

THE ISOLATION OF A TOXIC FACTOR FROM A
LOCAL CULTIVAR OF *PHASEOLUS VULGARIS*,
AND AN ASSESSMENT OF ITS RELATION TO
GROWTH DEPRESSION

by

ROBIN HUGH STEAD

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INTRODUCTION

The isolation of proteins has long been a major area of biochemical endeavour. The pioneer studies of Sumner, Northrop, Chibnall and others demonstrated the feasibility of manipulating proteins by chemical methods. Northrop's isolation of pepsin using rigorous criteria of purity based on solubility properties (1) remains one of the milestones in protein chemistry.

One of the reasons for the active interest in protein isolation has been the wide range of biological activity manifested by proteins. The unique specificity of action of enzymes, hormones and antibodies gives to them a key role in biochemical processes and poses fundamental questions as to the structural origin of such activity. Thus the ultimate objective in this, the separative or analytical phase of protein chemistry is an understanding of the mode of action of biological macromolecules. In other words the objective may be said to be the elucidation of a direct causal relationship between structure and function in proteins. Encouraging signs of progress towards this ideal are to be found in the detailed structure of lysozyme exposed by the X-ray diffraction studies of Blake, Koenig, Mair, North, Phillips and Sarma (2) which, coupled with the substrate affinity data of Pollock, Chipman and Sharon (3) provide a convincing explanation of the possible structural basis for lysozyme activity.

In only a few instances are conditions suitable for structural studies such as those on lysozyme. In most, the emphasis is still on the separation of proteins as homogeneous species - a prerequisite for any structural studies. This applies to the present investigation, in which the major objective was to isolate a protein in homogeneous form.

The incentive for isolation of proteins from legume seeds is provided by the potent biological activity of many proteins from this source. Raw legumes elicit a wide range of effects on living tissue, either when fed to laboratory animals, or when injected intraperitoneally, or when brought into contact with living cells *in vitro*.

The nutritional quality of legumes has been under discussion for some fifty years. The pioneer studies of Osborne and Mendel (4) on soybeans (*Glycine max*) revealed a problem which has not been fully solved to this day. The needs of an expanding world population provide an additional incentive to discover means of making legume proteins safe for human and animal consumption without resorting to costly processing procedures.

Osborne and Mendel observed that raw soybeans would not support the growth of laboratory rats, while heating improved their nutritional value. It is this growth-depressing effect which has eluded explanation for so long. Greater interest in recent years has brought a satisfactory description of one aspect of growth depression, but the problem has yet to be explained in its entirety.

Two observations have contributed most to our current understanding of the nature of growth depression. The first, that the effect could be counteracted by supplementation with cystine (5), led to the concept of non-availability of amino acids as the major cause of growth depression. Subsequently methionine was found to be the most limiting amino acid in soybean meal (6), while threonine and valine were next limiting (7). The lower protein digestibility of raw soybean meal (RSBM) (8) added substance to the concept that growth depression was caused by a deficiency and unavailability of essential amino acids.

The second aspect of the growth depression problem has its origin in a suggestion made by Westfall and Hauge (9) that since RSBM contains a trypsin inhibitor, unavailability of amino acids and poor digestibility may be caused by a curtailment of intestinal proteolysis through inhibition of tryptic activity. Unfortunately, experimental evidence accumulated which rendered this attractive hypothesis untenable (10 - 12). Nevertheless the role of the "trypsin inhibitor" (a protein concentrate, high in trypsin-inhibiting activity) in growth depression could not be disputed. Ham and Sandstedt's observation

that the trypsin inhibitor induced hypertrophy of the pancreas (13) brought to light the major physiological change which accompanies growth depression. Pancreatic hypertrophy was accompanied by excessive secretion of digestive enzymes (14), and elevated levels of intestinal nitrogen and protease (15). The observation that a chromatographically purified soybean trypsin inhibitor stimulated excessive pancreatic secretion (16) established beyond reasonable doubt that the effect is caused by the trypsin inhibitor.

Final demonstration of the role of the trypsin inhibitor in growth depression was achieved by controlling two variables previously encountered in comparative feeding trials. Khayambashi and Lyman (17) equalised food consumption and amino acid availability by tube feeding an amino acid diet which simulated RSBM. Addition of a small proportion of partially purified trypsin inhibitor caused growth depression, pancreatic hypertrophy and the other accompanying changes. The specificity of the trypsin inhibitor in stimulating hypertrophy and hypersecretion of the pancreas, and in depressing growth, was thus established. Final proof that amino acid wastage was the reason for trypsin inhibitor-induced growth depression was obtained by Barnes, Kwong and Fiala (18) and by Barnes, Fiala and Kwong (19) following an observation of the beneficial effect of penicillin. Restoration of normal growth occurred when penicillin was added to a RSBM diet, an effect which was shown to be due to the prevention of bacterial destruction of cystine-rich enzymes - particularly trypsin (18). In the absence of bacterial degradation, faecal cystine could be ingested by coprophagy and re-cycled to the upper intestinal tract. Prevention of coprophagy was found to abolish the beneficial effect of penicillin (19), providing elegant proof that the trypsin inhibitor-induced growth depression is the result of an amino acid deficiency induced by excessive pancreatic secretions. The beneficial effect of supplemental methionine and cystine could be entirely accounted for by the increased loss of cystine due to increased secretion of trypsin.

But trypsin inhibitors do not account for the total growth depression effect. Rackis (20) calculated that 30 - 50% of growth depression and nearly all of the pancreatic hypertrophy could be attributed to soybean trypsin inhibitor, while a low-molecular weight substance and the insoluble residue also showed antinutritional properties. Liener and Pallansch (21) and Liener (22) isolated from soybeans a growth depressor which showed potent haemagglutinating (HG) activity, while Jaffé's Phaseolotoxin A, a haemagglutinin isolated from black beans (*Phaseolus vulgaris*) depressed growth by 40% at a level of only 0.2% in the diet (23).

The haemagglutinins have been shown to cause a surprising variety of physiological responses, the nutritional significance of which is as yet unknown. The best-known of these was discovered by Nowell (24) who observed that the red kidney bean (*Phaseolus vulgaris*) phytohaemagglutinin isolated by Rigas and Osgood (25) could initiate mitosis in normally amitotic human leucocytes. Soybean haemagglutinin was reported by Liener (26) to be toxic when administered intraperitoneally to young rats, while Jaffé and Gaede (27) observed a similar toxic effect in the black bean (*Phaseolus vulgaris*) haemagglutinin. On the other hand, Rigas and Osgood (25) noted that the kidney bean haemagglutinin is non-toxic. Although the term toxicity is used by some authors (eg. 28,29) to describe the general antinutritional properties of legumes when ingested orally, use in the present study will be restricted to those instances in which death occurs following intraperitoneal administration.

As a result of the studies of Liener (26) and Jaffé and Gaede (27), HG activity and toxicity became synonymous. Since the haemagglutinins also depressed growth, all three effects were looked upon as being interrelated, and possibly caused by a common mechanism. Indeed, Jaffé was prompted to speculate that the haemagglutinin may inhibit growth by combining with the cells lining the intestinal wall, thus interfering with intestinal absorption (23,30).

A local variety of *Phaseolus vulgaris*, the Natal round yellow (NRY) bean, was found to be toxic when administered to young rats (31). Since it also exhibited high haemagglutinating activity, it was of potential value for a study of the toxicity - haemagglutination relationship. Separation of a crude protein extract by the author, using DEAE-cellulose chromatography, resulted in a concentration of a high proportion of HG activity in one peak and toxicity in another. Trypsin inhibiting activity was

detected at a low level in all fractions, but maximal activity was found in fractions which showed neither toxicity nor HG activity (32). The evidence indicated that toxicity and haemagglutination were unlikely to be caused by a common physiological mechanism, even though a single molecular species might be isolated which exhibited both effects. The isolation of a pure toxin and a pure haemagglutinin would add considerably to current understanding of the subject and open the way for studies on the role of each factor.

The isolation of a toxin from NRY beans was thus the main purpose of the present investigation. Although the identity and properties of the NRY haemagglutinin was also of some interest, the author chose to isolate the toxic factor because of the scarcity of published information regarding toxic proteins particularly from beans. The NRY toxin appeared to be a protein, or some moiety closely associated with proteins, for it could be salted out with ammonium sulphate, was non-dialysable and acetone-precipitable (31). Since the ratio of toxic and haemagglutinating activities of the toxin during the course of isolation was an important aspect of the problem, a quantitative method of estimating haemagglutinating activity (33) was used. Certain anomalous effects, observed by the author while using the haemagglutinin assay, prompted a closer investigation of the method. Certain changes were introduced as a consequence of the study, and the method is presented in modified form in Chapter II. A discussion of the factors which prompted the change from the original method together with data describing certain features of the haemagglutination reaction, is presented in Appendix 2

A further aspect which received attention was the implication of the toxic factor in the broader problem of growth depression. Although one aspect of growth depression on soybeans had been satisfactorily elucidated, the significance of toxic and haemagglutinating factors in the overall phenomenon remained to be explained. An examination of the contribution of these measurable activities (toxicity and haemagglutination) to growth depression by NRY beans would therefore be timely.

Many interpretational difficulties have arisen in this field through the use of impure mixtures in the study of physiological responses. It is the author's belief that only when all active factors have been isolated in homogeneous form, will their biological significance be established with certainty. The clarity which has been brought to the pancreatic hypertrophy aspect of growth depression is largely due to the use of crystalline, and finally chromatographically homogeneous trypsin inhibitor for physiological studies. Thus considerable attention has been directed in the present study, to the establishment of purity of the NRY toxin.

The preparation of homogeneous factors in sufficient quantity to sustain a comparative feeding trial is an ideal which was not wholly realised in the present study. Nevertheless a start was made in this direction by the development of a new method of fractionating legume extracts, using the synthetic polymer polyethylene glycol. The relative activities of fractions isolated by this method and their effect on growth, provide a basis for tentative conclusions regarding the number of factors operative in NRY beans.

It will become evident that the system under study is a complex one. The proteins exhibit properties which greatly increase the difficulties of purification and subsequent examination. Furthermore the study of growth depression and factors associated with it, reveals a complexity in the physiological sphere. Nevertheless the isolation of a pure toxin and the feeding of protein fractions to rats have added to existing knowledge concerning the nature of the toxin in beans and its possible physiological significance.

EXTRACTION AND PURIFICATION OF TOXIC FACTOR

1. INTRODUCTION

The factor responsible for intraperitoneal toxicity in NRY beans, isolation of which was one of the objectives of the present study, had been extracted from the bean in crude form (31) by a method originally used for the isolation of a soybean trypsin inhibitor concentrate (14). A similar procedure was used by Liener and Pallansch (21) to obtain a crude extract of soybean haemagglutinin. By contrast, an extract obtained from black beans by Jaffé and Gaede (27) using a similar procedure, was estimated to be 90% homogeneous.

In general, other documented isolation procedures for the preparation of active legume extracts do not differ much from the pattern outlined above (34 - 36) although, as indicated, the purity of the initial extract appears to vary with different legume varieties. Subsequent purification of the active extract, usually conducted on a large scale, is achieved by ion-exchange (37) or electrophoretic (36, 38) separation. The electrophoresis-convection (38) and Hannig free-flow preparative electrophoresis methods (36) have been used with success in this context. The former procedure was reported by Wada, Pallansch and Liener (38) to separate 5g of active extract, yielding 1g of purified soybean haemagglutinin. The advantages of such a purification step are considerable, and it facilitated the detailed study of soybean haemagglutinin by Lis, Sharon and Katchalski (39).

In the absence of facilities for preparative electrophoresis, the author used ion-exchange as a preparative tool in a manner similar to that reported by Rackis, Sasame, Mann, Anderson and Smith (37). The active extract was bound to a cation exchange resin, and the components eluted with discontinuous increases in chloride ions in a phosphate buffer. A fraction was obtained which showed a concentration of toxicity. The toxic fraction was further chromatographed by cation exchange, adsorption and molecular exclusion chromatography.

Certain unusual features were observed in the chromatographic properties of the toxic fraction during the final stages of purification. A study of the stability of the toxin indicated that changes were occurring in molecular size. These results are presented in a subsequent chapter. Together with the data concerning criteria of purity of the fractions, these results have bearing on the interpretation of data from the present chapter. A discussion of the implications of the fractionation results is thus deferred until the supplementary information from subsequent chapters has been presented.

2. MATERIALS AND METHODS

2.1 Composition of buffers

All buffers were made up by titration of the acidic and basic components of appropriate molarity to the required pH. When components other than the buffering salts were included in the buffers, these were added to the acidic and basic buffer salt solutions prior to titration.

Continuous changes of salt concentration in the eluting buffer were obtained by using the Büchler Varigrad, a 9-chambered gradient-forming apparatus. The appropriate buffer concentrations for a particular gradient were calculated from the tables published by Peterson and Sober (40).

Chromatography buffers were preserved by the inclusion of 10,000 I.U. of penicillin (sodium form) per litre. Buffers used for molecular exclusion chromatography contained sodium azide (.02%), because of the ease with which gel exclusion media became contaminated with micro-organisms.

2.2 Preparation of crude extract from Natal round yellow beans

In 1964 de Muelenaere (31) showed that an extraction procedure based on the Lyman and Lepkovsky method for preparing crude soybean trypsin inhibitor (14) yielded an extract from NRY

beans which was toxic to rats when administered intraperitoneally. The procedure was used therefore as a basis for further purification studies, modifications being introduced when evidence indicated that these were justified.

The procedure for preparing a toxic extract from NRY meal is outlined in Fig. 1. The extract isolated by this method is referred to as Extract C. Extracts A, B and D were obtained from slightly different procedures, details of which will be described in a later section. The differences between the procedure described in Fig. 1 and that of Lyman and Lepkovsky may be summarised as follows:

- i) The ratio of water to meal used in the extraction was reduced from 10:1 to 5:1. The more concentrated extract ultimately gave a higher yield of crude extract per unit volume of extraction solution handled.
- ii) Dialysis for removal of ammonium sulphate was continued for at least 40 h, whereas the original method specified only overnight dialysis. A dark brown non-toxic precipitate resulted from prolonged dialysis, the solubility properties of which suggests that its components could be classified as globulins.
- iii) The cold ether wash and air-drying were omitted. Instead, acetone was removed by dialysis in the cold, whereupon the extract was freeze-dried.
- iv) For ammonium sulphate precipitation the pH was adjusted to 6.8 prior to addition of the salt, whereas in the original method pH was not controlled. Furthermore the final concentration of ammonium sulphate was increased from 30g to 70g per 100ml of solution, and the salting-out step was not repeated. The three changes in section (iv) have been reported in a previous thesis (32) and will not therefore be discussed further.

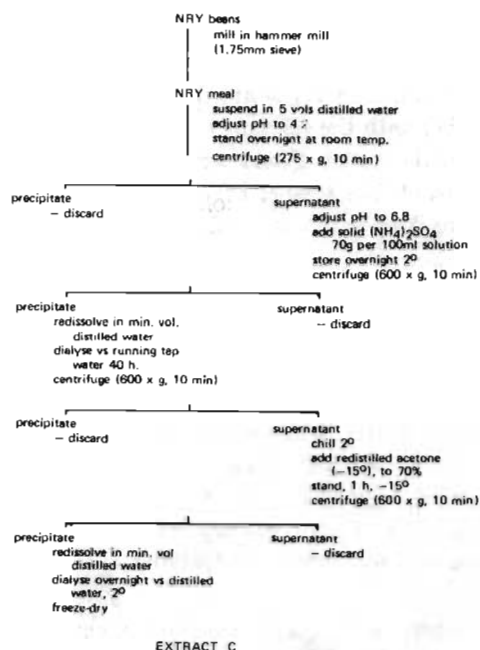


Fig. 1 - Procedure for isolating Extract C from NRY meal

2.3 Fractionation of toxic extract by ion-exchange chromatography

2.3.1 Preparation of cellulose fibres for use in column chromatography

Fibres of Whatman DEAE-cellulose (W. & R. Balston Ltd., Maidstone, Kent) or Bio-Rad CM-cellulose (Bio-Rad Laboratories, Richmond, California, U.S.A.; exchange capacity 0.71 meq/g, control No. 4690) were treated before use according to the procedure described by Peterson and Sober (41). In the case of

DEAE-cellulose the method was as follows:

Dry fibres were allowed to settle into 1 M-NaOH (1 litre per 100g of fibres) and stirred gently to minimise occlusion of air bubbles. Alkali was removed by vacuum filtration using a polythene Büchner funnel fitted with a fine-mesh stainless steel sieve (Labconco Cat. No. 55100). Suspension in alkali, stirring and filtration were repeated until the filtrate no longer showed yellow discoloration. The filter cake was suspended in 4 volumes of 1M-HCl, quickly filtered and suspended in 1M-NaOH, refiltered, and washed three times with distilled water on the filter funnel. Fibres used for the first time were then suspended in 6 volumes of distilled water, and fines removed by repeated settling and decantation of the supernatant, after which they were filtered and suspended in 3 volumes of starting buffer. The suspension pH was adjusted to the required value by addition of a molar solution of the acidic buffer component. The fibres were again filtered and suspended in 2 volumes of starting buffer, at which stage they were ready for packing into the column.

Fibres which had been used previously were suspended in 3 volumes of starting buffer after the alkaline wash and used without settling for removal of fines.

Preparation of CM-cellulose fibres differed from the above procedure in only two respects:

1. The alkaline wash solution consisted of 0.5M-NaOH-0.5M-NaCl instead of 1M-NaOH, since the carboxymethyl derivative swells markedly in alkali and is difficult to filter. Addition of sodium chloride to the washing solution counteracts the swelling tendency and allows easy filtration.
2. For the same reason the distilled water wash which followed the final alkaline wash was preceded by three washes with 0.5M-NaCl to ensure removal of alkali before the salt concentration was reduced.

2.3.2 Column preparation

Fibres were packed into the column as a 1:1 slurry in starting buffer. The glass columns, dimensions of which are given together with the relevant results, were fitted with a Quickfit conical socket at the inlet and an S13 ball at the outlet. Fibres were retained by a perforated porcelain disc covered with a thin layer of cotton wool. The smaller columns were packed with a 50cm extension tube, and during the final stages of packing with these columns, pressure (5 lb/in²) was applied to ensure adequate compaction of the fibres.

With the exception of the large column used for preparative DEAE-cellulose chromatography, columns were fitted with jackets to facilitate temperature control.

2.3.3 Fractionation of active extract by ion-exchange chromatography

Two grams of Extract C were suspended in 100ml of starting buffer (.01M-K-phosphate, pH7.6), and dialysed overnight against identical buffer. The dialysed suspension was centrifuged (1,400 xg, 10 min) and the supernatant applied to a column of DEAE-cellulose fibres (4 x 35cm), equilibrated with starting buffer. The sample was allowed to percolate into the column bed, residual protein was carefully washed from the walls, and the first eluting buffer was applied immediately (.01M-phosphate + .015M-NaCl, pH 7.6). Buffers, percolated through the preparative DEAE-cellulose column under gravity, were applied in the order described in Fig. 5.

Toxicity was found to be concentrated in fraction N. Consequently the effluent corresponding to this peak on the elution profile was pooled, dialysed for 15-20h at 20° against several changes of distilled water, freeze-dried, redissolved and desalted on a Biogel P-20 column, freeze-dried and stored as a white powder.

2.4 Preparation of Hydroxylapatite columns

Hydroxylapatite, or hydroxylated calcium phosphate, was prepared according to the method described by Levin (42). The procedure involves the formation of inactive calcium phosphate crystals, which are subsequently hydroxylated by treatment with alkali. Prepared in this manner, the crystals are well-formed and rigid, in contrast to the fine, flocculent crystals formed by the alternative, direct method.

The purpose of the first stage of Levin's method is to mix equal quantities of disodium hydrogen phosphate and calcium chloride as gently yet as thoroughly as possible. To this end, 2 litres of 0.5M- Na_2HPO_4 and 2 litres of 0.5M- CaCl_2 were placed in separating funnels above a beaker fitted with a mechanical stirrer. The length of the horizontal portion of the stirring rod was just less than the diameter of the beaker, so that efficient stirring could be maintained at low rotation speed. With the stirrer rotating at about 80 r.p.m. the contents of the two separating funnels were allowed to flow into the beaker at equal rates of 12 to 15ml per min. A white precipitate formed immediately. When mixing was complete the calcium phosphate was allowed to settle, the supernatant removed and the precipitate washed with 4 x 3L of distilled water, allowing the crystals to settle out each time. The beaker was filled with a further 3L distilled water and 100ml freshly prepared 40% (w/w) NaOH was added. Sufficient heat was applied to bring the suspension to the boil within 45 minutes. Boiling, as gentle as possible, was continued for an hour. The sediment was allowed to settle (about 5 min), the turbid supernatant aspirated off and 3 -4L distilled water added. The suspension was stirred for 5 min, allowed to settle for 5 min, and the supernatant removed. The washing, stirring and settling procedure was repeated three times.

To the washed precipitate was added 3 -4L of .01M-phosphate buffer, pH 6.8. The suspension was heated to boiling with constant stirring, then allowed to settle for 5 min. The supernatant was removed. This procedure was repeated twice with .01M-phosphate buffer, then twice with .001M-phosphate buffer. For the .001M buffer, boiling was continued for 15 min.

After the fifth boiling the hydroxylapatite was ready for use. The adsorbent is best stored in .001M-phosphate buffer, and Levin reports no change in its chromatographic properties after storage for about a year.

