.0) and the LD_{50} values were apparently not significantly different. CJ-7.6 was consequently used in subsequent studies and the material precipitated at pH 7.6 was given no further attention in this study.

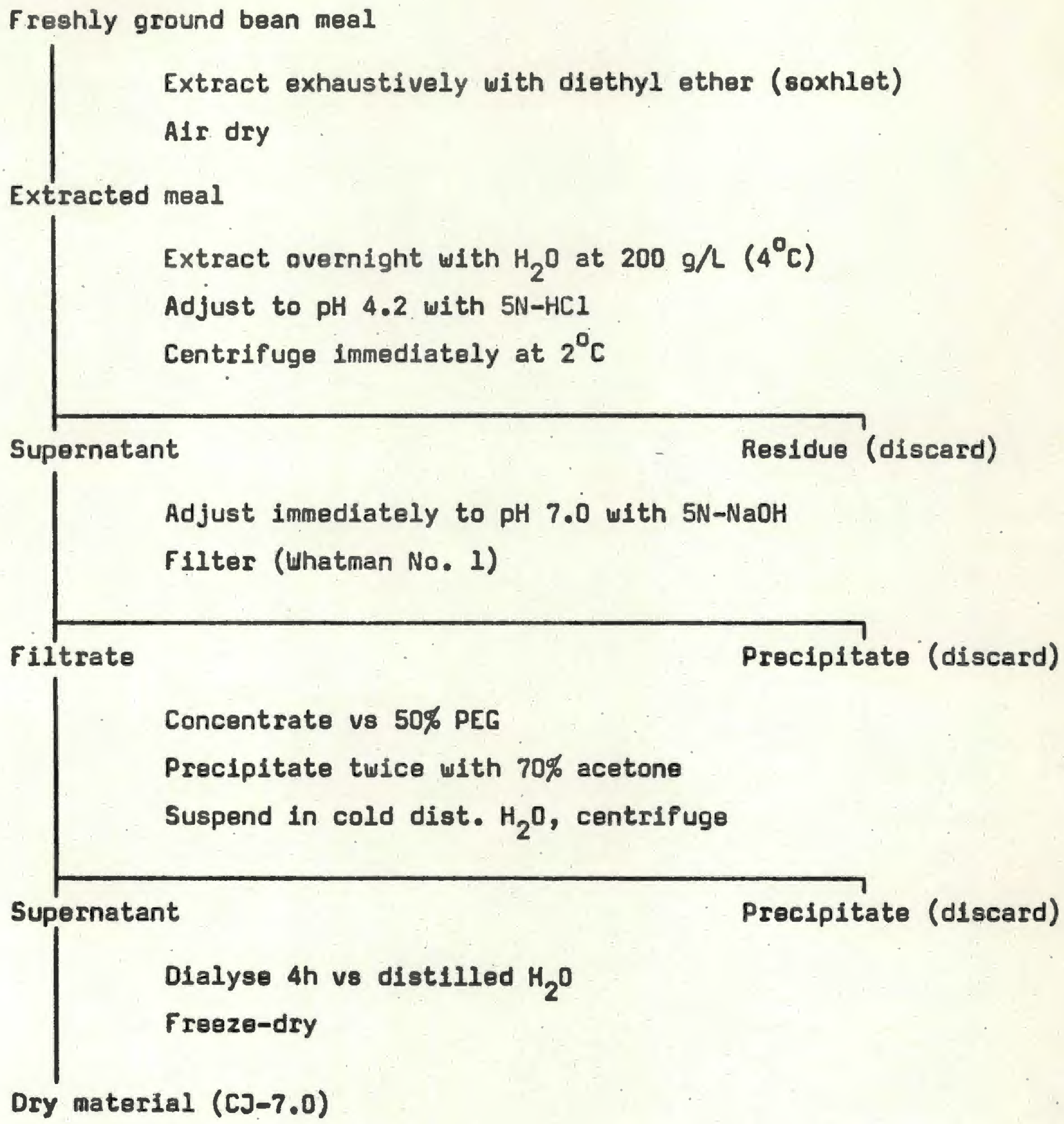


FIGURE 3 Extraction procedure I for the preparation of crude jack bean extract (18)

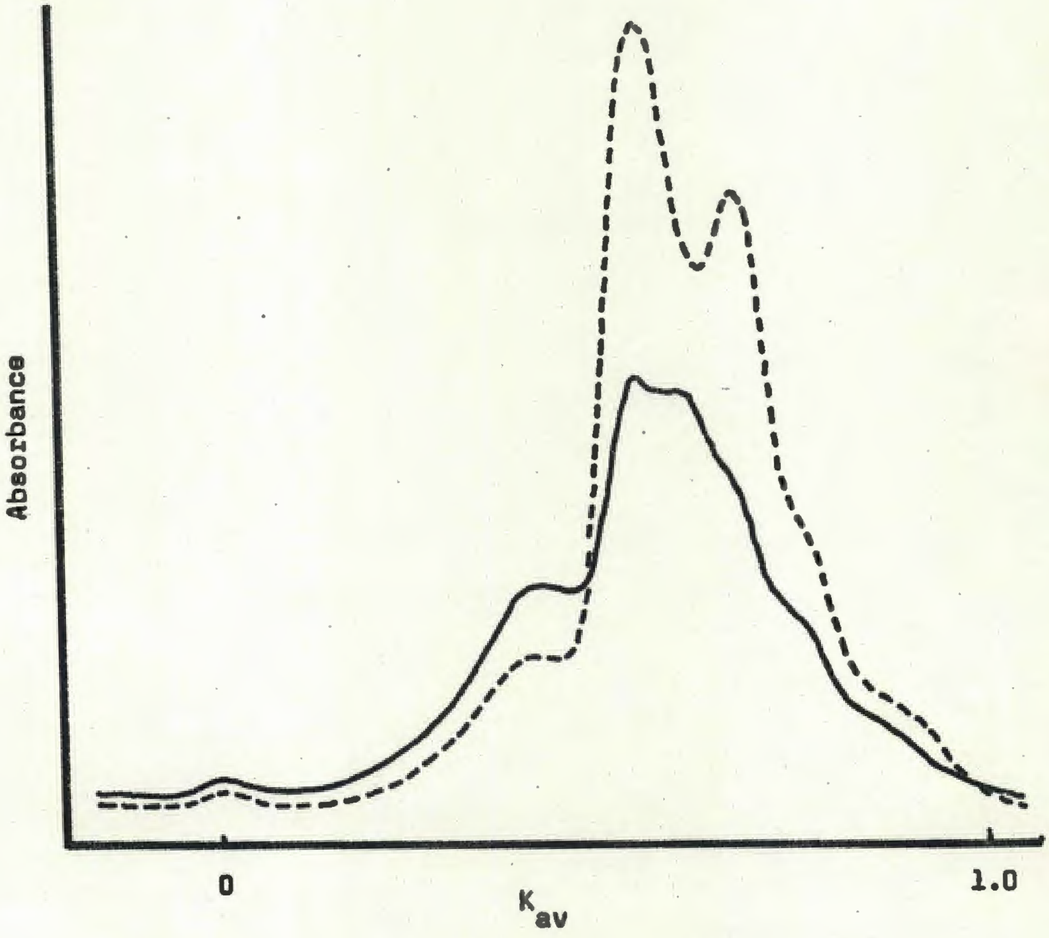


FIGURE 4 Elution profile of jack bean pH 7.6 precipitate on 8% chromium-formalin tanned gelatin.

---- = $A_{260\ nm}$; ——— = $A_{280\ nm}$;

Column, 1.75 x 118 cm; Buffer, 0.01M Tris, pH 7.6 containing 0.15M NaCl and 0.2% NaN_3

Acetone precipitation. Acetone precipitation of the protein effectively removes residual polyethylene glycol and also a yellow-coloured pigment, both of which are acetone soluble. The yellow pigment was UV-absorbing and, as it was present in relatively high concentration before acetone precipitation, was found to mask the UV-monitored protein peaks if it was not removed prior to the chromatography steps. The evaporated and freeze-dried acetone extract was not toxic, however, and consisted of low molecular weight material (Fig. 5) with $A_{260} > A_{280}$.

The acetone precipitation step was thought to contribute to the variable toxicity of the product, possibly due to partial denaturation of the precipitated protein components. An alternative method of acetone extraction immediately following soxhlet ether extraction was consequently attempted but, applied at this stage, acetone was apparently not effective in extracting the UV-absorbing pigment.

Effect of dialysis. The purpose of the dialysis step immediately preceding freeze-drying was to remove the bulk of the contaminating acetone and thus avoid damage to the freeze-drier. An apparent difference in toxic potency was observed on an occasion when the period of dialysis was increased from 4 h to 24 h. Consequently, a 24-hour diffusate was collected, acetone removed under reduced pressure in a rotary evaporator and the resulting solution freeze-dried. This material was not toxic, however, (Table 2) and consisted of low molecular weight material (Fig. 6).

It was known from previous studies (18) that the toxin under study was an albumin, soluble in distilled water, and it was concluded that the loss of activity on dialysis was possibly due to increased precipitation of the haemagglutinin, concanavalin A, which is a globulin, not soluble in distilled water. The latter fraction was found to contribute to the toxic potency of the extract (see section 3.2). However, for reasons of convenience and to avoid any remaining doubts, a 4 h dialysis period was used in subsequent experiments.

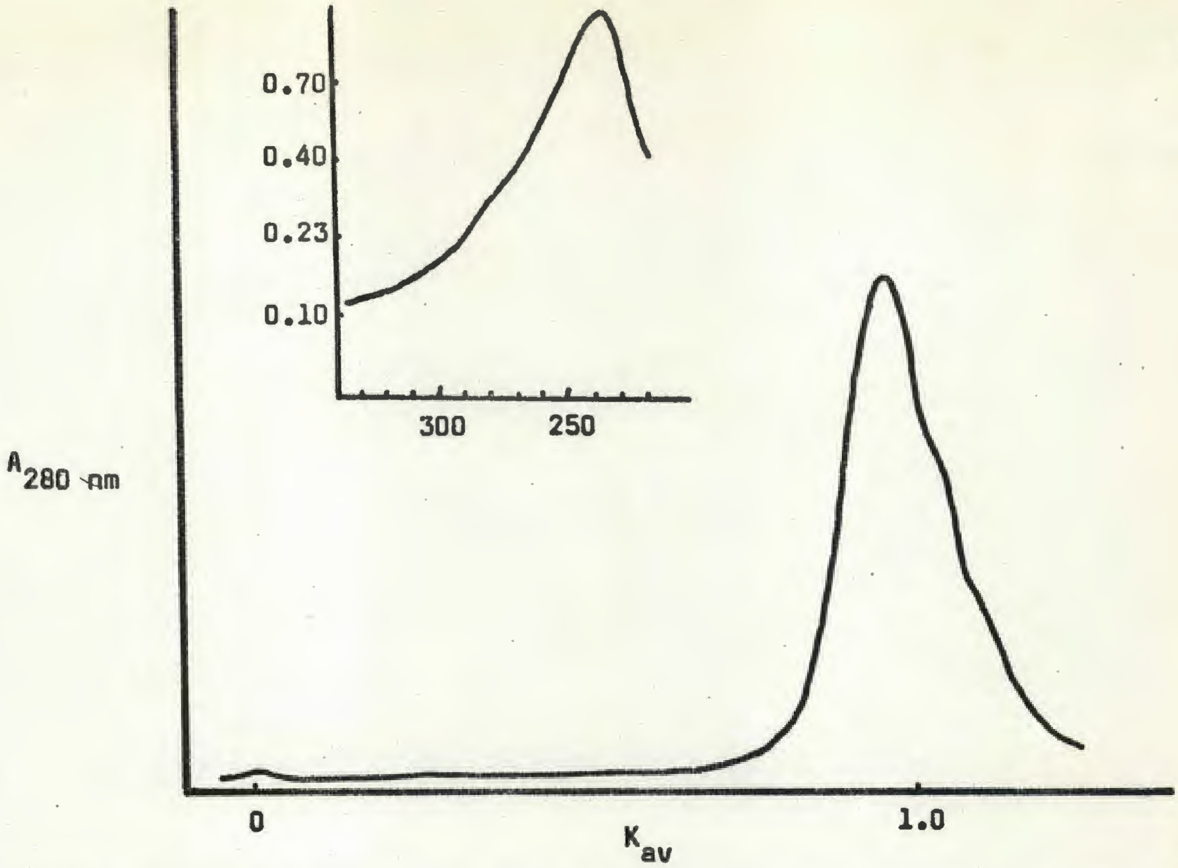


FIGURE 5 Elution profile of jack bean diffusate on Sephadex G-100. Column, 2.5 x 95 cm; buffer, 0.01M tris, pH 7.6 containing 0.15M NaCl and 0.02% NaN_3 (inset, UV-absorption spectrum)

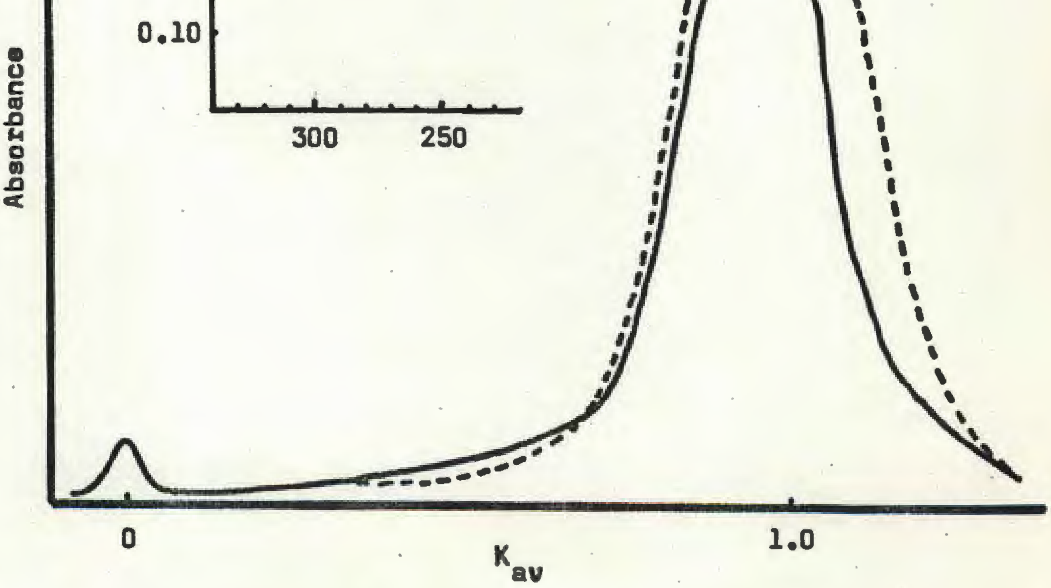


FIGURE 6 Elution profile of jack bean acetone extract on Sephadex G-100.

------ = $A_{260\ nm}$; ——— = $A_{280\ nm}$;

Column, buffer etc. as in Fig. 5

TABLE 2 Toxicity of certain jack bean fractions

Fraction	Toxicity at 250 mg/kg
pH 7.6 pptate	$^3/_3$
24 h diffusate	$^0/_3$
Acetone supernatant	$^0/_5$

3.1.2 Application of extraction procedure IA to jack beans, soybeans and Natal round yellow beans

On the basis of the investigations reported in section 3.1.1, extraction procedure IA (Fig. 7) was considered the most suitable for the preparation of a crude jack bean extract, and, for the purposes of comparison, this method was also applied to soybeans and Natal round yellow beans. The resulting extracts obtained from the three bean species were designated CJ-7.6, CS-7.6 and NRY-C2 respectively.

3.1.3 Preparation of crude extract from NRY-beans by Stead's method

An extract of NRY-beans was prepared by the original method developed by Stead (19) (Fig. 8) for comparison with that prepared by procedure IA. As this procedure had been applied to NRY-beans previously, the extract was designated NRY-C1.

3.1.4 Comparison of crude extracts

The crude extracts prepared from the various legumes are compared in Table 3. It may be seen that extract NRY-C1 was prepared in greater yield and was more toxic than NRY-C2. For these reasons, of the two NRY extracts, NRY-C1 was selected for further study.

It is apparent that there is no correlation, in the various extracts, between toxicity and haemagglutinating activity for while CJ-7.6 has the greatest toxic potency it has a relatively low haemagglutinating activity. Conversely,

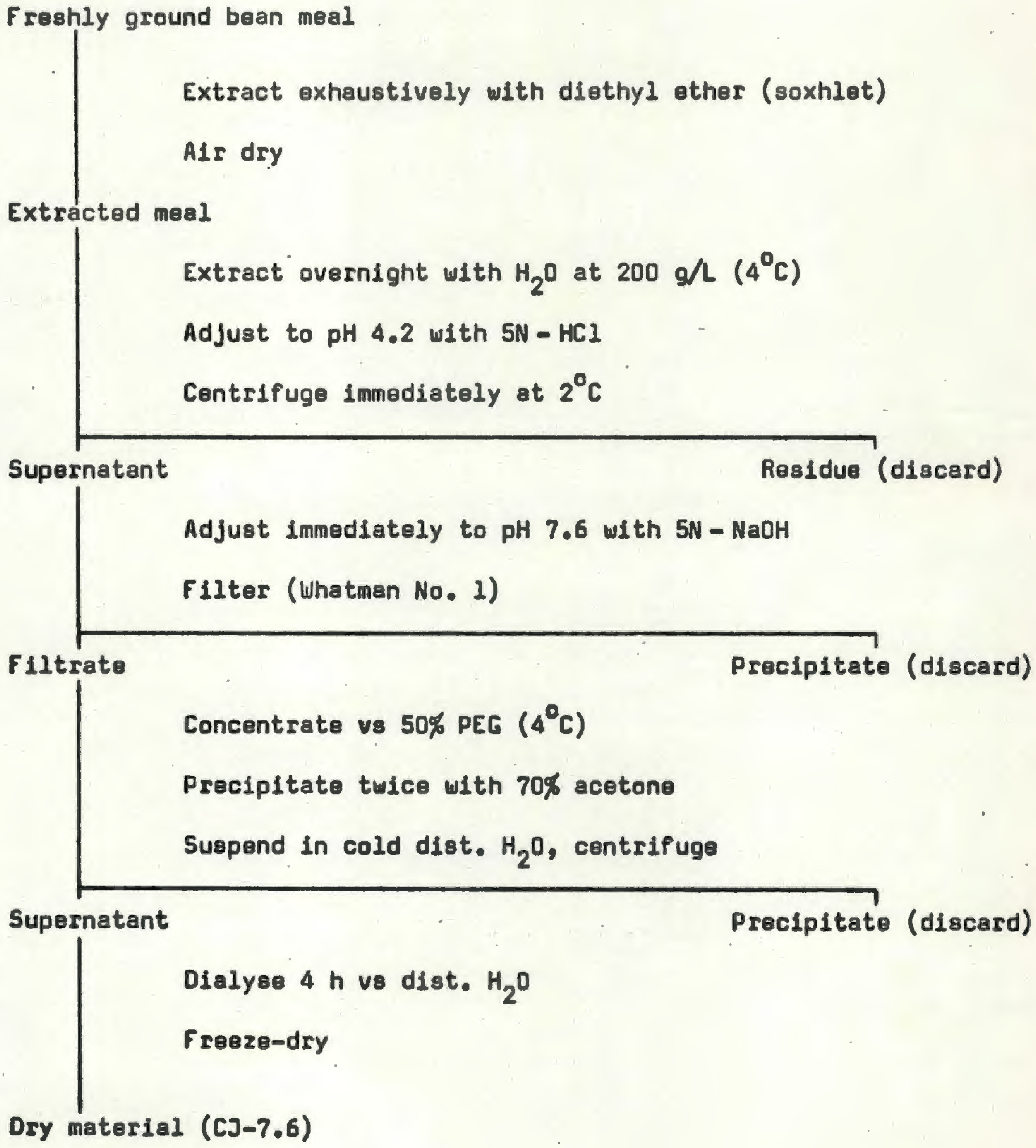


FIGURE 7 Extraction procedure IA for the preparation of crude extracts from the jack bean, the soybean and the Natal round yellow bean

Whole NRY bean meal

Suspend in 5 vols dist. H_2O
Adjust to pH 4.2 with 5N-HCl
Stand overnight at room temperature
Centrifuge (275 x g, 10 min)

Supernatant — Precipitate (discard)

Adjust pH to 6.8
Add solid $(NH_4)_2SO_4$ (70 g/100 ml) maintaining pH 6.8
Store overnight at 2°C
Centrifuge (600 x g, 10 min)

Precipitate — Supernatant (discard)

Redissolve in min. vol. dist. H_2O
Dialyse vs running tap water, 40 h
Centrifuge (600 x g, 10 min)

Supernatant — Precipitate (discard)

Chill to 2°C
Add redistilled acetone (-15°) to 70%
Stand, 1h, -15°C
Centrifuge (600 x g, 10 min)

Precipitate — Supernatant (recover acetone)

Redissolve in min. vol. dist. H_2O
Dialyse overnight vs distilled water, 2°C
Centrifuge and freeze dry supernatant

Dry material = NRY - C1

FIGURE 8 Procedure for preparation of crude NRY extract according to Stead (19)

NRY-C1, which has the highest haemagglutinating activity, is less toxic than CJ-7.6.

TABLE 3 Analysis of various crude legume extracts

Fraction	NRY-C1	NRY-C2	CJ-7.6	CS-7.6
Yield	18.0	15.0	10.0	10.0
% Protein	91.1	70.3	90.0	97.2
% Neutral sugars	17.0	19.0	8.0	2.8
HU/mg	612	236	250	449
LD_{50} (mg/kg)	250	500	80	480

3.2 The effect of removal of the jack bean haemagglutinin by adsorption onto Sephadex G-100

The jack bean haemagglutinin is adsorbed onto Sephadex G-100 (48, 72) and this provides a convenient way of removing this compound and also of assessing its contribution to the overall toxicity of the crude extract. With the latter object in mind, a number of crude jack bean extracts, listed in Table 4, were dissolved in buffer (0.01 M-Tris, pH 7.6, containing 0.15 M-NaCl) and passed through a short column (4 x 35 cm) of Sephadex G-100. The total UV-absorbing effluent was in each case pooled, desalted, freeze-dried and assayed for toxicity. The haemagglutinin was subsequently eluted with buffer containing 1M-glucose, dialysed and freeze-dried.

It was found that, while the haemagglutinin itself is not particularly toxic, in every case removal of the haemagglutinin resulted in a diminution of toxic potency (Table 4). Recombination of the haemagglutinin with the remainder of the eluted material resulted in an increased toxic potency, suggesting that the haemagglutinin and the remaining toxin(s) have a mutual potentiating effect so that a mixture of the two has a higher toxic potency than either of the components.

TABLE 4 Toxicity of various crude jack bean extracts, with and without haemagglutinin

Fraction	LD_{50} (mg)[1]	
	With Con A	Con A removed[2]
pH 7.0 extract (CJ-7.0)	3.4	> 5.0
pH 7.6 extract (CJ-7.6)	1.8	± 3.5
pH 7.0 extract, dialysed 24 h	0.9	± 4.3
pH 7.6 extract, dialysed 24 h	2.5	± 4.0
Concanavalin A[2]	> 5.0	
pH 7.6 extract, dialysed 24 h, plus concanavalin A (1 : 1, w/w)[2]	± 2.5	

1. x 50 = mg/kg
2. Insufficient material available for LD_{50} estimation. Values estimated from two levels of injection

3.3 Isolation of toxic fractions

3.3.1 Chromatography of jack bean extract (CJ-7.6)

Preliminary evaluation of the separation achieved on Sephadex G-100. As a means of evaluating the resolving power of the column, a sample (2 g) of CJ-7.6, dissolved in buffer, was chromatographed on a column (column 2) of Sephadex G-100 (4 x 95 cm) and samples (50 µl), corresponding to the apices of the six major peaks, were taken directly from the fraction collector tubes for analysis by disc gel electrophoresis. The elution profile, electropherograms and densitometric scans of the electropherograms are presented in Fig. 9.

Comparison of the elution profile obtained on this column with that obtained in a previous study (17) using a smaller (1.75 x 80 cm) column of Sephadex G-100 revealed that a better resolution was obtained on the larger diameter column.

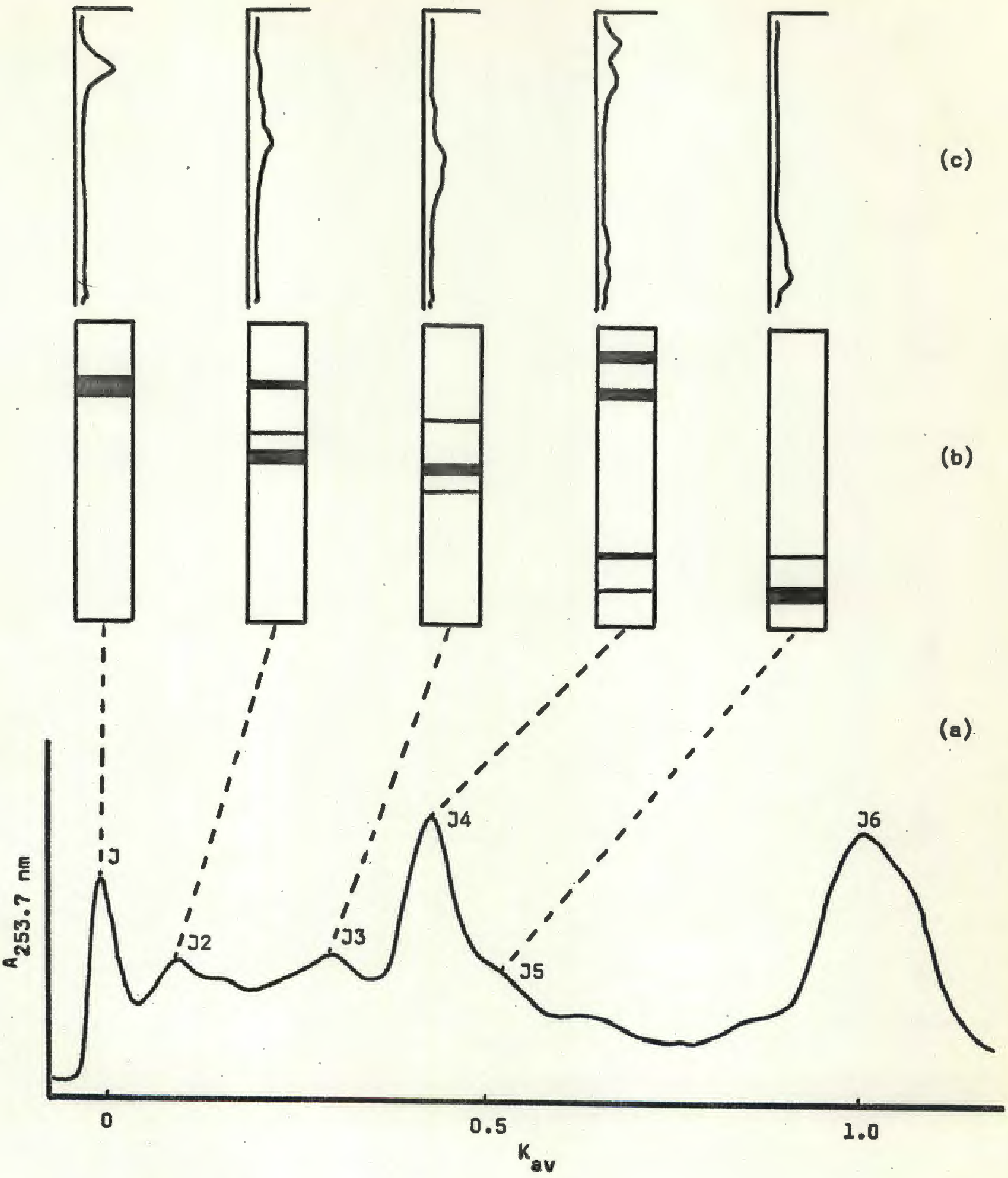


FIGURE 9 (a) Elution profile of CJ-7.6 on Sephadex G-100, column 2. Sample, 2 g CJ-7.6; column, 4 x 90 cm; Buffer, 0.01M tris, pH 7.6 containing 0.15M NaCl and 0.02% NaN_3 .

(b) Discontinuous slab gel electropherograms obtained from samples corresponding to the positions indicated on the elution profile.

(c) Densitometric scans of the gel electropherograms

This beneficial effect of increased column size has also been observed on a different protein system by Olson and Liener (73). Haglund (62) has interpreted the improved resolution on larger columns in terms of the mixing of sample zones caused by parabolic flow in the capillary tubing used to conduct the effluent into the photometer. He points out that for a given linear separation, the volume separation between two bands is larger on a larger diameter column and consequently the effect of parabolic flow (laminar flow) mixing is less than in the case of a smaller diameter column.

Analysis by disc gel electrophoresis revealed that the peaks were mainly heterogeneous but the first peak, corresponding to the toxin (fraction J; see below) yielded only a single band with an Rf value of 0.207. With the exception of two bands in peak J4, a trend in the electrophoretic bands, of increasing Rf values with increasing elution volume could be discerned. No bands were obtained from peak J6 and it is assumed that this consisted of small molecular weight material not fixed by 7% acetic acid.

Preparation of toxic fraction J. The toxic fraction J was prepared by chromatography and rechromatography on two different columns of Sephadex G-100. The first column (column 1) was a short column, used principally to remove the haemagglutinin, concanavalin A, which is adsorbed onto Sephadex G-100, and simultaneously to achieve a rough group separation. This is followed by chromatography on a long column (column 2, described above) of Sephadex G-100 to further purify the toxic fraction. An advantage of this two-column system is that only the short column requires the buffer change cycle needed to remove the bound haemagglutinin, so that this operation can be carried out in a minimum time.

For the preparation of large amounts of fraction J it was found convenient to have two identical short columns so that one could be regenerated while the other was being used. The toxic fraction obtained from two short column runs could be pooled before rechromatography on column 2.

The elution profile obtained by chromatography of CJ-7.6 (2 g) on column 1 is presented in Fig. 10 (a). Samples (50 µl) for analysis by disc gel electrophoresis (Fig. 10 (b)) were taken from fractions corresponding to the first peak. The fractions indicated in the figure were then pooled, concentrated by ultrafiltration and rechromatographed on column 2, yielding the elution profile presented in Fig. 11. Pooled fractions (Fig. 11) were desalted and freeze-dried, the freeze-dried material being denoted fraction J.

Removal of the haemagglutinin bound onto the resin of column 1 was effected by passage of one column volume of buffer containing 1 M-glucose as suggested by Agrawal and Goldstein (48). This method was preferred to the low pH method of Olson and Liener (72) as exposure of the buffer containing NaN_3 to low pH results in the liberation of the highly toxic gas, hydrazoic acid, HN_3.

3.3.2 Chromatography of soybean extract (CS-7.6)

As the soybean haemagglutinin does not bind onto Sephadex G-100 (74) the crude soybean extract was chromatographed directly on a long column of this gel (column 2, 4 x 95 cm). The elution profile, electropherograms and their densitometric scans obtained when a sample (2 g) of CS-7.6 was chromatographed on Sephadex G-100 and the effluent analysed by disc gel electrophoresis, are presented in Fig. 12.

The elution profile was essentially the same as that obtained for a similar extract by Catsimpoolas and Leuthner (74) and also has a marked resemblance to the elution profile obtained in the case of the jack bean (Fig. 9). Analysis of the effluent by disc gel electrophoresis, however, revealed that in general the various peaks yielded more bands than the equivalent peaks from the jack bean. Nevertheless certain common bands were observed; for example, peaks S3 and S4 contained major bands with Rf values of 0.49 and 0.89 respectively, corresponding to bands from the jack bean (peaks J3, J4 and J5).

The fractions corresponding to peak S, indicated in Fig. 12 were pooled, concentrated and desalted by ultrafiltration and freeze-dried, the freeze-dried material being denoted fraction S.