Hydroxylapatite was packed into a glass column under gravity. Because of the fragility of the crystals, flow rates were kept to a minimum, and reduced whenever back pressures indicated that the medium was becoming compacted

2.5 Preparation of molecular exclusion columns

2.5.1 *Dextran and agarose columns*

Dextran ('Sephadex', Pharmacia, Uppsala, Sweden) and agarose ('Sagavac', Seravac Laboratories, Cape Town, South Africa) were obtained from the manufacturers in a form which required no further treatment. Sephadex particles were suspended in buffer and allowed to swell for three hours. Air bubbles were removed by aspiration, and fine particles eliminated by successive settling and resuspension in a measuring cylinder. Sagavac spheres were supplied as a slurry which required only to be suspended in buffer.

Both media were packed into columns as a 2:1 slurry under gravitational flow.

2.5.2 *'Tanned' gelatin columns*

Polson and Katz (43) exploited the gelling properties of gelatin by tanning the protein with a chromium salt and formaldehyde. Tanned gelatin, in contrast to the natural form, is insoluble and almost entirely free of charged groups. The gel can be fragmented to form granules which have high pressure-resistance and are well suited for use in molecular exclusion chromatography.

The preparative procedure described by Polson and Katz requires initially, the isolation of a high molecular weight (MW) gelatin fraction from the commercial product. The high MW fraction is dissolved and gelled at a specific concentration, tanned with chromium salt, fragmented, re-tanned with formaldehyde and washed, before being used in a column.

Preparation of high molecular weight gelatin. Two hundred grams of Difco Bacto-Gelatin (Difco Laboratories, Detroit, Michigan) were dissolved in distilled water at 70°. Polyethylene glycol (360g of 6M species: Shell Chemicals(Pty) Ltd.) was dissolved in 200ml of hot distilled water and added to the gelatin solution. The mixture was maintained at 70° for four hours, after which time a yellow viscous layer had settled out. The supernatant was removed and discarded; the lower layer was slowly poured into 5L of water and ice, with gentle stirring. Gelatin formed a thick fibrous precipitate in the cold water.

The precipitate was transferred to a fine mesh nylon sieve and thoroughly washed with cold distilled water to remove polyethylene glycol. Water was removed by squeezing the precipitate in muslin, and further drying was achieved by washing several times with acetone. The gelatin was finally dried in a warm current of air, the high MW fraction being obtained in 20% yield.

Preparation of 'tanned' gelatin granules. High molecular weight gelatin was dissolved in hot distilled water in the desired concentration, then cooled to 50°. It was poured into a shallow dish floating on ice and water, allowed to set, and cut into small cubes with a scalpel. 'Kromex' chrome tanning salt (Marble Lime and Associated Industries, Ltd.; composition: 0.915 parts $\text{Cr}(\text{OH})_3$, 0.542 parts $\text{Cr}_2(\text{SO}_4)_3$, 1 part Na_2SO_4 , 0.119 parts $\text{C}_6\text{H}_{12}\text{O}_6$ as invert sugar), dissolved in physiological saline to a concentration of 4% and cooled to 40°, was added to the cubes of gel. The mixture was maintained at 20° for 24 hours. Excess Kromex solution was poured off and the gel fragments were washed with two 2-L changes of physiological saline containing .02% sodium azide. The gel was further fragmented in a Virtis homogenizer ($\frac{3}{4}$ speed for 10 min), and the granules washed with a further three 2-L changes of physiological saline-azide in a measuring cylinder.

At this stage the granules were tanned with formaldehyde. Chromium-tanned granules were taken up in 0.2M-tris buffer, pH 9.0. Three volumes of 38% formaldehyde (A.R.) were added, and the mixture was allowed to stand overnight at room temperature. Formaldehyde was removed by repeated washing with saline in a 2L measuring cylinder — a procedure which also removed undesirable fine particles.

The washed gelatin granules were packed into columns as a 2:1 slurry in .01M-phosphate buffer + .06M-NaCl, pH 7.6, containing .02% sodium azide as preservative. Before application of the first sample, columns were equilibrated with at least two column volumes of buffer. Although gelatin columns were packed under gravity, equilibration took place at a flow rate sufficient to develop a back-pressure of 2 - 5 lb/in², to ensure adequate compacting of the granules.

2.6 Collection and assay of chromatographic effluent

Effluents from chromatographic columns were collected in a Beckman Model 132 Fraction collector, using either the time or drop-counting mechanism. Mean tube volumes were determined by measuring the volume of randomly selected tubes. For molecular exclusion columns requiring accurate elution volumes, the volume of each tube was determined.

Effluents were routinely assayed for absorbance in the ultraviolet. The wavelength used was either 253.7 mμ, as emitted by the hydrogen lamp-filter system of the LKB Uvicord, or 280 mμ, selected by the Beckman DB prism spectrophotometer equipped with a flow-through cell.

When information of a more specific nature was required from a chromatographic separation, the effluent was assayed for protein, neutral sugars or sodium, as described below.

Lowry method for protein assay (44). To 1ml of column effluent was added 1ml of 'Reagent C' (50ml of 2% Na_2CO_3 in 0.1N-NaOH + 1ml of 0.5% CuSO_4 in 1% K-tartrate). After 10 minutes, 0.5ml of 'Reagent E' (Folin-Ciocalteu reagent diluted with distilled water to 1N in acid) was added, with immediate shaking of each tube after addition.

Between $\frac{1}{2}$ and 2½ hours after addition of 'Reagent E', the extinction of the tubes was read in a Spectronic 20 spectro-colorimeter (Bausch and Lomb) at 750mμ. A crystalline ovalbumin preparation (Seravac Labs., Cape Town) was used as a standard.

Dubois method for neutral sugar assay (45). To 1ml of column effluent was added 0.5ml of 5% (w/v) redistilled phenol and 2.5ml of conc. H_2SO_4 . Contents of the tubes were thoroughly mixed and allowed to stand for 10 min. They were shaken and placed in a water bath at 25 - 30° for 10 - 20 min. Thereupon the extinction was read immediately in the Spectronic 20 spectro-colorimeter at 490 mμ. Mannose (Koch-Light Labs., England) was used as a standard for the assay.

Assay of effluent for sodium. Samples of the effluent were diluted 100 or 1,000-fold to bring the sodium concentration into the range 0 to 100 parts per million. The exact concentration of sodium was determined in a flame photometer at 589 mμ, using suitable standards.

2.7 Concentration and desalting of chromatographic fractions

Two treatments were used for the fractions which resulted from chromatographic separations. The first, denoted Treatment A, was used throughout most of the present study. Towards the conclusion of the study a method was required to give rapid desalting and concentration of effluents without increasing the concentration of salts. Treatment B was an ultrafiltration procedure, based on an observation by Polson (personal communication) that narrow bore Visking dialysis tubing is able to withstand hydrostatic pressures of up to 20 lb/in². A pressurized membrane can thus be used for ultrafiltration and pervaporation.

Although all studies reported in the present chapter are concerned with fractions concentrated by Treatment A, related chromatographic procedures using Treatment B are reported in the following chapter. The two treatments are described together for purposes of convenience, and to facilitate comparison.

Treatment A. Contents of appropriate fraction collector tubes were pooled, dialysed overnight against two changes of distilled water (Visking 18/32 dialysis tubing), freeze-dried, redissolved in a minimum volume of distilled water and desalted on a Biogel P-20 column.

Treatment B. Fractions, pooled as described above, were placed in bags of Visking 8/32 dialysis tubing which had been moistened, tied and stretched at 20 lb/in² for 30 sec. The bags were suspended in a measuring cylinder of flowing distilled water and pressurised at 5 lb/in². Under these conditions, reduction in volume and removal of salts proceed simultaneously, the former effect maintaining a fairly high concentration gradient of salts, and hence a relatively high level of efficiency of the latter.

The effectiveness of the system is illustrated in Fig. 2, where sodium concentration and volume were measured in a solution containing .06M-NaCl in a pressurised dialysis bag immersed in flowing distilled water.

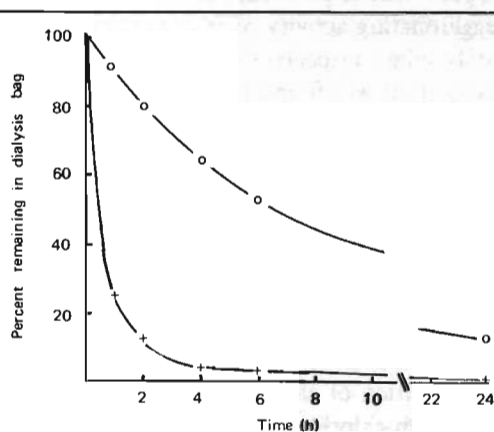


Fig. 2 — Changes in sodium concentration and volume during dialysis of buffer solution under pressure against flowing distilled water.
Solution: .01 M-K-phosphate buffer + .06M-NaCl
Membrane: Stretched Visking 8/32 tubing
Pressure: 5 lb/in²
Linear flow rate of distilled water: 15 cm/h
— o — o —: Volume
— + — + —: Sodium concentration (molarity)

The normal procedure was to dialyse under pressure for about 15 hours, then remove the bag from distilled water, and while still under 5 lb/in² pressure, stand it in a fast-flowing current of air to concentrate. The lower 1cm of the bag was immersed in distilled water to ensure removal of residual salts. The procedure proved extremely efficient, for within 24 hours a protein could be desalted 20 to 40-fold, using very little operator time. The salt-free concentrate (0.5 - 5ml) could be conveniently freeze-dried.

To facilitate the concentration of volumes of up to 100ml or the simultaneous handling of smaller volumes of different fractions, a glass manifold was constructed. The manifold (25ml capacity)

was fitted with a pressure inlet (S13 ball joint) and four outlet tubes suitably shaped for connection of the dialysis bags.

2.8 Assay of fractions for biological activity

2.8.1 *Intraperitoneal toxicity*

Protein fractions to be tested for intraperitoneal toxicity were dissolved in sterile physiological saline (0.9%). The required dose was administered by a single intraperitoneal injection (0.5 - 1ml) into adult mice (body weight $25 \pm 2g$). Mice were housed in groups according to treatments, and were inspected regularly for signs of physiological stress. Deaths were noted in the periods 0 - 12, 12 - 24, 24 - 48 and 48 - 72 hours after administration. Calculations of potency were based on total deaths recorded up to 72 hours.

Whenever possible, quantitative estimates of potency were obtained using the method described by Litchfield and Wilcoxon (46), which provides a statistically acceptable means of expressing the effectiveness of any administered substance producing a quantal response. It provides in addition a means of comparing data for significant differences and gives an estimate of the confidence limits applicable to each median effective dose, or LD₅₀. The principles of the method and an example of a calculation, are described in Appendix 1, together with all recorded toxicity data.

A potency factor was calculated from the LD₅₀ data for each sample. A sample was defined as having one mouse toxic unit (M.T.U.) of activity when it contained sufficient toxic activity to kill one mouse. Thus if a substance had an LD₅₀ of 5mg, 10mg would represent one M.T.U., since if 5mg were injected into each of two mice, theoretically one would survive and the other succumb.

2.8.2 *Haemagglutinating activity*

The author's modification of a photometric procedure described by Liener (38) was used to obtain a quantitative estimate of the haemagglutinating activity of fractions. While the experimental procedure differs from the original method in only minor respects, the method of evaluating activity represents a new approach. Furthermore, the assay itself was found to be subject to variation from several sources and certain anomalous effects were noted in connection with the haemagglutination phenomenon. These effects, together with the data upon which the new method of evaluating activity was based, are described in Appendix 2. The present section will be restricted to a description of the method of assay and means of evaluating activity which were developed as a result of the observations recorded in Appendix 2, and used routinely for the assay of chromatographic fractions.

Assay procedure:— Blood from 4 - 6 rabbits was drawn by ear vein puncture and collected into separate flasks. Two millilitres of anticoagulant solution (47) (sodium citrate, anhydrous, 2.0g; formaldehyde, 37%, 13.3ml; sodium chloride solution, 0.9%, 200ml), was included for every 15ml of blood, with regular swirling to ensure inhibition of clotting. An equal volume of Alsevers' solution (48) (glucose, 20.5g; sodium citrate, 8.0g; sodium chloride, 4.2g; citric acid, 0.55g; distilled water to 1 L) modified by the inclusion of 'Phenergan' (May & Baker) 300mg/L was added to each flask, and the suspension was centrifuged (225 x g, 20 min, 20°). The supernatant was aspirated off and replaced by the same volume of Alsevers' solution. The packed erythrocytes were gently resuspended and centrifuged as before, following which the supernatant was removed and replaced with Alsevers' solution. The resuspended erythrocytes from different rabbits were combined at this stage to form the stock erythrocyte suspension. A sample of the suspension was centrifuged in a calibrated tube (225 x g, 20 min, 20°), from which an estimate of the percentage(v/v) packed cells was obtained. The stock suspension was stored at 4°, and found to be stable for about 14 days.

A calculated sample of the stock erythrocyte suspension, based on the percentage of packed cells, was removed for each day's determinations. Erythrocytes were sedimented by centrifugation, the supernatant removed and the cells diluted to a concentration of 4% (v/v) by addition of buffered saline, pH 7 (sodium chloride, 8g; potassium phosphate, dibasic, 1.21g; potassium phosphate, monobasic, 0.34g; distilled water to 1 L). To the erythrocyte suspension was added one part in one hundred of a 10% (w/v) suspension of crude trypsin (British Drug Houses (Pty) Ltd., Poole, England) in .05N-HCl. The suspension

was incubated for one hour at 37°, after which the cells were sedimented and washed 3 to 5 times with physiological saline (0.9% w/v sodium chloride – subsequently referred to as 'saline') at 40°, until the supernatant was virtually free of red coloration. The washed erythrocytes were suspended in saline and their concentration adjusted by addition of saline until 1ml of suspension, when added to 1ml of saline, had an extinction value of $0.5 \pm .02$ on the Eel colorimeter. The colorimeter had been modified by means of a polythene adapter to accept test tubes with an external diameter of 12mm (I.D. 10mm). The blackened polythene adapter masked the tube completely, except for a small hole, 10mm square and 10mm from the base which enable extinction readings to be taken from a selected region of the tube. Thin-walled glass test tubes were calibrated by reading their extinction containing distilled water and were arranged into matched sets.

The standard trypsinated erythrocyte suspension (STES), adjusted to give an extinction of $0.5 \pm .02$ upon two-fold dilution with saline, was used for agglutination measurement. A fresh batch of STES was prepared daily from the stock suspension.

One millilitre of STES was added to serial dilutions of the test material in 1ml saline. Eight dilutions of each sample were prepared in duplicate in calibrated tubes, covering the dilution range 1:2, 1:4, 1:8 to 1:256. Tubes were also prepared containing 0.5ml STES + 1.5ml saline, and 1-ml STES + 1ml saline, the extinction values of which would correspond to 50% and 100% of cells in suspension (E50 and E100 respectively). Tubes containing 50% cells in suspension, were prepared in duplicate for each set of matched tubes, but only one tube containing 100% cells was necessary for each STES, since the E100 served only as a control on the settling properties of the STES and had no quantitative role in the calculation.

The tubes were maintained in a vertical position in a drilled wooden block, to minimise adhesion of sedimenting erythrocytes to the sides of the tube. The extinction of all tubes was read in the colorimeter 60 min., 90 min. and 120 min. after addition of STES to the test protein. Although it was not necessary to read at exactly these intervals, the time of reading was noted accurately for each group.

Calculation of haemagglutinating activity. Mean extinction values for the serial dilutions of each sample were plotted against the time at which extinction readings were taken, as illustrated in Fig. 3. Relevant E50 readings were included in the same graph.

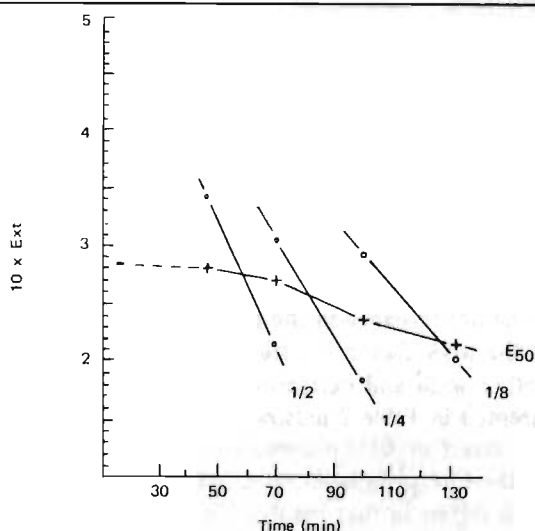


Fig. 3 - Example of graphical presentation of extinction data for washed, trypsinated rabbit erythrocytes in presence of serial dilutions of haemagglutinin.

The presence of haemagglutinin causes an increase in the sedimentation rate of erythrocytes, and hence the rate of decrease in extinction with time. At a certain time and with a suitable dilution of

haemagglutinin, the extinction of erythrocytes + haemagglutinin coincides with the extinction of 50% cells in suspension (E50).

The time of coincidence was estimated from the time at which the two graphs intercept. Times of intercept in the range 60 to 120 min. were recorded, together with the relevant dilution and hence the weight of haemagglutinin (see Table 1). These data were used in the nomogram (Fig. 4), to obtain an estimate of haemagglutinin units (HU) per milligram of sample.

Table 1 — Example of data used in calculation of haemagglutinating activity. Data obtained from Fig. 3.

Initial concentration mg/ml	Dilutions			wt. sample in tube ug	time of intercept min.	HG activity HU/mg	Mean activity
	initial	secondary	total				
0.5	1/10	1/2	1/20	25.0	58		
0.5	1/10	1/4	1/40	12.5	81	90	100
0.5	1/10	1/8	1/80	6.25	126	110	

One haemagglutinin unit is defined as the reciprocal of the weight (in milligrams) of sample required to cause 50% of cells to sediment out of suspension in 90 minutes under the conditions described. The nomogram provides a means of equalising data obtained from intercepts at times other than 90 min., and calculating HU from time of intercept and weight of haemagglutinin.

The range 85 to 95 minutes was taken as ideal for the calculation of valid HU values by this method. If, therefore, the extinction of a sample dilution coincided with E₅₀ within this range, the calculated value was accepted as the HU for the sample, even though coincidence may also have been achieved by other dilutions at other times. If no dilution had E = E₅₀ within the range 85 - 95 min., invariably the condition was satisfied by one dilution at $t > 95$ min. and another at $t < 85$ min. The arithmetic mean of these two values provided a good estimate of the value at 85 - 95 min., for deviations caused by extremes in either direction were cancelled. The data in Table 1 provide an example of the method of calculation.

3 RESULTS

3.1 Extraction of toxic factor from Natal Round Yellow Meal

As described under Materials and Methods, the original procedure for preparing an active extract from NRY meal was open to modification at three stages. Certain alterations to the original procedure, which have been discussed in a previous thesis, will not be given further consideration here.

The three stages in the preparative procedure in which change was indicated were: the ratio of water to solids, the dialysis time for removal of ammonium sulphate, and the ether wash. In addition it was of interest to ascertain whether a change in the pH of extraction from 4.2 to 7.0 would affect the total yield or properties of the toxic factor. No study was made of the effect of altering the water : solids ratio, but dialysis time, ether wash and extraction pH were investigated as variables in the initial extraction procedure. Data presented in Table 2 illustrate the effect of changes to these three aspects on total yield, solubility of the extract in .01M-phosphate buffer, pH 7.6, and intraperitoneal toxicity. Thus Extract A corresponds to the Ext ⁹³₄₂ isolated by Stead, de Muelenaere and Quicke (49), which is prepared in 3.2% yield. Extract B differs in that the dialysis period after salting out is increased from 12h to 40h, during which time a heavy brown precipitate forms. Removal of this precipitate reduced the yield to 1.7%, gave a solubility in phosphate of 42.5%, and an LD₅₀ of 9.8mg. A change in the method of drying, from ether-washing and air-drying, to dialysis and freeze-drying, increased the potency of the extract, LD₅₀ decreasing from 9.8 to 5.0mg, while total yield was unchanged and solubility increased to 65% (Extract C). Extract D differs from C only in the pH of initial extraction.

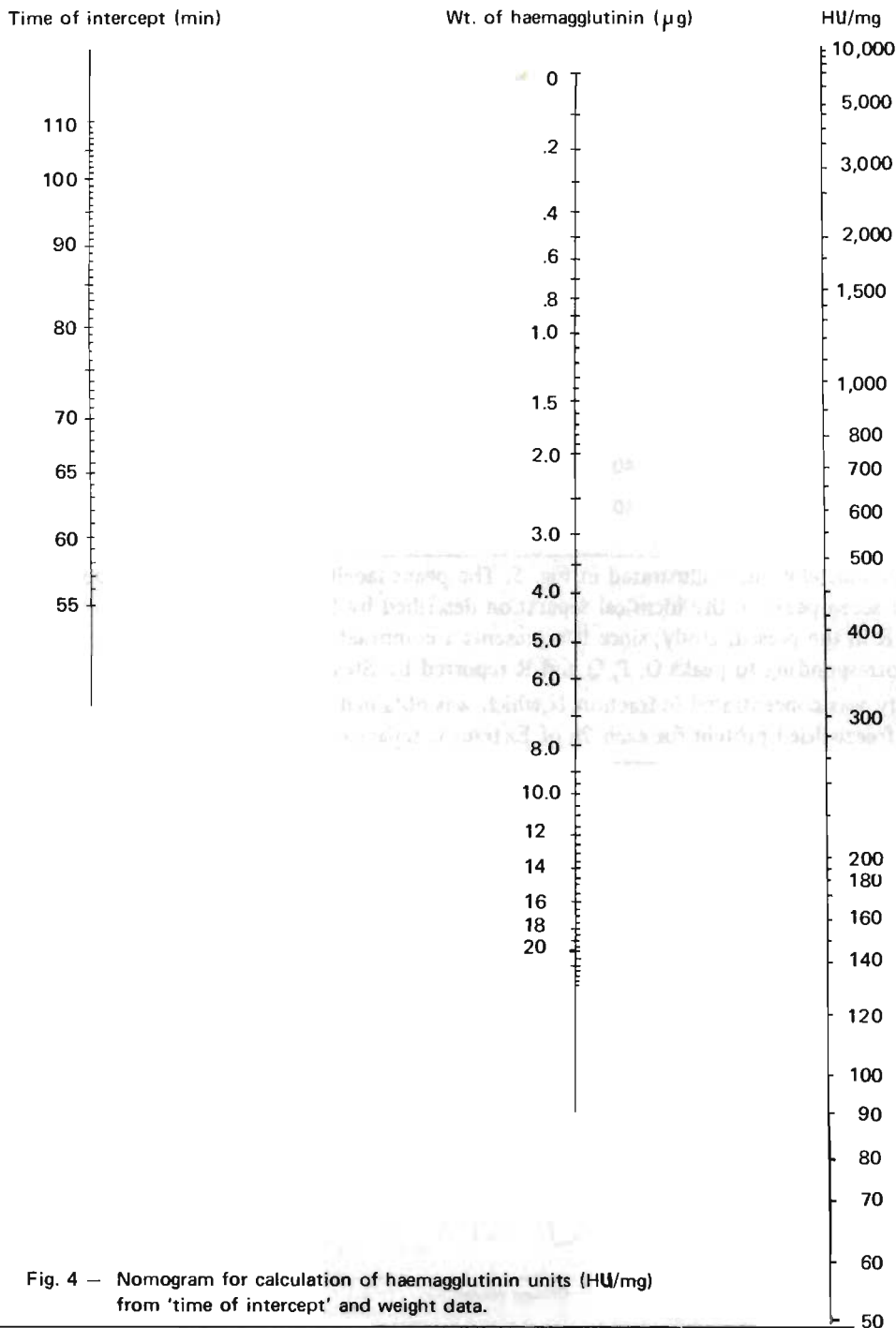


Fig. 4 — Nomogram for calculation of haemagglutinin units (HU/mg) from 'time of intercept' and weight data.

A change from pH 4.2 to 7.0 resulted in a slight decrease in yield, a slight increase in solubility and no significant change in LD₅₀ of the extract.

3.2 Preparation of toxic fraction N₁

3.2.1 Chromatography of crude extract on DEAE-cellulose

A typical elution profile describing the chromatography of NRY Extract C on DEAE-cellulose

Table 2 — The effect of changes in extraction conditions on total yield, solubility and toxicity of crude NRY extracts. The treatment consisted of either (1) an ether wash and air-drying procedure, or (2) a dialysis and freeze-drying procedure.

Solubility represents the percentage by weight of extract soluble in .01M-phosphate buffer, pH 7.6 when 2g of extract is suspended in 60ml of buffer.

Extract	Conditions			Yield g/100g meal	Solubility %	LD ₅₀ mg
	pH of extraction	dialysis time (h)	final treatment			
A	4.2	6 - 12	1	3.2	—	—
B	4.2	40	1	1.7	42.5	9.8
C	4.2	40	2	1.8	65.0	5.0
D	7.0	40	2	1.7	75.0	5.0

The discontinuous elution, is illustrated in Fig. 5. The peaks labelled L, M and N correspond with the three of seven peaks in the identical separation described by Stead *et al.* (49). The fourth peak is labelled O - R in the present study; since it represents a combination of all protein removable by NaCl, corresponding to peaks O, P, Q and R reported by Stead *et al.* (49).

Toxicity was concentrated in fraction N, which was obtained in a yield of approximately 116mg desalted, freeze-dried protein for each 2g of Extract C separated.

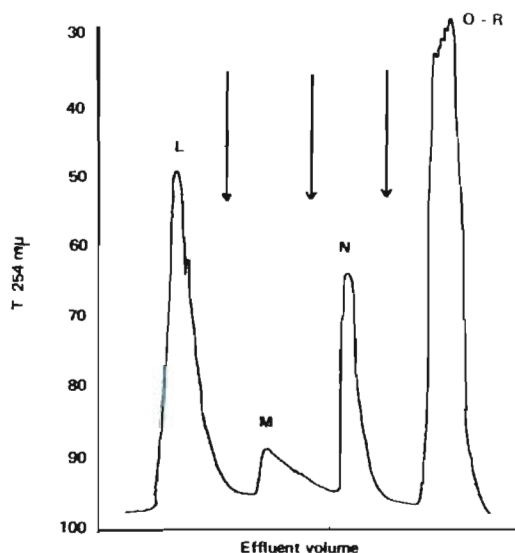


Fig. 5 — Ion-exchange chromatography of Extract C on DEAE-cellulose
Column dimensions: 4 x 35cm

Operating conditions:

Equilibrating buffer: .01M-K-phosphate, pH 7.6

Eluting buffers: Peak L .01M-K-phosphate + .015M-NaCl, pH 7.6

Peak M .01M-K-phosphate + .025M-NaCl, pH 7.6

Peak N .01M-K-phosphate + .060M-NaCl, pH 7.6

Peak O-R 2M-NaCl

Arrows indicate buffer changes

Sample size and volume: 2g Extract C in 60ml .01M-K-phosphate, pH 7.6

Flow rate: 120 to 150 ml/h

3.2.2 Desalting of fraction N and removal of low molecular weight component

As described in section 2.7 (Treatment A) of this chapter, fraction N was partially dialysed after chromatography, concentrated by freeze-drying and desalted on a polyacrylamide (Biogel P-20) column.

As illustrated in Fig. 6, fraction N is separated on Biogel P-20 into two components, one of which is totally excluded from the gel particles, while the other is included and appears in the same region of the elution profile as chloride.

The components are designated the symbols N_1 and N_2 respectively. That N_2 is not dialyzable can be deduced from the fact that prolonged dialysis, while it removes all trace of chloride from N_1 does not affect the proportion of N_2 .

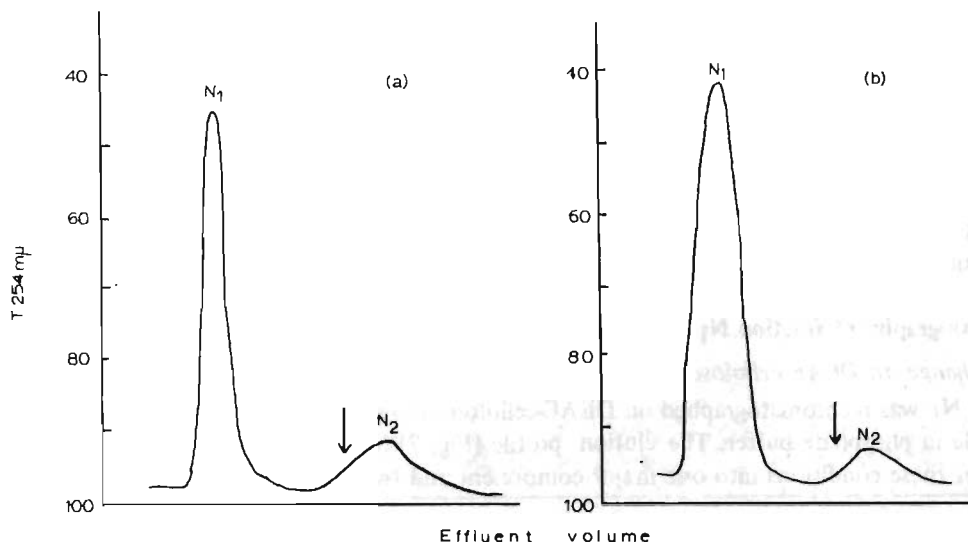


Fig. 6— Separation and desalting of fraction N on Biogel P-20. (a) N prepared from Extract C (extraction pH 4.2); (b) N prepared from Extract D (extraction pH 7.0). Arrow indicates first detection of chloride (Ammoniacal silver nitrate test).

Column dimensions:	2.5 x 25cm
Operating conditions:	Column equilibrated and run with distilled water
Sample size and volume:	50 to 150mg in 2 to 5ml
Flow rate:	80 to 120ml/h

The pH at which the crude fraction was extracted, appears to affect the proportion of N_1 to N_2 , for the preparation from Extract C (extraction pH 4.2) had a higher proportion of N_2 relative to N_1 , than that from Extract D (extraction pH 7.0).

Application of fraction N (from Extract C) to a Biogel P-100 column resulted in an elution profile almost identical to that in Fig. 6(a). Since Biogel P-100 has an exclusion limit in the region of 100,000, N_1 may be expected to have a molecular weight of 100,000 or greater. Peak N_2 , on the other hand, being retarded by Biogel P-20 and not obviously dialysable, would have an estimated molecular weight of about 10,000.

Fractions N_1 and N_2 were examined for toxicity and haemagglutinating (HG) activity. Toxicity and yield data were used to calculate the yield of mouse toxic units in each peak (Table 3).

Toxicity was concentrated in the high-molecular weight peak (N_1), for although the low molecular weight fraction (N_2) was toxic at between 4 and 5mg per mouse, the yield of toxic units was 1/30 of the yield in N_1 (0.6 units in N_2 and 21.4 in N_1).

No significant difference in toxicity was observed in the high molecular weight fractions extracted at pH 4.2 and 7.0.

Table 3 — Yield, toxicity and haemagglutinating activity of N₁ and N₂.

Mouse toxic units calculated as $\frac{\text{Yield}}{\text{LD}_{50}} \times \frac{1}{2}$				
Fraction	Yield mg/2g extract	Toxicity LD ₅₀	Mouse Toxic Units per 2g extract	HG activity HU/mg
N ₁ (pH 4.2)	116	2.7 (2.0 - 3.6) ⁺	21.4	50
N ₁ (pH 7.0)	116	3.2 (2.5 - 4.2)	18.1	
N ₂ (pH 4.2)	5.5	4.0 to 5.0 [*]	0.6	215

+ confidence limits for 19/20 probability

* This value is approximate, as insufficient data available for calculation of LD₅₀.

Both N₁ and N₂ were found to have a low level of haemagglutinating activity, with N₂ (215 HU/mg) some four times more active than N₁ (50 HU/mg).

Rechromatography of fraction N₁

1 Ion-exchange on DEAE-cellulose

Fraction N₁ was rechromatographed on DEAE-cellulose, using as eluant a continuous gradient of sodium chloride in phosphate buffer. The elution profile (Fig. 7) indicates that the fraction can be separated under these conditions into one major component and two minor fractions.

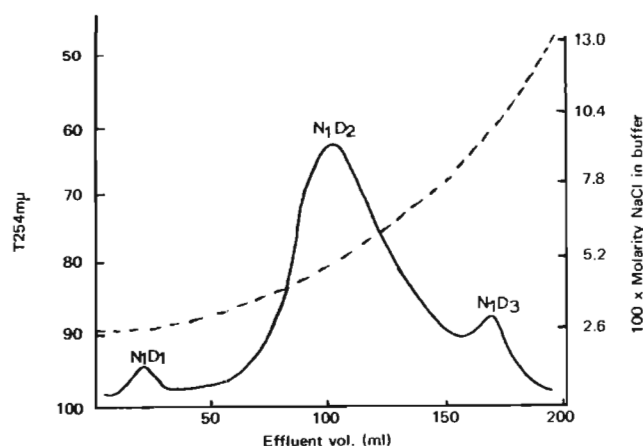


Fig. 7 — Ion-exchange chromatography of N₁ on DEAE-cellulose

Column dimensions: 1.9 x 32 cm

Operating conditions:

Equilibrating buffer: .01M-K-phosphate + .025M-NaCl, pH 7.6

Gradient device: Vessels 1 & 2:— 50ml equilibrating buffer

Vessel 3:— 50ml .01M-K-phosphate + .060M-NaCl, pH 7.6

Vessel 4:— 50ml .01M-K-phosphate + .130 M-NaCl, pH 7.6

Sample size and volume: 100 mg N₁ in 3ml equilibrating buffer

Temperature: ambient

— Transmission 253.7mμ

- - - Molarity NaCl in influent buffer (calculated)

3.3.2 Ion-exchange on CM-cellulose

Preliminary studies indicated that N_1 was loosely bound to the cation exchanger in the pH range 5 - 7. Below pH 5 retention on the column was stronger, to the extent that a higher concentration of salt was required for elution.

In order to achieve the elution of all components of N_1 from the CM-cellulose column, an integrated elution programme was devised, incorporating 8 chambers of the gradient former. A linear increase in salt concentration at pH 4 was followed by a pH change from 4.0 to 8.6, while a second increase in salt concentration up to 2M at pH 8.6 eluted the most strongly bound components. The elution profile in Fig. 8(a) illustrates the result of such a separation, the conditions of which (both calculated and measured) are described in Fig. 8(b).

Analysis of the effluent for protein indicated that one major component was eluted from CM-cellulose at a salt concentration of about 0.6M at pH 4.0. At least three minor components were detectable on the Lowry trace, the last of which would appear to be strongly bound to the column since it was eluted over a large effluent volume. The analysis for neutral sugars reveals that one of the minor components on the Lowry trace has a high sugar content. This component is eluted at the approximate void volume of the column and is therefore assumed to be non-cationic. The second minor protein peak, eluted immediately prior to the major peak, was also found to have a high sugar content. The other protein peaks gave a definite, though less pronounced, sugar reaction.

An analysis of the operating conditions (salt concentration and pH) revealed that changes in salt concentration closely paralleled the anticipated values calculated from the tables of Peterson and Sober (40). An unexpected decrease in effluent pH was noted in the region which corresponds to the point at which the major peak is eluted. This was presumably due to removal of Na^+ from the liquid to the solid phase to replace the cationic protein on the ion exchanger. Since at pH 4, sodium ions are present in acetate buffer in small proportion, a slight alteration in available sodium ions would be sufficient to reduce the pH. Rigas, Johnson, Jones, McDermid and Tisdale (50) report an identical change using a citrate buffer on an Amberlite IRC 50 cation exchanger.

The programmed change to a higher pH was delayed to beyond the theoretical effluent volume. This was not unexpected, however, for the buffering properties of an ion exchange column enable it to delay the effects of a pH change considerably.

The shape of the elution profile in Fig. 8(a) suggested that a separation might be more effectively achieved by combining a continuous gradient for the initial stage, to elute the carbohydrate and the major peak, with a discontinuous change from pH 4 to 8.6 to remove other components. The profile illustrated in Fig. 9 results from such a combination; it shows that three peaks are eluted in the initial continuous phase — the uncharged carbohydrate, the major component, and a third peak eluted over a large effluent volume. The discontinuous change from acetate to Tris-HCl buffer, pH 8.6 (both containing 1M-NaCl) produced a further two peaks. The system described in Fig. 9 was used routinely for preparing the fraction N_1CM_2 . The inclusion of .005M- $CaCl_2$ in the acetate buffers was made as the result of an observation by Morris and Morris (51) that calcium ions reduced losses due to irreversible binding of proteins to cellulose ion exchangers. The presence of $CaCl_2$ produced no noticeable change in the elution profile.

The fractions separated by cation exchange chromatography were tested for toxic and HG activity. Results of the assays are presented in Table 4, from which it appears that N_1CM_2 , the major protein component of N_1 , has both toxic and HG activity. N_1CM_1 the non-cationic component containing a high proportion of neutral sugars, is non-toxic at 4mg per mouse and has zero HG activity, while the same applied to N_1CM_3 .

N_1CM_4 and N_1CM_5 were tested together for toxicity and found to be innocuous at 5mg, while insufficient material was available for a study of HG activity.

Toxicity is thus concentrated in fraction N_1CM_2 which has an LD_{50} of 2.25 mg per 25g mouse and also shows residual HG activity (49HU/mg).

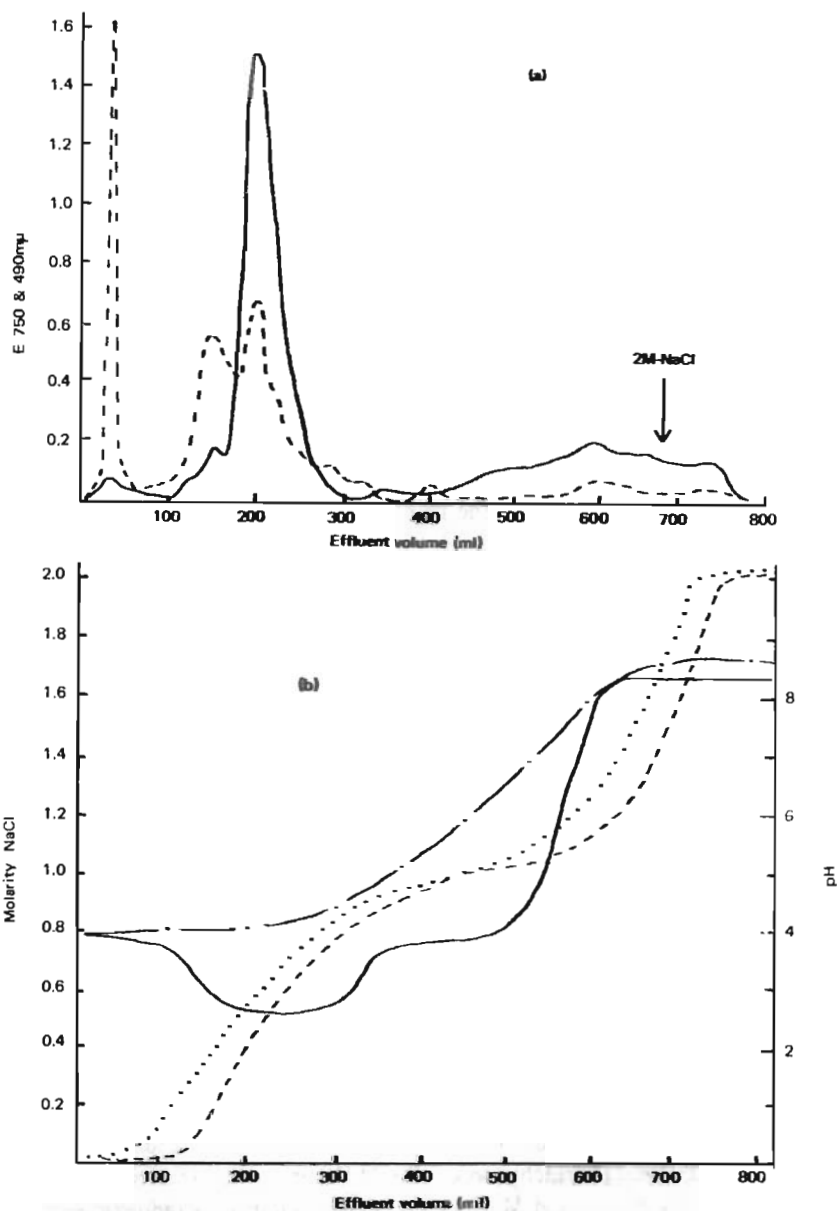


Fig. 8 - Ion-exchange chromatography of N_1 on CM-cellulose

a) Elution profile for protein (—, E750mμ) and neutral sugars (---, E490mμ)

b) Salt concentration (--- calculated; - - - measured) and pH (· · · calculated; — measured) of buffer

Column dimensions: 1.9 x 32cm

Operating conditions:

Equilibrating buffer: .01M-acetate, pH 4.0

Eluting buffers: A equilibrating buffer

B 0.1M-acetate + 1M-NaCl, pH 4.0

C 0.1M tris-HCl + 1M-NaCl, pH 8.6

D 2M-NaCl

Filling of gradient	Vessel	1	2	3	4	5	6	7	8
former (100ml/vessel): Buffer	A	A	B	B	B	C	C	D	

Sample size and volume: 50mg N_1 in 2ml equilibrating buffer

Temperature: ambient

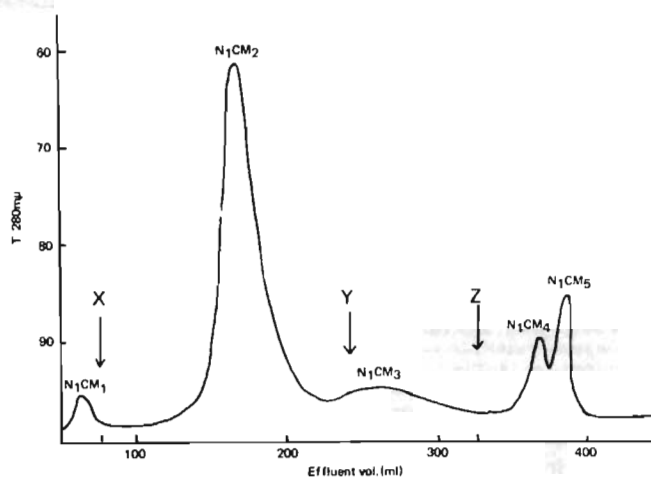


Fig. 9 — Ion-exchange chromatography of N_1 on CM-cellulose.

Column dimensions: 1.9×32 cm

Operating conditions:

Equilibrating buffer: .05M acetate + .005M- $CaCl_2$, pH 4.0

Eluting buffers: A equilibrating buffer

B 0.1M-acetate + 1M-NaCl + .005M- $CaCl_2$, pH 4.0

C 0.1M-tris-HCl + 1M-NaCl, pH 8.6

Filling of gradient Vessel 1 2
former (100ml/vessel): Buffer A B

Equilibrating buffer run to X; gradient former connected at X;
buffer B applied at Y; buffer C applied at Z

Sample size and volume: 50mg N_1 in 2ml equilibrating buffer

Temperature: 7°

Flow rate: 22.6ml/h

Table 4 — Yield, toxicity and haemagglutinating activity of fractions prepared by cation exchange chromatography of N_1 on CM-cellulose

Fraction	Yield mg/100mg N_1	Toxicity LD ₅₀	Mouse Toxic Units per	HG activity HU/mg
N_1CM_1	37	not toxic at 4mg	—	0
N_1CM_2	43	2.25	9.5	49
N_1CM_3	17	not toxic at 4mg	—	0
N_1CM_4	—	not toxic at 5mg	—	—
N_1CM_5	—	not toxic at 5mg	—	—

Rechromatography of N_1CM_2 . Fraction N_1CM_2 was rechromatographed on CM-cellulose, using a similar elution programme to that described in Fig. 9, except that the separation was not continued beyond pH 4 and 1M-NaCl.