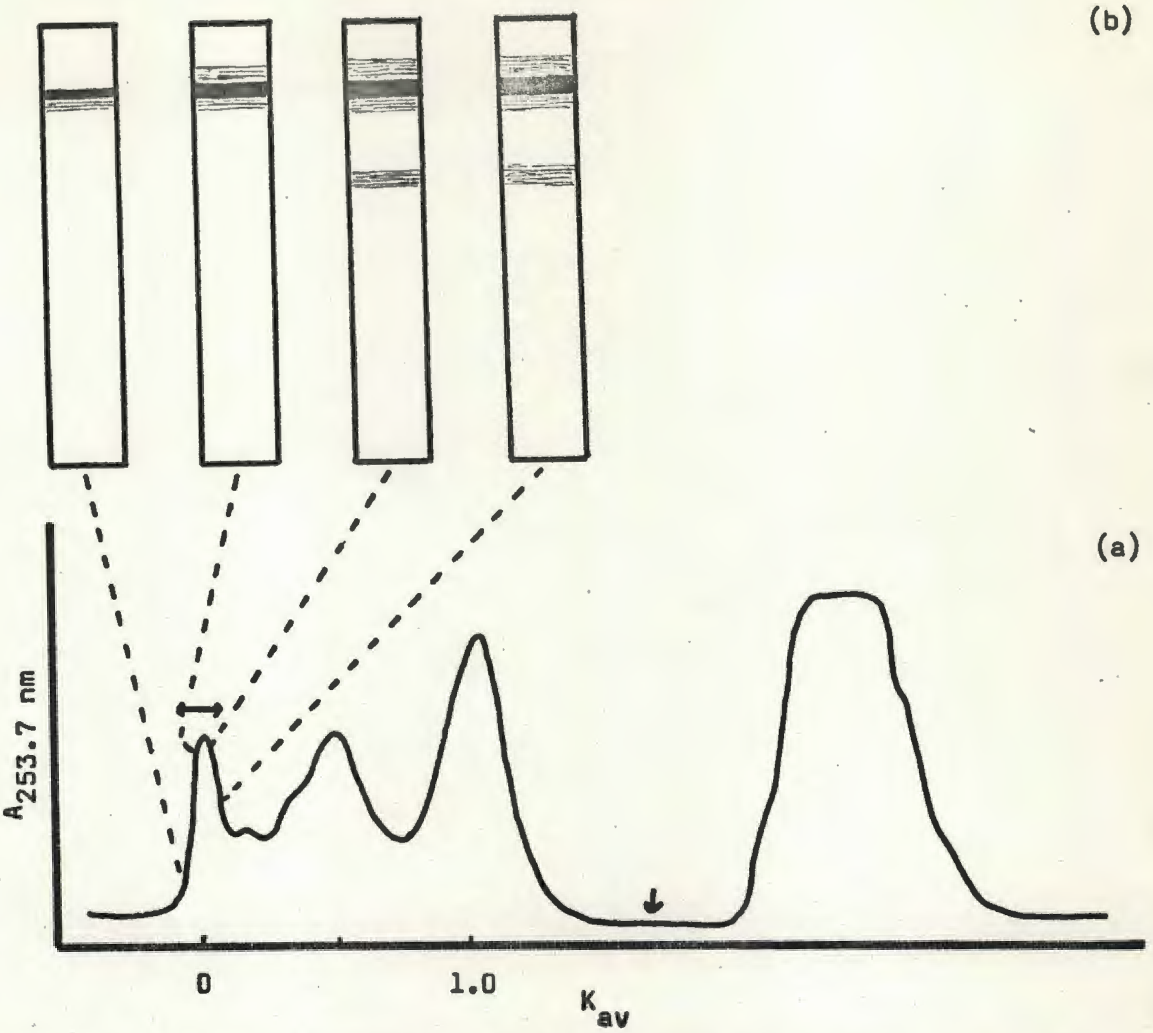


FIGURE 10 (a) Elution profile of CJ-7.6 on Sephadex G-100, column 1. Sample, 2 g CJ-7.6; Column, 4 x 35 cm; Buffer, 0.01M Tris, pH 7.6 containing 0.15M NaCl and 0.02% NaN_3 and, additionally, from point ↓, containing 1M glucose. ⟷ = Pooled fractions for rechromatography

(b) Discontinuous slab gel electropherograms obtained from samples corresponding to the positions indicated on the elution profile

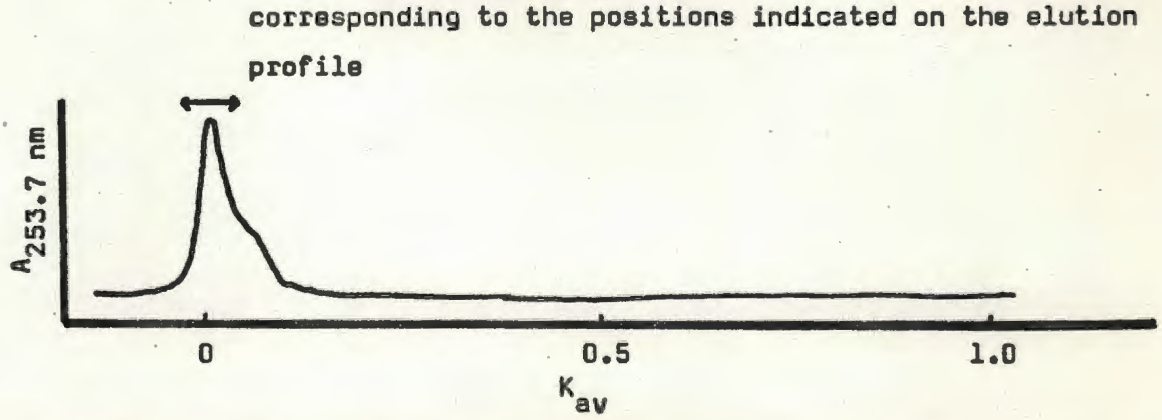


FIGURE 11 Rechromatography of CJ-7.6 peak 1 on Sephadex G-100, column 2. Sample, total yield from 4 g of CJ-7.6; Column, 4 x 90 cm; buffer, 0.01M Tris, pH 7.6 containing 0.15M NaCl and 0.02% NaN_3. ⟷ = Fractions pooled, desalted and freeze-dried

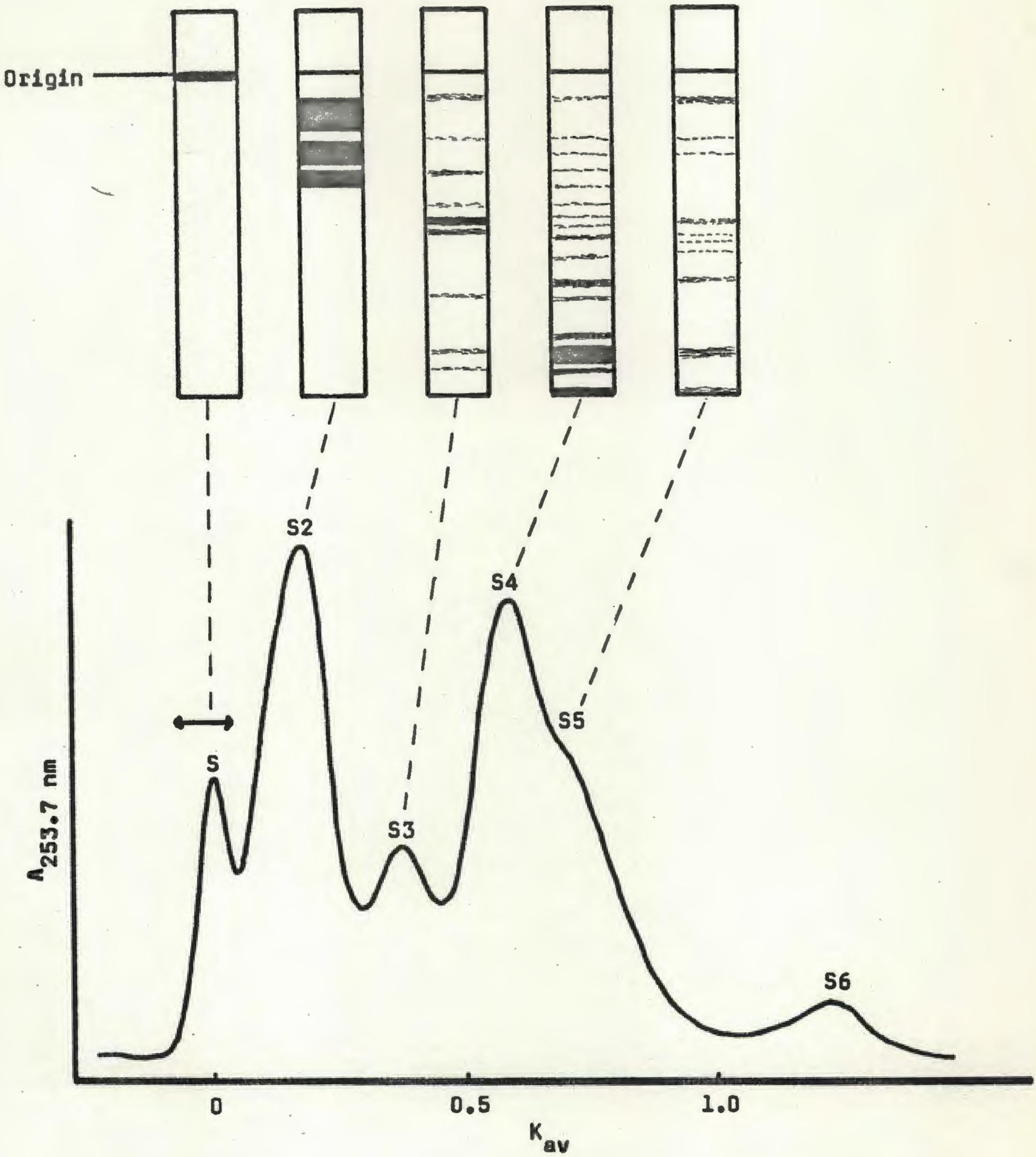


FIGURE 12 (a) Elution profile of CS-7.6 on Sephadex G-100.

Sample, 2 g CS-7.6; column 4 x 90 cm; buffer, 0.01M tris, pH 7.6, containing 0.15M NaCl and 0.02% NaN_3.

⟷ = Fractions pooled, desalted and freeze-dried.

(b) Discontinuous slab gel electropherograms obtained from samples corresponding to the positions indicated on the elution profile

3.3.3 Chromatography of NRY-bean extract (NRY - C1)

Chromatography on Sephadex G-100. Chromatography of the NRY-bean extract on Sephadex G-100 was attempted for comparison with the soybean and jack bean extracts. It was found, however, that at pH 7.6 the NRY extracts formed milky, viscous, colloidal solutions which would not readily flow into the Sephadex gel beds. Attempts to force the solutions into the gel beds by increased pressure resulted only in the bed being compacted.

It was found that NRY extract solutions could be clarified and converted to a less viscous form, either by treatment with 8 M-urea or by increasing the pH of the solution above pH 9.0. However, under these conditions the results would not be comparable to those obtained under the conditions used for the jack bean and soybean and consequently this avenue was not pursued further.

Chromatography on DEAE-cellulose. The NRY-bean extract (NRY - C1) was subjected to chromatography on DEAE-cellulose using essentially the procedure described by Stead (19) although this procedure was simplified by omission of the first buffer, the second buffer in Stead's system (i.e. 0.01M K-phosphate, pH 7.6, containing 0.015M NaCl) being used as the starting buffer.

Extract NRY-C1 (2 g) was suspended in starting buffer and dialysed overnight against identical buffer. The dialysed suspension was centrifuged (1,400 x g, 10 min) and the supernatant was applied to a column of DEAE-cellulose fibres (4 x 35 cm), previously equilibrated with starting buffer.

The elution profile obtained by stepwise elution of the NRY extract from the DEAE-cellulose is presented in Fig. 13. The fractions corresponding to the peak denoted fraction N were pooled, dialysed for 15 - 20 h at 2^{o}C against several changes of distilled water and freeze-dried.

Chromatography of fraction N on Biogel P20. The partially desalted and freeze-dried fraction N was resuspended in distilled water and subjected to

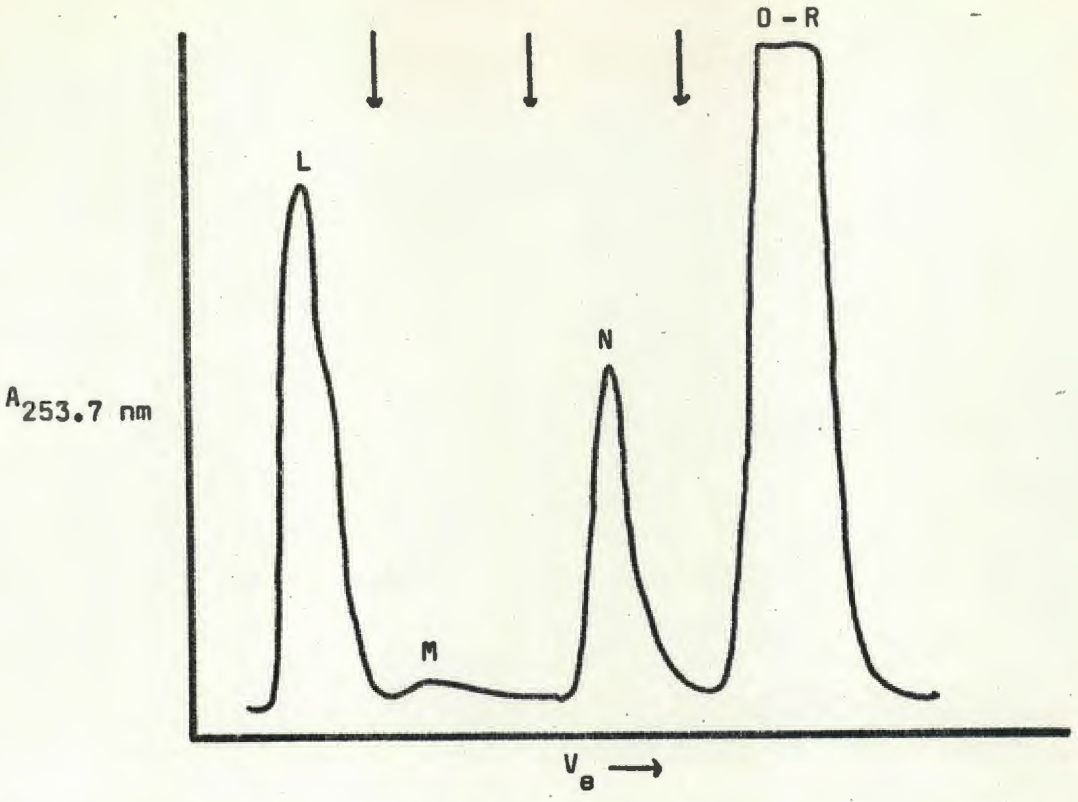


FIGURE 13 Elution profile obtained by stepwise elution of NRY-C from DEAE-cellulose. Sample, 2 g NRY-C;

column, 4 x 35 cm; buffers, starting buffer, 0.01M-K-phosphate + 0.15M NaCl, pH 7.6.

"M" buffer, 0.02M-K-phosphate + 0.25M NaCl, pH 7.6;

"N" buffer, 0.01M-K-phosphate + 0.60M NaCl, pH 7.6;

"0 - R" buffer, 2M NaCl.

Vertical arrows indicate buffer changes

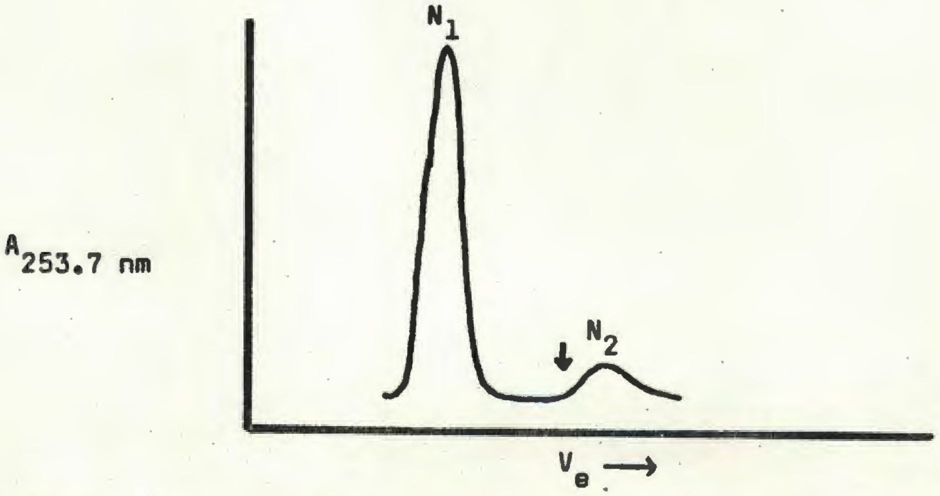


FIGURE 14 Chromatography of fraction N on Bio-gel P20.

Eluant, distilled water; ↓ = position of salt front chromatography on a column (2.5 x 30 cm) of Bio-gel P20 using distilled water as the eluant. The resulting elution profile, which was identical to that obtained by Stead (19), is presented in Fig. 14. The fractions corresponding to the peak designated fraction N_1 were pooled and freeze-dried.

3.3.4 Yields and biological activities of toxic isolates

The yields, haemagglutinating activity and toxicity of the various isolates are summarised in Table 5.

TABLE 5 Yields and biological activities of isolates

Fraction	J	S	N_1
Yield (mg/kg bean meal)	150	300	1000
Yield (mg/2 g crude extract)	30	60	111
HU/mg	0.0	0.0	49
Toxicity at 250 mg/kg	5/5	5/5	5/5

As it was intended to conserve material for the studies reported in Chapter 5, the LD_{50} values of the various toxins were not determined. However, from the limited number of injections which have been performed, the author is of the opinion that fraction N_1 is more toxic than fraction J which is more toxic than fraction S.

3.4 Discussion

A recurring problem in protein isolations involves the reproducibility of the procedure. In certain types of isolation, where the activity of interest can be quickly assayed, reproducibility is less important as the procedure can, in effect, be steered in successive runs to ensure maximal yields. However, in cases where the activity can only be assayed by a slow, tedious process it is important that the method be accurately reproducible as, of necessity, subsequent runs are often carried out without activity monitoring.

With these considerations in mind extraction procedure I was developed as a suitable "first approach" which could be applied with minimum previous knowledge of the protein system under study and which would, moreover, be accurately reproducible. Complete reproducibility was not achieved, however, as presumably the acetone precipitation step caused a variable amount of denaturation of the protein components. Provided that the large capital expenditure could be justified, removal of small molecular weight components and concentration of the extract solution could possibly better be effected by means of a large scale flow-through ultrafiltration system.

The reproducibility with which the extracts may be prepared is an important consideration for unless this reproducibility is known, the validity of comparisons between extracts prepared under different conditions cannot be assessed. The significance of the differences between the extracts prepared by different methods (Table 4) is consequently not known. On the other hand, the differences in the toxic potencies of the same extract before and after treatment with Sephadex G-100 (Table 4) may be considered highly significant as in this case the comparison is within groups rather than between groups.

Poor reproducibility in any step can be tolerated if this step is followed by a separating procedure which is not concentration dependent, and can thus accomodate differences in the proportions of the various components, and is also semi-analytical so that the effect of variations at the poorly reproducible step can be assessed. Molecular exclusion chromatography fulfils these requirements and hence was used in the case of jack bean and soybean extracts. Failure of this approach in the case of the NRY extract emphasises, however, that universally applicable methods are difficult to achieve.

In view of the failure of molecular exclusion chromatography in the case of the NRY-system, Stead's procedure of extraction and subsequent chromatography on DEAE-cellulose was used. This methodology is not optimal, however, as the extraction procedure involves ammonium sulphate precipitation, a technique which Dixon and Webb (75) have indicated may be poorly reproducible.

Furthermore, extended periods of dialysis against tap and distilled water were used, increasing the possibility of degradation of the proteins and contamination with metal ions. Despite these reservations, Stead's methods were found to be effective in this study and had the additional advantage that further findings could be related to the earlier work on the NRY-toxin.

It is of some interest to speculate why ion-exchange chromatography was successful in the case of the NRY extract while molecular exclusion chromatography was not. As has been mentioned earlier, molecular exclusion chromatography could not be used as the NRY extract, dissolved in pH 7.6 buffer, yielded an opaque, viscous colloidal solution. Increasing the pH to values greater than pH 9.0 or inclusion of 8 M-urea in the buffer, solubilised the colloidal material, yielding a clear solution. It would appear, therefore, that the visco-elastic nature of the solution is due to both ionic and urea-sensitive (hydrogen or hydrophobic bond) interactions. Solubilisation at values above pH 9.0 suggests the involvement of an ionising group having a pK_a value in the region of pH 9.0 to pH 10.0. This is the pK_a range of the lysine ε-amino group (76) and during chromatography on DEAE-cellulose at pH 7.6 the lysine-NH_3^+ group, if this is indeed involved in complex formation, would encounter competition from the $DEAE^+$ group which could thus disrupt the colloidal complex structure. It may therefore tentatively be suggested that ion-exchange chromatography is possible for the NRY extract because the open fibrous nature of the resin bed permits initial entry of the colloidal solution and that subsequently the colloidal complex is disrupted on the ion exchanger matrix.

The behaviour of the unfractionated NRY extract at high pH values and upon exposure to 8 M-urea is particularly noteworthy, and should be borne in mind for comparison with the behaviour of the fraction N_1 under similar conditions, to be described in Chapter 5.

The technique of analysing a protein mixture by chromatography followed by electrophoresis of the fractions, this being essentially a two-dimensional analysis, is currently finding favour and has been used by Okude and Iwanaga (77) and Tahagi and Iwanaga (78) amongst others. However, as far as the author is aware, the technique of applying samples directly from the column effluent fractions, without pooling and/or desalting and freeze-drying these fractions, is novel. By the use of this technique, which exploits the concentrating ability of the stacking phase of disc gel electrophoresis, the chromatographic effluent fractions can be studied quickly and directly so that possible modifications induced by desalting and drying steps are avoided. Analysis of a chromatographic effluent by disc gel electrophoresis also provides a sound basis upon which to decide which fractions should be pooled to obtain a maximum yield of a given fraction with minimal contamination from adjacent fractions.

The patterns obtained by disc gel electrophoretic analysis of the soybean Sephadex effluent fractions make an interesting comparison with the patterns obtained by Catsimpoolas and Leuthner (74) by iso-electric focussing of equivalent fractions. For example, in this study it was found that peak S apparently consisted of high molecular weight material which failed to penetrate the running gel. Catsimpoolas and Leuthner found that an equivalent fraction also would not focus isoelectrically in a 5% polyacrylamide gel, a result confirmed later in this study (see Chapter 5). It is also interesting that disc gel electrophoresis yielded a greater number of bands than iso-electric focussing, a phenomenon discussed in Chapter 5.

The mutual potentiating effect with respect to toxic potency of the jack bean toxin and haemagglutinin is an interesting phenomenon which provides an explanation for the loss of toxic units which accompanies separation of these components. A similar effect of diminished toxicity has been observed in the case of the separation of the toxin from other components of a Natal round yellow bean extract (19) and this could also be due to a mutual potentiating effect between the toxin and another component, possibly the haemagglutinin.

The mutual potentiating effect is not entirely unexpected in the case of a non-specific activity such as lethal toxicity as an assault by the first toxin might be expected to lower the animal's physiological resistance to a challenge by the second toxin. The effect cannot, therefore, necessarily be attributed to either a molecular interaction of the factor/cofactor type or to a common locus of action. The mutual toxicity enhancement of these two factors is, however, an important point in relation to the toxicity of the whole bean and the possible involvement of further factors in this overall toxicity cannot be excluded at this stage.

The methods described in this chapter have been successful insofar as the preparation of comparable extracts from the jack bean and the soybean are concerned. These extracts additionally proved to be very similar and equivalent fractions were prepared from each. Attempts to prepare a comparable extract from NRY-beans were not successful, however, and chromatography on Sephadex G-100 was also not feasible in this case. As it was obvious that the NRY-bean was different to the other beans, a compromise was reached and the proven method of Stead was used to prepare a toxic isolate from this bean.

CHAPTER 4

ATTEMPTED APPLICATION OF TANNED GELATIN IN THE ISOLATION OF THE JACK BEAN TOXIN

In the period immediately preceding commencement of the present project, Stead (19) had shown in this laboratory that tanned gelatin, a medium developed for molecular exclusion chromatography by Polson and Katz (38), had remarkable mechanical strength so that columns of this material, with an exclusion limit approximately equivalent to Sephadex G-200, could be operated virtually indefinitely at high flow rates and with high sample loads. Furthermore, from the results which he obtained by chromatography of two legume proteins of known molecular weight, Stead suggested that tanned gelatin appeared to have a resolving power greatly superior to that reported for agarose (see Fig. 27).

In view of these properties, tanned gelatin appeared extremely attractive as a chromatographic medium for the preparation of legume toxins. In particular, the tolerance of high sample loads seemed ideally suited to the preparation of the large amounts of material required for the quantitative toxicity assay (more than 100 mg of material is required for the assay of a toxin having an LD_{50} of 5 mg) while the good flow properties suggested that the chromatographic runs could be carried out expeditiously.

It was therefore considered that an investigation of tanned gelatin as a chromatographic medium was justified in the present context and this material was consequently employed in an attempted alternative procedure to isolate a toxic fraction from the jack bean. This chapter commences with a section on the evaluation of tanned gelatin as a medium for chromatography, a study which was considered necessary as very little information on this material has been published. The second section deals with the preparation of a toxic fraction from a jack bean extract by chromatography on tanned gelatin and the characterisation of this fraction. The latter studies yielded results which were not in accord with the information already available on the jack bean toxin (17, 18) and the third section of this chapter therefore deals with an investigation into the reasons for this disparity. For this purpose the separation obtained on tanned gelatin was more closely analysed by disc gel electrophoresis of the effluent fractions.

It will become apparent that, for several reasons, tanned gelatin is not as suited to the separation of legume proteins as had been anticipated. However, despite the fact that use of this gel was eventually abandoned, some interesting properties of the gel were discovered which could be of value in other applications.

4.1 Preliminary studies on tanned gelatin as a medium for exclusion chromatography

4.1.1 Selection of optimal gelatin concentration

Initially, use was made of Difco gelatin, fractionated according to the procedure described by Polson and Katz (38), but as this material could not be reproducibly prepared a change was made to an acid process gelatin made by Messrs Canada Packers Ltd. As the fractionation properties of a 4% gel prepared with this material differed considerably from those previously observed with 4% fractionated Difco gelatin, a systematic study of the relationship between gel concentration and exclusion limit was undertaken.

A series of gels was prepared, covering the concentrations 3%, 4%, 6%, 8% and 12%. Standard proteins of known molecular weight were subjected to chromatography on these gels and for each gel a standard curve relating V_e to log molecular weight was constructed, as suggested by Andrews (79, 80) (Fig. 15). The exclusion limit was defined as the molecular weight corresponding to the point of coincidence of an extrapolation of the linear portion of the curve with the horizontal line through the void volume (Fig. 15). The exclusion limit was

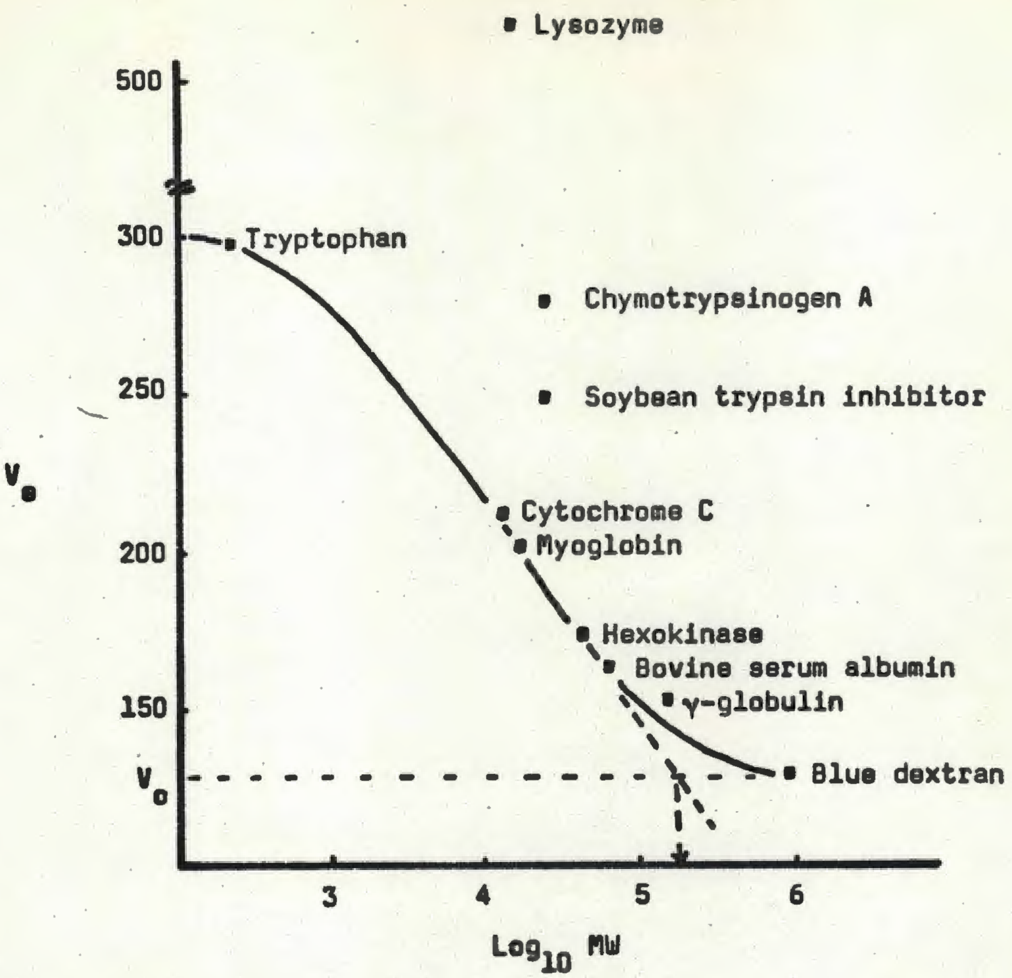


FIGURE 15 Relationship between V_e and $\log_{10}$ MW on 4% chromium-formalin tanned gelatin (Canada Packers Ltd.)

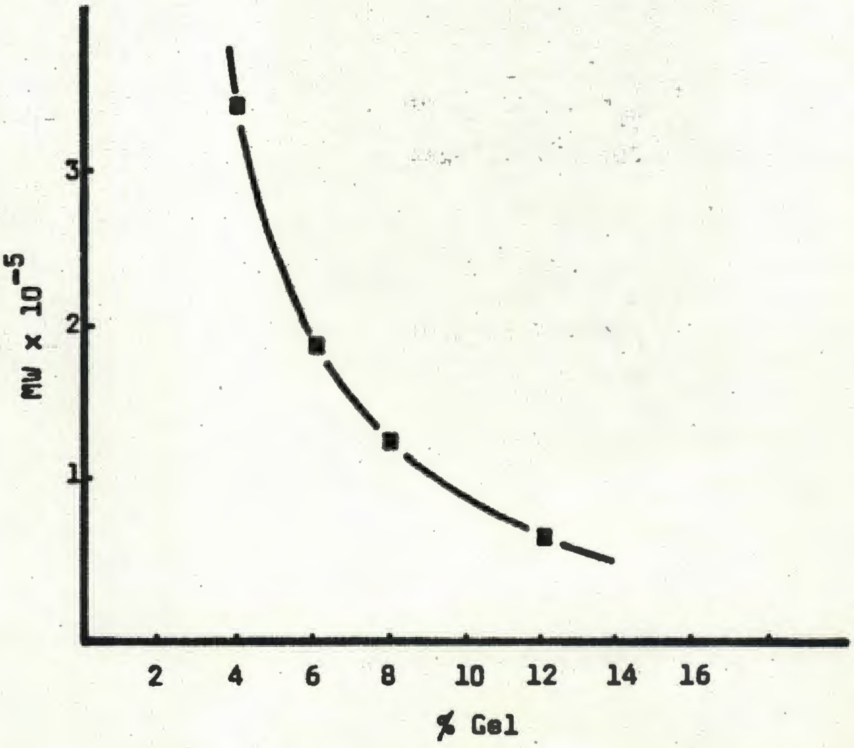


FIGURE 16 Relationship between gel concentration and exclusion limit on chromium-formalin tanned gelatin (Canada Packers Ltd, 275 bloom.)

determined for each gel and a curve relating the exclusion limit to the gel concentration was constructed (Fig. 16). From Fig. 16 it was decided that an 8% chromium-formalin tanned Canada Packers gelatin would be optimal for the separation of the toxin from the bulk of the contaminating material as the former is known to have a molecular weight of ± 150,000 (18).

An analytical separation of the crude jack bean toxin, CJ-7.6, on an 8% Canada Packers gel gave a separation consisting of a small excluded fraction, corresponding to the toxin, and a larger included fraction (Fig. 17). As this separation was considered satisfactory for the preparation of the toxin, an 8% Canada Packers gel was selected for preparative scale separations (see section 4.2.1).

4.1.2 Investigation of retardation phenomenon (see also ref. 41)

In establishing the relationship between the elution volume and the molecular weight of various standard proteins chromatographed on tanned gelatin gels (Fig. 15) it was observed that certain sample proteins, notably chymotrypsinogen A, lysozyme, soybean trypsin inhibitor and trypsinogen are retarded more than expected from their molecular weight so that, contrary to the conclusion drawn by Polson and Katz (38), separation is not solely dependent upon molecular size. This is an interesting observation as it has the consequence that the results of separations of unknown mixtures on tanned gelatin cannot, with confidence, be interpreted on the basis of molecular exclusion theory. This fact constitutes a potential disadvantage of the gel and therefore a study was undertaken, aimed at establishing the origin of the effect and possibly eliminating it.

Factors responsible for the retardation effect could possibly include ion-exchange interactions, co-ordinate bonding with the chromium or, as suggested by Dr A. Polson (personal communication) sulphydryl-disulphide exchanges between the gel and the sample protein. In an attempt to evaluate the contribution of these factors, chymotrypsinogen A was selected for study on an 8% gel. In

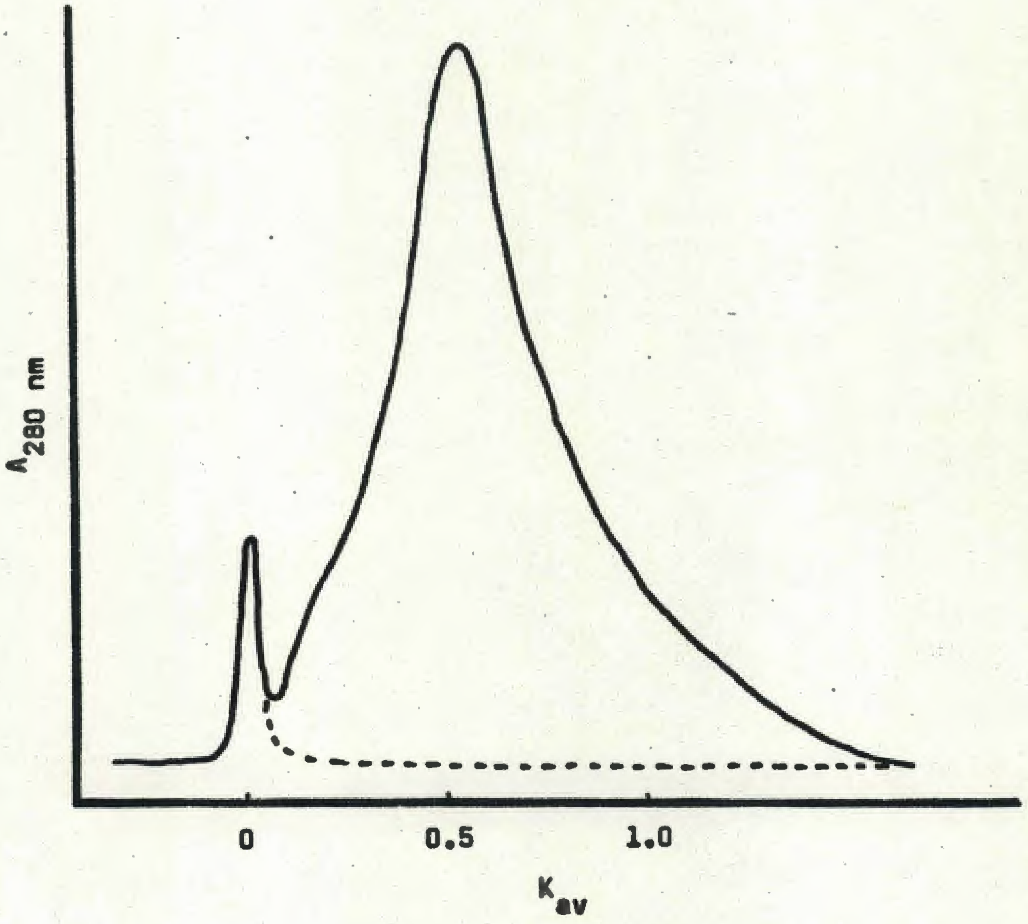


FIGURE 17 Separation of CJ-7.6 on 8% Canada Packers chromium-formalin tanned gelatin

———— : elution profile of CJ-7.6;

------ : elution profile of purified toxin (fraction 1A, ref. 18) successive experiments the effects of the following changes to the eluting buffer were investigated, the column being equilibrated in each case with the new buffer before addition of the sample:

(i) increasing the concentration of NaCl to 1.5 M;
(ii) addition of EDTA (0.01 M);
(iii) addition of 2-mercaptoethanol (1×10^{-3} M).