The elution profile (Fig. 10) indicates that when N_1CM_2 was rechromatographed, the non-cationic component appeared just as in the first cation exchange separation. This would suggest that the non-cationic component originated from the major toxic component (N_1CM_2), as a result of conditions to which it was exposed either between one chromatographic separation and the next, or during the ion-exchange process itself

3.3.3 Adsorption chromatography

Adsorption chromatography on hydroxylapatite has been found on occasions to resolve mixtures which are homogeneous by ion exchange criteria. Consequently adsorption chromatography was tested for efficiency in separating the components of fraction N_1 .

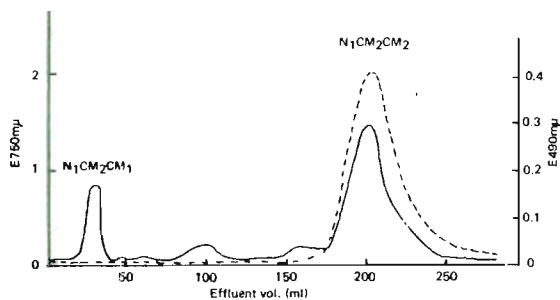


Fig. 10 — Rechromatography of N_1CM_2 on CM-cellulose
Effluent assayed for protein (—, E750m μ) and neutral sugars (---, E490m μ)
Column dimensions: 1.9 x 32cm
Operating conditions: as for first half of Fig. 9.

The elution profile is illustrated in Fig. 11. Fraction N_1 was resolved into two main components, the second of which showed an irregularity which would suggest that it is heterogeneous. Irregularity in the profile was observed in two consecutive separations, and could possibly be an artifact caused by channeling in the compacted crystals. Separation on hydroxylapatite was not pursued further, because the fragility of the crystals impaired the flow characteristics of the column which developed a high back pressure and was difficult to operate.

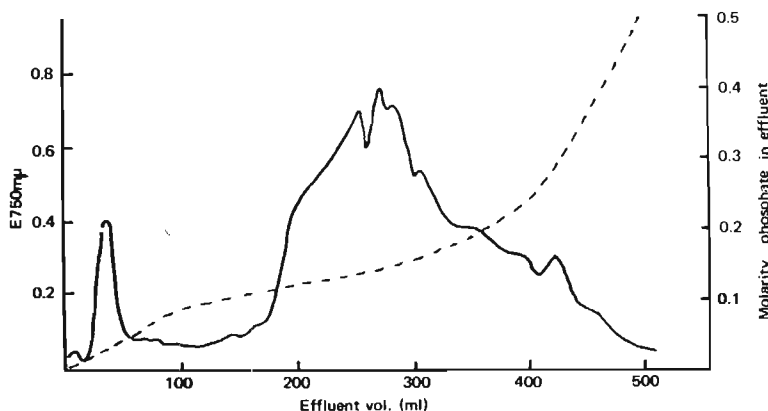


Fig. 11 — Adsorption chromatography of N_1 on hydroxylapatite
Effluent assayed for protein (—, E750m μ); ---, concentration of phosphate in effluent (calculated)
Column dimensions: 1.7 x 32cm
Operating conditions:—

Buffers: A .005M-phosphate, pH 7.0
B .100M-phosphate, pH 7.0
C .200M-phosphate, pH 7.0
D .250M-phosphate, pH 7.0
E .500M-phosphate, pH 7.0

Filling of gradient device (100ml/vessel)

Vessel	1	2	3	4	5	6
Buffer	A	B	C	A	D	E

Sample size and volume: 50mg in 2ml buffer A (equilibrated by dialysis against A)

Pump setting: 3.0

3.3.4 Molecular exclusion chromatography

Preliminary studies on media currently available for molecular exclusion chromatography indicated that fraction N_1 consisted of predominantly high molecular weight material. Since a high proportion of N had been found to be fully excluded by Biogel P-100, the exclusion limit of which is about 100,000 daltons, the molecular weight of N_1 was expected to be higher than 100,000 daltons.

Studies using dextran and agarose gels. The dextran gel Sephadex G-200 provided a means of distinguishing between four components in fraction N_1 . The profile illustrated in Fig. 12 shows one component at the approximate void volume of the column, the major peak at about 1/3 total eluted

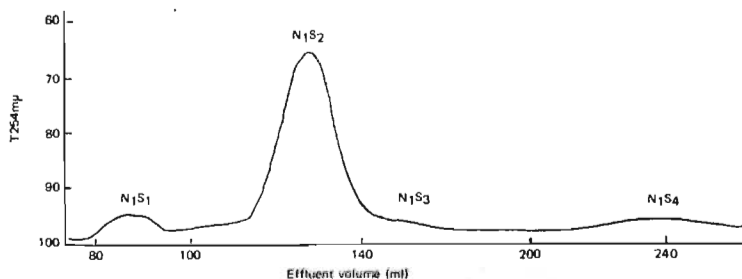


Fig. 12 - Molecular exclusion chromatography of N_1 on Sephadex G-200
 Column dimensions: 1.8 x 110 cm
 Operating conditions: Buffer: .01M-K-phosphate, pH 7.6
 Sample size and volume: 50mg in 1ml buffer
 Pump setting: 1.77
 Pressure: 5lb/in²
 Flow rate: 12ml/h
 Application of .01M-phosphate buffer + .015M-NaCl, pH 7.6 after 240ml
 had been eluted resulted in no further peaks.

volume, a third component which was eluted immediately after the main component, and a fourth, very attenuated peak at the maximum column volume.

The mechanical characteristics of the gel proved to be a limitation, for it is easily compressed and constant flow rates were difficult to maintain. The medium was therefore not ideally suited to the requirements of the present study, in which, for reasons which will become apparent later, a number of sequential column separations were required. Nevertheless, the commercial availability of large quantities of Sephadex enabled a limited number of preparative-scale separations to be made.

Agarose gel has properties of strength and inertness which render it suitable for exclusion chromatography. The high and reproducible flow rates reported for the spherical beads prepared from agarose are due to the unique hydrogen-bonded nature of the gel matrix.

A column packed with 6% agarose spheres separated N_1 in a manner similar to Sephadex, although the first two components were incompletely resolved (Fig. 13). A less concentrated gel (4% or 5%) would probably have retarded the major peak to a greater extent, and hence might have resolved the first two components. However the mechanical characteristics were again not ideal for the requirements of the present study.

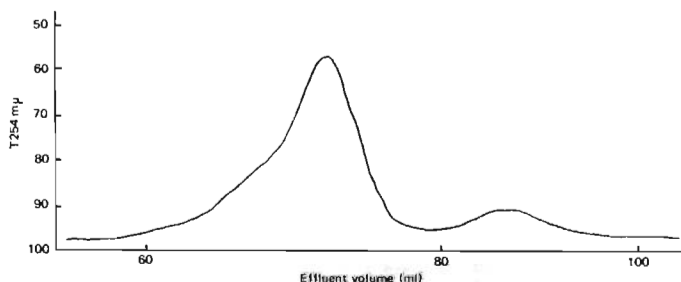


Fig. 13 - Molecular exclusion chromatography of N_1 on 8% agarose
 Column dimensions: 1.4 x 63cm
 Operating conditions: Buffer: .01M-phosphate + .015M-NaCl, pH 7.6
 Sample size and volume: 50mg in 1ml buffer
 Pump setting: 0.75
 Flow rate: 3.1ml/h

Studies using 'tanned' gelatin. In introducing 'tanned' gelatin as a medium for exclusion chromatography, Polson and Katz (43) indicated that the gel has a high degree of mechanical stability which makes it particularly suited to this purpose. A readily available, inexpensive starting material and relatively simple method of formation of the tanned product are additional attractive features.

A preparation of 4% 'tanned' gelatin in a 134cm column, resolved the first two components of fraction N₁, while the remaining components were evident as an irregularity on the trailing edge of the major peak (Fig. 14).

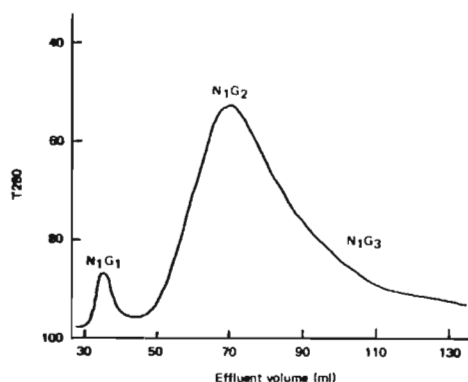


Fig. 14 — Molecular exclusion chromatography of N₁ on 4% tanned gelatin.
Column dimensions: 0.9 x 134cm
Operating conditions:
Buffer: .01M-phosphate + .06 M-NaCl, pH 7.6
Sample size and volume: 20mg in 0.5ml
Pump setting: 1.5
Flow rate: 12.8ml/h

Fractions obtained from Sephadex and 'tanned' gelatin columns were assayed for toxicity and haemagglutinating activity. The results (Table 5) indicate that the major peak in each case is toxic. The high molecular weight components (N₁S₁ and N₁G₁) and the lower molecular weight component N₁G₃ were non toxic, although the shortage of material precluded injection at dose levels higher than those reported.

Table 5 — Yield, toxicity and haemagglutinating activity of fractions prepared by molecular exclusion chromatography of N₁ on Sephadex G-200 and 4% tanned gelatin.

Fraction	Yield mg/100mg N ₁	Toxicity LD ₅₀ (mg)	Mouse Toxic Units per 100mg N ₁	HG activity HU/mg
N ₁ S ₁	16.6	not toxic at 3mg	—	0
N ₁ S ₂	57.0	2.0	14.2	84
N ₁ S ₃	2.7	—	—	0
N ₁ S ₄	—	—	—	0
N ₁ G ₁	21.2	not toxic at 3mg	—	0
N ₁ G ₂	54.5	± 2*	13.6	0
N ₁ G ₃	18.0	not toxic at 3mg	—	42

* Insufficient data available for LD₅₀ calculation. Consequently this value is an estimate from deaths recorded at two levels of injection (see Appendix 1 for toxicity data).

The LD₅₀ of peaks N₁S₂ and N₁G₂ appear to be similar although an accurate value of N₁G₂ could not be calculated due to shortage of data. It would appear, however, that the two gel exclusion media produce similar separations, for the yields of mouse toxic units do not differ markedly.

A difference between N₁S₂ and N₁G₂ is evident from the data for haemagglutinating (HG)

activity, for while N₁S₂ is mildly active (84 HU/mg) and the only Sephadex fraction to show activity, the corresponding 'tanned' gelatin peak N₁G₂ is inactive. In the case of the gelatin separation, a residual level of activity (42 HU/mg) was observed in the lower MW fraction (N₁G₃).

3.4 Summary of toxicity and yield data

The progress of purification of toxic activity may be assessed from the data in Table 6. Potency of the toxin (expressed in mouse toxic units/g) increased from the crude extract (100 m.t.u./g) to the DEAE-cellulose fraction N₁ (166 m.t.u./g) and again to the CM-cellulose (222 m.t.u./g), Sephadex and gelatin peaks (both 250 m.t.u./g). Considerable loss of activity was incurred in the preparative DEAE-cellulose chromatography stage, where total extracted units in N₁ decreased to some 14% of the total in Extract C. Of the subsequent separation procedures the two based on molecular exclusion proved more economical than cation exchange chromatography, for while the yield of units was 12.5% and 12.1% of total in Extract C for N₁S₂ and N₁G₂ respectively, N₁CM₂ retained 8.3% of the total.

Table 6 – Summary of toxicity and yield data for toxic fractions.

Fraction	Yield g/kg meal	Toxicity LD ₅₀	Potency m.t.u./g	Potency Purification	Total m.t.u.	Yield of m.t.u % of 1,700
Extract C or D	17.00	5.00	100	—	1,700.0	—
N ₁	1.50	3.00	166	1.7	250.0	14.0
N ₁ CM ₂	0.64	2.25	222	2.2	142.0	8.3
N ₁ S ₂	0.85	2.00	250	2.5	212.5	12.5
N ₁ G ₂	0.82	± 2.00	250	2.5	205.0	12.1

Chromatography of Extract C on DEAE-cellulose followed by molecular exclusion chromatography on either Sephadex or gelatin resulted in a 2.5-fold purification of toxic activity. Rechromatography on CM-cellulose, on the other hand, proved slightly less efficient, with a 2.2-fold enrichment being achieved.

4 DISCUSSION

The procedure used for preparing a crude protein extract from NRY meal was an improvement on the original method. Of the changes introduced, the most effective was the omission of ether-drying of the acetone-precipitate and substitution of dialysis for removal of acetone followed by freeze-drying. This modification decreased the LD₅₀ of the resultant extract from 9.8 to 5mg per mouse. A change in extraction pH from 4.2 to 7.0, the purpose of which was to avoid prolonged exposure to the potentially destructive acidic pH, resulted in no change in potency of the toxin.

The 'solubility' data quoted in Table 2 suggest that the crude extract shows pronounced insolubility, which is in fact the case with the particular extracts studied. It was found however that solubility tended to vary with different batches of beans. In some cases (when Extracts C or D were used) the insoluble precipitate which formed on centrifugation of the phosphate buffer suspension, was barely discernible. In other, apparently identical, preparations the precipitate was considerably larger as occurred with Extracts C and D from which data were taken for Table 2. The solubility of Extract B was determined on a sample which was about 18 months old and hence would be expected to be more insoluble than a fresh preparation. Nevertheless from observations over many preparations it can be stated with some confidence that omission of the ether treatment in the preparative procedure resulted

in an extract with higher solubility. This is not unexpected since ether, even in the cold, is a powerful denaturant of some proteins.

The heavy precipitate which forms after prolonged dialysis during the preparation of crude extracts is undoubtedly similar to the non-toxic globulin fraction obtained by Jaffé and Gaede (27) from the black bean under similar conditions. The extracts obtained by Jaffé and Gaede (51) and the present author, from two similar preparative procedures showed noticeable differences, which suggest that proteins from the black beans and the NRY bean (both *Phaseolus vulgaris*) have markedly different solubility properties. Jaffé and Gaede prepared a saline extract, dialysed to precipitate the globulin fraction, then salted out a protein with ammonium sulphate between 55 and 70% saturation. This fraction was shown to be 90% pure by electrophoretic and ultracentrifugal criteria. Previously reported studies on NRY meal indicated that fractionation with ammonium sulphate showed little selectivity between trypsin-inhibiting, HG and toxic activities (49). Furthermore, chromatography of NRY Extract A on DEAE-cellulose indicated that it could be separated into at least seven fractions and that the three activities were due to three different factors (49). A fourth factor, the enzyme lipoxygenase, has since been detected by the author in one of the seven fractions obtained by DEAE-cellulose chromatography (unpublished observations). The extract obtained from NRY beans is thus a mixture of many different proteins, only one of which is toxic (under the conditions studied), whereas the black bean extract consists of an almost pure toxic haemagglutinin.

Other reported extraction procedures are similar to the present study, since the initial extraction results in an impure mixture. Thus Wada *et al.* (38) and Takahashi, Ramachandramurthy and Liener (52), prepared extracts from soybeans and wax beans respectively which required subsequent purification by preparative electrophoresis-convection (38) or ion-exchange chromatography (52).

Preparative electrophoresis, such as the Hannig (53) system used by Pusztai (36), and Jaffé and Hannig (35) has been used with success to prepare in quantity a homogeneous or nearly homogeneous protein from the crude extract. The technique would appear to be uniquely useful at this stage of a protein purification procedure since its resolving potential is fully exploited despite the enlargement to a preparative scale. In addition the easy adaptation of electrophoresis to continuous operation allows a further increase in scale without sacrifice of separative efficiency. By comparison, ion exchange is less suited to the separation of large quantities of a crude extract. Discontinuous elution from the resin, as used in the present study, has the disadvantage that discontinuous buffer changes preclude the attainment of ideal conditions, such as might be obtained with a continuous buffer gradient. Thus preparative-scale operation is usually accompanied by a loss of resolution, and homogeneity of the product is never anticipated and seldom attained.

Preparative ion-exchange chromatography in the present context did not prove an economical step, in terms of toxic units isolated. Whereas Extracts C or D contained a total of 1,700 mouse toxic units per kg of NRY meal, with a potency of 100 units/g, fraction N₁ contained only 250 units per kg, albeit in higher potency (166 units/g). The 1.7-fold purification was thus achieved at a cost of some 1,450 toxic units. Nonetheless, preparative ion-exchange served a valuable purpose in isolating toxicity from other measurable activities. Thus Stead *et al.* (49) demonstrated that maxima for haemagglutinating activity and trypsin-inhibiting activity were found in other fractions eluted from the DEAE-cellulose column with discontinuous buffer changes, while the present author (unpublished data) detected the enzyme lipoxygenase in yet another fraction from the same separation. The loss of toxic units was thus compensated for by separation of the NRY toxin from other potentially toxic factors.

The obvious explanation for loss of toxic units in the preparative ion-exchange procedure is that more than one toxin was present in the crude extract, and ion-exchange has separated the activities. This explanation would appear unlikely, since all fractions eluted from the column were tested for toxicity (49), and while two other fractions were mildly toxic, they represented a negligible concentration of toxic units. Two other possible reasons for the loss, *viz.* that certain fractions were required in combination in order to be toxic, or that a toxic factor was irreversibly bound to the DEAE-cellulose, were not investigated. The method yielded a toxic protein of reproducible properties, which was

suitable for further study, and other aspects of the problem did not therefore receive further attention.

'Tanned' gelatin, which has only been recently introduced as a medium in gel exclusion chromatography, has shown remarkable mechanical properties in the author's laboratory. Indications are that it may extend the scope of gel exclusion considerably, particularly for large-molecule separations in the molecular weight range 200,000 and above. Although seldom mentioned, the tendency of gel media currently in use to collapse under pressure imposes a major limitation on the technique. The advanced state of gel exclusion theory and its mild effect on macromolecules render the technique ideal for protein separation. An improvement in mechanical properties of the media is thus of great potential value.

The gelatin granules used in the present study showed remarkable resistance to compression, and were able to withstand high flow rates for long periods. Since the optimal flow rate for separation was considerably less than the column maximum, the column could be used repeatedly without the need for repacking. In the present study the column of 4% 'tanned' gelatin granules was used for more than 30 consecutive separations, after which it had shown no signs of deteriorating. Mechanical resilience of this order is unique in gel exclusion media.

As might be expected from a new development in methodology, the limitations of 'tanned' gelatin have yet to be defined. A possible disadvantage has been brought to light by the observation that the 'tanned' gelatin fraction N_1G_2 has no haemagglutinating activity whereas N_1S_2 , a similar fraction isolated by gel exclusion on Sephadex, retains its activity. An unpublished observation in the author's laboratory indicated that a low concentration of chromium ions depressed the haemagglutinating activity of fraction N_1S_2 . Thus tanned gelatin prepared as described in this thesis may have free chromium ions, which could be bound to proteins during chromatography. Doubtless treatments could be devised to ensure prior removal of potentially hazardous ions, but this observation emphasized the need for caution in the development and application of 'tanned' gelatin.

Certain aspects of the chromatographic behaviour of the NRY toxin suggested that further investigation was necessary. The presence of N_1CM_1 , the non-cationic, high carbohydrate component was in itself unusual, since N_1 had been eluted from an anion exchanger. If N_1CM_1 was a normal contaminant, isolated in conjunction with N_1 , it would be expected to have a similar iso-electric point to N_1 . Since N_1CM_1 has no cationic charge at pH4, whereas N_1CM_2 is strongly charged (eluted at high salt concentration), the two would appear to differ considerably with regard to anionic charge as well. Thus N_1CM_1 is unlikely to be a normal contaminant of N_1CM_2 . The other possibility is that N_1CM_1 is derived from N_1 , that it is part of the N_1 molecule during anion exchange chromatography and is separated from it before or during the cation exchange stage.

The above suggestion is substantiated by the observation that N_1CM_2 , upon rechromatography on a cation exchanger under identical conditions, yields another peak of non-cationic, high-carbohydrate material (Fig. 10). Certain conditions to which the fractions were subjected either during the ion-exchange separation, or between successive separations, would thus appear to favour formation of fraction N_1CM_1 . The following chapter describes certain experiments, the results of which throw light on the nature of the relationship between N_1CM_1 and the toxin itself. An understanding of this relationship is necessary for a satisfactory assessment of the results of the present chapter, and an interpretation of the tests for homogeneity which are described in a subsequent chapter.

STABILITY CHARACTERISTICS OF THE TOXIN AND ORIGIN OF THE HIGH-CARBOHYDRATE COMPONENT

1 INTRODUCTION

The evidence presented in the previous chapter suggested that N₁CM₁, the high-carbohydrate component of N₁, was derived from N₁CM₂, the major component. This high-carbohydrate component was also noted when N₁CM₂ was rechromatographed on CM-cellulose under identical conditions. Thus one or more aspects of the procedure by which the protein was separated, desalted and concentrated prior to rechromatography, appeared to favour formation of the component.

The appearance of carbohydrate in association with protein is to be expected in a legume extract, as many legume proteins are found to contain carbohydrate, bound with varying degrees of firmness. In some cases the polysaccharide residue is covalently bound to the protein (36), while in others (25) it is easily dissociable. Takahashi *et al.* (52), using a wax bean (*Phaseolus vulgaris*) extract, report a situation almost exactly analogous to the present study, in which an uncharged, high-carbohydrate component was eluted from a DEAE-cellulose column. It was concluded that this represented non-covalently bound carbohydrate, and the fraction was discarded. Rigas and Osgood (25) isolated from red kidney beans (*Phaseolus vulgaris*) a phytohaemagglutinin containing 50% carbohydrate. Dissociation under acid conditions caused a loss of non-covalently bound carbohydrate, leaving a protein containing some 8% carbohydrate. In compositional studies, Takahashi and Liener (54) found the carbohydrate moiety of wax bean haemagglutinin to be a complex heteropolysaccharide comprising some 19 sugar residues. Soybean haemagglutinin has a carbohydrate moiety of similar size but less complex composition, mannose being the predominant sugar (39).

In spite of these documented observations, knowledge concerning the plant glycoproteins and the nature of the protein-carbohydrate link is still limited (39). The present chapter reports on studies designed to throw light on the nature of the protein-carbohydrate association in NRY toxin. An attempt was made to find conditions in which the toxin would be isolable in as natural or unmodified a condition as possible. The author is of the opinion that such conditions must be established with certainty so that changes in structure caused by subsequent modifications in procedure can be assessed in relation to the natural form. In the absence of such information, any product may only be regarded as a derivative of the naturally occurring compound. Inevitably, isolation of labile natural macromolecules involves the possibility of structural modification, and it is therefore imperative that an isolation procedure should be conducted under mild conditions. Any studies aimed at elucidating the 'smallest active component' are of limited value unless such a component can be related to its natural form.

With these considerations as a basis, a study was carried out using elution characteristics on a molecular exclusion column as criterion, in order to ascertain which conditions favoured the preservation of the toxic protein in unmodified form.

2 MATERIALS AND METHODS

2.1 General

Methods of chromatography, preparation of buffers, etc., were identical to those described in the previous chapter. Conditions to which protein fractions were subjected between chromatographic separations were of particular interest during this phase of the work. Consequently these conditions will receive greater emphasis than in the previous chapter. Dialysis and concentration were generally achieved by the ultrafiltration method described in Chapter III as Treatment B, although some variations in treatment were used. Such variations are described where necessary.

The chromatographic data to be included in this section were all obtained on a 4% tanned gelatin

column (0.9 x 134cm), using .01M-K-phosphate buffer + .06M-NaCl, pH 7.6, which, in the ensuing discussion, will be referred to as 'phosphate buffer'.

In certain instances, effluent from the column was assayed at 220mμ in the spectrophotometer. As indicated by Groves, Davis and Sells (55), it is possible to detect microgram quantities of protein at this wavelength. Such a procedure is justified when the material under investigation has been sufficiently purified, with minimal danger of interference by non-protein materials. Assaying at 220mμ enabled the column load to be reduced to 1mg, with a marked improvement in economy of material, and the chromatographic properties of a protein could be studied at lower concentrations than is possible with the conventional assay.

2.2 Estimation of molecular weights from molecular exclusion data

The theory of Laurent and Killander (56) was applied to certain gel exclusion data to provide an estimate of relative molecular weights. The Laurent-Killander treatment is based on a parameter (K_{av}) which represents the eluted volume of a solute (V_e) relative to the void volume of the column (V_o) and total column volume (V_t). This is in effect equivalent to the 'Rf' encountered in other forms of chromatography, and is calculated as follows:

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

Laurent and Killander describe a relationship between K_{av} and the Stokes' radius of spherical molecules:

$$\sqrt{-\ln K_{av}} = \alpha (\beta + r_s)$$

where r_s = Stokes' radius of spherical molecule

α = Constant made up from concentration of macromolecule
rods constitution the supporting network

β = constant made up from the radius of the macromolecule rods

Since for most proteins β is small compared to r_s , a plot of $\sqrt{-\ln K_{av}}$ against r_s will be linear. The slope of the plot should be dependent on gel concentration. Experimental confirmation of the linearity of the plot and the dependence of slope on gel concentration is found in the unpublished data of Sims, Bussey and Folkes (57) using agarose gel.

Since for a perfectly spherical molecule,

$$MW = 4/3\pi r^3 \rho, \text{ where } r = \text{molecular radius} \\ \rho = \text{density of molecule,}$$

$$\text{then } r \propto \sqrt[3]{MW},$$

and since r is closely related to the Stokes' radius r_s ,

$$r_s \propto \sqrt[3]{MW}, \text{ and } \sqrt{-\ln K_{av}} \propto \sqrt[3]{MW}$$

Thus the linear relationship also holds between $\sqrt{-\ln K_{av}}$ and $\sqrt[3]{MW}$. This provides a useful means of relating the elution properties of a solute to its approximate molecular weight.

Gel exclusion columns are normally calibrated by running standards of known molecular weight. In the present study this was not necessary, for approximate molecular weights of NG₁ (169,000) and NG₂ (113,000) had been calculated from sedimentation velocity and diffusion data (see Chapter V). Since the molecular weights to be estimated involved interpolation between NG₁ and NG₂, errors in calculation of molecular weights for either NG₁ or NG₂ would not affect the comparison. These were used to establish a straight line on the plot $\sqrt{-\ln K_{av}}$ vs $\sqrt[3]{MW}$. Molecular weights of the intermediate fractions were estimated from this relationship.

2.3 Estimation of void volume (V_o) and total volume (V_t) of gel column

A dyed dextran preparation known as Blue Dextran 2000 (Pharmacia, Uppsala, Sweden) was used for determination of void volume. The preparation consists of random polymers covering a

wide range of molecular weights, a certain proportion of which are fully excluded by the column. These are used to estimate the elution volume of a totally-excluded solute. Five milligrams of Blue Dextran, dissolved in phosphate buffer for several hours, was applied to the column in 0.1ml volume. The concentration of dextran in the effluent was estimated spectrophotometrically at 260m μ .

The total column volume was calculated from the dimensions of the column.

3 RESULTS

3.1 Molecular exclusions properties of N₁CM₁

Since it was of interest to ascertain whether fraction N₁CM₁ was related to N₁G₁, the former was applied to a 4% tanned gelatin column. The elution profile (Fig. 15) indicated that N₁CM₁ consists predominantly of a high molecular weight component with a high content of neutral sugars. A low molecular weight component, eluted at maximum column volume, is also present, while the sugar analysis reveals a third component eluted at an intermediate volume. On the basis of a similarity in molecular weight and high sugar content, it is likely that the major component of N₁CM₁ and N₁G₁ are identical.

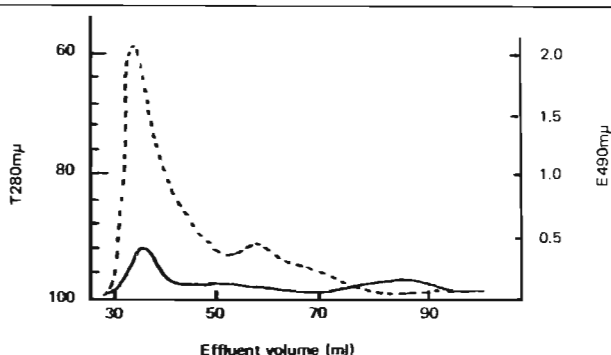


Fig. 15 -- Molecular exclusion chromatography of N₁CM₁ on 4% tanned gelatin. Effluent assayed for protein (—, T280m μ) and sugars (---, E490m μ)
Column dimensions: 0.9 x 134cm
Operating conditions:
Buffer: 0.1M-phosphate + .06M-NaCl, pH 7.6
Sample size and volume: 15mg in 0.5ml.
Pump setting: 1.5
Flow rate: 12.8ml/h

3.2 Evidence for formation of N₁G₁ from N₁G₂

Unequivocal evidence that N₁G₁ is derived from N₁G₂ is provided by the elution profiles in Fig. 16. A single solution of N₁ was made up in phosphate buffer at a concentration of 5mg/ml, and 0.2ml samples were used for each separation. Between runs the stock solution was stored at 0°C, and was thawed each time prior to removal of the 0.2ml aliquot.

The four profiles in Fig 16 indicate that the proportion of N₁G₁ relative to N₁G₂ increases under the conditions of storage in the cold, with intermittent freezing and thawing in the presence of phosphate and NaCl. Further, the change appears to occur via a number of intermediates, for the profiles show evidence of solutes with molecular weights between those of NG₁ and NG₂. This suggests that the change comes about by dissociation of molecular units from the major solute and their association to form a distinct species larger than the first, and with a different composition, particularly with respect to the high content of carbohydrate.

3.3 Estimation of molecular weights from elution volumes

If N₁G₁ was being formed by the association of smaller dissociation products from N₁G₂, the molecular weight of the four intermediate species (Gw, Gx, Gy and Gz — Fig. 16) should differ by some constant integral factor (provided that the monomers were of the same molecular weight). The

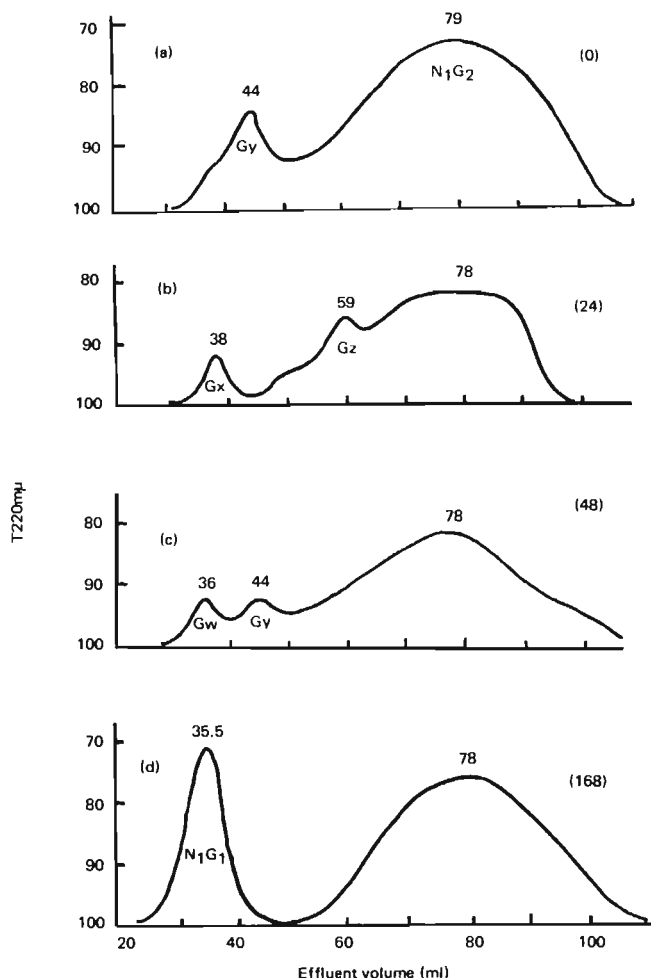


Fig. 16 — Molecular exclusion chromatography of aliquots from a single solution of N_1 , on 4% tanned gelatin
 Column dimensions: 0.9 x 134cm
 Operating conditions:
 Buffer: .01M-K-phosphate + .06M-NaCl, pH 7.6
 Sample: 5mg N_1 in 1ml phosphate buffer. 0.2ml used per run
 Pump setting: 1.5
 Flow rate: 12.8ml/h

Figures in perentheses refer to time (h) after dissolving in buffer. Solution maintained at 0° during the intervening periods. Numerals above peaks refer to eluted volume.

hypothesis was tested by applying the Laurent-Killander theory for estimation of molecular weights from gel exclusion data.

The plot of $\sqrt{-\ln K_{av}}$ vs $\sqrt[3]{MW}$ for N_1G_1 and N_1G_2 is illustrated in Fig. 17. It is immediately apparent that the slope of the line is much steeper than a corresponding plot for a 10% agarose gel (data taken from Sims *et al.* (57)).

Calculation of the molecular weights of G_w to G_z (Table 7) from the plot in Fig. 17 reveals that the various species do differ from one another, and from NG_1 and NG_2 , by a constant molecular weight factor. The ΔMW column of Table 7 shows that the difference is remarkably constant at 13,200 to 13,600, apart from the $G_w - G_x$ difference which is 11,000. The mean MW of peak N_1G_2 was lower under these conditions (104,500) than was observed with the normal N_1 (113,000). It is not possible on the basis of existing evidence to decide on a reason for the low value of N_1G_2 in this particular experiment. A possible cause might be the low sample concentration (5mg/ml) used in the series described in Fig. 16, compared with that generally used (40mg/ml). A low concentration would be

expected to favour the more dissociated state, so that at low concentration N_1G_2 may exist as a lower-MW, partially dissociated form. The treatment applied to the sample is unlikely to be a contributory factor, since the mean MW of N_1G_2 in the experiment described in Fig. 16 does not change with increasing time of treatment (Fig. 16, (a) to (d)).

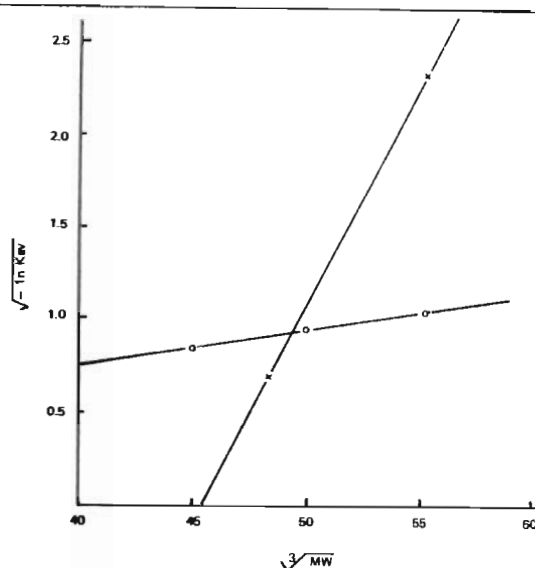


Fig. 17 — Laurent and Killander (56) plot of elution and molecular weight data for N_1G_1 and N_1G_2 on 4% tanned gelatin (—x—). The data of Sims *et al.* (57) for 10% agarose are included for comparison (—o—).

The constancy of the molecular weight difference between peaks Gw to Gz strongly suggests that they are intermediates in the formation of N_1G_1 from N_1G_2 . The former would appear to be formed from the latter by successive addition of a molecular unit of approximately 13,500 daltons.

Table 7 — Calculation of approximate molecular weights from molecular exclusion data.

Data in Part A are calculated from Fig. 14, and used to plot $\sqrt{-\ln K_{av}}$ vs $\sqrt[3]{MW}$ in Fig. 17. Molecular weights calculated from sedimentation/diffusion data. Data in Part B are calculated from elution volumes of peaks in Fig. 16, using the plot in Fig. 17.

$$K_{av} = \frac{V_e - V_o}{V_t - V_o}; \quad V_o = 35\text{ml}, \quad V_t = 85\text{ml}$$

Part	Peak	V_e	K_{av}	$-\ln K_{av}$	$\sqrt{-\ln K_{av}}$	$\sqrt[3]{MW}$	MW	ΔMW
A	N_1G_1	35.5	.004	5.5215	2.349	55.29	169,000	
	N_1G_2	66.2	.622	0.4748	0.6891	48.34	113,000	
B	N_1G_1	35.5	.004	5.5215	2.349	55.29	169,000	13,300
	Gw	36.0	.020	3.9121	1.978	53.80	155,700	11,000
	Gx	38.0	.060	2.8134	1.677	52.50	144,700	13,600
	Gy	44.0	.180	1.7148	1.308	50.80	131,100	13,400
	Gz	59.0	.480	0.7340	0.8567	49.00	117,700	13,200
	N_1G_2	78.0	.860	0.1508	0.3883	47.10	104,500*	

* The difference between this value for N_1G_2 and the value in Part A, is discussed in the text.

3.4 Conditions which promote fragmentation of N_1G_2

3.4.1 Treatment of N_1CM_2

After chromatography on CM-cellulose, peak N_1CM_2 was originally dialysed partially, concentrated by freeze-drying, desalted on Biogel P-20 and freeze-dried a second time. The resultant protein, even after rechromatography under identical conditions, gave a Schlieren profile on the ultracentrifuge which indicated the presence of a high molecular weight species which corresponds to N_1G_1 on the gelatin elution profile.

Additional evidence presented in Chapter II and the present chapter was in agreement with the hypothesis that N_1G_1 is formed from N_1G_2 . It thus appeared that some combination of conditions encountered in the procedure involving dialysis, freeze-drying, desalting and freeze-drying resulted in the formation of N_1G_1 . The most obvious possible causes are:

- i) high salt concentration, which would occur when protein and residual salt were redissolved after the first freeze-drying,
- ii) the freeze-drying stage itself.
- iii) the freezing stage (as opposed to freeze-drying) or a combination of high salt concentration and freezing/freeze-drying.

The concentration of protein is tentatively discounted as a causative factor, since formation of N_1G_1 was observed at both high (40mg/ml) and low 5mg/ml protein concentration.

Pervaporation of protein solutions in pressurised dialysis bags provides a means of concentrating protein solutions without freeze-drying. When evaporation is hastened by passing a current of air over the bag surface, volume decreases rapidly and the solution in the bag remains cool.

A sample of N_1CM_2 was dialysed against distilled water for 7 hours (Visking 8/32 bags), during which time the concentration of salts decreased about ten-fold. It was then pervaporated to reduce the volume twenty-fold from 20 to 1ml. The resultant concentration of acetate and NaCl in solution was thus approximately 0.16M and 1.6M respectively.

The sample was applied to a 4% tanned gelatin column, and had an elution profile as illustrated in Fig. 18. There is a small trace of N_1G_1 and a third peak N_1G_3 , at the maximum column volume. A likely explanation for this profile is that N_1G_3 represents a low-molecular weight units which have separated from N_1G_2 but have not reassociated to form N_1G_1 . This lends further weight to the hypothesis that a two-stage dissociation-association operates in the formation of N_1G_1 from N_1G_2 .

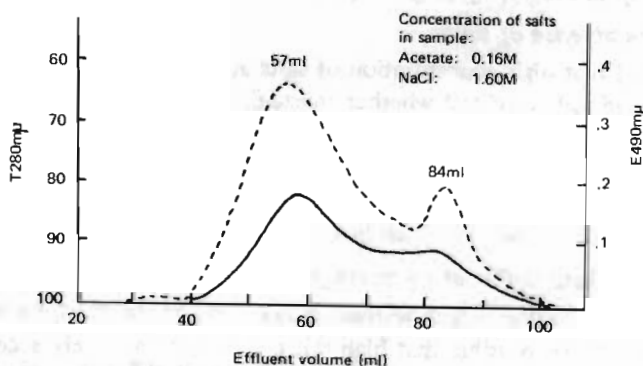


Fig. 18 - Molecular exclusion chromatography of N_1CM_2 on 4% tanned gelatin. Effluent assayed for protein (---, T280mμ) and sugars (—, E490mμ)

Column dimensions: 0.9 x 131cm

Operating conditions:

Buffer: .01M-phosphate + .06M-NaCl, pH 7.6

Flow rate: 12.8ml/h

Fraction N_1CM_2 dialysed 7h vs. running distilled water.

Concentrated by pervaporation

When N_1CM_2 was desalted by ultrafiltration (Treatment B, Chapter II), a procedure which avoids high salt concentration, the proportion of N_1G_3 was reduced considerably (Fig. 19), while N_1G_1 was again visible in only trace amounts. Thus salt concentration would appear to be one of the factors promoting fragmentation.

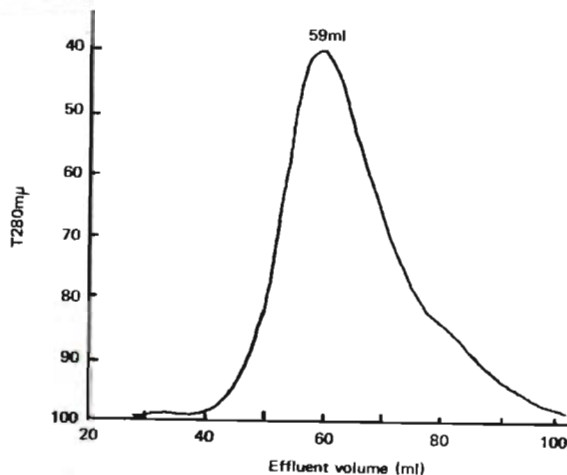


Fig. 19 — Molecular exclusion chromatography of N_1CM_2 on 4% tanned gelatin
Column dimensions and operating conditions: as above
Fraction N_1CM_2 desalted and concentrated by ultrafiltration (Treatment B)

The effect of freezing in the absence of high salt concentration, on the stability of N_1CM_2 was tested. Half of a concentrated, salt-free preparation was frozen to -15° , while the other half was chromatographed immediately. When the frozen preparation was thawed and chromatographed, its elution profile was identical to the control. Thus freezing at this stage in the absence of salts does not appear to have a detrimental effect on the stability of N_1CM_2 .

3.4.2 *Rechromatography of N_1G_2 after subjection to high and low salt concentrations*

Rechromatography of N_1G_2 on tanned gelatin caused a reduction in the proportion of N_1G_1 and N_1G_3 (Fig. 20 (a) and (b)). However, when this preparation was chromatographed again after being concentrated under conditions of high phosphate and sodium chloride, N_1G_1 and N_1G_3 were once again formed at the expense of N_1G_2 (Fig. 20 (c)).

3.4.3 *Freeze-drying in absence of salts*

Having established that high concentration of salts appeared to promote formation of dissociation products from NG_2 , it remained to test whether freeze-drying in the absence of salts was a further contributory factor.