None of these treatments reduced the retardation of the protein sample and it was concluded that retardation is probably not due to any of the factors in question.

Another possibility, that retardation might be due to hydrogen bond or hydrophobic bond interactions between the gel and the sample, was tested by adding urea to the eluting buffer. Addition of 6 M-urea caused a splitting of the chymotrypsinogen peak into two fractions, one of which appeared in the retarded position and one of which was not retarded relative to cytochrome C. This could indicate that the preparation used was not pure. Addition of 8 M-urea caused all of the chymotrypsinogen to be eluted before cytochrome C and it would appear, therefore, that the retardation is abolished by high urea concentrations. At the same time the addition of urea had the disadvantage that the major attractive feature of tanned gelatin, its mechanical strength and resistance to compaction, is also completely destroyed.

Although the effect of high urea concentrations indicates that the retardation effect is due to either hydrogen bond or hydrophobic bond interactions, it does not indicate which of these two effects is chiefly responsible. In an attempt to resolve this question, the polarity of the buffer was reduced by the addition of methanol to 20% (v/v) and a sample of lysozyme was chromatographed in this buffer (chymotrypsinogen A being not soluble in this buffer). The elution position of lysozyme was increased in the 20% methanol buffer, thus providing a strong indication that hydrogen bonding and not hydrophobic bonding is responsible for the retardation phenomenon. The non-involvement of hydrophobic bonding was confirmed by chromatography, in the absence of methanol, of tryptophan, phenylalanine and L-phenylalanine benzyl ester, all of which have hydrophobic side chains but were nevertheless not retarded.

4.1.3 Protease digestion of tanned gelatin

Tanned gelatin, being comprised of a largely protein matrix, would be expected to be susceptible to digestion by proteolytic enzymes. This is an important consideration for, if a particular sample should happen to contain a proteolytic enzyme, it might be difficult to distinguish between impurities in the sample and by-products caused by proteolysis of the gel matrix. Polson and Katz (38) have claimed that tanned gelatin is not digested by proteolytic enzymes but as a test method these authors simply observed the gel particles under a microscope to see if these were solubilised by the enzymes.

In this study, the effect of proteolytic enzymes during chromatography was regarded as of greater interest and, as a test system, two proteolytic enzymes, trypsin and α-chymotrypsin and their zymogens, trypsinogen and chymotrypsinogen A, were subjected to chromatography on tanned gelatin (Figs. 18 and 19). It may be seen from Fig. 18 that in the case of chymotrypsin, the area under the peak is very much greater than the area under the peak yielded by an equivalent amount of the zymogen, suggesting that the gel matrix is indeed digested by the enzyme, yielding additional UV-absorbing material. Although gelatin contains only a small percentage of aromatic amino acids (81) chymotrypsin would tend to selectively release peptides containing aromatic residues. Serial rechromatography of fresh batches of enzyme on the same gel yielded a diminished peak after the first run but this then remained virtually constant but still larger than the zymogen peak in subsequent runs (Fig. 18) suggesting that digestion continued with continuing exposure and that the gel apparently did not consist of an easily digested portion and a digestion-resistant portion.

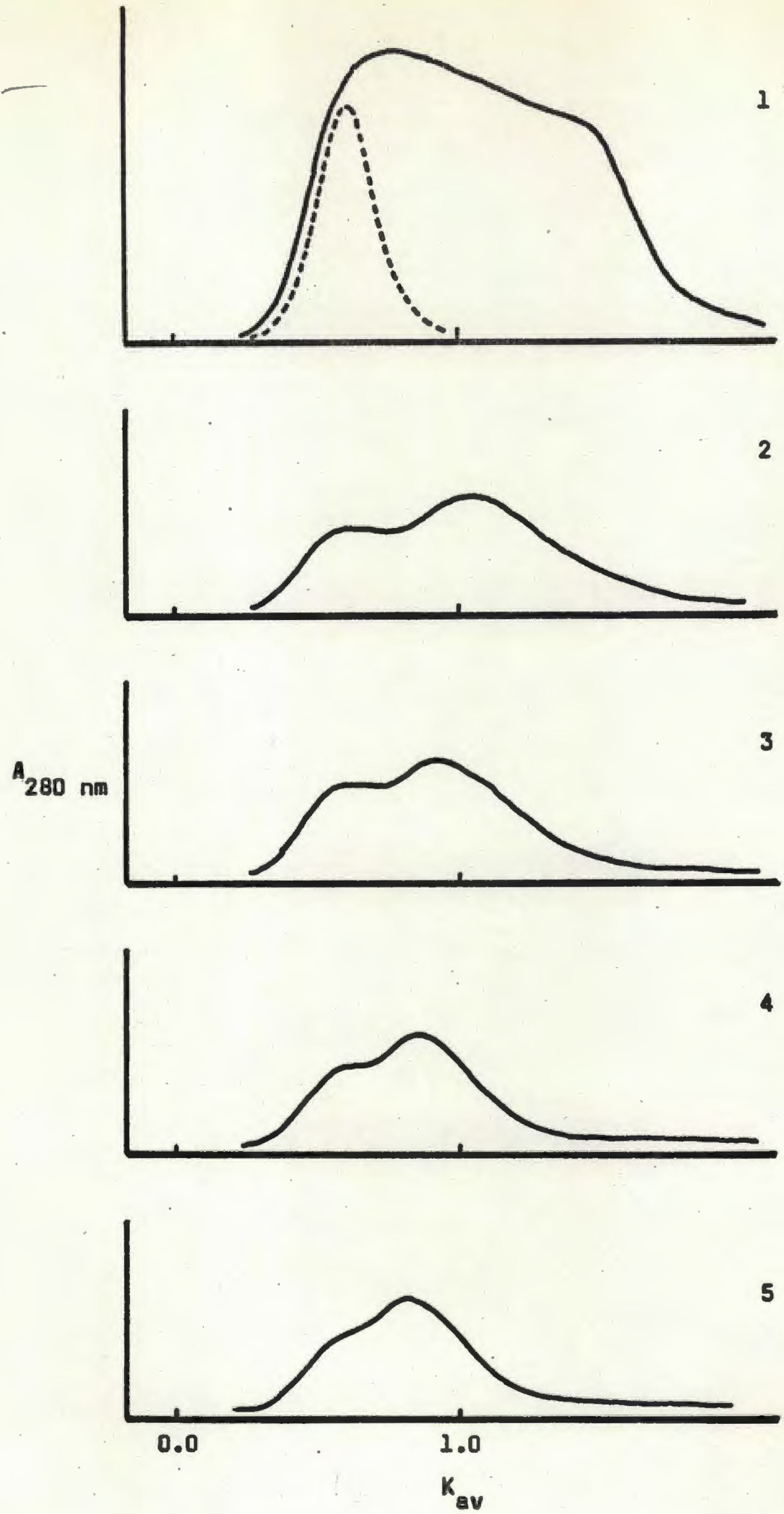


FIGURE 18 Chromatography of α chymotrypsin and chymotrypsinogen A on 4% chromium-formalin tanned gelatin.

——— = elution profile of α chymotrypsin; - - - = elution profile of chymotrypsinogen A. A fresh sample of α chymotrypsin (5 mg) was used for each chromatography run. Numbers 1 - 5 refer to the sequence of chromatographic runs carried out on a freshly prepared column of tanned gelatin

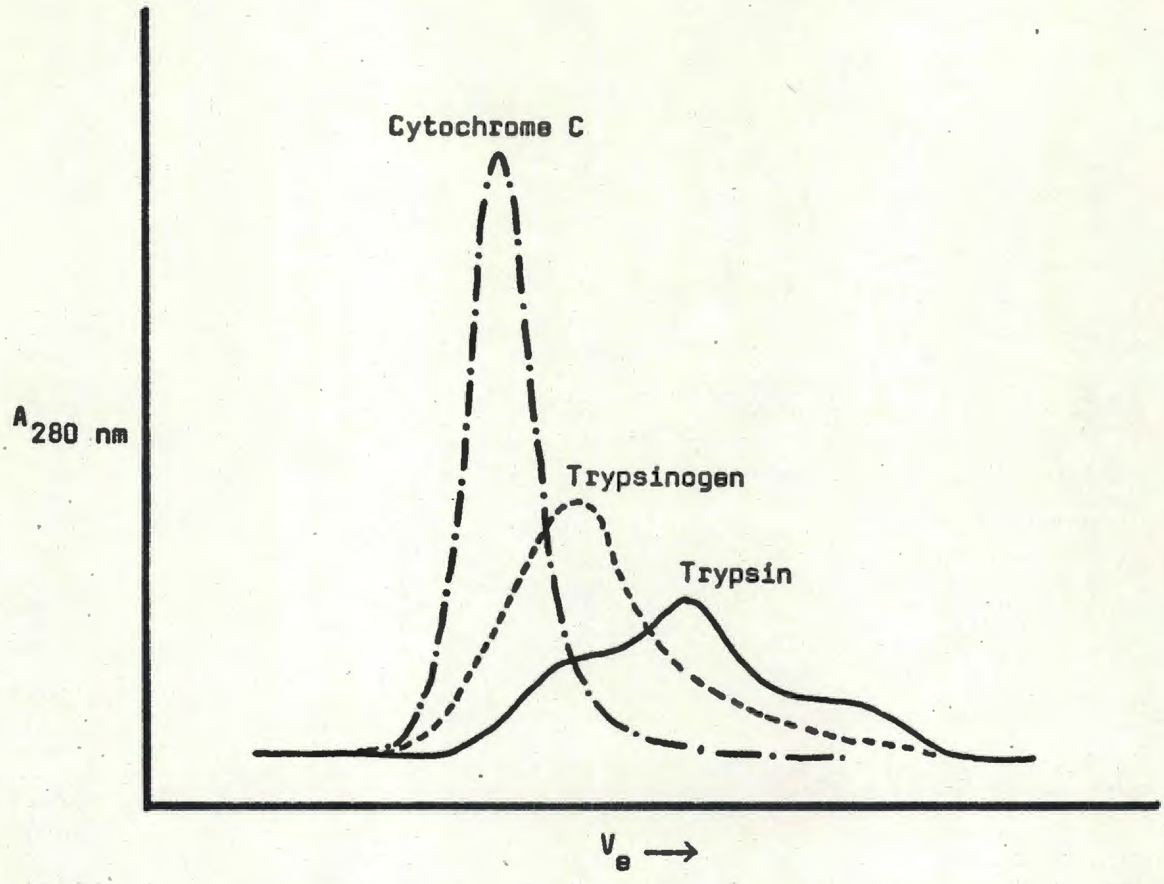


FIGURE 19 Chromatography of trypsinogen and trypsin on 4% chromium-formalin tanned gelatin.

——— = elution profile of trypsin; - - - = elution profile of trypsinogen; ·–·–·– = elution profile of cytochrome C reference peak. The order of chromatography was trypsin, trypsinogen, cytochrome C. A freshly prepared column of tanned gelatin was used

In the case of trypsin the enzyme peak was of a different shape compared to the zymogen peak but the area under the two peaks was not markedly different (Fig. 19). Trypsin has a more restricted specificity than chymotrypsin and furthermore would not tend to liberate peptides containing aromatic residues so that the smaller peak in the case of this enzyme, compared to chymotrypsin is not unexpected.

4.2 Use of tanned gelatin in the isolation of the jack bean toxin

Although tanned gelatin had been found to have certain disadvantages, notably in the retardation effect and the susceptibility of the gel to protease digestion, these were considered not to exclude the use of this material in the isolation of certain proteins. In fact, the retardation effect, in particular, could offer an advantage for separation in certain cases. A study on the usefulness of tanned gelatin in the isolation of the jack bean toxin was consequently undertaken.

4.2.1 Preparative chromatography on tanned gelatin

The elution profile obtained by chromatography of CJ-7.6 (2 g) on a preparative scale column of tanned gelatin is presented in Fig. 20. This profile was different to the analytical scale separation (section 4.1.1, Fig. 17) in that a better resolution was obtained. A similar phenomenon has been observed in the case of chromatography on Sephadex G-100 and is discussed on page 59. The overall elution volume was divided into six fractions, each of which was tested for haemagglutinating activity and toxicity. These activities were found to be separated as indicated in Fig. 20, toxicity being concentrated in the plateau region following the first peak. Although it would be expected, from previous results on Sephadex G-100 (16), that toxicity would be concentrated in the first peak, the observation that proteins can be retarded on tanned gelatin provides an explanation for the results obtained here. The

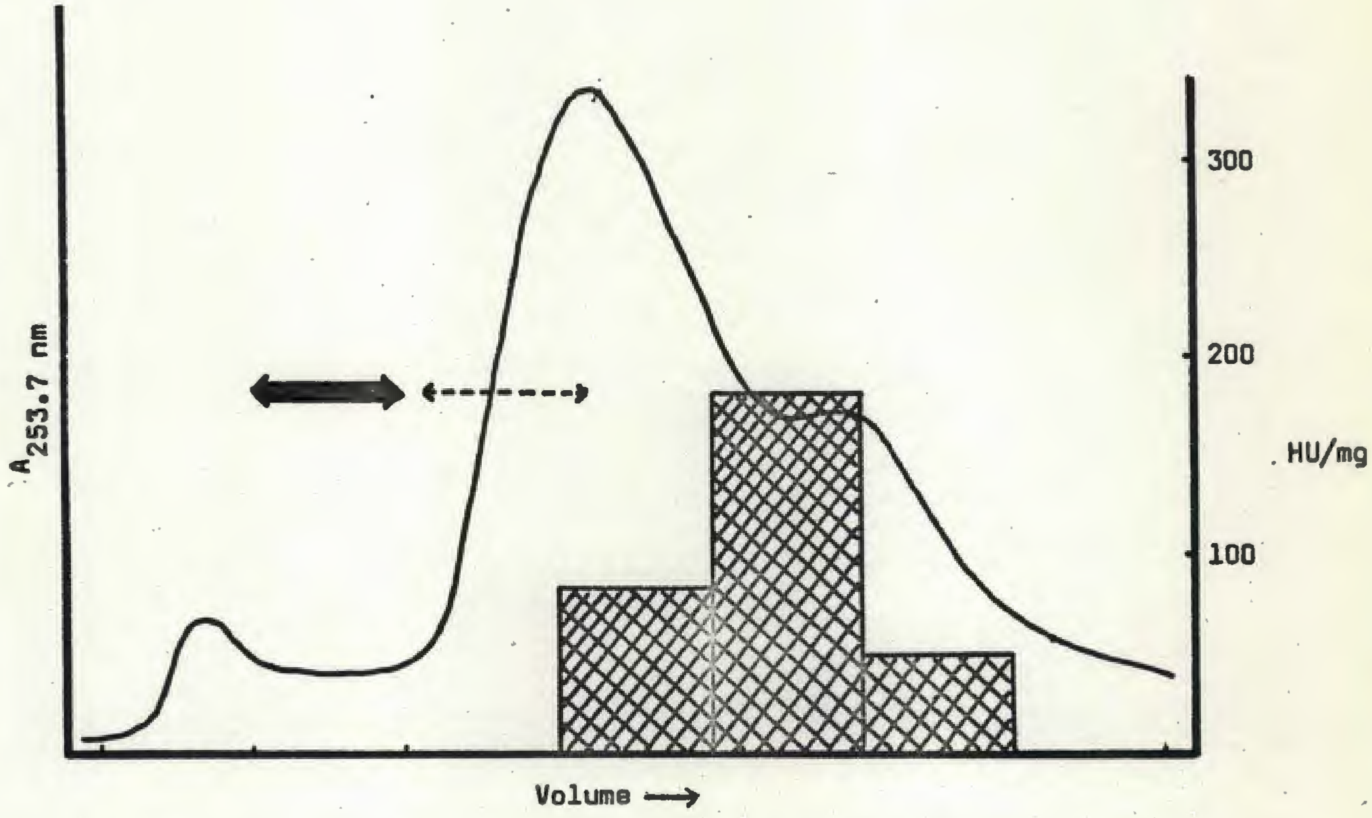


FIGURE 20 Preparative separation of CJ-7.6 on 8% chromium-formalin tanned gelatin.

Column, 4 x 150 cm; buffer, 0.01M tris, pH 7.6, containing 0.15M NaCl and 0.2% NaN_3; sample, 2 g CJ-7.6; ——— = absorbance at 253.7 nm; ⟺ = position of toxic activity (= fraction J_2); ← - → = position of post-chromatographic precipitate; cross-hatching indicates haemagglutinating activity fact that the haemagglutinin is not bound to the gel but is nevertheless separated from the toxin suggests that tanned gelatin has the advantage over Sephadex in that it does not need to be passed through a buffer change cycle to remove bound haemagglutinin, between successive chromatography runs. The haemagglutinin itself was eluted rather later than would be expected on the basis of its molecular weight but again this could be explained by the binding phenomenon mentioned earlier.

A further point of interest is the development of a precipitate in certain fractions after chromatography (Fig. 20). Formation of this precipitate suggests that protein-protein interactions might be modified by chromatography on tanned gelatin, as no equivalent precipitate was observed in the case of Sephadex (Fig. 9).

The toxic fraction, designated ⟷ in Fig. 20 as J_2, was selected for further study.

4.2.2 Characterisation of fraction J_2

Analytical chromatography on Sephadex. Chromatography of fraction J_2 on Sephadex resins yielded the elution profiles presented in Fig. 21. The best resolution was obtained on Sephadex G-200, in which case four peaks could be detected, but even here these peaks were poorly resolved. As only incomplete separation of fraction J_2 was achieved on Sephadex no attempt was made to carry out preparative separations on this material and the toxicity of the fractions was consequently not determined.

Gel electrophoresis. The electropherograms obtained by disc gel electrophoresis of fraction J_2 under different conditions are presented in Fig. 22. The most striking feature of these results is the demonstration that fraction J_2 is markedly heterogeneous, six major bands being detected in the high pH system

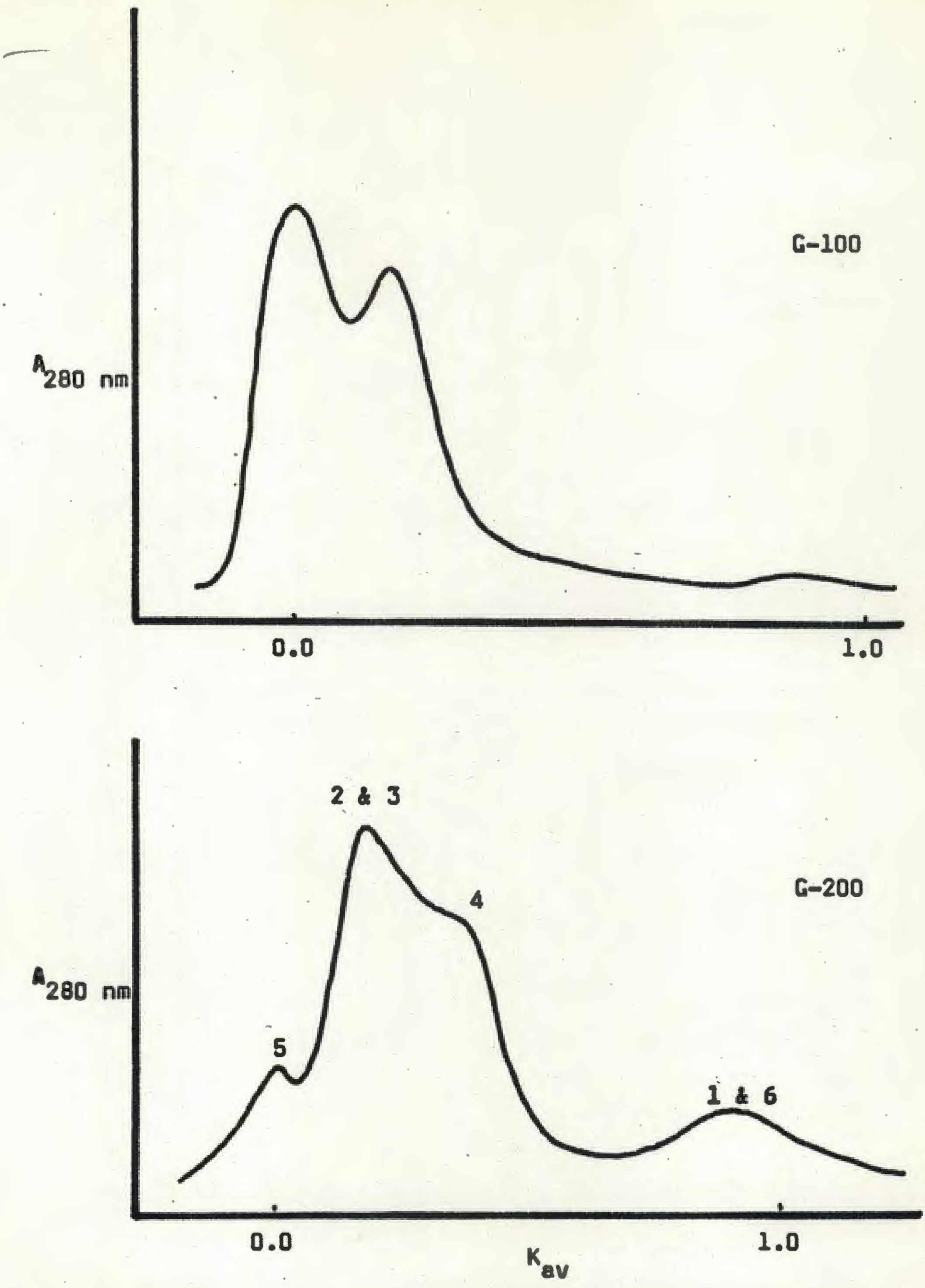


FIGURE 21 Rechromatography of fraction J_2 on Sephadex G-100 and G-200.

Columns, 2.5 x 90 cm; buffer, 0.01M tris, pH 7.6, containing 0.15M NaCl and 0.02% NaN_3.

Numbers 1-6 refer to numbers allocated to various antigens in an immunodiffusion experiment (see Fig. 23)

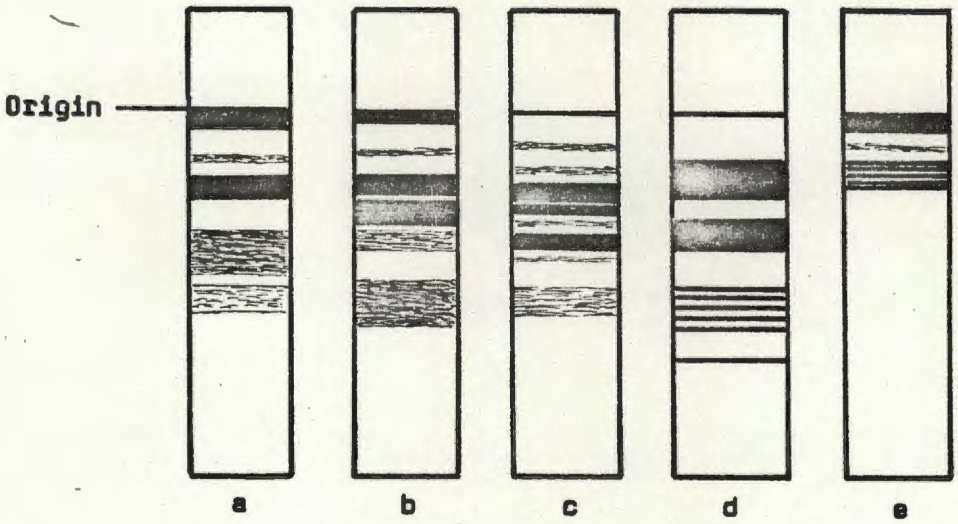


FIGURE 22 Disc gel electrophoresis of fraction J_2

a) low pH buffer system of Reisfeld *et al.* (53), 7.5% gel

b) as in (a) but with 8 M-urea, 7.5% gel

c) high pH buffer system of Davis (50), 7.5% gel

d) as in (c) but with 8 M-urea, 7.5% gel

e) as in (d), 15% gel

(Fig. 22 c) and five in the low pH system (Fig. 22 a) in the absence of urea. At high pH the bands were superimposed upon a diffuse background. Furthermore, regular multiple banding was observed at high pH in the presence of urea (Fig. 22 d). It would appear, however, that this effect was limited to only one of the components, as other fractions, not multiply-banded, were also observed.

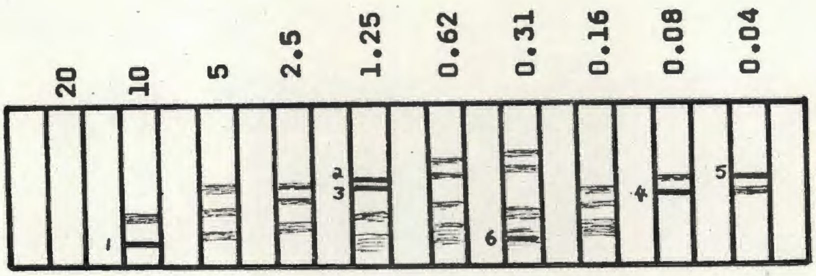
The number and distribution of the various bands was different at different pH values and in the presence and absence of 8 M-urea. This suggests that at least some of the components of fraction J_2 have a pH-sensitive oligomeric structure which could be dissociated by urea. Low pH appears to favour aggregation whilst disaggregation occurs at high pH.

Gel electrophoresis of fraction J_2 in sodium dodecyl sulphate yielded approximately thirteen bands, several but not all of which corresponded to bands also obtained in the case of fraction J (Fig. 40). It would appear therefore that fractions J and J_2 have certain subunits in common but that these fractions are not identical. The fact that a greater number of bands were obtained in the presence of SDS than in the presence of 8 M-urea suggested that urea does not bring about complete disaggregation of the oligomeric structure.

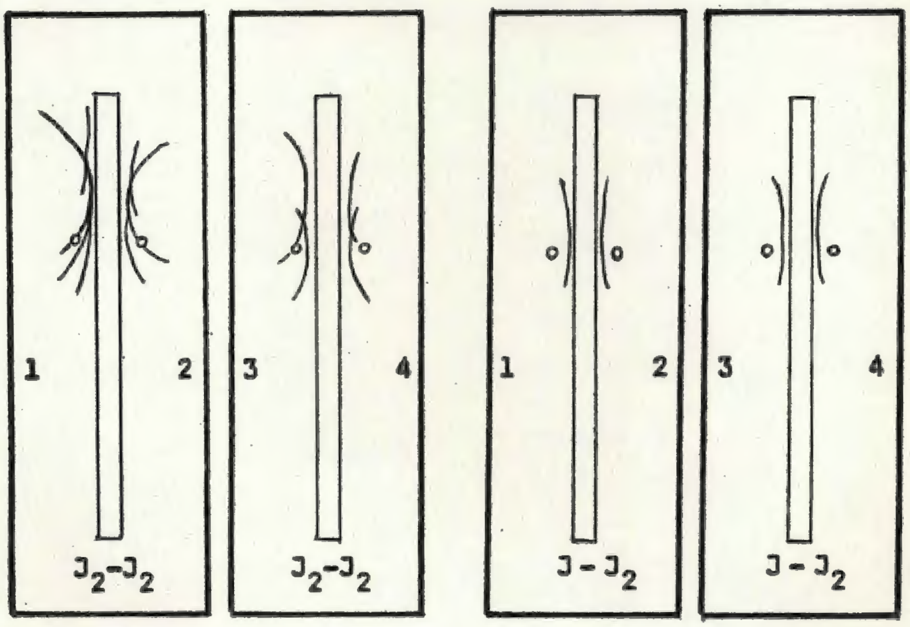
Immuno-diffusion. Immuno-diffusion of fraction J_2 against fraction J_2 antiserum, in the apparatus described by Polson (46), yielded a total of six bands, one of which was not ideally focussed and could not be accurately measured (Fig. 23).

The diffusion coefficients of the other antigens were; antigen 1, 3.35×10^{-5} cm^2 sec^{-1}; antigen 2, 5.19×10^{-7} cm^2 sec^{-1}; antigen 3, 6.60×10^{-7} cm^2 sec^{-1}; antigen 4, 6.87×10^{-7} cm^2 sec^{-1}; antigen 5, 3.77×10^{-7} cm^2 sec^{-1}. There is a possibility that the bands 2 and 3 might be due to a single antigen as double banding is a known phenomenon (82).

A particular point of interest in these results is antigen 1 which appears to have a small molecular weight, from its high diffusion coefficient. This antigen is present in fraction J_2 which was apparently of relatively high



<u>FIGURE 23</u> Immunodiffusion of fraction J_2 against J_2 antiserum in the apparatus of Polson (46). Antigen concentrations (mg/ml) are indicated above each well



<u>FIGURE 24</u> Immunoelectrophoresis of fractions J_2 and J against J_2 antiserum.

Numbers refer to antigen concentration, 1 = 60 mg/ml; 2 = 40 mg/ml; 3 = 20 mg/ml; 4 = 10 mg/ml molecular weight as it was eluted close to the void volume on a molecular exclusion/adsorption column (see Fig. 20). It would appear, therefore, that at least one of the components of fraction J_2 might have suffered a degree of dissociation after the chromatographic separation, thus giving rise to the small molecular weight antigen 1.

Antigens 2, 3 and 4, from the values of their diffusion coefficients, appear to have molecular weights of the order of 60,000 to 100,000 daltons, while antigen 5 is larger. The various antigens can therefore be tentatively correlated with the peaks obtained on Sephadex G-200 as suggested in Fig. 21.

Immuno-electrophoresis. Immuno-electrophoresis of fraction J_2 against J_2 antiserum revealed a total of four antigenic components (Fig. 24), somewhat fewer than were detected by immuno-diffusion.

Fraction J_2 antiserum was also tested for cross-reactivity with fractions J, S and N_1 (see page 114). Positive cross-reaction was obtained in the case of fraction J, indicating that fraction J and fraction J_2 have a common antigenic determinant (Fig. 24). This is not entirely unexpected as these two toxic fractions are derived from the same legume.

4.2.3 Rechromatography on tanned gelatin

Fraction J_2 was subjected to non-continuous recycling chromatography on a 3% tanned gelatin gel, this gel concentration being chosen as it was expected that fraction J_2 would have an increased elution volume and therefore be better separated on this material than on the 8% gel. The effluent from the preparative column corresponding to fraction J_2 was pooled and concentrated by ultrafiltration (UM-2 membrane, exclusion limit 1,000 daltons) to a volume of ± 2 ml. This sample was applied to the analytical column (3% gel) and after separation selected fractions were again pooled, concentrated and rechromatographed on the same analytical column. This procedure was repeated several times. The results of such a series of chromatographic runs are presented in Fig. 25

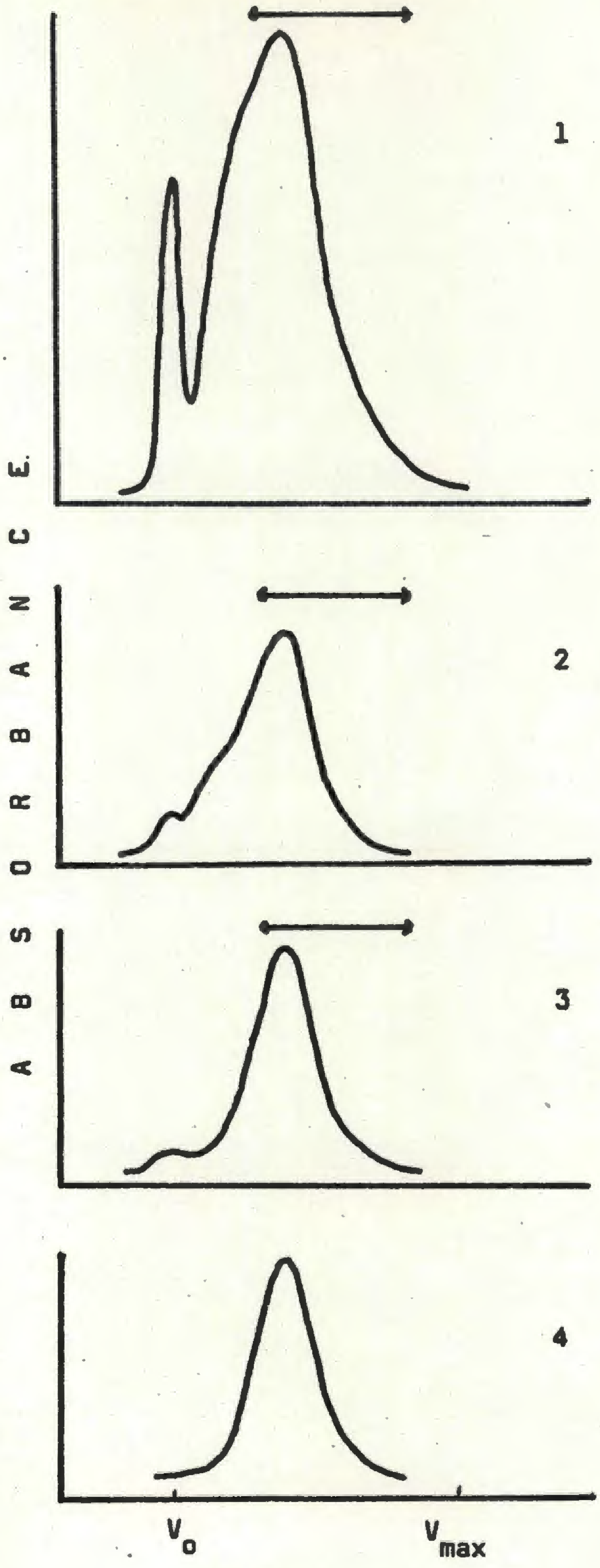


FIGURE 25 Rechromatography of fraction J_2 on 3% chromium-formalin tanned gelatin. ⟷ indicates fractions pooled and concentrated for rechromatography. Numbers 1 - 4 refer to the sequence of rechromatography

from which it may be seen that, from an original three peaks, after four rechromatography runs it was possible to obtain the major fraction in an apparently chromatographically pure state. The procedure used to obtain this fraction was time-consuming, however, and furthermore the end product was found to be non-toxic, as were the cut-off peaks.