One half of an NG_2 preparation (concentrated and salt-free) was freeze-dried, while the other half was used as a control. The elution profile of the freeze-dried sample was identical to that of the control. Freeze-drying as such does not, therefore, contribute directly to the instability of N_1G_2 .

3.4.4 *Exposure to phosphate buffer at room temperature.*

One of the remaining factors which warranted examination was the time for which the sample was exposed to buffer salts. It was possible that high salt concentration merely accelerated a change which occurred, albeit more slowly in the dilute buffers used in chromatography. Thus even if concentrations were kept low, a slow breakdown might occur which could have the same effect as the more dramatic fragmentation caused by high salts.

A preparation of N_1 was allowed to stand in phosphate buffer for 24h at room temperature. It was chromatographed to ascertain whether the proportion of N_1G_1 to N_1G_2 had changed. The preparation had been concentrated by Treatment A, and consequently its normal elution profile was identical to that in Fig. 14 (Chapter II). After standing for 24h at room temperature the profile was

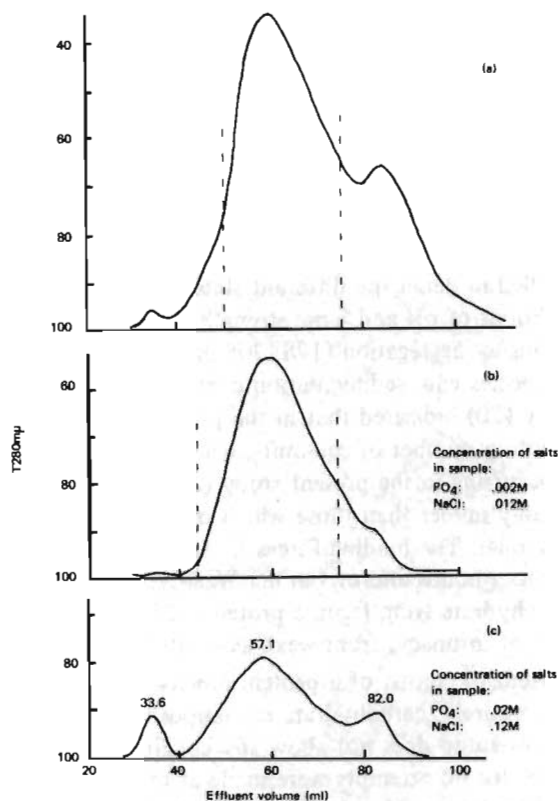


Fig. 20 - Molecular exclusion chromatography of N_1G_2 on 4% tanned gelatin. Successive rechromatography of major peak.

Column dimensions and operating conditions: As for Fig. 18

Fractions corresponding to area between dotted lines in (a) pooled, dialysed and concentrated by pervaporation. Rechromatographed - profile (b); major peak in (b) pooled, dialysed, concentrated by pervaporation and rechromatographed - profile (c).

indistinguishable from that at zero time (Fig. 14). Thus time, temperature and exposure to dilute phosphate buffer were not major factors in the fragmentation of N_1G_2 , although extremes of either time or temperature would undoubtedly induce an unfavourable change.

3.4.5 Effect of CM-cellulose chromatography on molecular weight of N_1G_2 .

The conditions which apply during CM-cellulose chromatography cause a change in the elution volume V_e of N_1G_2 , from 66.2 (N_1G_2 from N_1) to 59 (N_1G_2 from N_1CM_2). The latter state corresponds to G_z (Fig. 16), one of the intermediates formed between N_1G_2 and N_1G_1 , and has a molecular weight of 117,700 (Table 7). This is possibly one of the stable states of N_1G_2 , and whether it exists as the 113,000 or 117,700 species, seems to depend on the conditions to which it was subjected during preparation.

Estimates of the sedimentation coefficients of the major peak in N_1 and $N_1CM_2CM_2$ (described in Chapter IV) showed that the two were identical. Since at this stage in the study, all fractions were concentrated and desalted by Treatment A, this suggests that CM-cellulose chromatography *per se* is not responsible for the change in molecular weight.

4 DISCUSSION

It has become evident from the data in this chapter that dissociation of small-molecular weight units from the parent glycoprotein occurs in the NRY toxin. Under certain conditions the dissociation products are able to reassociate to form a high-carbohydrate component which is larger than the toxin from which it was originally derived. Freezing a dilute solution of toxin in phosphate buffer, thawing after varying lengths of time and separation by gel exclusion showed that the high-MW component is

formed via several intermediates. Estimates of the molecular weights of intermediates indicated that they increased in regular increments of approximately 13,500 daltons.

The nature and concentration of buffer salts appears to be a critical factor in the dissociation-association phenomenon. Thus high phosphate + NaCl concentration promotes the formation of both high and low-molecular weight species. High acetate + NaCl, on the other hand, favours the low-MW units, and trace amounts of the high-MW species are formed. Freeze-drying in acetate + NaCl promotes formation of the high-MW component, although freeze-drying in absence of salts has no effect.

Association-dissociation phenomena are not unknown in legume proteins. Wolf, Rackis, Smith, Sasame and Babcock (58) studied in detail the different states of the so-called 11S protein from soybeans. Under varying conditions of pH and ionic strength the 11S component dissociates into units of 7S and 2S, while states of higher aggregation (17S, 20S and 25S) were also noted. The 11S fraction was found to exist as several species with sedimentation coefficients differing appreciably from the mean. An electrophoretic study (50) indicated that in the presence of urea, the phytohaemagglutinin from navy beans is degraded into a number of sub-units, which could be reassociated to form the parent molecule. While the changes occurring in the present study (and that of Wolf *et al.*) are induced by conditions which are considerably milder than those which promote complete dissociation, the tendency to associate and dissociate is similar. The binding forces in the NRY system are almost certainly weak secondary forces such as hydrogen bonds and/or van der Waal-type attractions. Further reports of the removal of loosely-bound carbohydrate from legume proteins (25, 36, 39, 52), suggest that the two are associated with varying degrees of intimacy, from weak association to covalent bonding.

The NRY toxin would seem to consist of a protein moiety to which is attached residues which are predominantly, though not entirely, carbohydrate in composition. Some of the carbohydrate is easily removable, but the present study does not allow any conclusions as to the mode of attachment of all the carbohydrate residues, for no attempts were made at exhaustive removal of carbohydrate. The dissociated residues appear to be capable of reassociation in a series, the molecular weight of which increases above that of the toxin by a regular increment. The toxin itself is also capable of existence in at least three forms, depending on protein concentration and mode of preparation.

Although the molecular changes in the NRY toxin were found to be promoted by certain conditions, it did not prove possible to prevent their occurrence entirely. Thus even when a sample was concentrated and desalted rapidly and without accumulation of salts, successive rechromatography by molecular exclusion indicated a gradual decrease of the main toxic component, with formation of the higher- and lower- molecular weight products. Because of the difficulties in obtaining quantitative data for toxicity, it was not possible to discover whether toxic units were being lost during these changes, or whether the potency of the main component increased in inverse proportion to its decreasing recovery. Thus it is not known whether the changes involve a fragmentation of the toxic unit, or a loss of non-toxic peripheral material. Nevertheless, as outlined in the introduction to the present chapter, the objective of this study was to isolate a toxin in its natural form. Thus, even though the observed changes might have resulted in a toxin of higher activity, by virtue of the modifications the isolated toxin could only be regarded as a derivative of the natural toxin.

One aspect of the molecular weight calculations reported in the present chapter deserves mention, for it throws light on the properties of the recently-developed 'tanned' gelatin as a medium for molecular exclusion. It was observed that when elution volume data from a 4% 'tanned' gelatin column were plotted according to Laurent-Killander theory, and compared with a similar plot for agarose, the slope of the gelatin plot was considerably steeper than the corresponding agarose slope. This means that the change in K_{av} with change in molecular weight is much greater for gelatin than for agarose, or alternatively that for 4% gelatin there is a narrow range of molecular weights which are neither fully excluded nor fully included. This would suggest that the pore size of gelatin granules is more uniform than agarose spheres, since a narrow range of pore sizes would reduce the range of partially included molecular species. Furthermore, gelatin would appear to have greater resolution within a particular range of molecular weights, since a small difference in molecular weight would cause a greater difference in elution volume.

The information which came to hand towards the conclusion of this study, suggested that the problems of association and dissociation might have been avoided, had a buffer other than phosphate been used for chromatography. For some time the author had been aware of the disadvantages of phosphate in buffering systems. The only reason for retaining it was the lack of a suitable alternative in the range pH 6 to 8. Tris has poor buffering capacity below pH 7.5, and being a primary aliphatic amine, is more reactive than would be preferred for a buffering salt. The complexing properties of borate ions need no emphasis.

Good, Winget, Winter, Connolly, Izawa and Singh (59) describe several synthetic buffer salts designed to circumvent the difficulties of conventional salts used in the pH 6 to 8 range. While these would seem to have a number of commendable features, they would require testing under the specific conditions of this study before general use could be advocated. Further, their expense at present precludes widespread use in column chromatography. Nevertheless this timely development will doubtless have a profound effect on the pattern of buffer usage in biological studies.

As far as the present investigation is concerned, the main conclusion is that, while it has not been possible to isolate the toxic protein as a homogeneous solute by molecular exclusion, the reason for the difficulty and the nature of the effect have been well defined. It may thus be inferred with reasonable safety that the major component of N₁, N₁G₂, represents the pure toxin, even though the molecular exclusion criterion of purity has not been met in the conventional sense.

CRITERIA OF PURITY AND MOLECULAR PARAMETERS
OF THE TOXIC FACTOR

1 INTRODUCTION

The concept of purity in proteins is essentially a negative one. It depends on the failure to observe heterogeneity when the structural or functional properties of a protein are used as a basis for separation. Thus 'purity' in this context has varying depths of meaning, depending as it does on the number of criteria by which a protein has been judged to be homogeneous. Furthermore, homogeneity has no absolute meaning since it is always possible that some new criterion may be developed, which will reveal an order of heterogeneity within an apparently homogeneous preparation.

Nonetheless the principle of testing for purity is widely applied to protein preparations. Since no single criterion is sufficient to establish purity, several criteria, depending on different molecular parameters are generally chosen. In the first instance the methods used in the separation phase should produce a homogeneous fraction. The most commonly encountered methods (ion-exchange chromatography and molecular exclusion), each exploiting different molecular parameters, provide a suitable preliminary indication of the number of components in a system. Electrophoretic separation distinguishes between proteins on the basis of net surface charge, and the sensitivity of electrophoretic analysis can be increased by forcing the proteins to migrate through a sieving gel (as in disc electrophoresis). The antigenic properties of most proteins can be exploited by developing an antiserum and studying the number of immunologically active components in the system. Furthermore, immunoelectrophoresis offers the possibility of combining immunological and electrophoretic criteria of separation. Finally, the properties of proteins under the influence of high centrifugal forces provide a powerful index of their purity, and homogeneity in the analytical ultracentrifuge is a *sine qua non* of purity.

By virtue of its dependence on one aspect of the full range of properties exhibited by a protein, each criterion has certain advantages and certain limitations. Thus the charged state which is induced in a protein to ensure binding to an ion-exchange column, may induce fragmentation and production of artificial peaks. Alternatively, two different proteins may not be distinguished if they are inadequately bound to the stationary phase. The full power of molecular exclusion chromatography may be lost through choice of unsuitable media or through technical difficulties associated with column operation.

In all of the techniques mentioned, the method of sample detection plays a key role. Thus heterogeneity in an elution profile may be missed because the effluent was assayed for one chemical species and not another. Similarly an electrophoresis strip may reveal heterogeneity when stained by more than one method. The Schlieren optical system in the analytical ultracentrifuge, depending as it does on diffraction phenomena, has limited sensitivity. Immunological methods are fraught with difficulties, not the least of which arises from the concentration-dependence of the antigen-antibody reaction.

The few difficulties mentioned in the preceding paragraphs emphasize the degree to which the investigator depends upon secondary information to build up an image of a state which is essentially invisible. The progress of molecular biology has depended almost entirely on the ingenuity of the investigator in devising means of constructing a molecular picture from a series of effects which the molecule has produced. Evaluation of the purity of a protein preparation and the assessment of information regarding its molecular parameters, is a similar exercise in synthesis and extrapolation.

The properties of the NRY toxin have been studied by many of the accepted techniques for purity evaluation. An attempt was made to exploit the potential of each criterion and assess the reliability of the information obtained in each case. Although the stability of the NRY toxin was such that homogeneity could not be unequivocally established, the information suggested that a pure but unstable preparation had been obtained.

2 MATERIALS AND METHODS

2.1 Zone electrophoresis

2.1.1 Starch gel electrophoresis

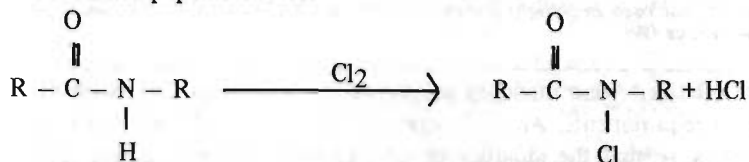
A modification of the original method of Smithies (60) was used to study the electrophoretic properties of NRY fractions. Separation was effected at low pH with an aluminium acetate buffer system as described by Elton and Ewart (61) using conditions of voltage and current density empirically determined for the proteins used in the present study.

The aluminium lactate buffer was prepared by activating 10g of pure aluminium foil by briefly immersing in 1% HgCl_2 , rinsing with distilled water and allowing to dissolve in about 1L of distilled water containing 176g lactic acid. The solution was filtered and diluted to 2L with distilled water.

Starch gel blocks were prepared as follows: 4.8g of partially hydrolysed starch (British Drug Houses (Pty) Ltd., Poole, England) was suspended in 40ml of aluminium-lactate buffer and heated to just below boiling. Air bubbles were removed by brief evacuation and the solution was poured into a perspex former (4 x 14 x 0.6cm) containing suitably placed filter paper wicks. The slot former was set in position and air bubbles removed. When the gel had set the surface was sealed with molten beeswax. Sample was applied to the cooled gel, the sample holes were sealed with wax, the wicks placed in buffer tanks, and electrical potential applied. The upper gel surface was cooled with ice during electrophoresis.

When the electrophoretic phase was complete, the gel was sliced horizontally. The lower gel section, removed from the perspex former, was used for staining and observation of zone positions.

Staining of zones. A new staining method was developed by the author, based on the chlorination principle described by Rydon and Smith (62). Gaseous chlorine is able to replace the hydrogen of the secondary amine in the peptide bond:



Chlorine is able to replace iodine from a compound, due to its greater electronegativity. Thus by adding KI to a chlorinated protein in the starch gel, Cl replaces I from KI, releasing free iodine which immediately reacts with the starch gel. Since the stain is activated by every peptide bond, it is extremely sensitive, and specific for secondary amines. A disadvantage of the conventional staining procedures with starch gel is that they show high background staining — a feature which was encountered by the author in preliminary studies. The chlorine-KI stain shows no background staining, and protein zones appear as deep blue-black bands. A disadvantage of the stain is that the colour fades within two to four hours, so that the result must be recorded promptly. Since starch gels are difficult to manipulate and store, prompt recording of results is a feature of normal procedure and the extra requirement does not add significantly to existing limitations.

While gaseous chlorine was used by Rydon and Smith to stain proteins on paper chromatograms, a liquid source of active chlorine was more suitable for staining in a gel medium. Sodium hypochlorite proved adequate for this purpose. The staining procedure was as follows:

After electrophoresis, the gel was immersed in 5% (v/v) sodium hypochlorite ("JIK" commercial bleach, containing 3.2 – 3.5% active chlorine) for 30 min, with intermittent agitation. Excess hypochlorite was removed by washing in running tap water for 3h. The washed gel was immersed in 5% (w/v) KI for a few minutes, then rinsed briefly in distilled water.

2.1.2 Electrophoresis on cellulose acetate membranes

Cellulose acetate membranes, developed by Kohn (63) as media for zone electrophoresis, offer considerable advantages over starch gel. The membrane is chemically inert, and shows negligible

background stain. It is easily manipulated, stained and stored. Because of its microporous structure, it is quickly impregnated with buffer, or with suitable reactants for specific staining reactions.

Because of the advantages and improvements in simplicity of handling, cellulose acetate was used as an adjunct or as an alternative to starch gel throughout most of the present study.

Preparation of membrane. Prior to electrophoretic treatment the membrane (2.5 x 12cm, Sartorius-Membranfilter, Germany) was marked with carbon ink to indicate the origin, identity of sample and other relevant data. It was placed on the surface of the appropriate buffer solution in a petri dish, until fully impregnated with buffer. When no air spaces remained in the membrane, it was momentarily immersed in buffer, removed and blotted once, quickly but thoroughly between sheets of fine grade filter paper. The protein sample was immediately applied from a capillary tube as a single strip, and the membrane placed in a sealed perspex box connected by filter paper wicks to the buffer vessels (see Plate 1). If several strips were being run simultaneously, a multiple vessel, adapted from the conventional paper electrophoresis tank, was used.

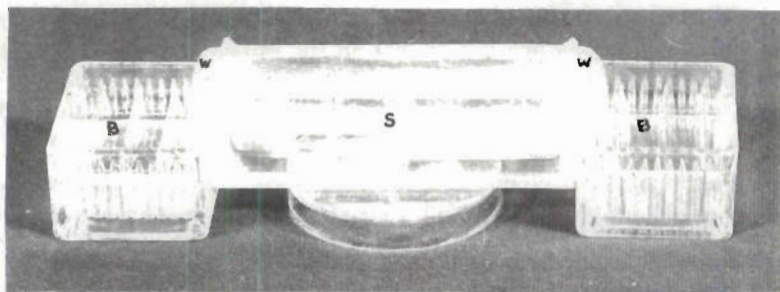


Plate 1 — Apparatus for electrophoresis of proteins on single cellulose acetate membranes. Apparatus includes electrophoretic strip (S) enclosed in airtight perspex container, connected to buffer vessels (B) by means of filter paper wicks (W).

Buffers. Electrophoretic buffers were made up according to Bloemendal (64) with modifications to ionic strength according to requirements. An ionic strength of 0.1 M was found to be suitable for cellulose acetate electrophoresis, so that the quantity of salts given by Bloemendal was doubled in the present study. Buffer pH was measured accurately at least 24h after preparation, and bacterial growth prevented by addition of 2ml of 1% aqueous merthiolate per litre.

Electrophoresis. Electrophoresis was conducted in a refrigerator at 20°, under conditions of voltage, current and time which will be described together with the relevant results.

Staining of membranes. Staining procedures included the nigrosin and amido black stains for protein, and the periodic acid-Schiff's base stain for glycoproteins. While the amido black staining method was obtained from Dr. A. Polson (personal communication), the other two were described in the membrane manufacturer's catalogue.

1. Nigrosin. Nigrosin has the advantage of sensitivity, so that low-concentration impurities are detectable. However, the intensity of stain is not proportional to protein concentration, and Nigrosin-stained strips are not used for densitometric scanning or estimation of relative concentrations. Following the electrophoretic separation, strips were dried at 110° for 10 minutes, and placed on the surface of a .002% solution of Nigrosin in 2% acetic acid. When fully impregnated the strip was immersed in the solution and allowed to stand overnight. Excess stain was washed from the strip with distilled water.

2. Amido Black. Although less sensitive than Nigrosin, Amido Black is suitable as a general purpose stain because the intensity of colour is proportional to protein concentration. This facilitates comparison of components with respect to concentration and permits densitometric scanning.

The electrophoretic strip was dried at 110° for 10 min. It was placed on the surface of Amido Black staining solution (0.55g of Amido Black + 0.55g of HgCl₂ in 55ml of ethanol: distilled water: glacial acetic acid (25:25:5); filter before use). When fully impregnated it was immersed for 15 min, and

excess stain washed from the strip with ethanol: distilled water: glacial acetic acid (25:25:5).

3. Periodic acid-Schiffs base stain. After electrophoresis, the strip was dried in an oven (110°) for 20 min, and immersed in a dish of 96% ethanol for 10 min. It was transferred for 8-10 min to the periodic acid solution (2.5g of periodic acid + 50ml of 0.2M-sodium acetate + distilled water to 500ml), and rinsed in .001N-HCl. The strip was immersed in a 10% KI solution in distilled water, whereupon both strip and solution turned brown. A few drops of saturated ammonium thiosulphate were added and within minutes both strip and solution became colourless. After a second rinse in .001N-HCl the strip was transferred to the Schiffs reagent (Fuchsin base (2g) dissolved in 400ml of distilled water + 10ml of 2N-HCl and 4g of potassium metabisulphite. Active charcoal + 10ml of 2N-HCl were added to the solution, which was filtered and stored in a dark bottle). Finally the strips were rinsed three times (15 min each) in 0.1N-HNO₃.

Stained membranes were dried and pressed between filter paper sheets, after which they were easily filed.

Estimation of electroendosmosis. Cellulose acetate, in common with other stabilizing media for zone electrophoresis, bears a slight nett potential, known as the zeta potential (65). This results in a hydration of buffer cations with consequent bulk movement of buffer towards the cathode during electrophoresis. The effect is known as electroendosmosis. When making quantitative measurements of the migration of solute it is necessary to make allowance for the contribution of electroendosmosis to total migration. For estimation of the extent of buffer flow, polyethylene glycol, an uncharged randomly coiled, synthetic polymer is placed on the impregnated cellulose acetate membrane and subjected to the same electrophoretic conditions as the protein.

Polyethylene glycol (6M, supplied by Shell Chemicals) was applied at the origin in a 10% solution, and normal electrophoretic conditions were applied. At the conclusion of the run, the strip was immersed in 10% (w/v) trichloroacetic acid for 5 min. It was rinsed briefly with distilled water, placed on a clean glass plate and pressed firmly against the plate so that all air bubbles were removed. Plate and membrane were immersed in a clearing solution consisting of dioxane : isobutanol (7 : 3). Care was taken at this stage to prevent the membrane from being dislodged from the glass plate by the stresses developed during swelling in the clearing solution.