A possible reason for the loss of toxic activity might have been the number of concentration steps involved in the non-continuous recycling procedure which extended the overall period required for preparation of the final product into several days. Consequently an attempt was made to carry out a separation in a shorter time by a continuous recycling procedure, using a Pharmacia G25/100 column equipped with flow adaptors. This procedure was also found to be impractical, however, as the apparatus required constant surveillance throughout a 36-hour period while the total yield from 50 mg of fraction J_2 was only 5 mg, sufficient for injection into only one mouse. The procedure was not investigated further.

4.2.4 Analysis of separation on tanned gelatin by disc gel electrophoresis

UV-analysis of the effluent from a chromatographic column provides a means of determining the distribution of total protein concentration but it is a non-specific method, incapable of distinguishing between individual molecular species. For this purpose an analytical method of greater specificity is required.

A suitable method of great sensitivity, and with broad-spectrum applicability, is the disc gel electrophoretic technique of Ornstein (49) and Davis (50). A particular advantage of disc gel electrophoresis for the analysis of a chromatographic effluent is that the concentrating effect of the stacking phase enables very dilute samples to be used so that samples can be taken directly from the fraction collector tubes without prior concentration and desalting. In this way secondary effects due to sample handling after chromatography can be minimised

It was reasoned that analysis of the overall separation by disc gel electrophoresis would enable the distribution of each protein species in the chromatographic effluent to be ascertained. In the present context this was considered desirable as the toxic fraction (fraction J_2) obtained by chromatography on tanned gelatin had proved to bear little relation to the fraction (fraction J) obtained using Sephadex G-100 (see Chapter 5). For example, fraction J_2 contained several proteins of a similar size which could not be adequately separated on Sephadex gels whereas fraction J did not contain equivalent contaminants. It was therefore of particular interest to ascertain the distribution of proteins in the effluent from tanned gelatin with a view to determining the position of the fraction corresponding to fraction J. Non-dissociating conditions were chosen for the electrophoretic separation as it was desired to establish the distribution of intact proteins rather than that of individual subunits.

A sample of CJ-7.6 (2 g) was dissolved in buffer (0.01 M tris, pH 7.6 containing 0.15 M NaCl and 0.2% NaN_3), cleared by centrifugation and separated on a preparative scale column (4 x 150 cm) of 8% tanned gelatin. The effluent from the column was monitored at 253.7 nm and collected in approximately 20 ml fractions. Samples (50 µl) for electrophoresis were taken directly from the fraction collector tubes with the aid of a micro-syringe (Hamilton Co., Whittier, Calif. U.S.A.) and were subjected to electrophoresis in the slab gel apparatus described in section 2.12.2, using the high pH system of Davis (50).

The elution profile obtained by chromatography of fraction CJ-7.6 on 8% tanned gelatin and the electropherograms obtained from the eluted material are presented in Fig. 26. This may be compared with the separation on Sephadex G-100 presented in Fig. 9, section 3.3.1. In the case of tanned gelatin, samples for electrophoresis were taken from all of the fraction collector tubes containing UV-absorbing material although for clarity only five representative electropherograms are presented in Fig. 26. These five pherograms nevertneless represent all of the bands obtained from the eluate from tanned gelatin.

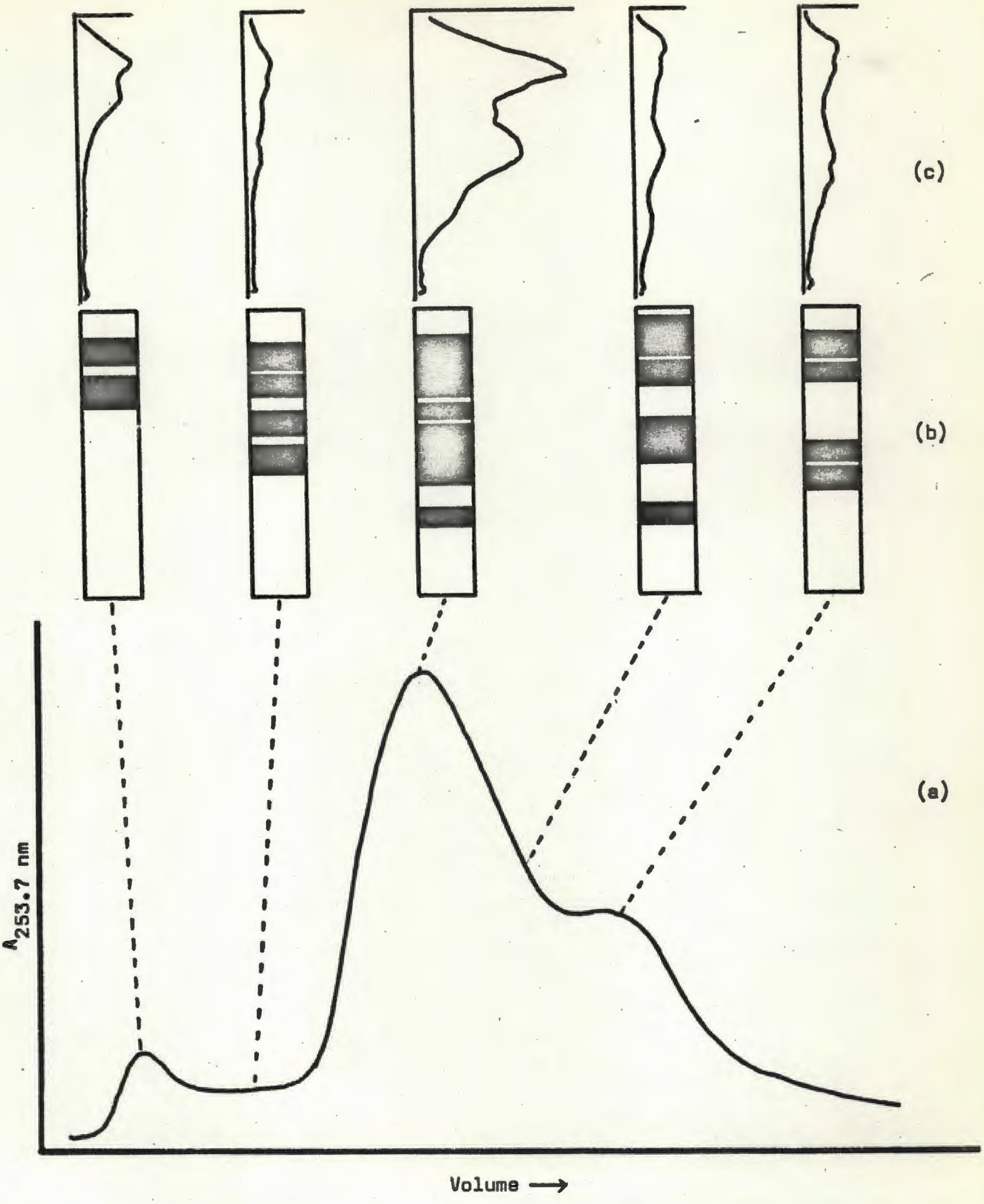


FIGURE 26 a) Elution profile of CJ-7.6 on 8% chromium-formalin tanned gelatin. Operating conditions as in Fig. 20.

b) Discontinuous slab gel electropherograms obtained from samples corresponding to the positions indicated on the elution profile.

c) Densitometric scans of the discontinuous slab gel electropherograms

Comparison of the electropherograms obtained on the eluates from tanned gelatin and Sephadex reveals that the tanned gelatin eluate yielded relatively broad, diffuse bands contrasting with the sharp, well defined bands obtained from the Sephadex eluate fractions. In addition, comparison of the Rf values of the various bands (Table 6) reveals that the Sephadex effluent yielded bands corresponding closely to those of the unfractionated material. In contrast, the tanned gelatin effluent yielded bands which largely did not correspond to those of the unfractionated material. It is also noteworthy that in the case of tanned gelatin several bands with a mobility of about 0.2 were obtained, whereas in the Sephadex eluate only the band due to the toxin and one other band had mobilities of this order.

TABLE 6 Electrophoretic Rf values of proteins from CJ-7.6 before and after chromatography on Sephadex G-100 and 8% tanned gelatin (TG)

Band No.	Unfractionated	Chromatographed on Sephadex (Fig. 9)	Chromatographed on TG
1		0.118	
2			0.121
3	0.132		0.138
4			0.146
5			0.167
6			0.184
7		0.207	
8			0.223
9	0.239	0.235	
10			0.240
11			0.245
12			0.283
13		0.326	
14			0.357
15		0.363	
16			0.391
17	0.453	0.452	
18		0.488	
19			0.500
20			0.513
21	0.528	0.556	
22			0.592
23			0.719
24			0.730
25	0.767	0.779	
26	0.893	0.890	

4.3 Discussion

From the results presented in this chapter it will have become apparent that tanned gelatin has proved to be disappointing as a chromatographic medium. The expectation of a generally superior resolving power, based upon Stead's plot of $\sqrt{-\ln K_{av}}$ vs $\sqrt[3]{MW}$ for two characterised legume proteins (Fig. 27) can now be seen to have been based upon a compound error. On the one hand the fraction N_1G_1 was eluted at a position close to the void volume ($K_{av} = 0.004$) at which point the approximate linear relationship between $\sqrt{-\ln K_{av}}$ and $\sqrt[3]{MW}$ has been shown not to apply, the experimental points being displaced upwards in this region (see Appendix 1). On the other hand it would appear, since the elution volume is greater than expected, that the fraction N_1G_2 was retarded on tanned gelatin, resulting in the experimental point for this protein being displaced downwards. The slope of the line joining these two points is consequently steep, indicating an excellent resolving power. This is in fact true for these two fractions but is not applicable to proteins which are both not retarded and the experimental points of which also fall within the linear portion of the curve, i.e. proteins in general. It may be seen, in fact, from Fig. 27 that tanned gelatin per se has a resolving power of the same order as that of agarose while from Fig. 15 it is clear that the granulated material, used in this study, has a relatively poor resolving power due to the large void volume, i.e. the low packing density. This factor is possibly partly responsible for the relatively poorer resolution obtained on tanned gelatin compared to Sephadex G-100.

The observation that certain proteins can interact with tanned gelatin and be retarded during chromatography is a disadvantage in certain respects as the results obtained by chromatography of unknown mixtures cannot be interpreted on the basis of molecular exclusion theory. This retardation effect would appear to be due to hydrogen bonding since it is eliminated by high concentrations of urea and is enhanced by a reduction in the polarity of the buffer.

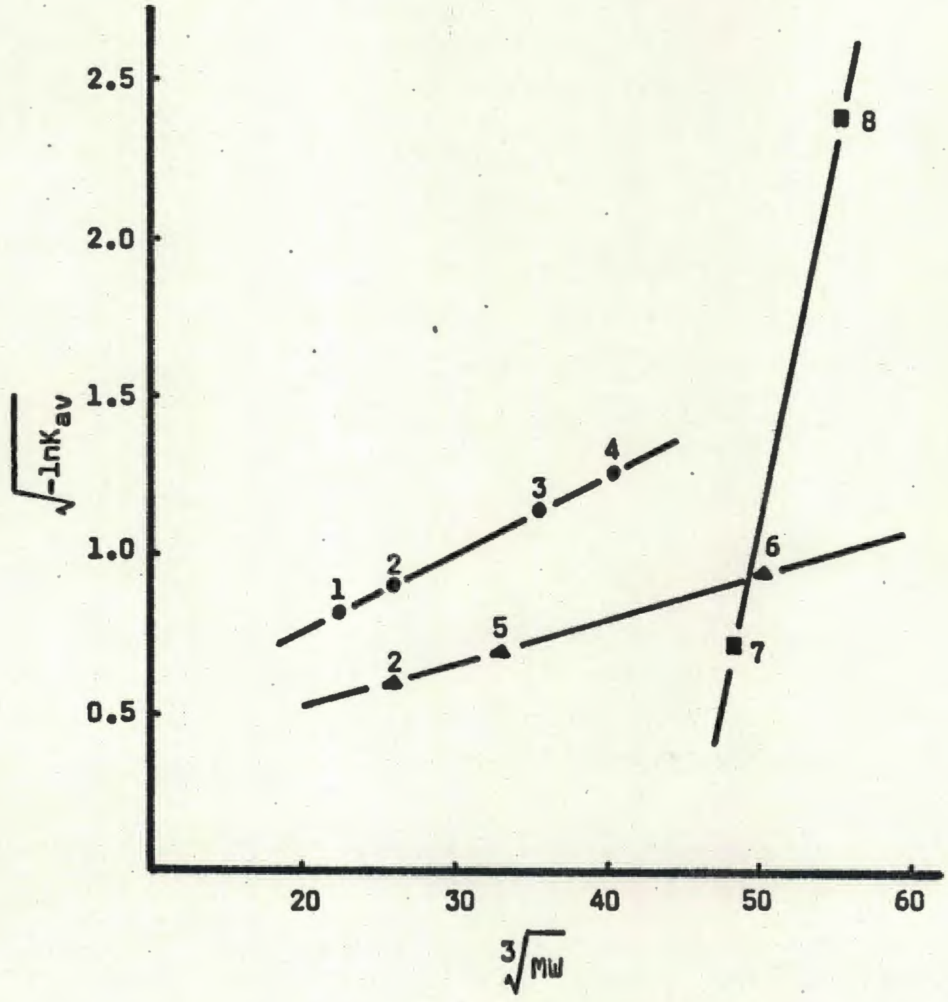


FIGURE 27 Comparison of the separating abilities of two different tanned gelatin gels and an agarose gel.

● - ● = 4% chromium-formalin tanned gelatin (Canada Packers Ltd.);

■ - ■ = 4% chromium-formalin tanned gelatin (Difco), data from Stead (19);

▲ - ▲ = 10% agarose, data from Sims et al. (88).

1 = Cytochrome C; 2 = myoglobin; 3 = hexokinase; 4 = bovine serum albumin; 5 = horseradish peroxidase; 6 = yeast alcohol dehydrogenase; 7 = N_1G_2; 8 = N_1G_1

To establish how tanned gelatin could function as a hydrogen bonding medium it might be of value to consider briefly the structure of collagen and gelatin. Collagen, from which gelatin is prepared, is comprised of three polypeptide chains, each of which is coiled in itself into a poly-L-proline II type of left-handed helix while the three chains are also coiled around one another, forming a right-handed triple helix. In this triple helix the individual chains are bound together by inter-chain hydrogen bonds. Boiling of a collagen preparation, in either an acidic or basic medium, results in the collapse of the ordered structure of collagen and in solvation of the individual polypeptide chains. In the model of Harrington and von Hippel (83), gelation of the hot solution of gelatin by cooling is thought to be due to the reformation of poly-L-proline II type helices along regions of the individual peptide chains. These are thought to subsequently associate to regenerate local regions of triple helix, again stabilised by interchain hydrogen bonds. An overall randomly ramified structure, composed of regions of triple helix linked together by single polypeptide chains is thought to result (Fig. 28). The structure of the resulting gelatin has been found to be dependent upon the rate of cooling (Fig. 29).

In the model outlined above, the regions comprised of single polypeptide chains would be particularly rich in potential hydrogen bonding groups and during chromatography these could pair in a dynamic manner with complementary groups on the sample protein and cause it to be retarded. The degree of retardation experienced by a given protein would thus be a function of the number of potential hydrogen bonding groups arrayed upon its surface. Of course, in an aqueous medium water would compete with the sample protein for any hydrogen bonding sites on the gel matrix and this possibly explains why the samples are only retarded and are not immovably bound.

In itself, the retardation phenomenon does not rule out the use of tanned gelatin and may in some cases offer an advantage (e.g. in the isolation of lysozyme). However, in the present study several indications were obtained that the sample proteins had undergone changes in structure (either secondary,

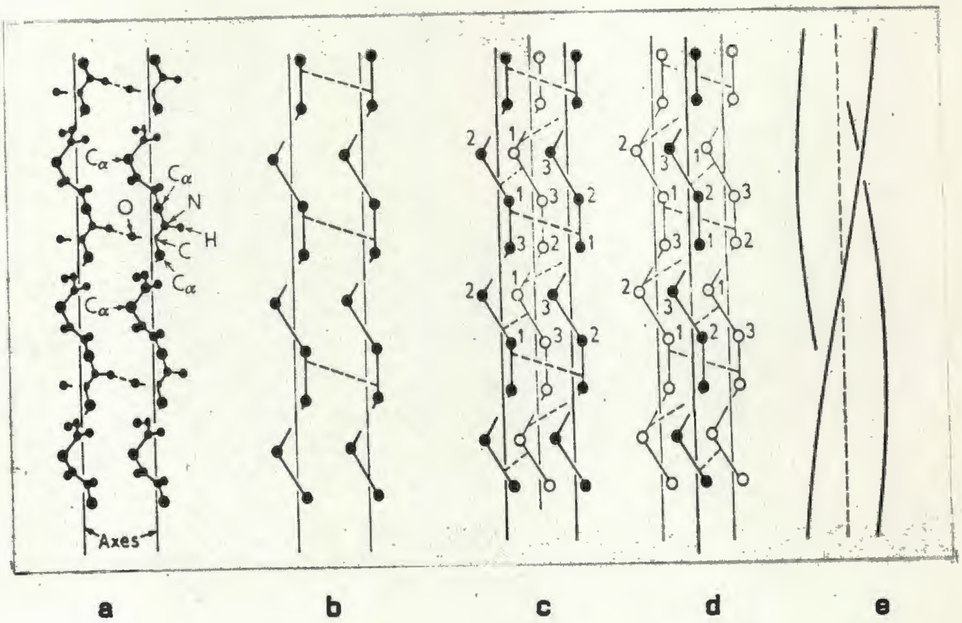


FIGURE 28 Diagram illustrating formation of collagen I and collagen II models from polyglycine II structure.

a) Two strands of polyglycine with atoms represented by dots and NH ... O = C hydrogen bonds by dashed lines;

b) simplified version of (a) with only α carbon atoms shown;

c) the third chain shown with open circles lies behind the two in (b) to make a collagen I arrangement. The α- carbons are numbered with that of glycine as number 1. The N-terminal ends of the chains are at the bottom of the diagram.

d) The third chain shown with solid circles lies in front of the two in (b) to make a collagen II arrangement.

e) Solid lines represent the axes around which the polyglycine chains are coiled. These axes are wound about the central axis of the triple helix to form a ropelike structure. From Rich and Crick (84).

Note: The poly-L-proline II structure is very similar to the polyglycine II structure and leads to the same model (85)

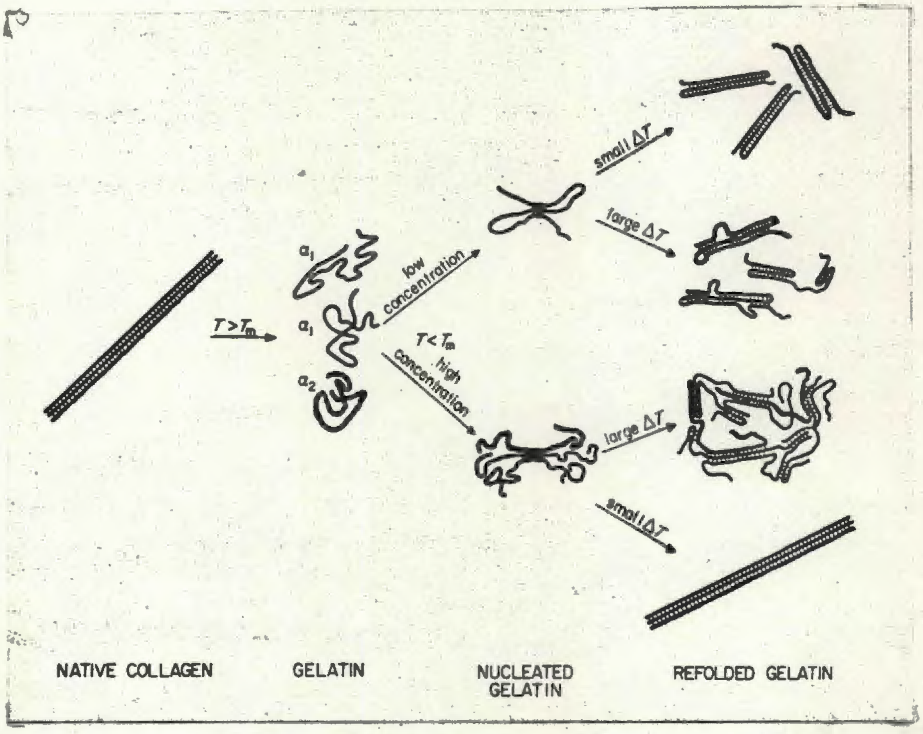


FIGURE 29 Suggested scheme for the concentration and temperature dependent pathways for helix formation in α chains derived from collagen. From Harrington and Rao (86)

tertiary or, most likely, quaternary structure) during chromatography on tanned gelatin. These indications included

(i) the formation of a precipitate in certain eluate fractions;

(ii) the difference between the toxic fractions from Sephadex and gelatin; and

(iii) the modification of the electrophoretic pattern of the crude jack bean extract induced by chromatography on tanned gelatin.

Although there is no experimental evidence to suggest that the retardation phenomenon is directly linked to the apparent effect of tanned gelatin on the structure of the sample proteins, it is conceivable that, in the case of oligomeric proteins, interaction with the gel matrix could lead to disaggregation as some subunits might associate preferentially with the gel matrix. Under the dynamic conditions pertaining during chromatography, reassociation of subunits could again occur although now in a different manner due to the spacial separation of different subunits, brought about by the differential binding to the stationary phase. In this way new aggregates, either hybrids of different parent subunits or families of molecules comprised of different numbers of subunits from the same parent molecule, could conceivably be formed.

In terms of this hypothesis it is assumed that relatively non-specific association between subunits is possible. Although in general subunit associations show a high degree of specificity, at least in enzymes, Cook and Koshland (87) have demonstrated that under suitable conditions hybrid oligomers can be formed. In the case of legume proteins, many of which are storage proteins, having, in general, a marked tendency to aggregate, non-specific aggregation could also be more pronounced.

To recapitulate the results presented in this chapter the advantages and disadvantages of tanned gelatin as a chromatographic medium, in terms of the present study, may be tabulated as follows:-

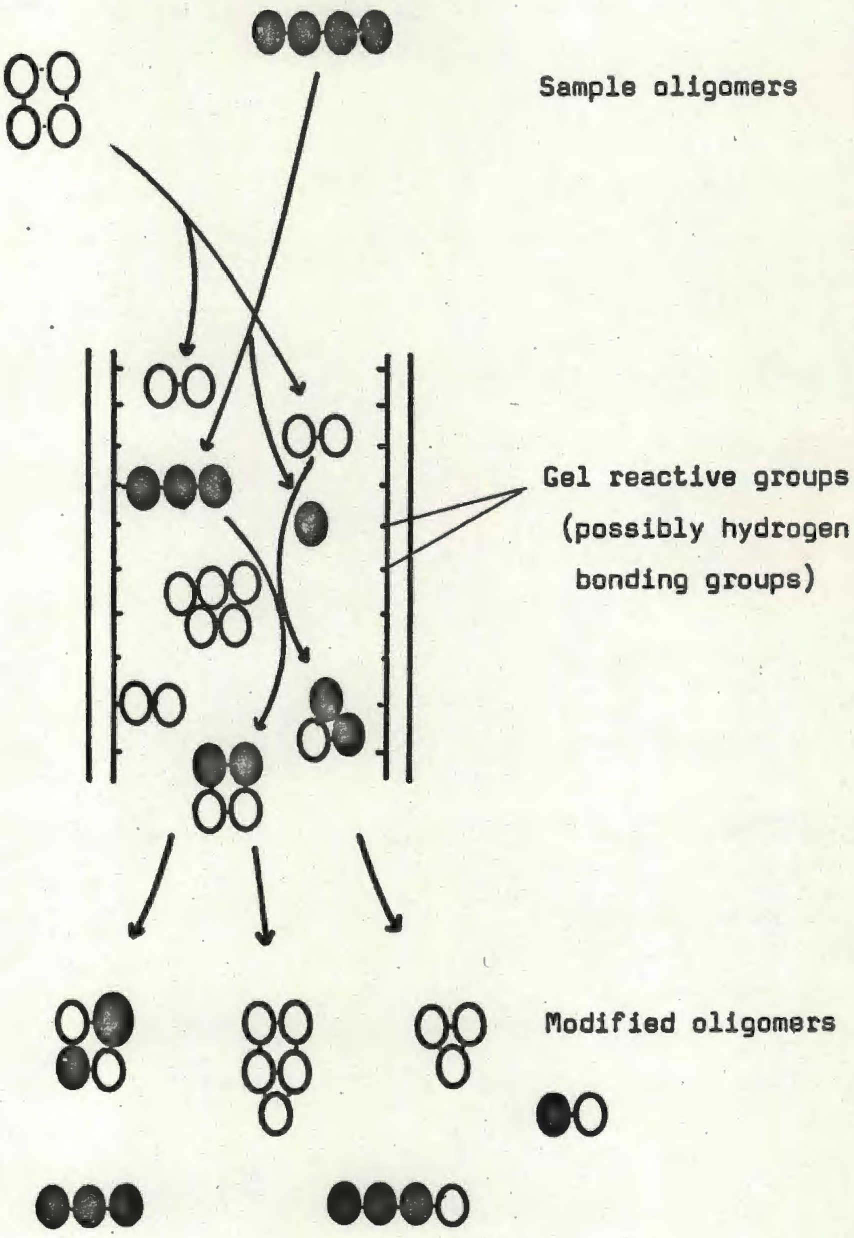


FIGURE 30 Schematic presentation of the mechanism of subunit scrambling on chromium-formalin tanned gelatin

Advantages : (i) Mechanical strength - high flow rates and high sample loads possible;

(ii) low cost - easily prepared in large amounts in a range of pore sizes.

Disadvantages: (i) Low packing volume - poor resolution;

(ii) retardation effect - interpretation of results difficult;

(iii) susceptibility to digestion by proteases - possibility of confusing digestion products with sample;

(iv) sample modification effect - impossible to obtain pure active toxic fraction.

Although tanned gelatin was considered unsuitable for the purposes described here it might prove useful in other studies. For example, the observation that egg white lysozyme is markedly retarded on tanned gelatin suggests that this resin could be used to advantage in the isolation of this enzyme. Furthermore, the observation that tanned gelatin can apparently function as a hydrogen bonding resin suggests that an investigation is warranted into the potential applicability of this material in the separation of nucleic acids.

As a result of the findings reported in this chapter, in particular the fact that it was not possible to obtain a pure toxic fraction, the use of tanned gelatin gels was abandoned.

CHAPTER 5

CHARACTERISATION OF TOXIC PROTEIN ISOLATES

Characterisation of the toxic protein isolates was undertaken with two objectives in mind. The major purpose was to ascertain the degree of similarity between the three toxic preparations isolated respectively from the Natal round yellow bean, the jack bean and the soybean. It was reasoned that at this stage establishing the features common to these toxic preparations would offer the best prospect of obtaining a greater insight into the nature of these compounds and the origin of the toxic effect. The methods used to achieve this primary goal were also compatible with the requirements of the second, though less immediate, objective which was to evaluate the practicability of further purification of the toxins and if possible to establish the best procedure to be followed for this purpose in each case.

The various isolates were accordingly characterised in a series of parallel studies, their degree of homogeneity was ascertained and the feasibility of further purification was established. The objectives of this study were thus attained and the results obtained provide a sound basis for further studies on these toxins.

5.1 Chemical analysis

5.1.1 Analysis for nitrogen, neutral carbohydrate and phosphorus

The results of analysis of fractions N_1, J and S are presented in Table 7.

TABLE 7 Protein (N x 6.25), neutral sugar (mannose equivalents) and phosphorus content of toxic protein isolates (air dry)

Component	g per 100 g		
	N_1	J	S
Moisture	6.3	10.8	10.1
P	0.8	0.0	0.0
Protein	75.2	34.1	26.0
Neutral sugars	28.0	42.0	46.0

A notable feature of these results is the observation that fractions J and S contain a higher proportion of carbohydrate than protein. Furthermore, in these two fractions the protein and carbohydrate do not account for all of the material, indicating possibly that they also contain a proportion of unidentified material. That this material is not phytate is indicated by the absence of phosphorus.

In the case of fraction N, on the other hand, protein, neutral sugars, phosphorus and moisture amount to more than 100%, most likely indicating that the value for protein is an overestimate. A value of approximately 65% protein is regarded as more probably correct.

5.1.2 Amino acid analysis

Little difference in the results was observed between 24 h, 48 h and 72 h hydrolysates. Analysis of a 48 h hydrolysate of each of the isolates yielded the results presented in Tables 8 and 9.

TABLE 8 Amino acid residues, g/100 g protein (N x 6.25)

Amino Acid	N	J	S
LYS	3.18	4.39	5.56
HIS	0.83	2.23	2.15
NH_3	1.10	0.76	1.55
ARG	3.22	3.58	4.25
ASP	10.81	7.36	9.65
THR	5.35	4.61	5.27
SER	5.06	4.04	6.12
GLU	5.89	6.84	9.77
PRO	2.22	2.83	3.74
GLY	2.80	3.04	4.10
ALA	2.71	4.45	5.33
½ CYS	-	1.00	1.54
VAL	5.37	4.40	5.55
MET	0.03	1.44	0.88
ILE	3.39	4.51	4.21
LEU	5.96	5.60	7.14
TYR	2.14	2.36	4.15
PHE	5.73	2.65	3.97
TOTAL	65.79	66.09	84.93

TABLE 9 Amino acid composition (moles/100 moles)

Amino acid	N	J	S
LYS	3.71	5.22	4.97
HIS	0.90	2,47	1.79
NH_3	10.26	7.19	11.09
ARG	3.07	3.49	3.12
ASP	14.02	9.75	9.62
THR	7.90	6.95	5.97
SER	8.67	7.07	8.06
GLU	6.80	8.08	8.68
PRO	3.42	4.44	4.41
GLY	7.32	8.11	8.24
ALA	5.69	9.54	8.59
½ CYS	-	0.69	0.79
VAL	8.09	6.77	6.41
MET	0.04	1.67	0.76
ILE	4.47	6.08	4.26
LEU	7.86	7.54	7.24
TYR	1.96	2.21	2.91
PHE	5.81	2.74	3.09

The results reveal that, expressed in terms of amino acid composition as a percentage of sample protein (N x 6.25), the amino acids and ammonia do not account for all of the nitrogen. Tryptophan, which could contribute to the total nitrogen, was not measured but most proteins have a low tryptophan content (89). The UV-absorption spectrum of fraction N_1, however, has a shoulder at 290 nm (Fig. 47), which would tend to indicate the presence of relatively significant amounts of tryptophan (90). In the case of the jack bean a proportion of the total nitrogen could be due to certain unidentified peaks which were found to be eluted from the long column some time after tyrosine and phenylalanine. The main unidentified peak was eluted after 306 mins, compared to 155 mins for phenylalanine. The area under this peak was several fold greater than the amino acid peaks but as this was not identified the material could not be quantitated. Amino sugars were not identified in any of the isolates.

It is also possible that these glycoproteins were particularly resistant to acid digestion so that not all of their constituent amino acids were liberated by the procedure used. Jollès (91) has noted that in many glycoproteins the amino acids and sugars do not account for all of the dry weight and has suggested that the deficit is due to a resistance to hydrolysis and to interaction between the amino acids and reducing sugars. It may be speculated that in glycoproteins the sugars could protect the peptide bonds and increase their resistance to acid hydrolysis.

The results indicate that fraction N_1 contains no half-cystine and virtually no methionine while fractions J and S contain a low proportion of these amino acids. This means that disulphide bonds are not possible in the case of fraction N_1 and are unlikely to be a major consideration in the case of fractions J and S.

N-terminal amino acid determination. Although, in a control experiment, the dansylation procedure was found to give satisfactory results with chymotrypsinogen, no N-terminal amino acids could be detected in any of the three isolates. This is not an unknown phenomenon in glycoproteins and has been reported for K-casein by Jollès (91), α_1 - acid glycoprotein by Jeanloz (92) and egg albumin by Neuberger and Marshall (93).

5.2 Analytical molecular exclusion chromatography

5.2.1 Chromatography on Sephadex G-100

The elution profiles obtained by chromatography of the three legume isolates on Sephadex G-100 in the presence or absence of 8 M-urea and at different pH values are presented in Figs. 31, 33 and 34.

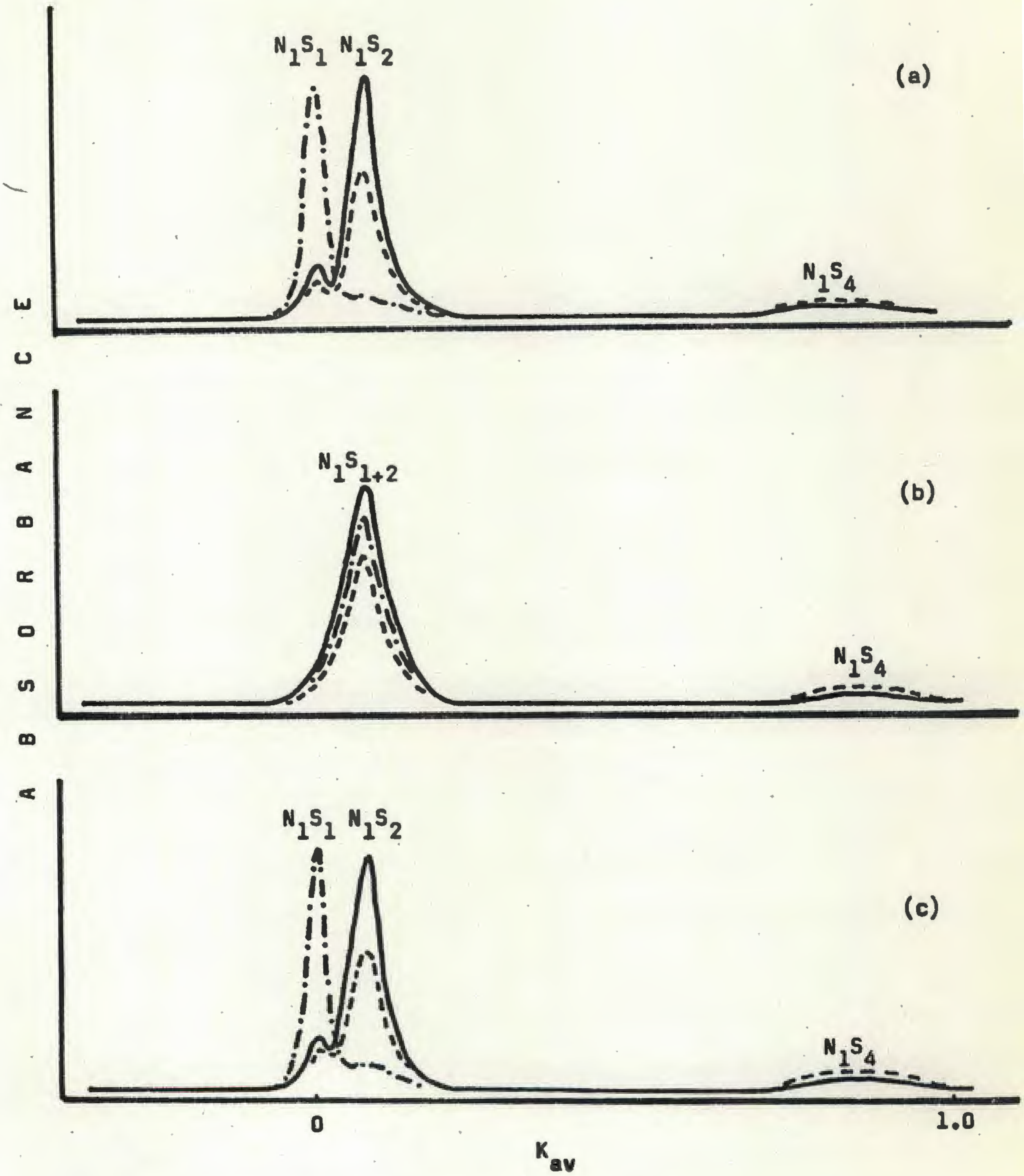


FIGURE 31 Chromatography of fraction N_1 on Sephadex G-100. Sample, 15 mg fraction N_1; Column, 2.5 x 95 cm; Buffers, with or without 8 M-urea:

(a) 0.05M acetate plus 0.15M NaCl, pH 4.0;

(b) 0.01M tris plus 0.15M NaCl, pH 7.6;

(c) 0.05M glycinate plus 0.15M NaCl, pH 9.5.

——— = $A_{280\ nm}$; ------ = $A_{260\ nm}$; -·-·- = $A_{490\ nm}$ after reaction for carbohydrate

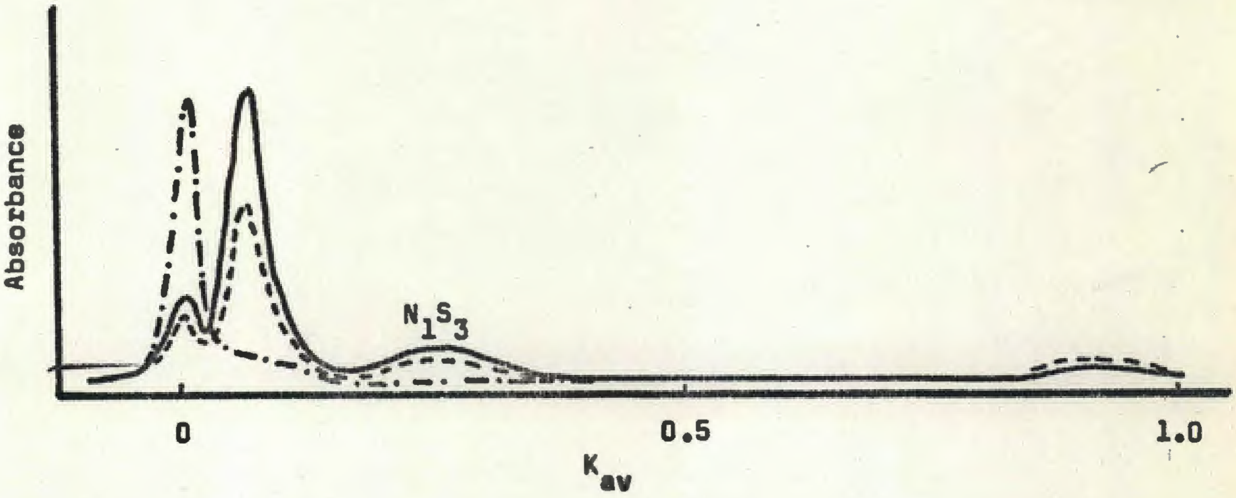


FIGURE 32 Chromatography of an aged sample of fraction N_1 (stored as freeze-dried powder at room temperature for ± 7 months) on Sephadex G-100.

Sample, 15 mg; column, 2.5 x 95 cm;

Buffer, 0.01M tris, pH 7.6, containing 0.15M NaCl and 0.2% NaN_3

——— = $A_{280\ nm}$; ------ = $A_{260\ nm}$; -·-·-· = $A_{490\ nm}$ after reaction for carbohydrate

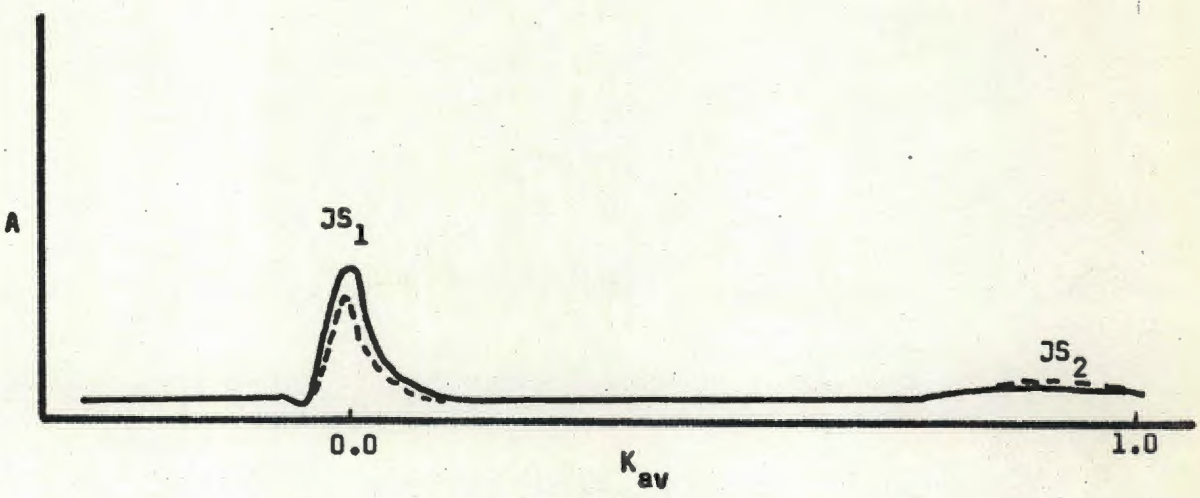


FIGURE 33 Typical elution pattern obtained with fraction J on Sephadex G-100 at pH 4.0, 7.6 or 9.5, in the presence or absence of 8 M-urea. Sample, 15 mg; column, 2.5 x 95 cm; buffers: as for Fig. 31. ——— = $A_{280\ nm}$; ------ = $A_{260\ nm}$. Absorbance at 490 nm after reaction for carbohydrate parallels the UV-absorption profile but is not shown

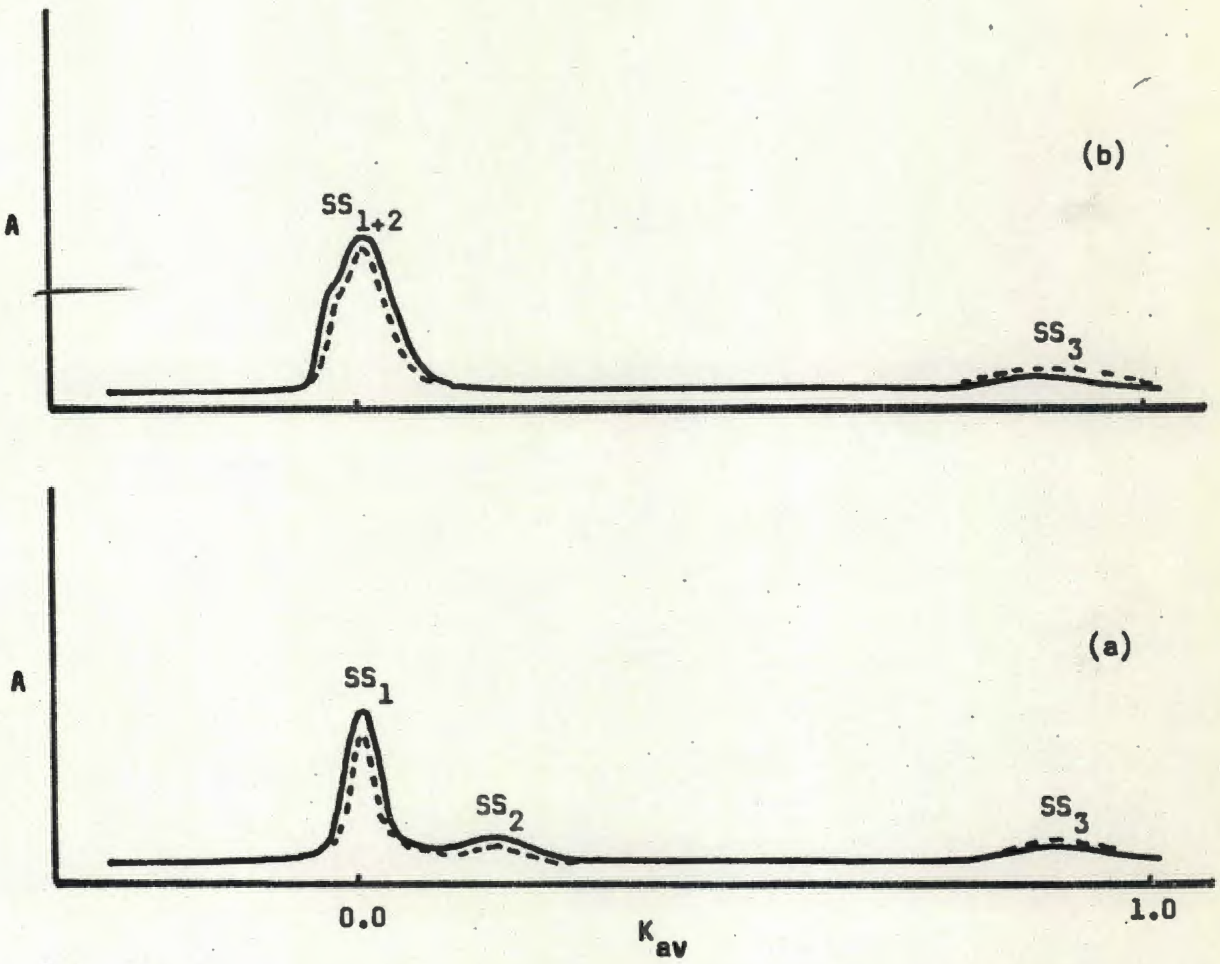


FIGURE 34 Elution pattern obtained with fraction S on Sephadex G-100 at pH 4.0, 7.6 or 9.5, in the presence or absence of 8 M-urea. Sample, 15 mg fraction J; column, 2.5 x 95 cm; buffers: as for Fig. 31.

——— = $A_{280\ nm}$; ------ = $A_{260\ nm}$; absorbance at 490 nm after reaction for carbohydrate parallels the UV-absorption profile but is not shown

In the case of fraction N_1 (Fig. 31) which, in contrast to fractions J and S, had not been previously chromatographed on Sephadex, three peaks were obtained at both high and low pH values. The peak eluted at V_o had a high carbohydrate content but low UV-absorbance (N_1S_1), in contrast to the second peak with relatively little carbohydrate but high UV-absorbance (N_1S_2). A minor peak with $E_{260\ nm} > E_{280\ nm}$ eluted just before V_{max}. Significantly, at pH 7.6, at which pH the original separation on DEAE-cellulose had been carried out, the two high molecular weight peaks (polysaccharide and protein) were fused into a single peak which was eluted at $V_e > V_o$.

Chromatography at pH 7.6 of an aged sample of fraction N_1 (stored as a freeze-dried powder at room temperature for 7 months) yielded the profile shown in Fig. 32. This is essentially similar to the profile obtained from freshly prepared material chromatographed at pH 4.0 or pH 9.5 except that a new peak with a K_{av} value of 0.25 was found.

The major peak obtained with fraction J was eluted virtually at V_o at all pH values but the shape of this peak suggested that the material was not homogeneous. In the case of fraction S a single major peak, eluted at V_o, was found at pH 7.6 while at pH 4.0 and 9.5 this was split into two peaks. In both J and S the carbohydrate and UV-absorption peaks were coincident.

All three legume fractions had two points in common. The elution profiles were not altered by the addition of 8 M-urea to the buffers and in each case a small peak with an absorbance at 260 nm slightly greater than at 280 nm was eluted just before the total column volume. This material, on the basis of its absorption characteristics and elution position, apparently corresponds to the "acetone extract" obtained from the jack bean (Fig. 5), which was shown to be non-toxic.

As fraction N_1S_2 was not totally excluded from Sephadex G-100 estimation of the molecular weight of this fraction from its elution position was feasible. A standard curve relating K_{av} to log MW was constructed using 4 standard proteins (Fig. 35) and from this curve the molecular weight of fraction N_1S_2 was estimated

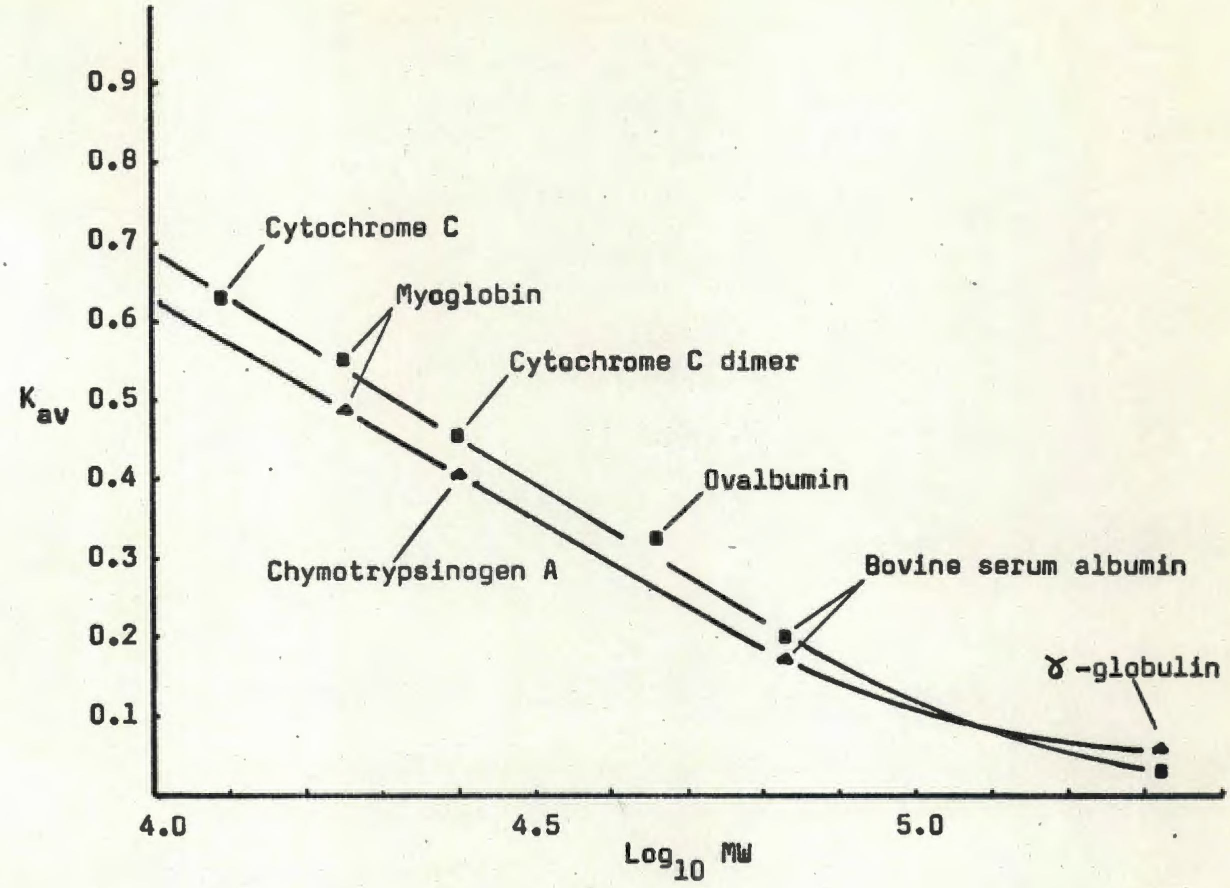


FIGURE 35 Calibration curve for estimation of molecular weights on Sephadex G-100.

▲ = data from present study; ■ = data from Andrews (37) as being approximately 130,000 daltons. For comparative purposes the data of Andrews (37) is presented in the same figure. However, as fraction N_1S_2, being a glycoprotein, is of a somewhat different nature to the proteins used as standards the value of 130,000 daltons must be regarded as a tentative estimate. Similar considerations apply to the estimation of the diffusion coefficient of fraction N_1S_2 from a standard curve of $\frac{1}{D}$ vs K_{av} (37) (Fig. 36). The value obtained for the diffusion coefficient by this method was $4.0 - 5.0 \times 10^{-7}$ cm^2 sec^{-1}.

5.2.2 Chromatography on Sepharose 4B

As fractions J and S were fully excluded from Sephadex G-100 an attempt was made to chromatograph them on Sepharose 4B, which has a larger pore size. These fractions were apparently adsorbed onto the Sepharose, however, as no UV-absorbing peaks were detected in the effluent.

Agrawal and Goldstein (48) have eluted concanavalin A, which is adsorbed onto Sephadex, with 1 M-glucose and this approach was used in an attempt to elute fractions J and S from Sepharose 4B using 1 M-galactose, but this proved unsuccessful. The limited pH range in which the Sepharose gel is stable precluded the use of the low pH method of Olson and Liener (72).

5.3 Immunoelectrophoresis

Immunoelectrophoretic analyses of the isolates N_1, J and S and the fraction J_2, obtained by chromatography on tanned gelatin, against the corresponding rabbit antisera were carried out in the combinations indicated in Table 10.

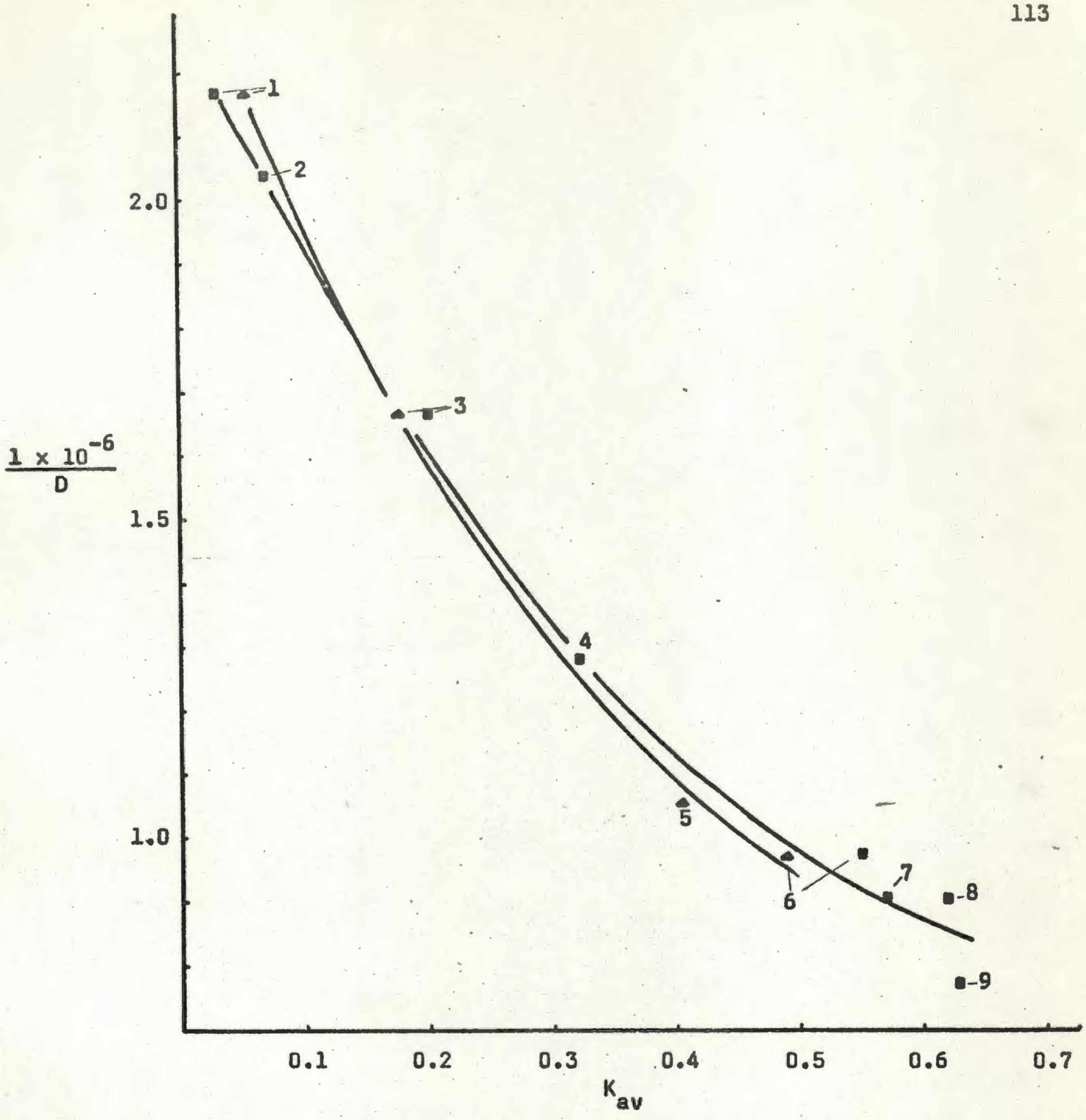


FIGURE 36 Relationship between K_{av} and diffusion coefficient (D) on Sephadex G-100.

▲ = data from present study; ■ = data from Andrews (37)

1 = γ-globulin; 2 = bovine serum albumin dimer;
3 = bovine serum albumin; 4 = ovalbumin;
5 = chymotrypsinogen A; 6 = myoglobin;
7 = lactalbumin; 8 = ribonuclease; 9 = cytochrome C

TABLE 10 Immunological crossreactivity of various legume fractions (+ = positive reaction; - = negative reaction)

Antigen / Antiserum	J	N_1	S	J_2
J	+	-	-	+
N_1	-	+	-	-
S	-	-	+	-
J_2	+	-	-	+

Each antigen was tested against all of the antisera at four different concentration levels, 60, 40, 20 and 10 mg/ml. As indicated in Table 10 no crossreactivity between the fractions from different legumes was observed. A measure of crossreactivity between fractions J and J_2, both of which are jack bean fractions, was, however, observed.

The patterns obtained by immunoelectrophoresis of the fractions N_1, J and S, each against its corresponding antiserum are presented in Fig. 37. Fractions N_1 and J were both relatively strong antigens which yielded clear bands. Fraction N_1 yielded only a single band at all concentration levels, indicating that this fraction is immunologically homogeneous. Fraction J, on the other hand, yielded two bands but these manifested a marked degree of crossreactivity, suggesting that the two antigenic components of fraction J are closely related. Fraction S was apparently only weakly antigenic and yielded only a single faint band.

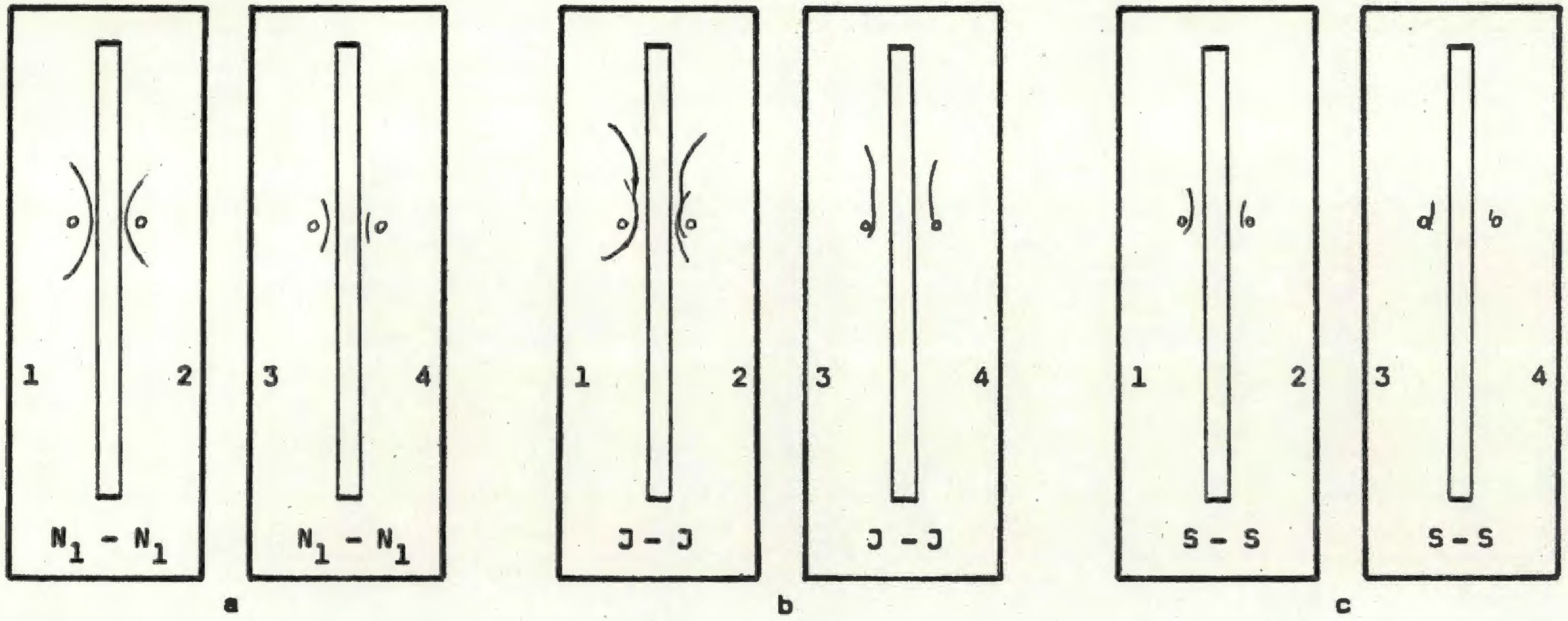


FIGURE 37 Immunoelectrophoretic patterns of various legume fractions.

a) Fraction N_1 vs N_1 rabbit antiserum;

b) fraction J vs J rabbit antiserum;

c) fraction S vs S rabbit antiserum.

Numbers refer to antigen concentrations, 1 = 60 mg/ml; 2 = 40 mg/ml; 3 = 20 mg/ml; 4 = 10 mg/ml

5.4 Immuno double-diffusion

Immuno double-diffusion of each of the fractions against its corresponding antiserum, in the apparatus described by Polson (46), yielded one band in the case of fraction N_1, two bands in the case of fraction J and three bands in the case of fraction S. The positions of each of these bands and the diffusion coefficients calculated for each of the antigens are presented in Table 11. Also presented in Table 11 are the results obtained by double diffusion of fraction N_1 against a saline solution of concanavalin A. The other two isolates did not precipitate with concanavalin A.

TABLE 11 Diffusion coefficients (Dg) of the antigens in various legume fractions measured by immuno double-diffusion. Db indicates the diffusion coefficients of the antibodies. Units are $cm^2\ sec^{-1}$

System	N_1/N_1	N_1/Con A	J/J Band 1	J/J Band 2	S/S Band 1	S/S Band 2	S/S Band 3
Xg	1.13	0.725	0.93	1.00	1.00	1.20	0.85
Xg^2	1.277	0.526	0.86	1.00	1.00	1.44	0.72
Xb	0.87	1.275	1.07	1.00	1.00	0.80	1.25
Xb^2	0.757	1.626	1.14	1.00	1.00	0.64	1.56
$\frac{Xg^2}{Xb^2}$	1.687	0.323	0.75	1.00	1.00	2.25	0.46
Db	4.6×10^{-7}	7.35×10^{-7}	4.6×10^{-7}	4.6×10^{-7}	4.6×10^{-7}	4.6×10^{-7}	4.6×10^{-7}
Dg	7.76×10^{-7}	2.38×10^{-7}	3.45×10^{-7}	4.6×10^{-7}	4.6×10^{-7}	1.035×10^{-6}	2.12×10^{-7}

A single band was obtained by diffusion of fraction N_1 against concanavalin A but the diffusion coefficients calculated for the immunoreactive component and the component reacting with concanavalin A were markedly disparate.

Two precipitin bands were obtained in the case of fraction J, both components having diffusion coefficients lower than the antigen of fraction N_1. The two components of fraction J have been shown by immunoelectrophoresis to exhibit a measure of immunological crossreactivity and this raises a doubt concerning the accuracy of measurement of the diffusion coefficient of the larger component for, if both components react with the same antibody, after contact with the first component the antibody will have a different concentration and rate of diffusion. The observed diffusion coefficient of the slower component will therefore be too high.

Fraction S yielded three precipitin bands, two apparently due to large molecules (diffusion coefficients 4.6×10^{-7} and 2.12×10^{-7} cm^2 sec^{-1}) while the third is due to a relatively small molecule (diffusion coefficient 10.35×10^{-7} cm^2 sec^{-1}). This smaller antigen could possibly correspond with the peak SS_3 although no equivalent antigen has been detected in the case of fraction J, which nevertheless manifests a corresponding peak JS_2.

5.5 Disc gel electrophoresis

The electropherograms obtained by disc gel electrophoresis of fraction N_1 and J under various conditions are presented in Figs. 38 and 39. Fraction S was also subjected to electrophoresis under the same conditions but in each case no distinct bands were obtained in the running gel.

Fraction N_1 yielded the most interesting results upon disc gel electrophoresis. At low pH a single major band and two relatively minor contaminants were found. The proportions and positions of these bands were not affected by the presence of 8 M-urea in the separating gel. At high pH (Fig. 38c) three sharp bands were found, separated by a constant Rf interval and superimposed on a diffuse background which was continuous with a fourth somewhat diffuse band of higher mobility. The presence of 8 M-urea increased the overall Rf values of the bands but now five sharp bands, again separated by a constant Rf interval, and a diffuse zone of higher mobility were found. An increase in the gel concentration to 15% packed the previous bands together, eliminated the diffuse zone and brought a further seven bands into focus. Five of these bands were again separated by a constant Rf increment.

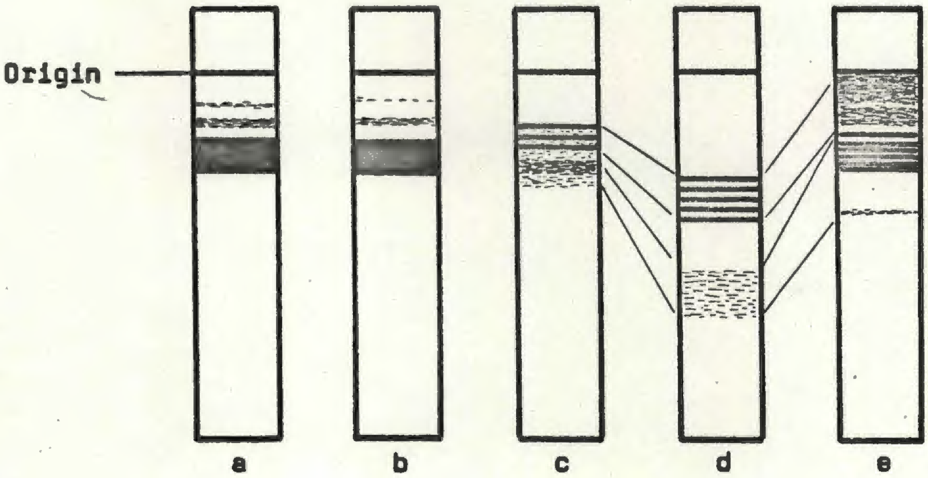
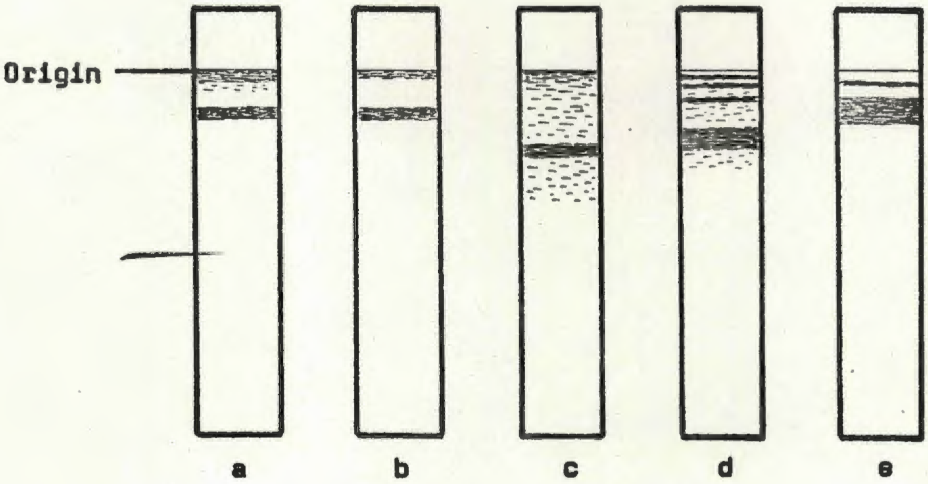


FIGURE 38 Disc gel electrophoresis of fraction N_1.

a) Low pH buffer system of Reisfeld *et al.* (53), 7.5% gel;
b) as in (a) but with 8 M-urea, 7.5% gel;
c) high pH buffer system of Davis' (50), 7.5% gel;
d) as in (c) but with 8 M-urea, 7.5% gel;
e) as in (d), 15% gel.
Lines joining adjacent gels indicate proposed relationship between bands



<u>FIGURE 39</u> Disc gel electrophoresis of fraction J.

a) Low pH buffer system of Reisfeld <u>et al</u>. (53), 7.5% gel;
b) as in (a) but with 8 M-urea, 7.5% gel;
c) high pH buffer system of Davis' (50), 7.5% gel;
d) as in (c) but with 8 M-urea, 7.5% gel;
e) as in (d), 15% gel

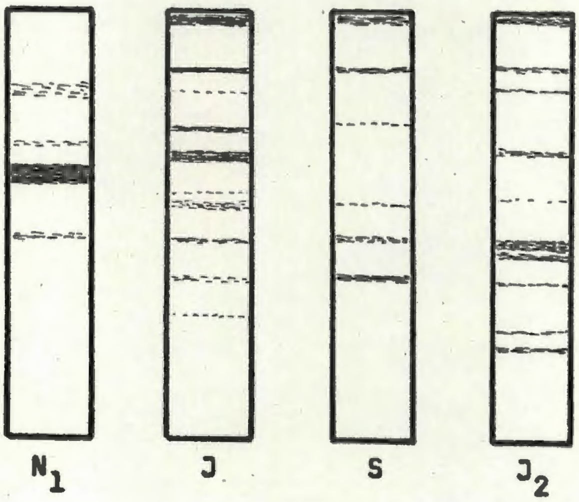
Fraction J (Fig. 39) did not manifest regular multiple banding. At low pH only a small proportion of the protein of this fraction migrated cathodically, although this proportion was increased in the presence of 8 M-urea. At high pH, in the absence of 8 M-urea a single major band with an Rf value of 0.20 was found but this was superimposed upon a diffuse background. The density of this background was found to vary in different preparations and, as has been discussed in Chapter 3, this background is thought to be derived from the major band. At high pH in the presence of 8 M-urea, the Rf value of the major band was slightly reduced (to 0.19) and a further three bands with very low mobilities were also found. When the gel concentration was increased to 15%, however, only two bands were obtained.