The cleared membrane was dried in an oven at 110° for about 5 min. Properly controlled, this stage causes the membrane to melt briefly and become firmly bound to the glass plate. As drying occurs (within 4 hours) the polyethylene glycol becomes visible as a white zone on the transparent membrane. The membrane can be removed from the plate by soaking in warm water, after which it is conveniently stored.

2.1.3 Disc electrophoresis

Ornstein (66) and Davis (67) described the original method of disc electrophoresis using polyacrylamide columns. The procedure consists of two stages. In the first stage, the sample is concentrated into a zone of molecular dimensions by selecting leading and trailing buffer ions which have mobilities higher and lower respectively than the protein ions. This, by the regulating function of Kohlrausch (66) causes the protein to be concentrated into the junction between the leading and trailing ions. In the second stage, a pH increment causes a change in mobility of the trailing ion, such that it migrates faster than the sample ions, leaving the latter to separate according to charge in a uniform voltage gradient. The molecular-sieving properties of polyacrylamide gel impose a frictional resistance on larger molecular species, so that an additional factor is imposed to facilitate separation.

In spite of the empirical approach of Williams and Reisfield (68), disc electrophoresis is difficult to exploit effectively in a protein system of unknown mobility. Nonetheless the molecular sieving properties of polyacrylamide can be used to aid electrophoretic separation without prior knowledge of solute properties. By omitting the initial "steady state stacking" phase in which concentration of the sample zone takes place, and applying the sample with care to the surface of the electrophoretic gel, the second phase (separation) can be used in a continuous buffer system. A sharp sample zone is obtained and the separation is suitable for exploratory purposes.

A simple apparatus, made by the author, was used for the study of the NRY toxin by disc electrophoresis. Since the study was exploratory in nature, the apparatus was used for single samples only. An increase in the scale of operation for the simultaneous electrophoresis of several samples was not indicated by the pilot experiment.

Polyacrylamide gel was prepared as described by Bosman (69) as follows: Cyanogum 41 monomer (British Drug Houses, Ltd., Poole, England) (1.25g) was dissolved at room temperature in 25ml of .05M formate buffer, pH 3, prepared according to Bloemendal (54). Ammonium persulphate (.05g) was weighed into a small beaker. Five drops of crosslinking agent β -dimethylaminoethyl cyanide was added to the same beaker which was tilted to keep the two reagents separate. The monomer solution was added to the contents of the beaker, thoroughly mixed by stirring and poured into a vertical glass tube (1.0 x 13cm) suitably sealed at one end in such a manner as to allow the gel to form a flat face at the mouth of the tube. Sufficient solution was poured into the tube to form a column approximately 10.5cm in length, leaving the upper 2.5cm of the tube empty. Distilled water was carefully pipetted onto the upper liquid surface, the tube mouth was sealed with a stopper and the tube was allowed to stand for 2 hours for polymerisation to occur. Addition of water above the polymerisation mixture ensured that the gel had a flat upper surface which facilitated formation of an undistorted sample zone.

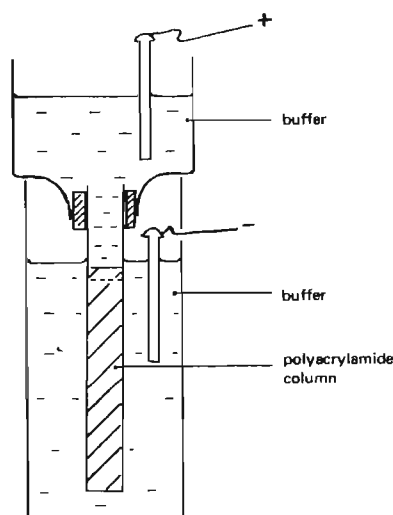


Fig. 21 — Apparatus for disc electrophoresis of individual samples. Containers consist of suitably adapted polyethylene bottles.

The glass column was opened at either end when polymerisation was complete, rinsed with buffer and fitted into the buffer vessels as illustrated in Fig. 21. The vessels were filled with buffer and the apparatus chilled to 20°. Sample (0.1ml) containing 20mg dry weight of material per ml, was carefully layered on the gel surface with a capillary pipette, and the potential was applied. Overheating was prevented by maintaining the apparatus in a refrigerator during electrophoresis.

At the conclusion of electrophoresis, the polyacrylamide column was extruded from the glass tube. It was stained with amido black (as used for cellulose acetate membranes), destained with the EtOH : H₂O : HAC solution used with cellulose acetate and stored in a stoppered tube in the same solution.

2.2 Immunodiffusion

2.2.1 Preparation of antisera

Antisera were prepared by injecting a series of dilute antigen solutions into adult rabbits, with a two-day interval between injections. The procedure as follows:

Three intramuscular injections of 2mg dry weight of material in 1ml of sterile physiological saline

were followed by two intravenous injections of the same quantity and finally by two intravenous injections of 1mg dry weight of material in 1ml of sterile physiological saline. Rabbits were bled by ear vein puncture one week after the last injection. Rabbits bled more than one month after receiving the primary immunisation treatment described above, received a booster injection (1mg dry weight of material in 1ml of sterile physiological saline, administered intravenously), one week before being bled.

2.2.2 Double diffusion

The Ouchterlony (70) double diffusion principle was used for studying the antigen-antibody reaction. The arrangement of wells followed the usual pattern of a central antibody well surrounded by antigen wells at a constant distance from the centre. Sewell (71) recently introduced a system of arranging wells in the form of interlocking hexagons. This is particularly suitable when a number of different antigens are being tested against a standard reference antigen, as several comparisons can be made simultaneously. A pattern based on that suggested by Sewell was used for certain comparisons in the present study.

Agarose (Seravac Laboratories, Cape Town) was suspended in buffered saline, pH 7 (NaCl, 8g; K_2HPO_4 , 1.2g; KH_2PO_4 , 0.3g; distilled water to 1 L) containing 1ml 1% merthiolate per 100ml, to a concentration of 1% (w/v). The agarose was melted by heating over a boiling water bath, then poured onto a glass plate (7.5 x 15cm) to a depth of 2-3mm. The plate had been prepared by covering the surface with a thin layer of molten agarose and drying in an oven at 100°. The layer of dried agarose improves adhesion between gel and glass. A polythene sheet covered the solution while cooling and setting occurred, after which the gel was covered by a film of distilled water.

The pattern of wells (0.5cm diam, 1cm apart) was cut from the gel with a cork borer and the plugs removed with a dissecting needle. Excess distilled water was removed from the wells and the antigen (dissolved in physiological saline) and antiserum samples added to appropriate wells. The plates were incubated in a sealed humid box at 37° and examined daily from the 3rd to 14th days for development of precipitin bands.

2.2.3 Quantitative gel diffusion

In 1958 Polson (72) developed a method for quantitative study of the precipitin reaction. Antigen (at different concentrations) and antibody are allowed to diffuse toward each other from opposite ends of an agarose column in a suitably designed apparatus.

The apparatus consisted of three rectangular Perspex bars, 20 x 2 x 1.25cm, with a narrow section acting as a lid (Plate 2). Holes (0.4cm diam and 1.8cm apart) were drilled through the lid and through two sections. The holes in the third section were drilled to about $\frac{3}{4}$ of its depth, to form cylindrical cavities, one end of which was closed. Opposing surfaces of the various sections were machined and polished so that, when greased and clamped together, they formed an air-tight seal. The apparatus was assembled (Plate 2, position A) and excess grease removed from the holes with cotton wool. Position A, with all holes in apposition, allowed access from the lid to the cavities in the third section. These cavities were filled with antiserum, and closed by displacement of the second and third sections with respect to one another (Position B). Agarose (0.3% (w/v) in buffered saline), melted and cooled to just above its gelling temperature, was introduced into the holes of the second section, which in turn was closed off by displacement w.r.t. the first (Position C). Serial dilutions of antigen in physiological saline (or in buffered saline) were introduced into the holes of section 1, and the holes were closed by displacement of the lid. Finally, all sections except the lid were once more brought into apposition (Position D), so that antigen and antibody were simultaneously brought into contact with opposite ends of the agarose column, and sharp interfaces were formed between them. The apparatus was held together with clamps or rubber bands, to prevent separation of sections and loss of moisture from the holes.

When antigen and antibody diffuse toward one another in an agarose column a precipitin band will form at that position where the two meet in optimal proportions. The reaction is described by the following equation (72):

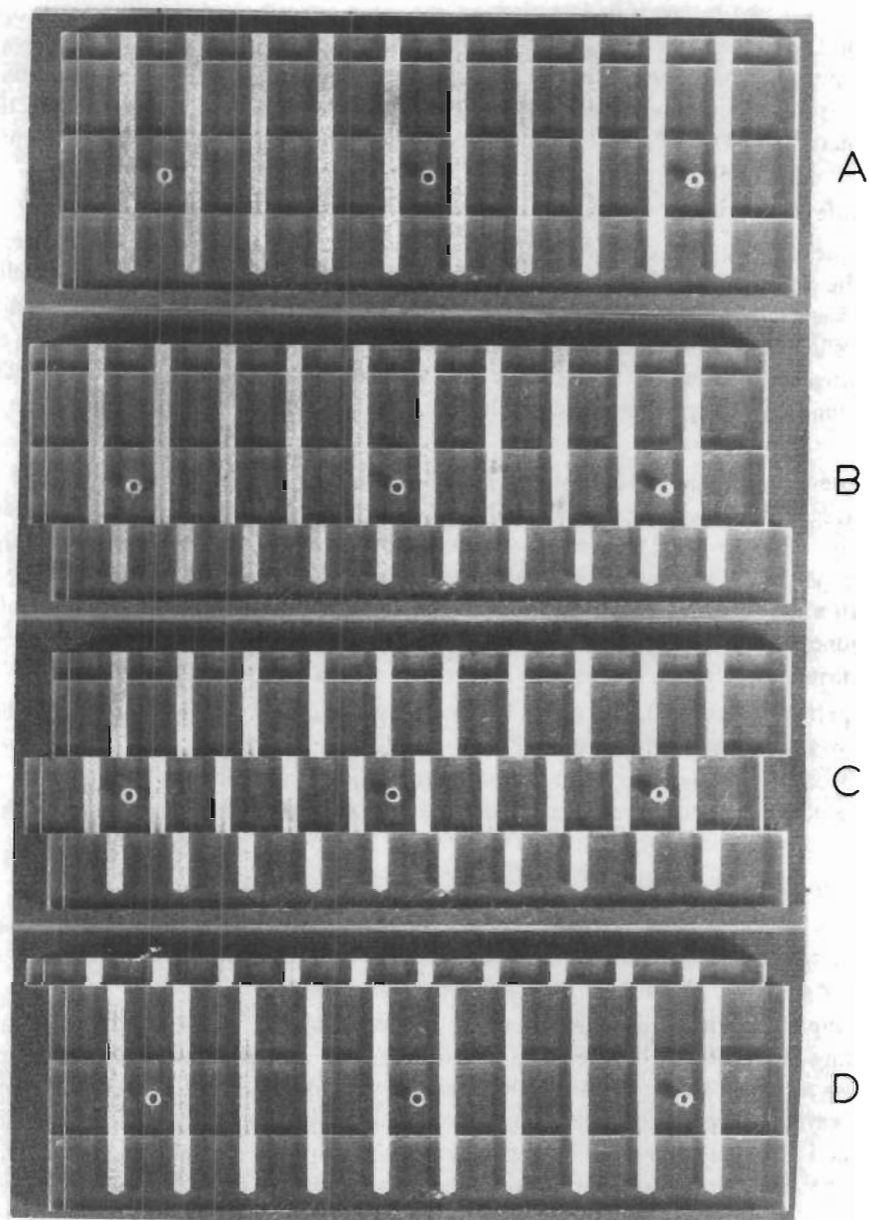


Plate 2 — Apparatus for determination of diffusion coefficients by quantitative gel diffusion. See text for details regarding assembly and filling.

$$\log \frac{C_{go}}{C_{bo}} = \log \frac{C_g}{C_b} + \frac{X_g}{\sqrt{\pi D_g t}} - \frac{X_b}{\sqrt{\pi D_b t}} \quad \dots \quad (1)$$

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 from antigen and antibody meniscus respectively, where precipitin band is formed
 D_g and D_b = diffusion coefficients of antigen and antibody respectively
 t = diffusion time in seconds
 $X_g + X_b$ = H (total length of agarose column)

When the initial concentrations of antigen and antibody are optimal for the precipitin reaction to occur

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

$$\text{Therefore } \frac{X_g}{\sqrt{\pi D_g t}} = \frac{X_b}{\sqrt{\pi D_b t}}$$

$$\text{and } \frac{X_g^2}{X_b^2} = \frac{D_g}{D_b} \dots \dots \dots (2)$$

Thus at optimal proportions the time factor is eliminated, and the position of the precipitin band relative to the menisci depends solely on the ratio of the diffusion coefficients of antigen and antibody.

Since the majority of antibodies are classified as γ -globulins, the diffusion coefficient may be taken as $4.6 \times 10^{-7} \text{ cm}^2/\text{sec}$ (Polson, A., personal communication), and an approximate coefficient for the antigen may be obtained from accurate measurements of X_g and X_b at optimal proportions.

From equation 1 it can be seen that excess of antigen will cause migration of the precipitin band away from the antigen well, and *vice versa*. In practice this appears as a diffuse broadening of the band away from the well containing excess antigen or antibody. When antigen and antibody are present in optimal proportions, the precipitin band is sharp.

Measurement of distances X_g and X_b at optimal proportions with a microcomparator gives an accurate estimate of the relative position of the band. Using equation 2, the diffusion coefficient can be calculated from the measurements.

Polson's method includes a graphical procedure for estimating X_g and X_b when optimal proportions occur between two chosen antigen dilutions. In the present study it was possible to estimate the distance with sufficient accuracy without the graphical refinement, for with the major antigen optimal proportions coincided with one of the chosen antigen dilutions.

2.3 Immunelectrophoresis

The behavior of a protein in immunelectrophoresis is accepted as one of the important criteria by which homogeneity is judged. By combining a separation based on electrical charge in one dimension, with a distinction according to diffusion coefficient and antigenic specificity in another, immunelectrophoresis has demonstrated extraordinary sensitivity, particularly in the analysis of serum proteins.

Conventionally, the electrophoretic and immunological stages are both performed in agarose gel, although Kohn (73) described a method whereby an electrophoretic separation on cellulose acetate could be followed by immunodiffusion on agar. Since the method uses only small quantities of antigen, and the antisera produced in response to the NRY proteins were not particularly strong, Kohn's technique was not successful in the present study. It was therefore necessary to develop electrophoretic conditions such that NRY fraction N₁ would migrate in agarose gel. In addition it was important to find concentration conditions which were optimal for the immunodiffusion phase.

Preparation of plates. Microscope slides (2.5 x 7.6cm), prepared as described in section 2.2 for immunodiffusion plates, were covered with molten agarose (1% w/v) in appropriate buffer, to a depth of 2-3mm. Filter paper wicks were attached and moulded according to the Grabar-Williams technique (74), as illustrated in Fig. 22. The sample was placed in a well (1mm diam) which was covered with a small microscope cover slip to prevent drainage or evaporation. During electrophoresis the plates were covered with a thin polythene sheet to prevent evaporation. The placement of wicks and sample well, and the location of plates during electrophoresis, are illustrated in Fig. 22.

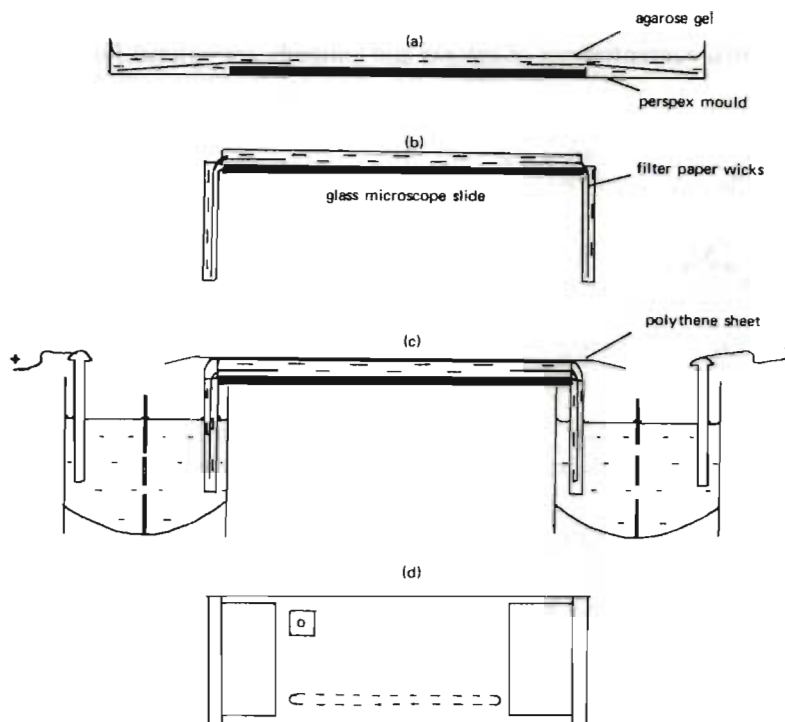


Fig. 22 — Preparation of agarose plates for immunoelectrophoresis

- (a) Agarose poured on microscope slide; wicks in perspex mould
- (b) Wicks cut and bent
- (c) Cuts filled and slide attached to buffer tanks
- (d) Top view of slide showing location of sample well and cover slip. Dotted line indicates location of antiserum slot (prepared at conclusion of electrophoretic phase).

Electrophoretic conditions. Details of voltage, current and time are included with the relevant results. The concentration of sample applied depended upon whether the plates were to be stained or used for immunodiffusion, the respective concentrations being 30mg/ml and between 1 and 20mg/ml respectively.

Application of antiserum. When the electrophoretic phase had terminated, the wicks were cut from the slide. The antiserum slot was cut with a sharp scalpel and the sliver of gel within the slot removed with a dissecting needle. Antiserum was added to the slot and the slide was placed in a sealed humid petri dish. Development of bands took place at 37° for between 2 and 7 days.

Staining of slides. Companion slides were run under identical conditions to those used for immunodiffusion, so that the position of the antigen band could be ascertained by staining. Thus after electrophoresis the slide to be stained was dried in an incubator at 37° overnight. It was immersed in amido black stain (identical to that used for cellulose acetate — see 2.1) for 30 min, and destained with ethanol : water : glacial acetic acid wash used previously.

2.4 Ultracentrifugation

2.4.1. Density gradient ultracentrifugation

The preparative ultracentrifuge has found wide application in protein isolation studies. Used in a semi-analytical role together with density gradients and the swinging-bucket rotor, it provides useful information on the homogeneity of protein preparations with respect to sedimentation rate and buoyant density.

As with its analytical counterpart, the preparative ultracentrifuge may be used at high gravitational

forces to study the rate of sedimentation of a solute zone (rate zonal ultracentrifugation) or at low forces sufficient to create a balance between the centrifugal force in one direction and the buoyancy of the solute in the other (equilibrium zonal ultracentrifugation). In spite of the low maximum resolution rating (25,000 r.p.m.) of the rotor used in the present study, the NRY toxic protein was unlikely to reach equilibrium conditions in a sucrose gradient, and the rate zonal state was assumed to apply. The experimental procedure was based on a method described by Martin and Ames (75).

Buffers: Acetate buffers (.02M, pH 5.6) containing 5% and 20% sucrose (w/v) were constituted by titration of acidic and basic components (.02M) containing the appropriate percentage of sucrose.

Gradients: Linear sucrose gradients (5% to 20% in .02M-acetate buffer) were prepared using a small apparatus constructed in this laboratory (Fig. 23). The apparatus was checked by including a few grains of

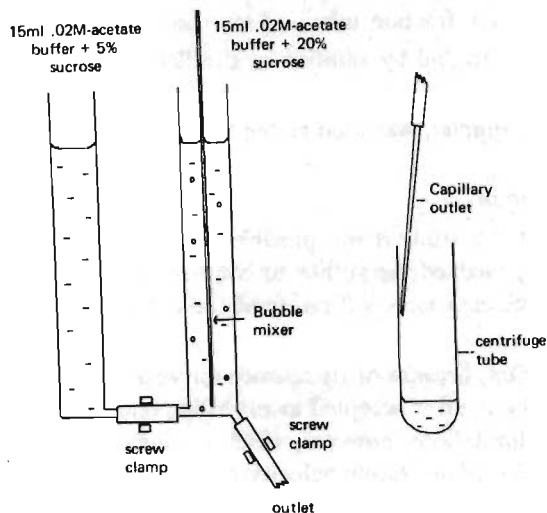


Fig. 23 — Device for formation of sucrose gradients

2:6 dichlorophenol indophenol in the mixing vessel. Analysis of the change in colour intensity (Spectrophotometer at 660 mμ) of fractions collected from the tube showed that the change was satisfactorily linear. Tubes containing gradients were chilled to 2° before use.

Centrifugation: The sample (3.2mg in 0.4ml of .02M-acetate buffer) was carefully layered above the gradient and the tube fitted to the chilled rotor (SW-25) of the Spinco Model L preparative ultracentrifuge.

The rotor was accelerated slowly (minimum setting on rotor speed control) for 30 seconds, after which the control was set to the required speed. The following data were recorded for each experiment:

- Time of acceleration to full speed
- Running time and revolutions per min
- Total revolutions over running time
- Deceleration time

The rotor was allowed to coast to rest at the conclusion of the run, with no assistance from the electrical brake.