5.6 Gel electrophoresis in SDS

The results of gel electrophoresis in buffers containing SDS are presented in Fig. 40. Fraction N_1 yielded a single major band and three very faint bands due to trace components. The major band did not correspond to any of the bands obtained with fractions J and S. Fraction J yielded a total of ten bands but of these only three could be regarded as major bands. Fraction S yielded approximately seven bands and, interestingly, four of these bands corresponded to bands obtained in the case of fraction J.

5.7 Iso-electric focussing

Iso-electric focussing was only successfully accomplished in the case of fraction N_1. In this case only a single band was obtained by iso-electric focussing in a 5% polyacrylamide gel following the procedure of Catsimpoolas (64). The iso-electric point of the material comprising this band was unambiguously established as pI = 6.8 by the plateau region induced in the pH gradient when sample was included in the gel used for measuring the gradient (Fig. 41 a). This plateau coincided with the position of the band obtained by precipitation



<u>FIGURE 40</u> Gel electrophoresis of fractions N_1, J, J_2 and S in the presence of sodium dodecyl sulphate

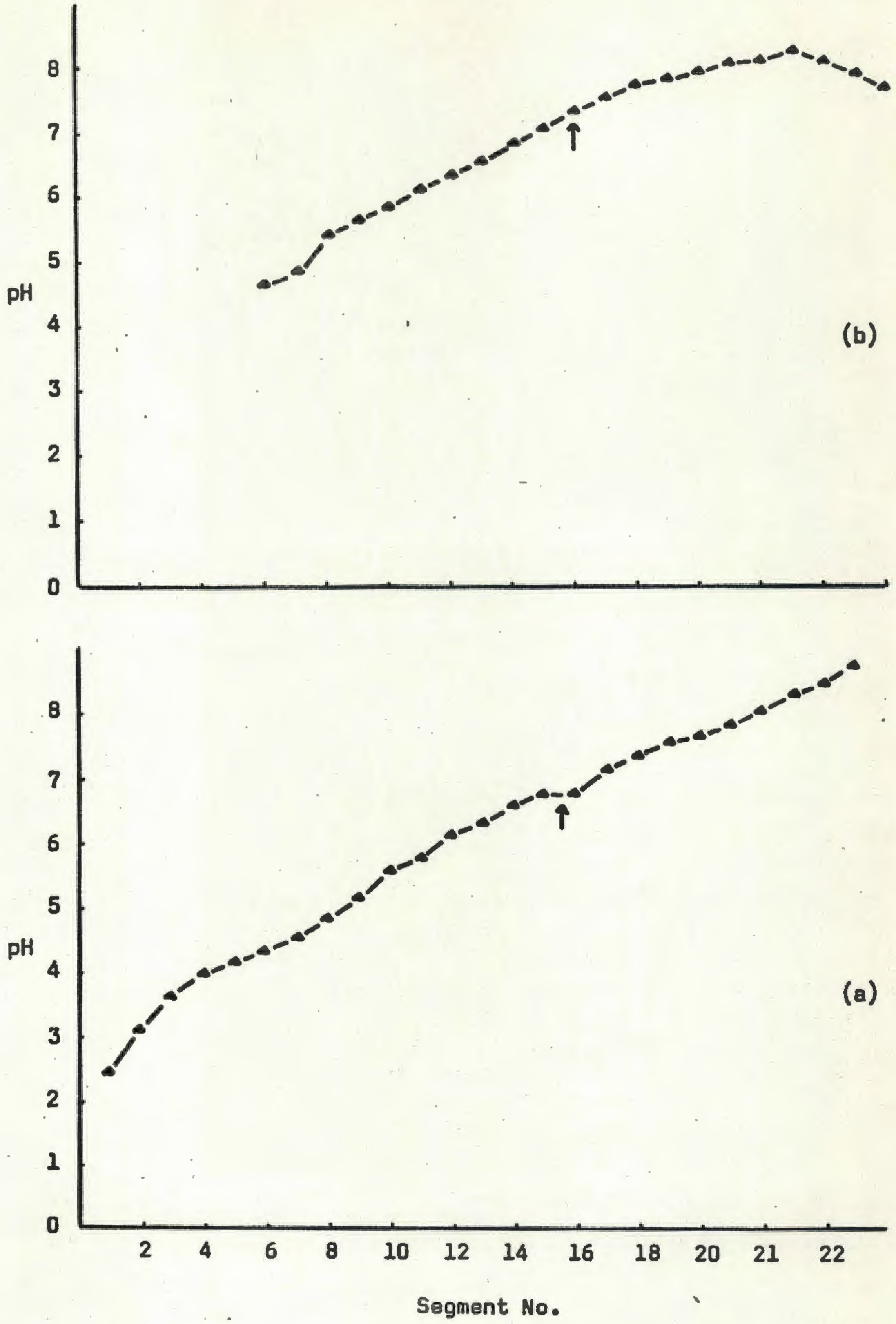


FIGURE 41 Iso-electric focussing of fraction N_1 in 5% polyacrylamide gel,

(a) in the absence of urea;

(b) in the presence of 8M urea.

Arrows indicate the position of the single focussed band in each case with trichloroacetic acid, in an identical gel run concurrently. Subsequent staining of the gel after removal of the ampholytes failed to reveal any further bands.

Samples run in the presence of 8 M-urea also yielded only a single band although this band was focussed at a slightly higher pH (pH 7.3) than that run in the absence of 8 M-urea (Fig. 41 b).

An attempt was made to conduct iso-electric focussing in agarose gels which, being macroreticular, would be expected to impose minimal steric resistance to the migration of macromolecules. In each case, however, as the run proceeded the gels suffered distortion and either became detached from the glass tubes or else split into two.

5.8 Analytical ultracentrifugation

The schlieren profiles obtained by sedimentation of fractions N_1, J and S in a Beckman model E analytical ultracentrifuge are presented in Figs. 42, 43 and 44. Although fraction N_1 shows a small shoulder, this preparation is virtually homogeneous and in this case the sedimentation coefficient was determined at four different concentrations and a least squares linear regression was used to estimate S^{o}_{20} w (Fig. 45). The value obtained by this method was S^{o}_{20} w = 6.65 S.

As fraction N_1 was virtually homogeneous, estimation of the molecular weight of this fraction, by the conventional sedimentation equilibrium method (66), was considered feasible. The multisample cell described by Yphantis (67) was used to determine apparent molecular weights at three sample concentrations, the concentration distribution across each segment being measured at 280 nm using the photoelectric scanner. Three replicate determinations were performed at each of two speeds. A linear relationship between log C and r^2 was obtained in each case but the apparent molecular weight was found to be markedly concentration dependent. A plot of the reciprocal of the mean apparent molecular weight against the sample concentration according to Munk and Cox (94) was

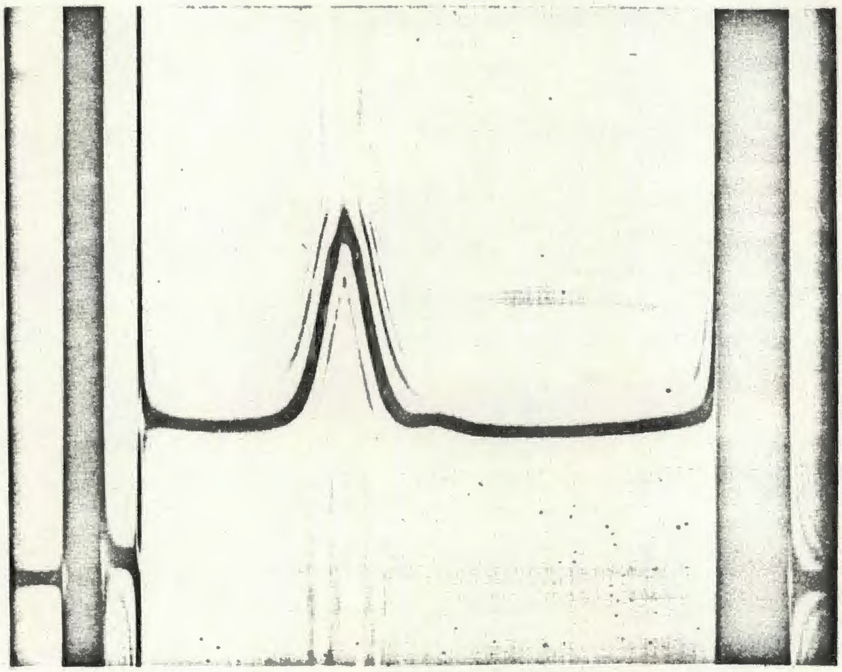


FIGURE 42 Schlieren profile of fraction N_1 in model E ultracentrifuge.
Sample concentration, 5 mg/ml in 0.01M tris containing 0.2M NaCl, pH 7.6;
rotor velocity, 60,967 r.p.m.;
temp, 20^oC.
Photograph taken 36 min after zero time

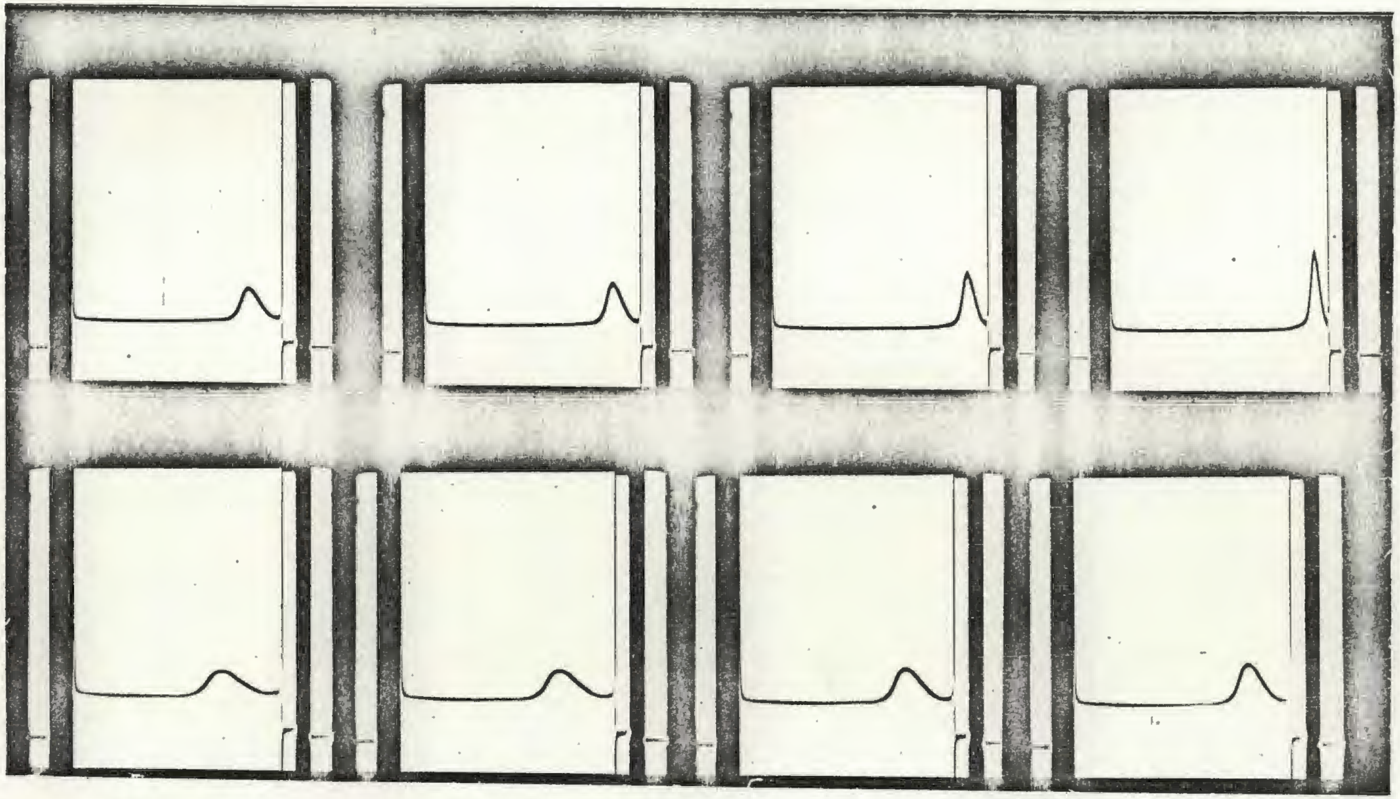


FIGURE 43 Sedimentation of fraction J in model E ultracentrifuge.

Sample concentration, 5 mg/ml in 0.01M tris containing 0.2M NaCl, pH 7.6; rotor velocity, 56,385 r.p.m.; temp, 20°C.

Photographs taken at 4 min intervals.

Sedimentation is from right to left

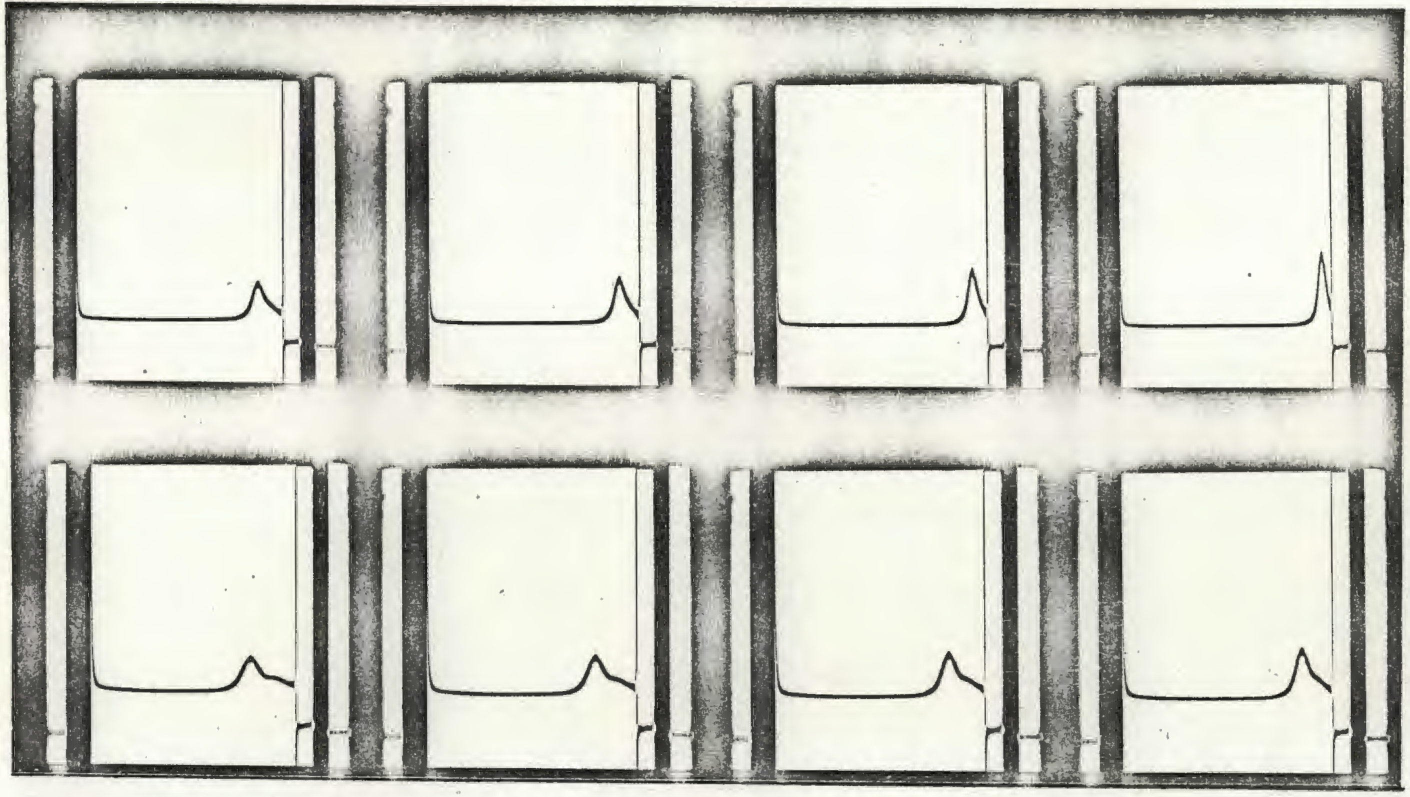


FIGURE 44 Sedimentation of fraction S in model E ultracentrifuge.

Sample concentration, 5 mg/ml in 0.01M tris containing 0.2M NaCl, pH 7.6;

rotor velocity, 56,420 r.p.m.; temp, 20°C.

Photographs taken at 4 min intervals.

Sedimentation is from right to left

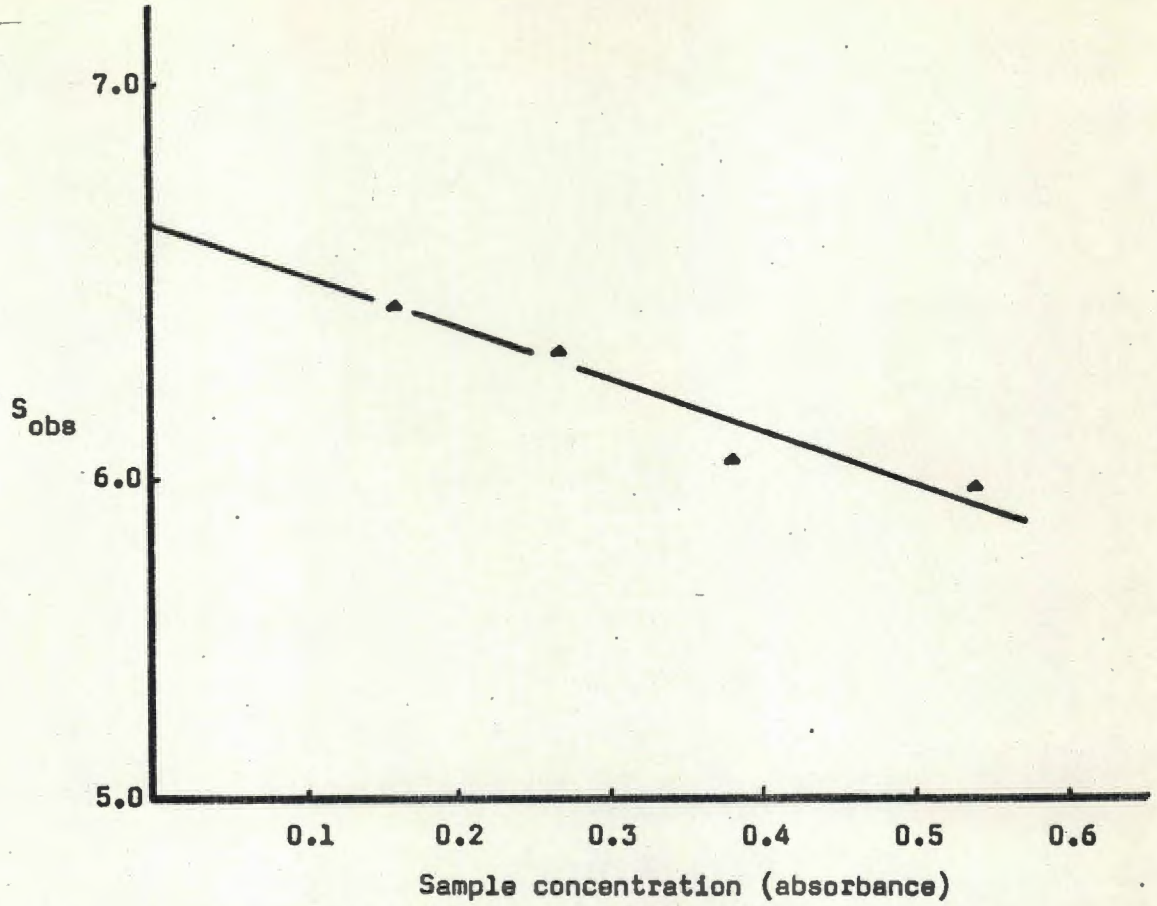


FIGURE 45 Concentration dependence of sedimentation coefficient of fraction N_1 . The straight line represents a least-square regression

linear, for the three values determined, and could be extrapolated to give an apparent molecular weight at zero concentration of 148,000 daltons (Fig. 46).

In the calculation of both the sedimentation coefficient and the molecular weight, a value of 0.70 $cm^3 g^{-1}$ was assumed for the partial specific volume of fraction N_1. This figure is considered not unreasonable in view of the carbohydrate content of fraction N_1 (cf ref. 95) but as the composition of the carbohydrate moiety has not been established, the value of $\bar{v} = 0.70$ must be regarded as a tentative estimate. Lack of sufficient material has prevented direct measurement of the partial specific volume.

Although fractions J and S were not homogeneous it was possible to estimate the mean sedimentation coefficient in the former case and the sedimentation coefficient of the major component in the latter case. The values obtained were; fraction J, $S_{obs} = 7.9$ S and fraction S, $S_{obs} = 6.0$ S. Fractions J and S were observed to also contain a proportion of insoluble material as a rapid clearing of the solution was observed in the early stages of centrifugation.

The molecular weights of the components of fraction J and S could be estimated by the sedimentation and diffusion method (66) using the values for the diffusion coefficient obtained by immuno double-diffusion. By this method, correlating the higher value of the sedimentation coefficient with the lower value of the diffusion coefficient (Table 11), the components of fraction J were estimated to have molecular weights of 140,000 daltons and 190,000 daltons respectively while the faster sedimenting species in fraction S was estimated to have a molecular weight of 234,000 daltons. The sedimentation coefficient of the slower sedimenting species in fraction S could not be quantitatively determined but by visual inspection the molecular weight of this species was tentatively estimated as approximately 80,000 - 100,000 daltons. This is in approximate agreement with the value estimated for peak SS_2 by molecular exclusion chromatography (Figs. 34 and 35).

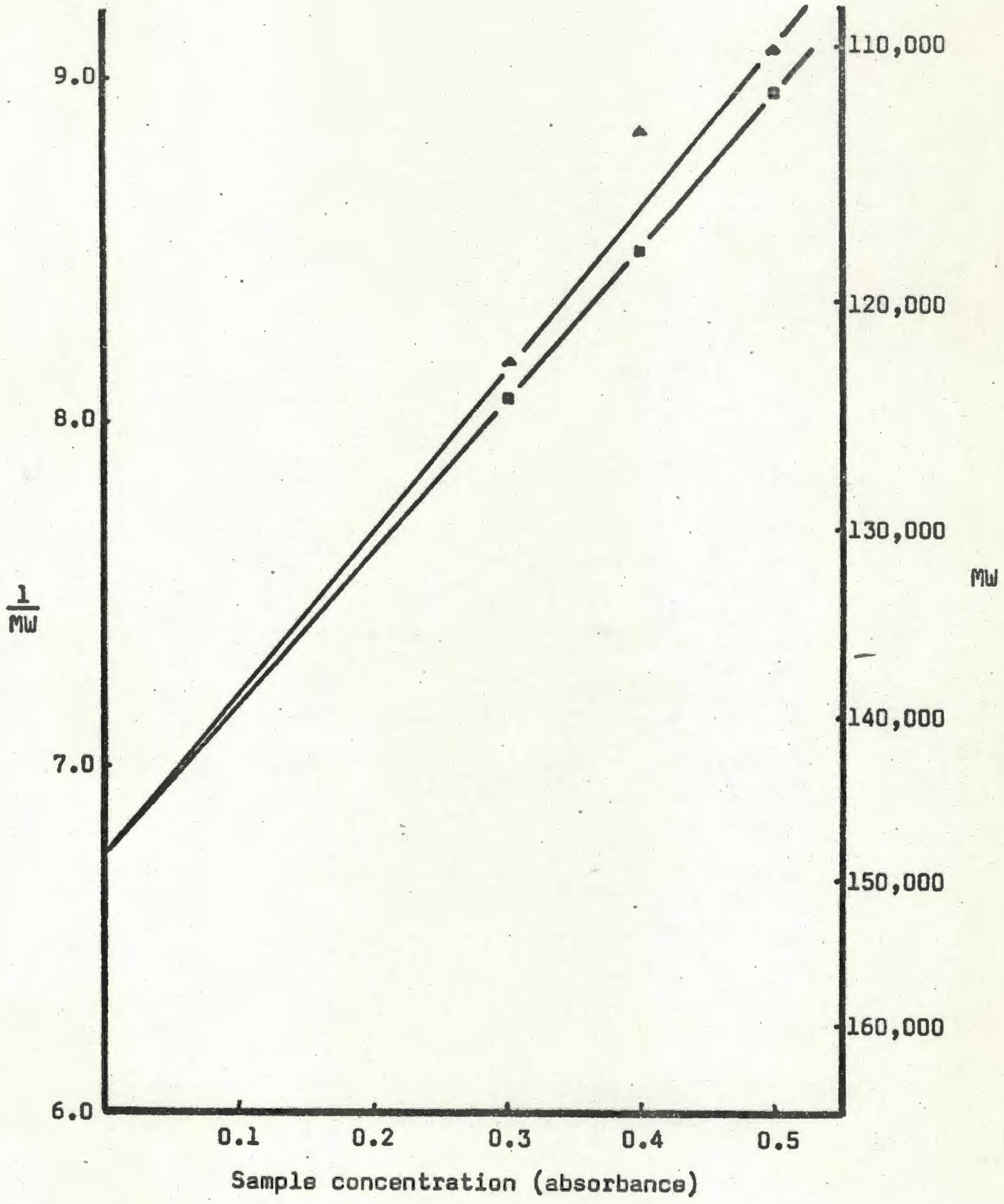


FIGURE 46 Concentration dependence of apparent molecular weight of fraction N_1 determined by sedimentation equilibrium analysis.

▲ = 7,346 r.p.m.; ■ = 8,087 r.p.m.

5.9 Spectral analysis

The UV-absorption and circular dichroism spectra of fractions N_1, J and S are presented in Figs. 47, 48 and 49.

The UV-absorption spectra of all three fractions were characteristic of proteins, each having a peak at 280 nm and also absorbing strongly below 230 nm.

The CD spectrum of fraction N_1 (Fig. 47) was characterised by a broad positive band in the region of 280 - 290 nm and a complex negative band at 235 nm. No detectable differences were observed in the CD spectrum at pH 4.0, pH 7.6 and pH 9.5. In 8 M-urea the CD spectrum showed a diminution of the bands at 280 - 290 nm and 235 nm and the appearance of a new negative band below 220 nm. Again, in 8 M-urea the spectrum was not sensitive to changes in pH. The changes induced by 8 M-urea were apparently not reversible for after exposure to 8 M-urea, which was subsequently removed by dialysis, the freeze-dried material was poorly soluble, although it did not precipitate during dialysis.

The CD spectra of fractions J and S (Figs. 48 and 49) were similar to each other but different from N_1. Each was characterised by a shoulder in the region 220 - 230 nm and a large negative band below 220 nm and in each case the spectrum was not altered by the addition of 8 M-urea and/or changes in pH. The spectra of fractions J and S were similar to that of fraction N_1 in 8 M-urea.

5.10 Histological examination of intoxicated tissues

The livers, kidneys, spleens and brains of animals lethally intoxicated respectively with each of the three legume toxins were fixed in formalin, sectioned, stained and examined in the microscope.

In the case of fraction N_1 the major symptom was a gross engorgement of the spleen. This was coupled with a slight degree of tissue damage manifest in the kidney but the liver and brain were apparently normal. Fractions J and S on the other hand apparently cause fatty infiltration and necrosis of the

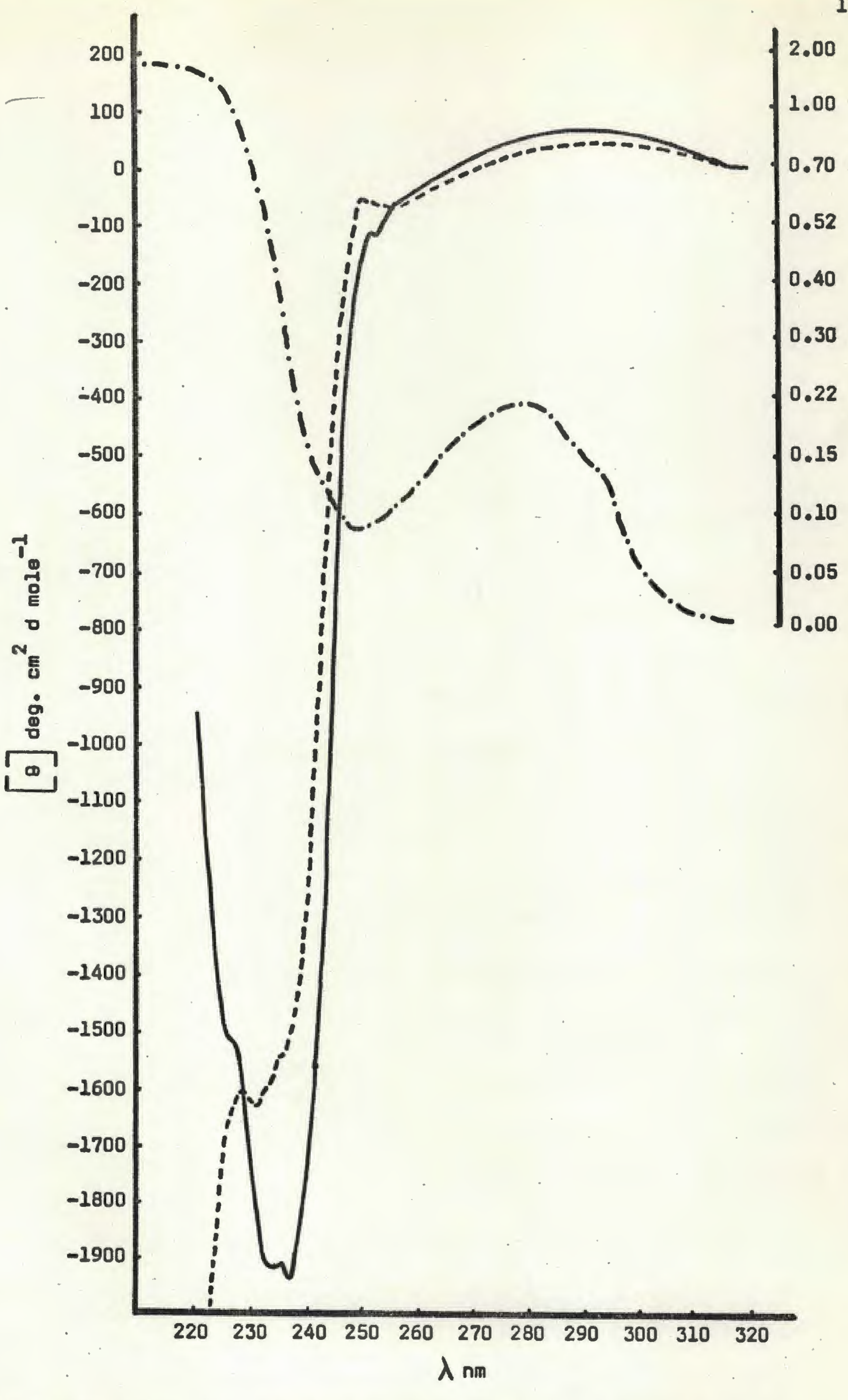


FIGURE 47 Absorption and circular dichroism spectra of fraction N_1.
•-•-• = absorption spectrum; ——— = CD spectrum;
- - - = CD spectrum in the presence of 8M urea.
Spectra were identical at pH 4.0, pH 7.6 and pH 9.5

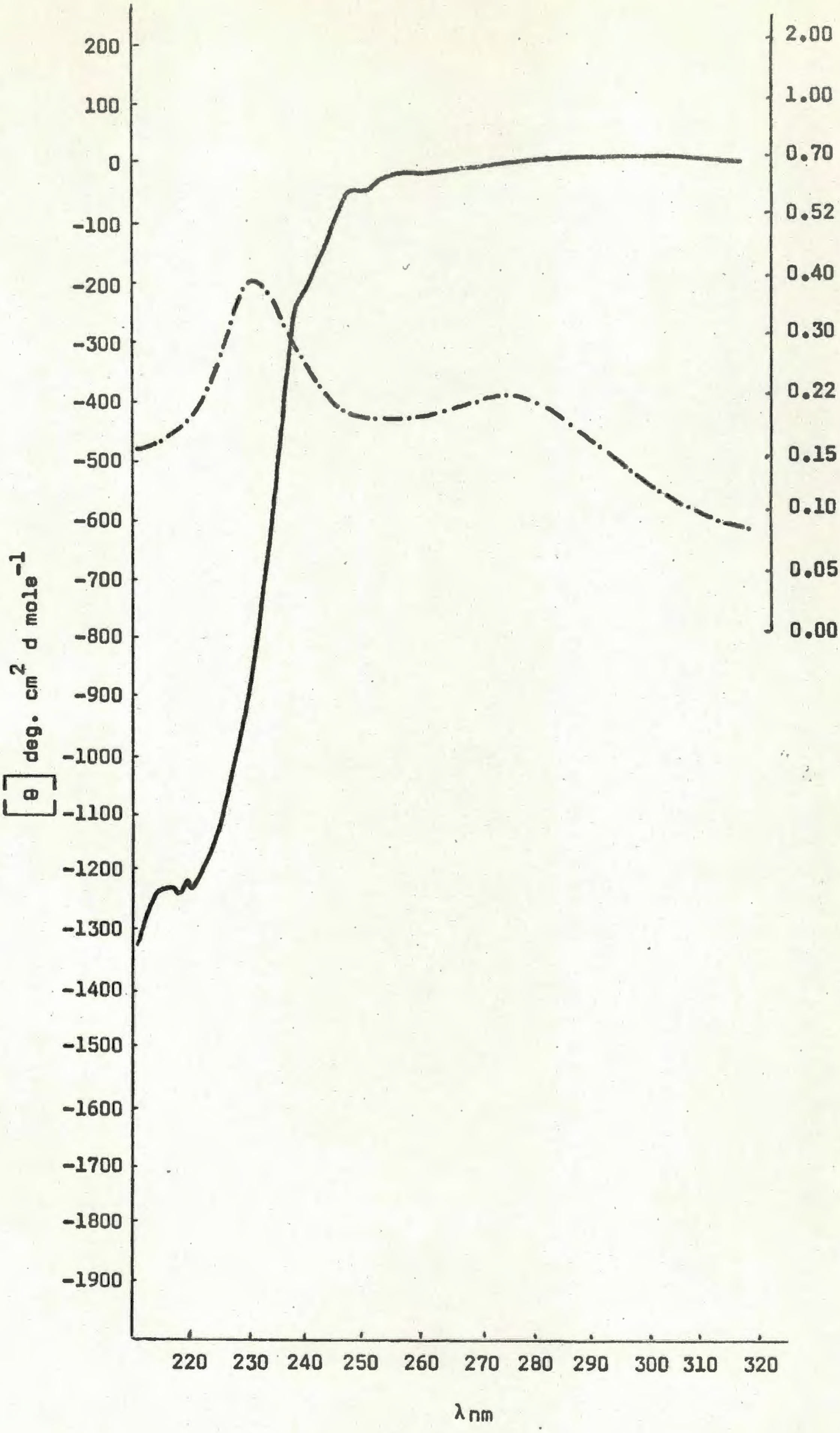


FIGURE 48 Absorption and circular dichroism spectra of fraction J.
-·-·- = absorption spectrum; ——— = CD spectrum

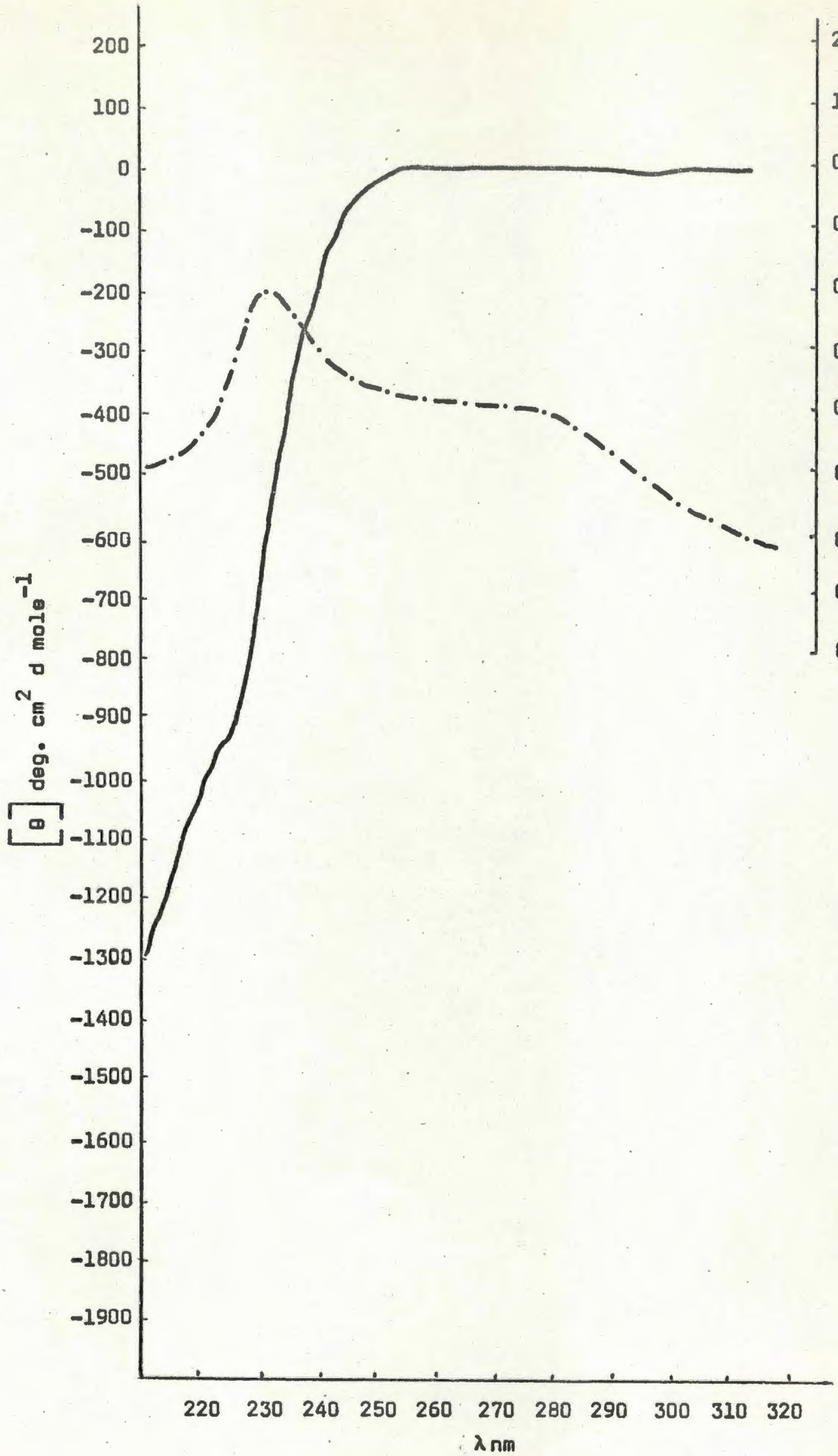


FIGURE 49 Absorption and circular dichroism spectra of fraction 5.

•-•-• = absorption spectrum; ——— = CD spectrum liver while the spleen, kidney and brain are relatively unaffected. It would appear, therefore, that fraction N_1 and fractions J and S have a different locus of action in eliciting the toxic response. Fraction N_1 would appear to be essentially a blood toxin while fractions J and S are hepatotoxins.

5.11 Discussion

Fraction N_1 has proved to be markedly different to fractions J and S in a number of respects. Furthermore, of the three legume isolates, N_1 has given the most interesting and positive results and consequently this fraction is discussed at greater length than the others.

5.11.1 Natal round yellow bean toxin

The Natal round yellow bean toxin has two structural aspects which warrant consideration, namely the relationship between the carbohydrate and the protein moieties and the structure of the protein moiety itself.

With regard to the former aspect, the results of molecular exclusion chromatography suggest that the majority of the carbohydrate of fraction N_1 is non-covalently associated with the protein as these two components can be separated at pH extremes. The fact that the carbohydrate, after separation from the protein, is eluted from Sephadex G-100 at a position corresponding to a higher molecular weight than that of the original protein/carbohydrate hybrid additionally suggests that aggregation of carbohydrate sub-units occurs under these conditions. This reaction is apparently not influenced by high urea concentrations as the elution profiles were identical in the presence or absence of 8 M-urea.

The results presented here tend to confirm Stead's previous results (19) concerning the formation of the high molecular weight carbohydrate material. Using freezing and thawing, followed by analysis on tanned gelatin, all at pH 7.6, Stead observed that formation of the high molecular weight carbohydrate material took place via a number of intermediates. Unfortunately, as Stead used chromatography on tanned gelatin as an analytical system, it is not possible to decide whether the effects observed were due to the freezing and thawing or to interaction with the tanned gelatin during chromatography (see Chapter 4). In the present study, in which formation of the high molecular weight carbohydrate material was induced by pH extremes, no intermediates were observed although formation of the carbohydrate material as a derivative of the protein/carbohydrate complex was confirmed.

In general, therefore, it would appear that fraction N_1 is unstable and tends to split spontaneously into separate carbohydrate and protein moieties. This reaction occurs most quickly under adverse conditions such as freezing and thawing or chromatography on tanned gelatin or under pH extremes but also apparently occurs, although more slowly and in a slightly different manner, upon storage in the freeze-dried state (Fig. 32). An indication that the reaction may occur in solution at neutral pH is provided by the results of ultracentrifugation and immuno-diffusion. It is suggested that the small fast sedimenting peak observed during ultracentrifugation (Fig. 42) represents the aggregated carbohydrate material. Furthermore, the marked disparity in the diffusion coefficients of the immunoreactive component and the component reacting with concanavalin A (i.e. the carbohydrate moiety), as measured by immuno-diffusion, suggests that complete dissociation of the protein and carbohydrate moieties had occurred in the time taken for the experiment (± 2 weeks). The conditions pertaining during double-diffusion are particularly mild so that these results support the hypothesis that dissociation of the carbohydrate from the protein, and subsequent self-association of the carbohydrate, is a spontaneous reaction. The measured value of the diffusion coefficient of the carbohydrate moiety (2.38×10^{-7} cm^2 sec^{-1}) suggests that this is a large molecule and substantiates the evidence from molecular exclusion chromatography indicating that aggregation of carbohydrate subunits does indeed occur.

It should perhaps be noted here that the relationship found between the protein and carbohydrate moieties of fraction N_1 is closely paralleled by the situation existing in the case of a haemagglutinin from the black bean, Phaseolus vulgaris (7). Discussing the muco-phytohaemagglutinin (MPHA) from this bean, Rigas and Osgood (7) have stated that, "this material, in an environment of low pH, dissociates into two parts, one a protein haemagglutinin and the other an inactive polysaccharide." Stead (19) has also presented evidence that the carbohydrate of fraction N_1 is non-toxic and that the toxic potency of the protein moiety is not reduced by the splitting off of the carbohydrate, a finding which was corroborated by the observation made in the present study that ageing of fraction N_1 causes the carbohydrate to split off without a parallel decrease in toxic potency. It would appear therefore that in fraction N_1, as in the MPHA from Phaseolus vulgaris, the carbohydrate is not involved in the activity of the preparation. In view of the similarity between the carbohydrates of fraction N_1 and the MPHA of Phaseolus vulgaris it is tempting to speculate that the carbohydrate of these two fractions, from two different varieties of P. vulgaris, may be a ubiquitous non-specific protein-complexing agent found in association with several proteins in this species.

The apparent autoaggregation of the carbohydrate of fraction N_1 is extremely interesting, for while non-covalent association of protein and carbohydrate has been reported (e.g. ref. 7, 72) the author is not aware of any studies concerning the formation of carbohydrate "oligomers" of this nature. It would appear therefore that further, more detailed, studies of the carbohydrate and the forces involved in its aggregation are warranted.

With regard to the structure of the protein moiety, the results are not as unambiguous as the various techniques have given conflicting indications regarding the existence or non-existence of a quaternary structure in this component.

The results of disc gel electrophoresis, for example, indicate that fraction N_1 is largely homogeneous at low pH but this technique, applied at high pH, provides evidence of a complex system. Several hypotheses can be advanced to accomodate the regular multiple banding phenomenon observed at high pH. Firstly, and most simply, it might be speculated that the molecule is oligomeric and that high pH, acting in concert with the effect of 8 M-urea, occasions disaggregation of the subunits, each band representing a distinct subunit species, differing from the other subunits in a fundamental parameter such as the amino acid composition. This hypothesis has, for example, been invoked by Rigas _et_ _al_. (98) to explain the eight uniformly spaced bands obtained upon starch gel electrophoresis of the haemagglutinin from _P_. _vulgaris_ in 8 M-urea at pH 9.0.

An advantage of this hypothesis is that the difference between the various bands is ascribed principally to a charge difference and so the uniform spacing of the bands can be attributed simply to an equal charge difference between the various bands. A hypothesis involving a difference in size between the components is more difficult to accomodate as the relationship between mobility and molecular weight is logarithmic. To give rise to a series of bands differing by a constant increment, therefore, the various components would have to bear a logarithmic relationship to one another, in respect of molecular weight, which, over six bands, represents an extremely large difference between the largest and the smallest components.

The hypothesis invoking a difference in charge, which is compatible with the existence of a quaternary structure, is therefore considered more likely correct. However, evidence opposing this view is provided by the results of iso-electric focussing. Only a single band was obtained by this technique in 8 M-urea, suggesting the absence of a quaternary structure or that, if a number of subunits exist, they must be of a very similar composition to all have the same iso-electric point.

In considering the polymorphism of glycoproteins, the possibility of qualitative or quantitative differences in the carbohydrate moiety should not be overlooked. Fraction N_1 contains some firmly bound carbohydrate, in addition to that which is non-covalently bound (see Fig. 31 and ref. 19), and it is possibly differences in this carbohydrate which give rise to the multiple banding (polymorphism) manifested by this fraction on disc gel electrophoresis. Such differences are considered not unlikely as it has been suggested (95) that the synthesis of the carbohydrate moieties of glycoproteins is not under direct genetic control but is only indirectly controlled through the specificity of the synthesising enzymes. Absolute precision in the synthesis of the carbohydrate moieties is therefore not to be expected.

Consider once again the apparent disparity between the results of disc gel electrophoresis and iso-electric focussing. Assuming that fraction N_1 protein does not have a quaternary structure, the multiple banding on disc gel electrophoresis can be reconciled with the results of iso-electric focussing if the polymorphism of the protein is ascribed to differences in the carbohydrate moiety, in particular in the content of an ionic component bearing an equal number of positive and negative charges. However, it must be pointed out that this argument could also explain the results if polymorphic subunits were present so that the question of the presence or absence of a quaternary structure cannot be resolved on the basis of the results of disc gel electrophoresis and iso-electric focussing.

At this stage it should perhaps be mentioned that regular multiple banding upon gel electrophoresis has been observed in the case of other glycoproteins, for example α_1-acid glycoprotein, which exhibits multiple banding in the range pH 2.0 to pH 4.0 (96), certain other plasma proteins, multiply banded at pH 8.6 (97) and the haemagglutinin of _P. vulgaris_, multiply banded at pH 9.0 (98). The observed polymorphism of the α_1-acid glycoprotein and the haemagglutinin of _P. vulgaris_ has been ascribed to differences in nett charge (96, 98).

Evidence from other studies throws some light on the structure of the protein moiety of fraction N_1. For example, the elution profile of fraction N_1S_2 on Sephadex G-100 was found to be identical in the presence or absence of 8 M-urea, suggesting either the absence of a quaternary structure or that the quaternary structure is relatively stable. Tanford (99) has presented evidence of several proteins which are not denatured by 8 M-urea and the suggestion of a stable quaternary structure in the case of fraction N_1S_2 is therefore not entirely untenable. However, circular dichroism studies (Fig. 47) have provided clear evidence that 8 M-urea occasions a shift in the secondary and/or tertiary structure towards a random coil. The maintenance of quaternary structural integrity under these conditions is therefore considered unlikely and the results may be taken to indicate the absence of a quaternary structure.

Further evidence suggesting the absence of a quaternary structure is provided by gel electrophoresis in the presence of sodium dodecyl sulphate (SDS). This technique yielded only a single major band corresponding to a molecular weight of between 90,000 and 160,000 daltons, which is in agreement with the value of 120,000 to 150,000 daltons estimated for the intact molecule by molecular exclusion chromatography and ultracentrifugation. This assumes, however, that SDS would occasion disaggregation of the subunits, if any. SDS was found to dissociate the 22 oligomeric proteins tested by Weber and Osborne (58) but the author is uncertain if these results can be extrapolated to glycoproteins.

The balance of evidence presented so far would appear to favour the view that the protein moiety of fraction N_1 does not have a quaternary structure, at least when newly isolated. However, an effect of ageing should be noted. The availability of molecular weight and sedimentation velocity data enables an estimate to be made of the diffusion coefficient of fraction N_1, by means of the Svedberg equation (p. 44). By this means the diffusion coefficient of fraction N_1 was calculated as 4.0 to 4.5 x 10^{-7} cm^2 sec^{-1} for molecular weights of 140,000 to 120,000 daltons. This is in agreement with the value of 4.0 - 5.0 cm^2 sec^{-1} estimated by molecular exclusion chromatography but is markedly different to the value of 7.76 x 10^{-7} cm^2 sec^{-1} measured by immunodiffusion. Interestingly, however, the value of 7.76 x 10^{-7} cm^2 sec^{-1} is very close to the value of 7.0 x 10^{-7} cm^2 sec^{-1} estimated for peak N_1S_3 (Fig. 32) by the standard curve of $1/D$ vs K_{av} (Fig. 36). The molecular weight of the peak N_1S_3 was estimated from the standard curve of K_{av} vs log MW (Fig. 35) as 50,000 to 52,000 daltons.

It will be remembered that peak N_1S_3 was observed only after ageing of a sample of N_1 and it could be suggested from the evidence presented above that, during the time taken for the immuno-diffusion experiments (2 - 3 weeks), not only does the carbohydrate moiety split off, but the protein also splits into fragments. As 130,000 is not an integral multiple of 50,000 it is difficult to decide the number of fragments, but this is either 2 or 3.

Is fraction N_1 pure? By this stage it will be realised that this is a more complex question than it would at first appear. Fraction N_1 is not pure in the sense that it contains protein, carbohydrate, non-covalently bound carbohydrate and the material of peak N_1S_4. The protein and carbohydrate moieties appear to be complexed when initially isolated but subsequently dissociate. The low-carbohydrate protein is therefore a derivative of the form initially isolated but on the basis of the available evidence, it is impossible to decide whether the carbohydrate/protein complex is the naturally occurring form or whether it is an artifact generated during the isolation procedure. Fraction N_1 would appear to be comprised very largely of the carbohydrate and protein derived from the initial complex, however, and in this sense is largely pure. Molecular exclusion chromatography, ultracentrifugation, immuno-diffusion, immunoelectrophoresis, iso-electric focussing, electrophoresis in SDS and disc gel electrophoresis at low pH all indicate that fraction N_1 is also largely pure in respect of protein constituents and contains only few trace impurities. The impurity present in greatest amount would appear to be the non-protein material of peak N_1S_4. Although this material has been demonstrated to be non-toxic and is unlikely to be relevant to the topic of this study it should be noted that Goldberg _et_ _al_. (100) and Haber _et_ _al_. (101) have implicated non-protein factors from a haemagglutinin preparation of _P_. _vulgaris_ in the stimulation of RNA and DNA synthesis in lymphocytes.

The overall conclusion is that the muco-protein complex initially isolated is virtually pure but is unstable and readily breaks into both carbohydrate and protein fragments. The results obtained in the present study suggest that it would be possible to obtain a pure glycoprotein fraction from fraction N_1 by molecular exclusion chromatography at low pH in the absence of urea. It is impossible to predict the stability of this glycoprotein, however, and it would be necessary to investigate this aspect fully.

5.11.2 Jack bean and soybean toxins

In the case of the jack bean fraction J the results are not as clear cut. Immunological techniques, viz. immuno-diffusion and immunoelectrophoresis indicate that there are two major protein components in fraction J while several other techniques, i.e. column chromatography, disc gel electrophoresis and ultracentrifugation have provided evidence of more than one component. However, immunoelectrophoresis provides evidence that the two antigenic components are related as the precipitin arcs due to these components merge (Fig. 37). Similarly, disc gel electrophoresis of fraction J at various stages of isolation (Figs. 9, 10 and 39) indicates that the major band gives rise to further bands after concentration and freeze-drying. These techniques thus provide evidence that fraction J is unstable and consequently the apparent heterogeneity observed by the various techniques might in fact be due to dissociation of the parent protein.

Fraction J has given no manifestation of the evenly-spaced banding phenomenon observed with fraction N_1 at high pH in 8 M-urea. However, under these conditions fraction J has proved to be anomalous in a different respect. At high pH in 8 M-urea, three bands with low mobilities were observed in the 7.5% gel but corresponding bands were not present in the absence of 8 M-urea. In the absence of urea some material was observed at the origin, however, and it is possible that a proportion of this material could have been solubilised in 8 M-urea, thus giving rise to the additional bands.

The jack bean and soybean fractions J and S have proved to be similar in several respects. These fractions are both apparently hepatotoxins, are similar in respect of protein and carbohydrate fractions, and have similar absorption and CD spectra, while neither could be iso-electrically focussed in the pH range 3.0 to 10.0 and both were apparently adsorbed onto agarose. However, immunoelectrophoresis, immuno-diffusion and ultracentrifugation have indicated that these fractions are not identical.

Of the two, fraction S has proved to be the more enigmatic as no clear bands were obtained by disc gel electrophoresis under various conditions and only a very faint band was obtained in immunoelectrophoresis. At this stage, therefore, it is not possible to comment on whether or not the observed heterogeneity of fraction S is due to the presence of impurities or whether it is due to fragmentation of a parent molecule. An indication that dissociation of at least one molecular species does occur is given by the appearance of the peak SS_2 during molecular exclusion chromatography at pH extremes. The molecular weight of this fraction was estimated from the standard curve for Sephadex G-100 (Fig. 35) as ± 60,000 daltons, so that the material of 234,000 daltons, estimated by ultracentrifugation, could be a tetramer.

5.11.3 Some general considerations

At this stage it may be asked why ion-exchange chromatography was not used in purifying and/or characterising the various fractions. The reason for this omission is that both fraction N_1 and a fraction approximately equivalent to fraction J have previously been studied by ion-exchange chromatography (18, 19). In the case of fraction N_1 no further purification was achieved by gradient elution rechromatography on DEAE-cellulose while on CM-cellulose at low pH a separation of the carbohydrate and protein moieties was observed (19). On the basis of the results of molecular exclusion chromatography presented here this effect can now be attributed more to the low operating pH than to an effect of the CM-cellulose resin.

In the case of the jack bean fraction (18), no significant purification was achieved on CM-cellulose and, on the other hand, none of the fractions recovered after chromatography on DEAE-cellulose were toxic, either individually or in combination. Simple exposure to DEAE-cellulose, without chromatography, did not reduce toxicity, thus ruling out irreversible binding of a toxic factor. The situation observed in the case of DEAE-cellulose is therefore closely paralleled by that observed in the case of tanned gelatin in this study (Chapter 4).

It is possible that in the case of both DEAE-cellulose and tanned gelatin loss of toxic activity was due to the separation of two or more components of the toxic system which cannot subsequently recombine in the original manner. Alternatively, chromatography on these media could possibly lead to structural modification of the toxic component, particularly at the quaternary structure level.

At this stage no suggestions can be made regarding a method for the further purification of fractions J and S although it would appear that some advantage would be gained by the substitution of Sephadex G-200 for the Sephadex G-100 used in this study. Possibly the most profitable avenue to follow in the further study of these compounds in view of their high carbohydrate content would be that of changing from the methods of protein chemistry to those of carbohydrate chemistry.

The results presented in this chapter have been largely successful in that they have given a greater insight into the nature of the three legume toxins. The Natal round yellow bean toxin, in particular, has proved to be most interesting and very similar to the haemagglutinin from the same species (98). This toxin was, however, markedly different to the toxins from the jack bean and the soybean which in turn were very similar.

CHAPTER 6

GENERAL DISCUSSION

From the evidence presented in the preceding chapters it would appear that the toxins isolated from the soybean and jackbean are similar, in respect of both their gross physico-chemical characteristics and their physiological activity, while the NRY-bean toxin has quite distinct properties. Nevertheless all three isolates have one feature in common, namely, they all contain both protein and carbohydrate, and in this final chapter an attempt will be made to discuss these toxins in the light of our present knowledge concerning naturally-occurring carbohydrate-protein macromolecules. In the course of this discussion the NRY-bean toxin will receive the most attention as this isolate has given results which in certain respects are the most interesting and which can moreover be more easily related to previously published work on similar molecules.

A consideration of existing schemes for the classification of high molecular weight substances containing polypeptide and/or polysaccharide affords a useful starting point as this also introduces the problem of the nature of the carbohydrate-protein linkage. In his classification scheme, which is based primarily upon the nature of this linkage, Jeanloz (103) has recognised three classes, viz.,

1) pure polysaccharides
2) compounds containing a carbohydrate component attached to a polypeptide component through a weak link (salt bridge, hydrogen bonding etc.) and,
3) compounds containing a carbohydrate component attached to a polypeptide component through a strong (covalent) linkage.

Gottschalk (104), while acknowledging the existence of two major classes involving carbohydrate and protein, represented respectively by the chondroitin sulphate-protein complex and the α_1 acid glycoprotein of human plasma as prototypes, has questioned the classification based upon the nature of the linkage. He presents evidence that in the chondroitin sulphate-protein type complex the linkage is actually covalent and that the ease with which the carbohydrate and protein moieties may be separated in dilute alkali is due to the fact that the O-glycosidic linkages to serine and threonine are alkali labile.

Gottschalk (104) has therefore proposed a different scheme based upon the structural features of the carbohydrate. The chondroitin sulphate type of polysaccharide, for example, is, he points out, a linear structure composed of small repeating units. The carbohydrate moriety of what he calls "glycoproteins" on the other hand is composed of one or more heterosaccharides with a relatively low number of sugar residues and lacking a serially repeating unit. Gottschalk further notes that there appears to be a predilection for a branched structure in the carbohydrate of these molecules. While he has rejected the classification based upon the nature of the carbohydrate-protein link, Gottschalk nevertheless mentions a covalent bond between the carbohydrate and protein as a requirement in his definition of a glycoprotein.

In the present author's view the classifications suggested by Jeanloz (103) and Gottschalk (104) are not mutually exclusive and the system proposed by Gottschalk could constitute a subdivision of the third group in Jeanloz's scheme. Further divisions and subdivisions in this scheme are also necessary to accommodate the results obtained in the case of the NRY-bean toxin in this study. Furthermore, Marshall and Neuberger, in a review of the structure and metabolism of glycoproteins (106), have pointed out that a given molecule might contain polysaccharide chains attached to the polypeptide chain by different types of linkage so that this possibility should be accommodated in any classification.

A difficulty attending any classification scheme concerns the use of the word "glycoprotein". Should this term be used for all carbohydrate-protein complexes, for covalently linked complexes only, or exclusively for the group of molecules with one or more covalently bound heterosaccharides, lacking a serially repeating unit? Confusion arises as different authors use the term in different senses. For the purposes of this discussion a distinction will be made between non-covalently linked and covalently linked carbohydrate-protein complexes. The former will be referred to simply as carbohydrate-protein complexes while the term "glycoproteins" will be used for covalently linked carbohydrate-protein complexes.

With the above considerations in mind the following classification of macromolecules containing carbohydrate is proposed:

1. Pure polysaccharide
2. Non-covalently linked carbohydrate-protein complexes
3. Covalently linked carbohydrate-protein complexes (glycoproteins)
    (a) labile linkage - chondroitin sulphate-protein type (proteoglycans (105))
    (b) stable linkage - glycoproteins as defined by Gottschalk (104)
4. Polysaccharide - glycoprotein hybrid complexes.

This scheme, which is based upon those of Jeanloz (103) and Gottschalk (104), could have further subdivisions if the possibility of the protein moiety having a quaternary structure is taken into account.

It is important to note that the classification schemes of Jeanloz and Gottschalk are based upon the carbohydrate-protein complexes and glycoproteins from animal tissues and it might be asked to what extent these are similar to the corresponding molecules from plant tissues. In animal glycoproteins, other than collagen, three main types of linkage between carbohydrate and protein have been recognised (106), viz. a linkage in which the amide group of asparagine is linked to the anomeric carbon atom of 2-acetamido-2-deoxy-D-glucose, an O-glycosidic linkage between serine and 2-acetamido-2-deoxy-D-galactose or D-xylose and an O-glycosidic linkage between threonine and

2-acetamido-2-deoxy-D-galactose.

In the case of the soybean haemagglutinin the linkage is very likely of the first type as the carbohydrate, which is present as a single polysaccharide chain, is thought to be linked to an asparaginyl residue through the anomeric carbon atom of N-acetylglucosamine (108). The carbohydrate of the wax bean haemagglutinin, on the other hand, consists of more than one polysaccharide chain (8) but, although the protein has been shown to be rich in aspartic acid and serine residues, the means by which the carbohydrate chains are attached to the protein has not been elucidated. It is therefore clear that further evidence is needed before it can be categorically stated that plant glycoproteins are either similar or different to animal glycoproteins in respect of the number of polysaccharide chains and their means of attachment.

In animal glycoproteins the carbohydrate moiety is commonly composed of mannose, galactose, fucose, glucosamine, galactosamine and sialic acid. Not many plant glycoproteins have been characterised but three legume seed glycoproteins which have been studied (8, 107, 108) contained some or all of these sugars except galactosamine and sialic acid. In the wax bean haemagglutinin (8) glucose, arabinose and xylose were additionally found and arabinose and xylose were also found in the so-called glycoprotein I from haricot beans (107). Again, therefore, although plant and animal glycoproteins have some common sugars there is insufficient evidence to decide whether or not there might be characteristic differences between glycoproteins from these two sources.

In the absence of any evidence suggesting major differences between the glycoproteins or carbohydrate-protein complexes of plants and animals, the classification schemes devised for the molecules from animals may tentatively be assumed to apply also to those from plants. It is difficult, however, to assign the jackbean and soybean toxins described in this thesis to any particular class in this scheme for although these have been shown to contain both carbohydrate and protein the nature of the association between these groups is unknown. The NRY-bean toxin on the other hand would appear to contain both covalently bound and non-covalently bound carbohydrate and is therefore placed in the carbohydrate-glycoprotein hybrid class.

An unusual aspect of the carbohydrate moiety of the NRY-toxin is the apparent autoaggregation of the non-covalently bound carbohydrate "subunits" to form an aggregate larger than the parent molecule. At this stage not much is known about the higher structural orders of carbohydrates. However, Rees (109) has discussed the mechanism of formation of polysaccharide gels and has concluded that the interlocking of the polysaccharide chain segments in the so-called junction zones occurs by a mechanism best described as crystallisation. The fact that the monosaccharide residues are already ordered, along the length of the polysaccharide chain, presumably enables this "crystallisation" to occur at a lower overall concentration than is usual for the monosaccharides themselves. The apparent insensitivity of the formation of the carbohydrate oligomer from fraction N_1 to high urea concentration and to changes in pH is compatible with the involvement of a crystallisation mechanism in this reaction as crystallisation is usually highly stereospecific and involves the cooperation of a number of forces. Furthermore, as carbohydrates are usually neutral molecules their crystallisation is usually not pH-dependent.

Although "crystallisation" and helix formation have been involved in the aggregation of polysaccharides, the exact nature of the forces involved in these processes remains to be completely explained. Strahs (110), however, has invoked hydrogen bonding as the primary force in carbohydrate crystal structure and has also noted that there is virtually a complete absence of water in carbohydrate crystals. Hydrogen bonds would tend to be strengthened by the absence of water and could therefore be a major factor in the crystal structure.

While the aggregation of the carbohydrate "subunits" themselves can be adequately explained by crystallisation, the way in which these are bound to the protein moiety when first isolated requires further consideration. Covalent bonds are considered unlikely due to the ease with which the carbohydrate and protein components can be separated at low or high pH. The separation at high pH could be attributed to the rupture of an alkali-labile O-glycosidic bond through serine or threonine but this does not explain the separation at low pH. Another possibility is that the binding could be electrostatic with the carbohydrate and protein components bearing an opposite charge at pH 7.6 while at pH extremes the charges would become similar, thus promoting disaggregation. However, the most likely possibility is that the non-covalently bound carbohydrate is associated with the covalently bound carbohydrate by the same mechanism as subsequently occasions the auto-aggregation of the non-covalently bound carbohydrate itself. In this way the insensitivity of the association to high urea concentrations is explained while the effect of pH can be attributed to an effect upon the tertiary or quaternary structure of the protein moiety imposing strain upon the carbohydrate bonding forces and promoting disaggregation.

Several glycoproteins have now been isolated from different varieties of P.vulgaris and for the purposes of comparison the physical parameters of these glycoproteins are presented in Table 12.

TABLE 12 Comparison of various glycoproteins from P.vulgaris

Common name	Activity	MW	$S^{o}_{20}W$	pI	Sub-units	Reference
Red kidney bean	haemagglutinating	128,000	7.2	6.5	8	98
"	erythroagglutinating	150,000	7.1	6.5	4	111
"	leucoagglutinating	140,000	6.8	5.0	4	111
Black bean	toxic/ haemagglutinating	126,000	6.04	5.9	-	112
Wax bean	haemagglutinating	132,000	5.37	5.5	-	8
Haricot bean	antigenic	140,000	7.59	5.4	4	113
NRY-bean	toxic	145,000	6,65	6.8	2-3	this thesis

From Table 12 it may be seen that these molecules have a distribution of physical parameters but that they are not entirely dissimilar in their overall properties. The NRY-bean toxin has physical parameters falling within the range of the published properties of the haemagglutinins from three varieties of P.vulgaris and, as has been mentioned (p.136), is similar to the muco-phytohaemagglutinin from the red kidney bean (90) in its association with non-covalently bound carbohydrate.

It is therefore again tempting to speculate that the toxin from the NRY-bean might be related to the haemagglutinins and possibly other glycoproteins from P.vulgaris in some manner. It is possible, for example, that these various molecules might represent secondary (post-ribosomal) modifications of essentially the same parent molecule which, in the case of glycoproteins, could involve either the protein component, the polysaccharide, or both. In this regard it should be pointed out that there is a possibility that the NRY-bean toxin could, in fact, be identical to the haemagglutinin from the same bean, but with the haemagglutinating site blocked. This is considered not impossible as Kornfeld and Kornfeld (117) have shown that the activity of the haemagglutinin from red kidney beans (another variety of P.vulgaris) can be abolished by reaction with a specific glycopeptide.

All of the glycoproteins of P.vulgaris which have been analysed have proved to have a similar amino acid composition characterised by a high proportion of aspartyl and seryl residues, which are potential carbohydrate attachment points (106), and the virtual absence of sulphur-containing amino acids. In general less attention has been paid to analysis of the carbohydrate moieties of these glycoproteins but in those cases which have been analysed (8, 113, 114) the polysaccharides have proved to be different. The polysaccharide moiety is therefore considered to be a more likely area of difference between the different molecules.

It must be emphasised that activities such as haemagglutinating activity, leucoagglutinating activity, antigenicity and toxicity must each be the adventitious consequence of a particular molecular structure, related to a specific physiological function in vivo as these activities in themselves appear to have no physiological significance in the legume seed (a possible exception here is mitogenic activity). It may be asked, therefore, what effect the addition of polysaccharide has upon the molecular properties of the protein and what physiological rôle this could have?

The addition of a polysaccharide to a protein at asparaginyl, seryl or threonyl residues would result in a nett increase in the hydrophilicity of the molecule but the physiological usefulness of this property is not known. However, it could be that the addition of polysaccharide increases the tendency to aggregation by carbohydrate-carbohydrate interactions as outlined above. Many of the adventitious activities of legume seed glycoproteins can be explained in terms of a tendency to "aggregate" with other polymers. Kornfeld and Kornfeld (117), for example, have shown that haemagglutination, brought about by the haemagglutinin from red kidney beans, is due to interaction with an oligosaccharide on the red blood cell membrane while Bourrillon and Font (118) have shown that the active part of the haemagglutinin from Robinia pseudoaccacia is a glycopeptide. There is a possibility therefore that haemagglutination might in certain cases be due to a carbohydrate-carbohydrate interaction. That this is not universally true, is shown by the jack bean haemagglutinin, concanavalin A, which is a protein, devoid of carbohydrate (72, 73), but which reacts with carbohydrate groups (47). Even in this case, however, the activity can be ascribed to an "aggregation" reaction. Toxicity, as manifest by the glycoproteins described in this thesis could also be due to "aggregation", possibly with a vital cell component. The apparent interaction of the soybean and jackbean toxins with agarose could be significant in this respect as it is indicative of a tendency on the part of these toxins to associate with certain carbohydrates.

The physiological usefulness of aggregation mechanisms may be explained in terms of the storage of protein material in the seed. A major part of seed protein is storage (ergastic) protein which in the intact seed is located within particulate bodies known as aleurone grains (115) and utilisation of certain ergastic proteins during germination has been shown to involve a disaggregation of the protein into subunits (116). Considering this process in the reverse direction, it may be speculated that, during formation of the seed, a tendency for the protein subunits and the proteins or glycoproteins themselves to aggregate might be an indispensable requirement as only by an aggregation mechanism could the storage molecules be "packed" into the aleurone grains without these undergoing destructive osmotic swelling.

Conclusion. The findings reported in this thesis have served to reinforce the earlier suggestion (16, 17) that the toxicity of isolated bean proteins is not essentially related to haemagglutinating activity although in the case of the jackbean a mutual potentiating effect between the haemagglutinin and the remaining toxin(s) was observed. The possibility of this relationship existing between the haemagglutinins and other toxins in the soybean and NRY-bean has not been determined.

The toxic isolates from the jackbean and soybean described here apparently belong to a class of legume carbohydrate-protein molecule which has not previously been described. The NRY-bean toxin on the other hand, by virtue of the linkages between the protein and carbohydrate moieties, does not fit into any of the previously documented classes of protein-carbohydrate macromolecule. Further studies on these compounds should therefore prove of interest but in future work emphasis should possibly be placed on the nature of the carbohydrate groups and their attachment to the protein moieties. In the case of the jackbean and soybean preparations the nature of the material not accounted for in terms of carbohydrate and protein might also receive attention.

While the present study leaves a number of unanswered questions it is hoped that the information gained will pave the way for future studies upon the biochemistry and the physiological and pharmacological properties of this interesting group of plant proteins which are toxic to animals.

SUMMARY

Two previous studies carried out in this laboratory had resulted in the isolation and partial purification of glycoprotein isolates from the Natal round yellow (NRY) bean (a variety of *P. vulgaris*) and the jackbean (*Canavalia ensiformis*), both of which were toxic when administered to rats or mice by intraperitoneal injection but which elicited only low haemagglutinating activity. In view of the general lack of information on this class of compound it was decided to undertake a comparative study of the properties of these two toxic proteins and of a similar preparation isolated from the soybean.

A procedure involving aqueous extraction, pH fractionation and acetone precipitation was successfully used to prepare extracts from the jackbean and the soybean but this procedure was not as effective in the case of the NRY-bean. In the latter case, therefore, an alternative procedure was used which included, additionally, an ammonium sulphate precipitation step.

In the course of the preparation of the jack bean extract it was discovered that the haemagglutinin and the toxin(s) from this bean have a mutual potentiating effect so that a mixture of the two is more toxic than either of the components alone.

A toxic isolate was prepared from each of the crude extracts. Purification of the jackbean and soybean toxins was achieved by chromatography on Sephadex G-100 but again this method could not be used for the NRY-bean and in this case recourse was made to stepwise elution chromatography on DEAE-cellulose. The toxic isolates from the jackbean and soybean were found to be devoid of haemagglutinating activity while that from the NRY-bean contained a very low level of haemagglutinating activity.

The amounts of material which could be prepared by chromatography on Sephadex G-100 were found to be limiting and consequently, with a view to preparing larger amounts of material, the possibility of using tanned gelatin

as a chromatographic medium was investigated. Tanned gelatin was found to have mechanical properties suitable for the preparation of large amounts of material but it was found to interact with sample proteins, causing these to be retarded during chromatography and in the case of the jackbean extract at least, evidence was obtained that the sample proteins were modified during chromatography. Tanned gelatin therefore proved unsuitable for the preparation of the jackbean toxin and was not investigated further.

The three toxic isolates, prepared by chromatography on Sephadex G-100 or DEAE-cellulose were compared and characterised by a number of physicochemical methods. It was concluded that the jackbean and soybean isolates were not homogeneous although in the former case the two major components were found to be antigenically related. The NRY-bean toxin on the other hand was found to contain only trace impurities but it was found that non-covalently bound carbohydrate tended to spontaneously split off from the parent molecule and subsequently aggregate to form a complex larger than the parent molecule. This reaction apparently occurs more readily at pH extremes.

The measured values for the molecular parameters of the NRY-bean toxin were S^{o}_{20w}, 6,65 S; diffusion coefficient, 4.0-5.0 $cm^2\ sec^{-1}$; molecular weight, 145,000 daltons and pI, 6.8. The two major components of the jackbean toxin both had S_{20w} values of approximately 7.9 S but were distinguished by having diffusion coefficients of 4.6 and 3.45 $cm^2\ sec^{-1}$ respectively, corresponding to molecular weights of 140,000 and 190,000 daltons. The respective values for the soybean toxin were S_{20w}, 6.0 S; diffusion coefficient, 2.12 $cm^2\ sec^{-1}$ and molecular weight, 234,000 daltons. A distinguishing feature of the jackbean and soybean isolates was their high proportion of carbohydrate, which was more than half as much again as that of the NRY-bean toxin, and their relatively low protein content. All three isolates contained a high proportion of aspartyl residues and the NRY-bean isolate in particular also contained a relatively high proportion of seryl residues.

In its overall properties the NRY-bean toxin proved to be very similar to a haemagglutinin isolated from another variety of Phaseolus vulgaris while the jackbean and soybean toxins, although being similar to one another, apparently do not fit into any of the previously described class of compound.

APPENDIX 1

ESTIMATION OF MOLECULAR WEIGHTS BY MOLECULAR EXCLUSION CHROMATOGRAPHY

The supposition that separation by molecular exclusion chromatography is dependent upon molecular size differences has led many workers to put forward theoretical models to explain the relationship between elution volume and molecular size and to propose methods of using molecular exclusion chromatography for the estimation of molecular sizes (see reviews by Andrews (37) and Ackers (119).

An empirical method for the estimation of molecular sizes which is currently favoured due to its simplicity, is that proposed by Andrews (79, 80) in which a standard curve, relating the elution volume (V_e) to $\log_{10}$ MW, is constructed using data obtained by chromatography of a number of standard proteins (Fig. 51). An advantage of Andrews' plot is that the properties of a given gel column can be easily visualised from the standard curve. A disadvantage of the method is that the parameter V_e is dependent upon many column variables so that the standard curve can only be applied to a particular column.

Laurent and Killander (120) have introduced a parameter known as K_{av} (the availability constant) which refers to the fraction of the gel phase which is available to any given solute molecule (Fig. 50) and can be defined as follows:-

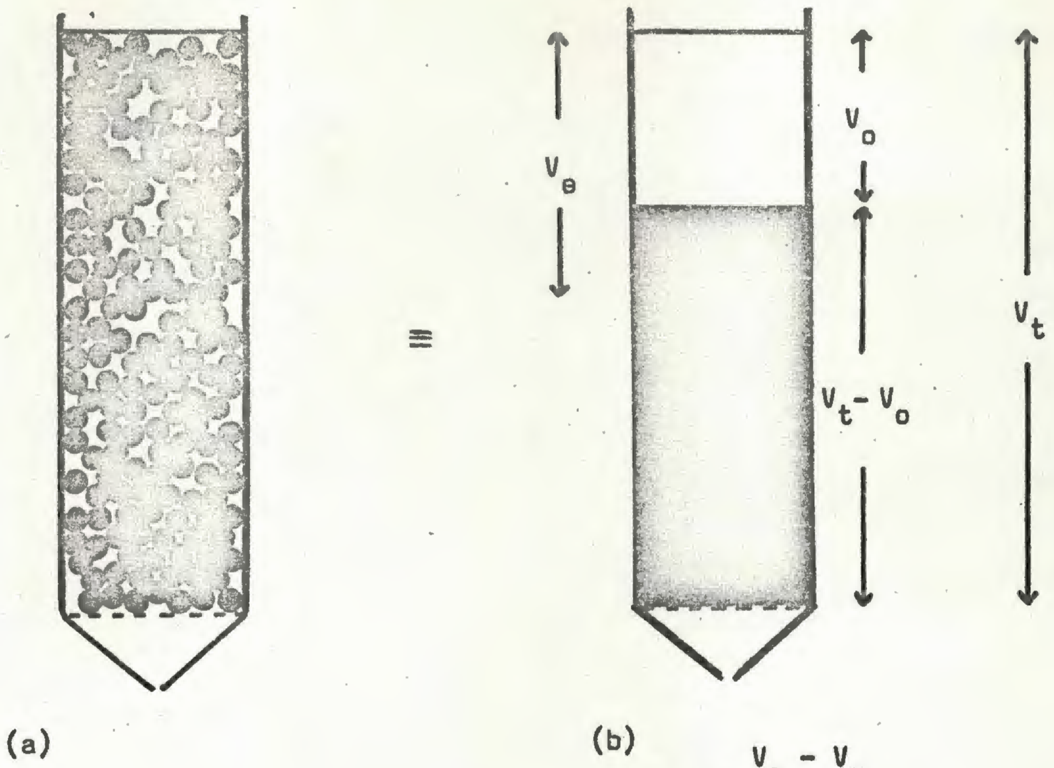
$$K_{av} = \frac{V_e - V_o}{V_t - V_o} \quad \ldots\ldots\ldots\ldots \quad [1]$$

where V_e = elution volume of a given solute;

V_o = void volume in the gel bed;

V_t = total volume of gel bed.

An advantage of the term K_{av} is that for a given solute, chromatographed on a given gel, K_{av} is constant and is independent of the dimensions of the column or of packing variables. In the case of a standard curve relating K_{av} to MW,



$$K_{av} = \frac{V_e - V_o}{V_t - V_o}$$

FIGURE 50 Illustration of the term K_{av}

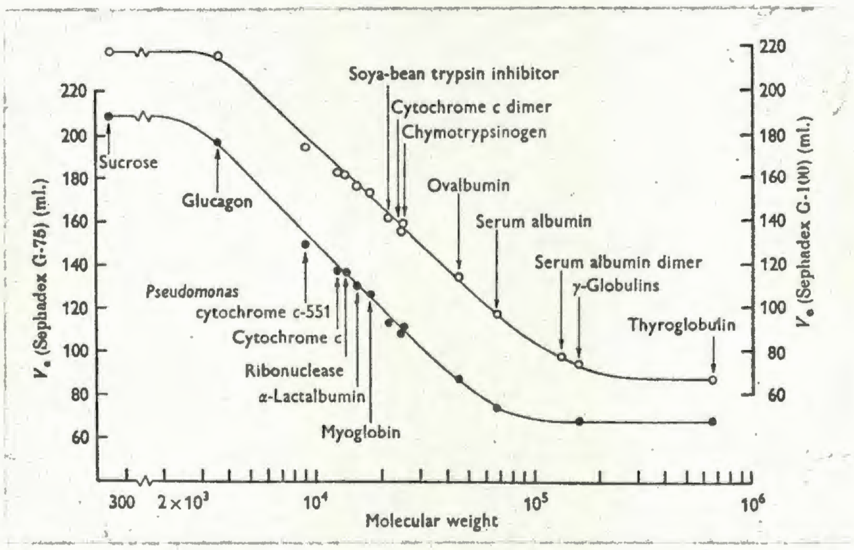


FIGURE 51 Relationship between V_e and $\log_{10}$ MW on Sephadex G-100. (From Andrews (37))

therefore, it is in effect the gel which is standardised. Fischer (36) has combined the advantages of simplicity inherent in Andrews' plot with the advantages of the term K_{av} and has presented standard curves relating K_{av} to $\log_{10}$ MW for Sephadex G-75, G-100 and G-200.

From equation [1] it follows that

$$V_e = (V_t - V_o)K_{av} + V_o \quad . \; . \; . \; . \; . \; . \; . \; . \; . \; . \quad [2]$$

and hence a plot of V_e vs K_{av} is linear and can be easily plotted from the values when $V_e = V_o$ and $V_e = V_t$. This provides a convenient method of determining K_{av} values from V_e values for a given column.

Although K_{av} has the range $0 \leq K_{av} \leq 1$ it is not the same as the Rf value used in other forms of chromatography. The gel chromatography Rf value (i.e. with a moving origin) is given by the expression

$$Rf = \frac{V_t - V_e}{V_t - V_o} \quad . \; . \; . \; . \; . \; . \; . \; . \; . \; . \quad [3]$$

Hence

$$Rf = 1 - K_{av} \quad . \; . \; . \; . \; . \; . \; . \; . \; . \; . \quad [4]$$

A limitation of standard curves relating V_e or K_{av} to log MW is that the relationship is empirical and when plotted graphically is curvilinear. An alternative method, having a theoretical basis is therefore sometimes used (19, 41, 121). Ogston (122) has calculated the spaces available to a spherical molecule in a uniform random suspension of rigid fibres. Laurent and Killander (120) have applied Ogston's model to the situation pertaining during gel chromatography and have related K_{av} to the Stokes radius of a molecule as follows:

$$K_{av} = \exp\left[- \pi L(r_s + r_r)^2\right] \quad . \; . \; . \; . \; . \; . \; . \; . \quad [5]$$

where r_s = Stokes radius of sample molecule;

r_r = radius of macromolecular rods constituting the gel network;

L = concentration of macromolecular rods.

Sims and Folkes (121) have extended the theory of Laurent and Killander but their work has only been published in a manufacturer's catalogue and is consequently presented here in some detail. These authors have expressed equation [5] more simply as

$$\sqrt{-\ln K_{av}} = \alpha(\beta + r_s) \quad \ldots\ldots\ldots\ldots \quad [6]$$

where α and β are constants ($\alpha = \sqrt{\pi L}$, $\beta = r_r$) . Equation [6] can be written in the form

$$\sqrt{-\ln K_{av}} = \alpha r_s + \alpha\beta . \quad \ldots\ldots\ldots\ldots \quad [7]$$

Considering $\sqrt{-\ln K_{av}}$ and r_s as variables, equation [7] is an equation for a straight line, with slope α and ordinate intercept $\alpha\beta$. Now, in the case of spherical molecules

$$MW = \frac{4}{3}\pi r^3 \rho$$

where r = molecular radius;

ρ = density of molecule.

Hence,

$$r \propto \sqrt[3]{MW}$$

$$\text{i.e.} \quad r = k\sqrt[3]{MW}$$

where k is constant for molecules of equal density.

And, since for spherical molecules $r \propto r_s$,

$$r_s \propto \sqrt[3]{MW} .$$

Equation [7] can therefore be written in the form

$$\sqrt{-\ln K_{av}} = \alpha_1 \sqrt[3]{MW} + \alpha\beta \quad \ldots\ldots\ldots\ldots \quad [8]$$

This is again a linear relationship and a plot of $\sqrt{-\ln K_{av}}$ vs $\sqrt[3]{MW}$ therefore provides a convenient means of relating K_{av} to MW.

In an extremely interesting theory, based upon thermodynamic considerations and involving a minimum number of assumptions, Hjerten (123) has presented equations relating V_e and MW. These equations are too complex to be of practical value in the estimation of molecular weights, however, and for this purpose Hjerten has suggested a first approximation plot of $-\ln K_{av}$ vs $MW^{2/3}$, which he has pointed out is virtually linear in the range in which secondary factors can be ignored, i.e. for non-extreme values of K_{av}.

Although this has not previously been referred to in the literature it is interesting to note that Sims and Folkes' plot of $\sqrt{-\ln K_{av}}$ vs $\sqrt[3]{MW}$ in fact expresses the same relationship between MW and K_{av} as Hjerten's plot of $-\ln K_{av}$ vs $MW^{2/3}$ and the latter relationship may be derived from the former by squaring of the terms. This is interesting as the two relationships were derived by entirely different means, one being based upon a steric model while the other was derived from thermodynamic considerations. The compatibility of the two models therefore indicates that these are probably different but fairly accurate representations of the real situation.

A factor which has not been clearly stated by authors who have presented data graphically in terms of the variables of equation [7] (37, 120, 124) and equation [8] (19, 41, 125) is that for real molecules these equations have only a limited range of validity. Thus it is apparent that V_e can equal both V_o and V_t, and hence K_{av} can equal both 0 and 1, for finite positive values of MW and also of r_s. Now, when $K_{av} = 1$, $\sqrt{-\ln K_{av}} = 0$ and when $K_{av} = 0$, $\sqrt{-\ln K_{av}} = \infty$. Therefore, within a range of positive finite values of MW or r_s,

$\sqrt{-\ln K_{av}}$ must have the range $0 \leq \sqrt{-\ln K_{av}} \leq \infty$. Experimental evidence of this may be derived from the results of Andrews (37) (Fig. 51) which have been transformed and presented in terms of the variables $\sqrt{-\ln K_{av}}$ and $\sqrt[3]{MW}$ in Fig. 52. It is apparent from Fig. 52 that, provided sufficient points are considered, the relationship is actually curvilinear.

The curve is approximately linear in the range $0.25 \leq K_{av} \leq 0.75$ $(1.18 \geq \sqrt{-\ln K_{av}} \geq 0.54)$ and hence within this range errors in the estimation of molecular weights are small. However, this is approximately the same range of linear reliability as the plot of K_{av} vs log MW and consequently it would appear that, apart from the questionable merits of a theoretical basis, there is little to be gained by the use of the more complicated $\sqrt{-\ln K_{av}}$ vs $\sqrt[3]{MW}$ plot. Similar arguments can be used in the case of the linear plots of $\sqrt[3]{Kd}$ vs $\sqrt{MW}$ and $\sqrt[3]{Kd}$ vs $\sqrt[3]{MW}$ of Porath (126) and Andrews (37) respectively.

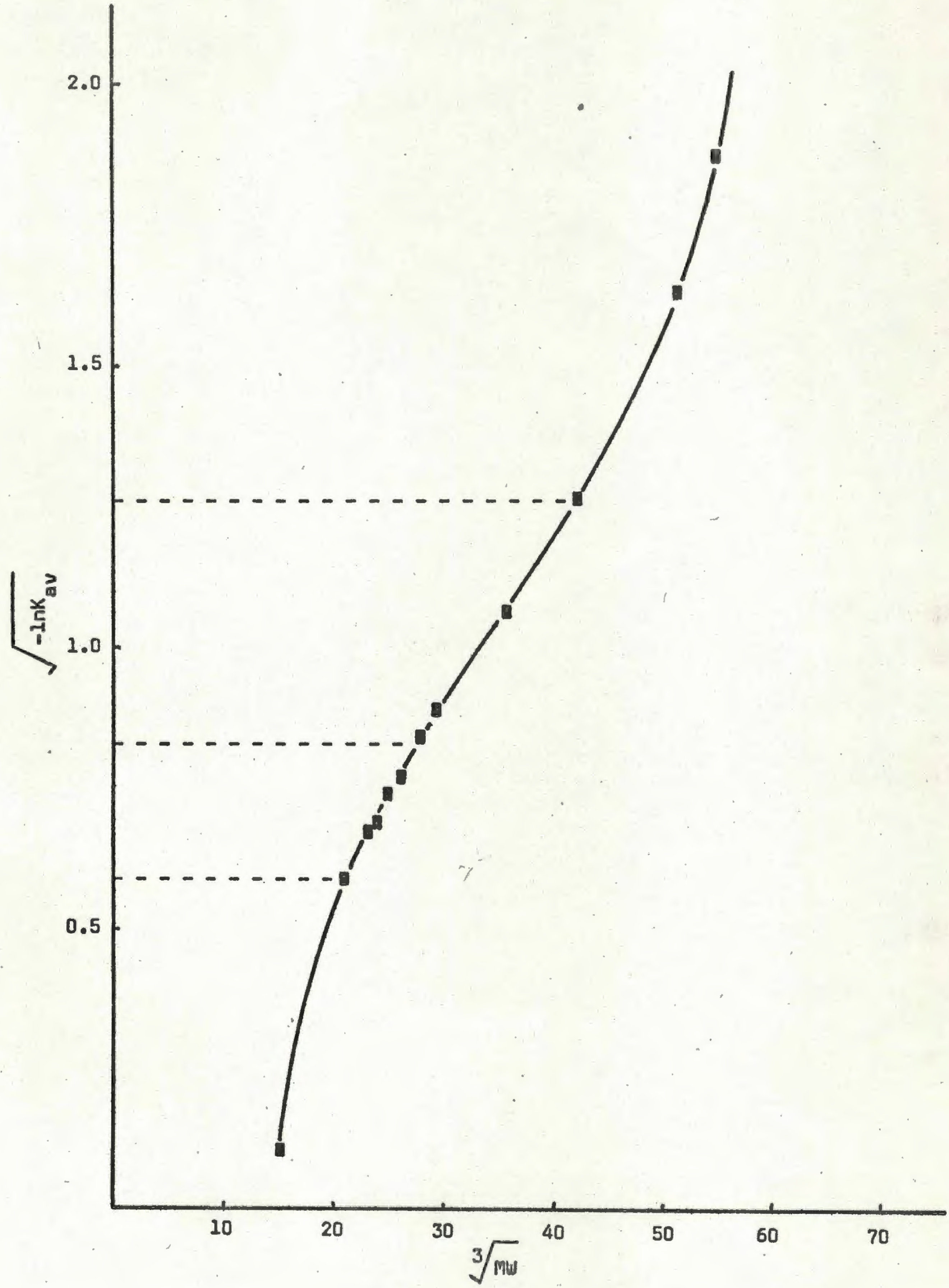


FIGURE 52 Relationship between $\sqrt{-\ln K_{av}}$ and $\sqrt[3]{MW}$ on Sephadex G-100 (data from Andrews (37))

APPENDIX 2

COMPUTER PROGRAMME FOR THE CALCULATION OF SEDIMENTATION COEFFICIENTS

```
LOG DRIVE   CART SPEC   CART AVAIL  PHY DRIVE
  0000        000D        000D        0000

V2 M10   ACTUAL 16K  CONFIG 16K

// XEQ LIST2

// FOR
*ONE WORD INTEGERS
*LIST SOURCE PROGRAM
*IOCS (CARD,1132 PRINTER)
C----    RUN ON AN I.B.M. 1130 COMPUTER WITH 8K CORE.
C----      1132 LINE PRINTER AND 1442 CARD READER.
C----            COMPUTER CENTRE - UNIVERSITY OF NATAL.
C     CALCULATION OF SEDIMENTATION COEFFICIENTS
C
      DIMENSION TIME(50),Y(50),CONC(20),SW(20),NAME(20),CS(50)
      PI=3.14159265
      READ(2,100) NEXP,NAME
 100  FORMAT(I2,2X,20A2)
C---- NEXP - NUMBER OF EXPERIMENTS
C----  N   - NUMBER OF OBSERVATIONS.
C----  RPM - REVOLUTIONS PER MINUTE.
C----  FACT - CORRECTION FACTOR.
C----  CONS - CONCENTRATION.
      DO 10 JEXP=1,NEXP
      READ(2,101) N,RPM,FACT,CONS
 101  FORMAT(I2,F8.0,F8.4,F6.1)
      DO 2 J=1,N
      READ(2,102) TIME(J),CS(J)
 102  FORMAT(F10.0,F10.2)
C----  TIME - TIME IN MINUTES.
C----  CS - RADIAL DISTANCE IN CMS.
      Y(J)=ALOG(CS(J))
 2    CONTINUE
      CALL FIT(N,TIME,Y,A,B,SER)
C----  THIS FITS THE STRAIGHT LINE  Y=A+B*TIME
      W=RPM/60.*(2.*PI)
C----  W - OMEGA  ROTATION IN RADIANS PER SECOND.
      WRITE(3,103) N,RPM,W,FACT,CONS
 103  FORMAT(1H1,I2,' OBSERVATIONS   R.P.M.=',F7.0,5X,'W=',F10.1,5X,
     1       'FACT=',F8.4,5X,'CONCENTRATION=',F5.1/)
      WRITE(3,104)
 104  FORMAT(7X,'TIME',7X,'CMS',6X,'LOG',7X,'EST',6X,'DIFF'/)
      DO 3 J=1,N
      YEST=A+B*TIME(J)
      DIFF=Y(J)-YEST
      WRITE(3,105) TIME(J),CS(J),Y(J),YEST,DIFF
 105  FORMAT(1X,F10.0,F10.2,F10.4,F10.4,F10.4)
```

```
  3    CONTINUE
C----   SOBS - OBSERVED SEDIMENTATION CO-EFFICIENT.
       SOBS=B/(60.*W*W)*1.E13
C----   S20W - CORRECTED SEDIMENTATION CO-EFFICIENT AT 20 DEG.
       S20W=FACT*SOBS
C----   COMPUTED VALUES SAVED FOR SUBSEQUENT REGRESSION.
       SW(JEXP)=S20W
       CONC(JEXP)=CONS
       WRITE(3,106) B,SOBS,S20W,SER
  106  FORMAT(1H0,'SLOPE=',F8.5,5X,'SOBS=',F6.2,5X,'S20W=',F6.2,5X,
     1        'STD.ERROR=',F8.5)
  10   CONTINUE
       CALL FIT(NEXP,CONC,SW,A,B,SER)
C----   THIS FITS THE STRAIGHT LINE  SW=A+B*CONC
C----           TO ENABLE US TO FIND INTERCEPT AT ZERO.
       WRITE(3,107) NAME
  107  FORMAT(1H1,10X,20A2/)
       WRITE(3,108)
  108  FORMAT(7X,'CONC',6X,'S20W',7X,'EST',6X,'DIFF'/)
       DO 4 J=1,NEXP
       YEST=A+B*CONC(J)
       DIFF=SW(J)-YEST
       WRITE(3,109) CONC(J),SW(J),YEST,DIFF
  109  FORMAT(1X,F10.2,F10.2,F10.2,F10.2)
  4    CONTINUE
       WRITE(3,110) A,B,SER
  110  FORMAT('0','S20W AT CONC=0',F7.3,5X,'SLOPE=',F8.4,5X,'STD.ERROR=
     1        F8.4)
C----   THE PROCEDURE IS REPEATED WITH THE RECIPROCALS.
       DO 5 J = 1,NEXP
       SW(J) =  1./ SW(J)
  5    CONTINUE
       CALL FIT(NEXP,CONC,SW,A,B,SER)
       WRITE(3,111)
  111  FORMAT(1H0,6X,'CONC',5X,'RECIP',7X,'EST',6X,'DIFF'/)
       DO 6 J = 1,NEXP
       YEST = A + B * CONC(J)
       DIFF = SW(J) - YEST
       WRITE(3,112) CONC(J),SW(J), YEST,DIFF
  112  FORMAT(1X,F10.2,3F10.6)
  6    CONTINUE
       SREC = 1. / A
       WRITE(3,110) SREC,B,SER
       STOP
       END
```

APPENDIX 3

COMPUTER PROGRAMME FOR THE CALCULATION OF MOLECULAR WEIGHTS

```
// FOR
*ONE WORD INTEGERS
*LIST SOURCE PROGRAM
*IOCS (CARD,1132 PRINTER)
C----     RUN ON AN I.B.M. 1130 COMPUTER WITH 8K CORE.
C----       1132 LINE PRINTER AND 1442 CARD READER.
C----             COMPUTER CENTRE - UNIVERSITY OF NATAL.
C.....  CALCULATION OF MOLECULAR WEIGHTS -
      DIMENSION R(50),DO(50),R2(50),Y(50),NAME(20)
      DIMENSION NNRUN(40),WTMS(40),SERS(40)
C       R  - MEASURED R
C       DO - OPTICAL DENSITY
C       R2 - ( R/MF + 5.7 ) ** 2
C       Y  - LN(OD)
C       NAME - NAME OF EXPERIMENT - 40 CHARACTERS
      KOUT=3
      KIN=2
      CR=8.313E07
      TOPI=2.*3.14159265
C----   NN- NUMBER OF EXPERIMENTAL RUNS.
      READ(KIN,119)NN
 119  FORMAT(I2)
      DO 99 JN=1,NN
      READ(KIN,100)NCOMP,NRUN,N,NAME
 100  FORMAT(3I4,20A2)
      READ(KIN,101)FM,T,RPM,DEN
 101  FORMAT(4F12.4)
C----   FM - MAGNIFICATION FACTOR.  TO BE DIVIDED BY 1.62
C----   T - ABSOLUTE TEMPERATURE.
C----   RPM - REVOLUTIONS PER MINUTE.
C----   DEN - BOUYANCY CONSTANT.
      WW=(RPM/60.*TOPI)**2
C----   WW - OMEGA SQUARED.
      FMM=FM/1.62
      READ(KIN,106)(R(J),DO(J),J=1,N)
 106  FORMAT(2F8.3)
      DO 4 J=1,N
      R2(J)=(R(J)/FMM+5.7)**2
      Y(J)=ALOG(DO(J))
 4    CONTINUE
C
C       LINEAR REGRESSION OF Y ON R**2
      CALL FIT(N,R2,Y,A,B,SER)
C
      WRITE(KOUT,113) NCOMP,NRUN
  113 FORMAT(1H1,'COMPOUND',I4,5X,'RUN NO.',I4)
      WRITE(KOUT,102) NAME,FM,T,WW,DEN
 102  FORMAT(1X, 20A2,2X,'MAGNIFICATION FACTOR =',F6.2,'/1.6',4X,
     *      'TEMP.=',F6.2/43X,'W2=',E12.5,4X,'DENOM=',F8.5)
```

```
      WRITE(KOUT,103)
 103  FORMAT(6X,'R',6X,'R**2',6X,'O.D.',4X,'LN(OD)',7X,'EST',6X,'DIFF'/
      DO 6 J=1,N
      YE=A+B*R2(J)
      DIFF=Y(J)-YE
      WRITE(KOUT,104) R(J),R2(J),DO(J),Y(J),YE,DIFF
 104  FORMAT(1X,F8.2,F8.2,F10.3,F10.5,F10.5,F10.5)
 6    CONTINUE
C----  B   - SLOPE OF THE GRAPH USING NATURAL LOGS.
      SLOPE=B/ALOG(10.)
C----  SLOPE - SLOPE OF THE GRAPH USING LOGS TO THE BASE 10.
      F1=CR*T/(DEN*WW)
      WTM=2.*F1*B
C----  WTM - MOLECULAR WEIGHT.
      WRITE(KOUT,105) F1,WTM,SER,SLOPE
 105  FORMAT(1H0,'F=',F10.2,5X,'MOL WT.=',F10.2,5X,'STD. ERROR',F8.5,
     1       5X,'SLOPE=',F8.5)
C
C----  COMPUTED VALUES SAVED FOR SUMMARY AT THE END.
C
      WTMS(JN)=WTM
      SERS(JN)=SER
      NNRUN(JN)=NRUN
 99   CONTINUE
C----  SUMMARY OF CALCULATIONS.
      SUM=0.
      SS=0.
      WRITE(KOUT,112)
 112  FORMAT('1SUMMARY'//1X,'  COMP   RUN    MOL WT    ST ERR')
      DO 7 K=1,NN
      SUM=SUM+WTMS(K)
      SS=SS+WTMS(K)**2
      WRITE(KOUT,110)NNRUN(K),WTMS(K),SERS(K)
 110  FORMAT(7X,I6,F10.1,F10.4)
 7    CONTINUE
      AVE=SUM/FLOAT(NN)
      SIG=SQRT((SS-SUM*AVE)/FLOAT(NN))
      WRITE(KOUT,111)AVE,SIG
 111  FORMAT(1H0,'AVE=',F10.2,10X,'STD.DEV.=',F8.2)
      STOP
      END
```

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