Each tube was removed from the rotor and the contents collected by piercing the tube base with a hypodermic needle to which was attached a length of 1/16" teflon tubing. Leakage of fluid from the junction between tube and needle was eliminated by affixing a small square of linen-backed adhesive plaster to the base of the tube and piercing both plaster and tube with the needle. The tube contents were collected automatically in 15-drop (0.7ml) fractions. Distilled water (1.3ml) was added to each tube and the rack was chilled to 10° before analysis of tube contents.

Assay of fractions. Fractions were assayed for protein content by the Lowry modification of the

Folin Ciocalteu reaction, as described in Chapter II, section 2.6. For the present purpose, the procedure differed slightly from that described in Chapter II, in that the volume of reagents was doubled, and reagents were chilled to 10° before use.

During earlier examinations of fractions obtained after centrifugation an anomalous reaction was observed with the Lowry assays. Even in the absence of protein (i.e. with sucrose-containing buffer alone) colour was formed in such a manner as to give the appearance of many random peaks when plotted graphically. A systematic study of the effect of sucrose in the presence of tris and acetate on the colour formed between protein and Lowry reagents (unpublished data) revealed that sucrose does depress colour-formation. This alone however, does not account for the random colour formation, which the author is unable to explain at present. Two treatments were, however found to eliminate the artifact:

- (i) dialysis of each tube against distilled water for 8h (by sealing a piece of dialysis membrane across the mouth of each fraction tube and inverting the rack in water),
- (ii) dilution of the sample to 2ml by addition of distilled water, and conducting the reaction at 10°.

The second method, being simpler, was used as the standard procedure for ultracentrifuge effluent analysis

2.4.2 Analytical ultracentrifugation

Towards the conclusion of this study it was possible to study a few samples on an analytical ultracentrifuge. This opportunity enabled the author to confirm the results obtained on the preparative ultracentrifuge and calculate sedimentation coefficients of the major components in the toxic preparation.

Analytical ultracentrifugation, because of its reliance on well-defined physical parameters and the soundness of its theoretical background is accepted as providing one of the absolute criteria of homogeneity. It is not without limitations, however, which are imposed mainly by limitations in methods of detection. Thus while sedimentation velocity using Schlieren phase-plate optics is the most commonly encountered procedure for testing the homogeneity of a protein, it is limited by the Schlieren detection system and the fact that velocity determinations do not depend directly on molecular weight. Ease and rapidity of operation are mitigating factors which result in the wide applicability of this method as a criterion of purity.

Samples in the present study were examined in a Spinco Model E analytical ultracentrifuge using the An-D rotor which was equilibrated to 20° before use. A wedge-window cell which displaces the optical image, was used in conjunction with a normal cell and facilitated the study of two samples simultaneously. Sample protein (0.5% w/v), was dissolved in M/15 phosphate buffered saline (0.9% w/v) pH 7.1.

Calculation of sedimentation coefficients and molecular weights. Sedimentation coefficients were calculated from the photographic plates which recorded the position of the sedimenting peak at exactly timed intervals. The distance from the peak maximum to the reference hole was measured for each interval with a microcomparator.

The sedimentation coefficient was calculated from the rate of increase in a factor x , which represents the distance from the axis of rotation to the peak maximum. This distance x , was calculated for each time interval from the following formula:

$$x = 7.3 - \frac{y}{21}$$

where x = the distance from the axis of rotation,

y = the distance from the peak maximum to the reference hole,

the factor 21 accounts for magnification in the optical system, and 7.3 is the distance (in cm) from the axis of rotation to the reference hole.

The sedimentation coefficient, defined as the velocity attained in a unit centrifugal field, is given by:

$$s = \frac{dx/dt}{\omega^2 x}$$

where x = as described above

t = time in seconds

ω = the angular rotor velocity in radians

Since the angular velocity is calculated from rotor revolutions as

$$\omega = 2\pi r,$$

where r = rotor speed in revolutions per second,

The sedimentation coefficient is calculated as

$$s = \frac{2.303 \Delta \log x}{t(2\pi r)^2} \quad \dots \dots \dots (3)$$

$\log x/t$ is obtained by plotting $\log x$ against t and calculating the slope of the straight line. This also serves as a check on the accuracy of the distance measurements, for marked deviations from linearity will be observed as a result of slight inaccuracies in measurement. $\Delta \log x/t$ is then used to calculate the sedimentation coefficient from equation 3.

Molecular weights were estimated from sedimentation velocity and diffusion data using the Svedberg relationship:

$$M = \frac{RTS}{D(1-\bar{v}\rho)}$$

where R = universal gas const.
 T = absolute temperature
 S = sedimentation coefficient
 D = diffusion coefficient
 \bar{v} = partial specific volume of solute
 ρ = density of medium

The value of R was taken as 8.314×10^7 erg/mole/degree. \bar{v} was assumed to have a value of 0.72, and the density of the medium was calculated from Svedberg and Pedersen's (76) tables to be 1.005g/ml. The diffusion coefficient D was calculated from quantitative gel diffusion measurements, results of which are presented in section 3.2.2 of the present chapter.

Details of the calculation are presented in Appendix 3.

2.5 Amino acid analysis

Analyses for amino acid content were performed on the crude extract, DEAE-cellulose fraction N_1 and two rechromatographed fraction N_1CM_2 and N_1S_2 . Fraction N_1CM_2 was obtained from a similar separation to that described in Fig. 9, Chapter II. Although the conditions were not identical (fraction used for analysis was eluted at pH 5.6 instead of 4.0) the relative positions and heights of the peaks were the same and there was no reason to doubt that N_1CM_2 was any different from that obtained by the procedure reported in Fig. 9.

Hydrolysis of samples. The hydrolysis procedure described in the manual for the Beckman 120B amino acid analyser was followed in the present study. Concentration of the sample after hydrolysis was achieved by rotary evaporation in contrast to the vacuum desiccator procedure described in the manual.

Protein samples (1.5mg) were weighed into pyrex tubes (15 x 160mm), and concentrated HCl (the exact normality of which had been determined by titration) and distilled water were added in proportion to give 1ml of 6N-HCl. The neck of each tube was constricted in an oxygen flame, following

which the contents were evacuated on a high-vacuum pump and frozen in acetone-dry ice. The tubes were sealed under vacuum and hydrolysed in an oil bath at $110^{\circ} \pm 1^{\circ}$ for 22, 46 and 70h. They were removed from the bath, chilled, opened and filtered if necessary. Hydrochloric acid was removed on a rotary evaporator (evaporation bath at 60° , condensation bath frozen in acetone-dry ice), care being taken to stop evaporation just before the sample was reduced to complete dryness. The process was repeated twice by redissolving the sample in distilled water and re-evaporating. The concentrated sample was dissolved in 5ml of the sodium citrate buffer (0.20N, pH 2.2), described in the manual. Samples were stored at -15° when not required for immediate analysis.

Hydrolysed protein samples (1ml) were analysed for amino acid content in a Beckman 120B amino acid analyser, using the system described by Moore, Spackman and Stein (77). A summary of the analysis conditions is presented in Table 8.

Table 8 – Summary of conditions for amino acid analysis

Condition	Acidics/Neutrals	Basics
Resin type	50A	15A
Dimensions of resin	0.9 x 54cm	0.6 x 11.5cm
Buffer flow rate	40ml/h	40ml/h
Ninhydrin flow rate	20ml/h	20ml/h
Back pressure buffer	55-60 p.s.i.	100 p.s.i.
Back pressure Ninhydrin	27 p.s.i.	28 p.s.i.
Sample size	0.3mg in 1ml	0.3mg in 1ml
Buffers and times	pH 3.21 Na-citrate (0.20N) 0 - 2h 20min pH 4.29 Na-citrate (0.20N) 2h 20min to 5h 30min	pH 5.28 Na-citrate (0.20N) 0-75min
Temperature	55°	55°

2.6 Neutral Sugar analysis

The phenol-sulphuric acid method, as described in section 2.6 of Chapter II, was used for determination of the content of neutral sugars in certain of the NRY fractions.

3 RESULTS

3.1 Zone Electrophoresis

3.1.1 Starch gel electrophoresis

The electrophoretogram in Fig. 24 illustrates the separation effected on starch gel. Approximately seven species are distinguishable in Extract C. The fractions prepared by rechromatography of N_1 show evidence of being electrophoretically homogeneous, although N_1CM_2 (prepared by CM-cellulose chromatography) appears to be more pure than N_1D_2 (prepared by DEAE-cellulose chromatography).

3.1.2 Electrophoresis on cellulose acetate membranes

Electrophoresis of N_1 on cellulose acetate membranes (CAM) over the pH range 4 to 10.75 revealed that the fraction migrated as a single major component throughout (Plate 3).

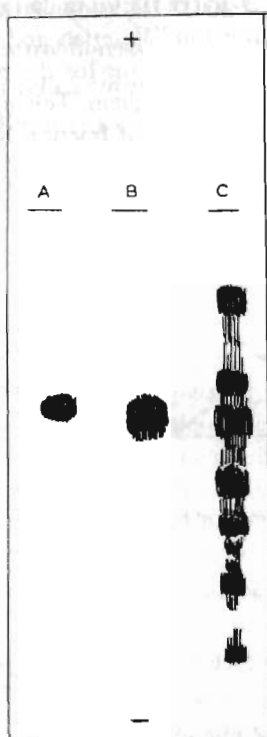


Fig. 24 — Starch gel electrophoresis of NRY fractions

Samples: A N_1CM_2 (Fig. 9), 10ml at 20mg/ml
 B N_1D_2 (Fig. 7), 10ml at 20mg/ml
 C Extract C (Fig. 1), 10ml at 20mg/ml

Conditions: Aluminium lactate buffer, pH 2.95
 (diluted 1 : 1 with distilled water)

Stain: Hypochlorite — KI

* Fraction N_1CM_2 was eluted from CM-cellulose at pH 5.0. From relative peak positions it would appear identical to N_1CM_2 eluted at pH 4.0 (Fig. 9).

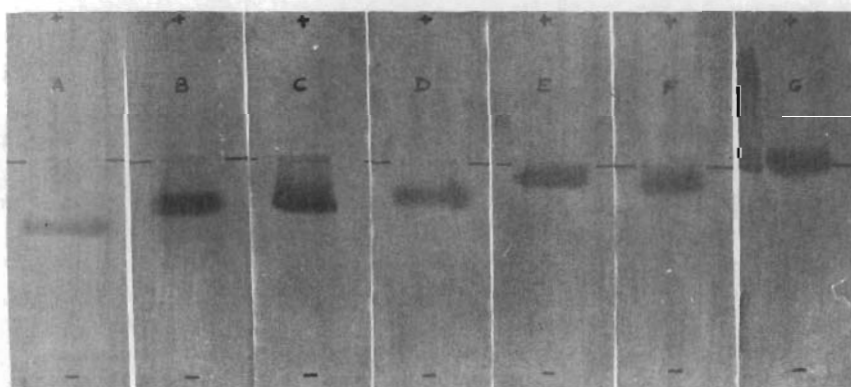


Plate 3 — Electrophoresis of N_1 on CAM in the pH range 3.85 to 10.75

Buffers: Ionic strength of all buffers: 0.1M

Membrane A: acetate, pH 3.85

Membrane B: acetate, pH 5.10

Membrane C: phosphate, pH 5.55

Membrane D: tris-HCl, pH 9.07

Membrane E: NaOH-glycine, pH 9.45

Membrane F: NaOH-glycine, pH 10.75

Conditions: 40V, 35-4mA (membrane C : 3.25mA), 2h

Sample: 40mg of N_1 per ml in electrophoresis buffer

Stain: Arnido black

The extent to which electroendosmosis had contributed to the migration of the protein component was estimated by studying the migration of polyethylene glycol (PEG) under identical conditions. The membrane was cleared after electrophoresis as described in "Materials and Methods", and the migration of the white PEG zone measured. When the migration patterns for the protein and PEG were compared (Fig. 25) a pH of zero migration was obtained for the protein. This pH, 8.2, is a tentative, empirical estimate of the iso-electric point of the major component of fraction N₁.

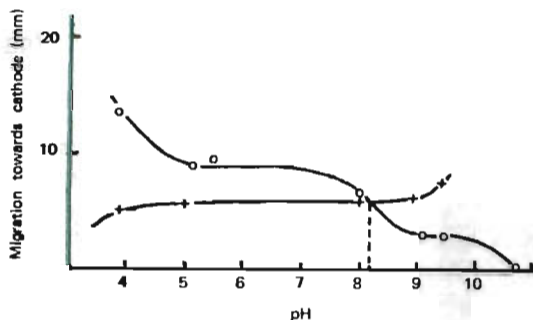


Fig. 25 — Empirical determination of iso-electric point of major component of N₁. Migration data obtained from strips illustrated in Plate 3.

—○—○— migration of protein zone
 —+—+— migration of polyethylene glycol under identical conditions.

A detailed examination of the zones in Plate 3 revealed the presence of a minor component, faintly visible in membranes E and F in the photograph and also in the original membrane A, though not visible in the photograph. Electrophoresis in a higher voltage gradient and staining with Nigrosin confirmed the presence of the minor component, while the periodic acid-Schiff's base stain showed that both components were glycoproteins (Plate 4).

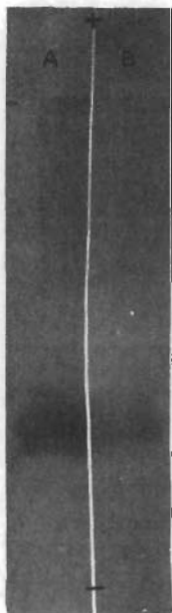


Plate 4 — Electrophoresis of N₁ on CAM in high voltage gradient, with comparative staining for proteins (A) and glycoproteins (B)

Buffer: 0.1M acetate, pH 3.85
 Conditions: 300V, 2mA, 1h
 Sample: 16mg N₁ per ml of electrophoresis buffer
 Stain: Membrane A: Nigrosin
 Membrane B: periodic acid-Schiff's base stain for glycoproteins

A comparison of fraction N₁ with two rechromatographed fraction N₁CM₂ and N₁S₂ under similar conditions of electrophoresis and staining indicated that the minor component of N₁ was not present in either of the other two fractions (Plate 5). Both N₁CM₂ and N₁S₂, therefore, were homogeneous electrophoretically under these conditions.

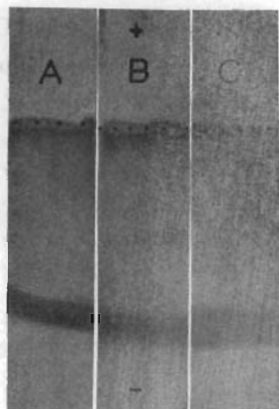


Plate 5 – Electrophoresis of N_1CM_2 (A), N_1 (B) and N_1S_2 (C) on CAM.
 Buffer: 0.1M acetate, pH 3.85
 Conditions: 240V, 8mA, 1.5h
 3 membranes run simultaneously
 Sample: 16mg per ml of electrophoresis buffer
 Stain: Nigrosin

3.1.3 Disc electrophoresis

A study of fraction N_1 by disc electrophoresis indicated (Plate 6) that the fraction migrates as a single component through a 5% polyacrylamide gel at pH 3.



Plate 6 – Disc electrophoresis of N_1 in 5% polyacrylamide gel
 Buffer: 0.05M formate, pH 3
 Conditions: 140V, 50mA, 1h
 Stain: Amido black

3.2 Immunodiffusion

3.2.1 Double diffusion

Double diffusion of N_1 over the concentration range 20mg/ml to .016mg/ml against N_1 -antiserum indicated that at least four groups of antigenic components are present in the fraction (Fig. 26). One component is evident in the high concentration range (i.e. is weakly antigenic), precipitating slightly closer to the antigen well than the antibody well. Also in the high concentration range are about four bands precipitating almost together and very close to the antibody well. Two further components, reacting approximately mid-way between the two wells, can be seen in the 2.5 and 5mg/ml range.

If the precipitin bands are designated numbers 1 - 4 as illustrated in Fig. 26, it can be seen that N_1 consists of four antigenic groups, one of high molecular weight (150-200,000 – antigen 1) which is weakly antigenic. Groups 2 and 3 have a slightly lower MW (80 - 140,000) and are strongly antigenic, while the fourth group is low-MW in nature and weakly antigenic. Antigens 2 and 3 were not visible in the higher concentration range, since the precipitate is solubilised in regions of antigen excess.

It was observed that in antisera from different rabbits, group 4 was not always present and groups 1, 2 and 3 were able to precipitate in the same concentration range.

An experiment was designed to test the fractions which resulted from a separation of N_1 on

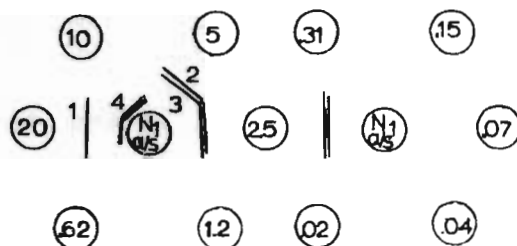


Fig. 26 — Double diffusion of N_1 (serial dilutions from 20 to .016mg/ml) against N_1 -antiserum. Wells (5mm diam.) cut 10mm apart. Figure accurately drawn from completed gel, as bands were too faint to be photographed.

Sephadex G-200, at different concentration levels against N_1 at two corresponding concentration levels for comparison of each fraction. The results of the experiment are illustrated in Plate 7. As can be seen from the plate, the wells are arranged in three adjacent groups of concentric wells on the upper section of the plate, with a fourth group below the horizontal line. Thus each column fraction diffuses at four concentrations against N_1 -antiserum and is simultaneously compared with N_1 at two corresponding concentrations.

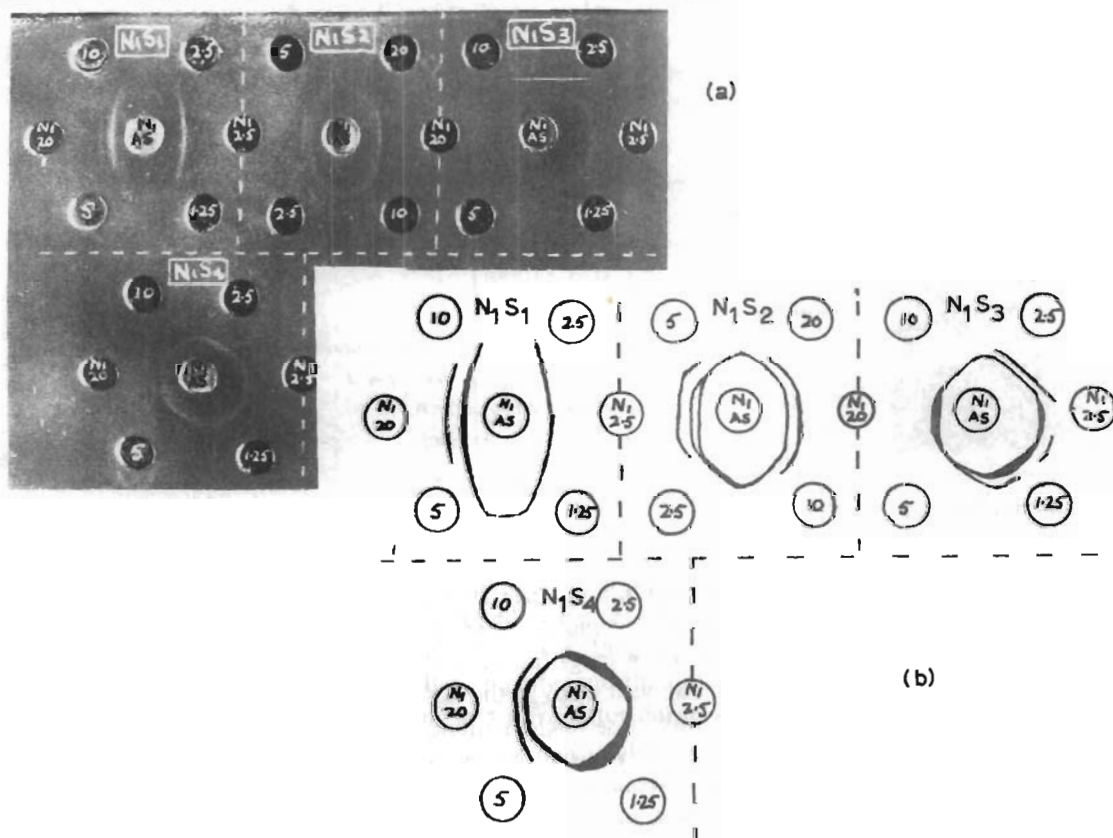


Plate 7 — Double diffusion of fractions N_1S_1 to N_1S_4 obtained from separation of N_1 on Sephadex G-200 (elution profile Fig. 12) against N_1 antiserum. N_1 is included as standard antigen at 20mg/ml and 2.5mg/ml. (a) Photograph, with (b) sketch included for identification of bands.

N_1 and N_1 antiserum are in 2nd and 5th horizontal rows of wells. Remaining wells contain fractions. Figures refer to concentration (mg dry weight per ml).

The results indicate that with the exception of fraction N_1S_1 , all fractions showed precipitin bands corresponding with all bands formed by fraction N_1 . Clear reactions of identity are visible in each case. This suggests that N_1S_2 , N_1S_3 and N_1S_4 share a common antigenic site with all of the antigens in N_1 . Thus, although N_1S_3 and N_1S_4 are low MW species they react strongly with precipitin band 1, which, from its position in the N_1 - N_1 antiserum system, is formed by a high MW component in N_1 . N_1S_3 in particular shows strong antigenic activity on a dry weight basis, for the precipitin bands formed from the 10 and 5mg/ml wells are in a typical state of antigen excess.

The high MW fraction N_1S_1 showed no antigenic activity whatsoever. The distortion and linking of the major precipitin band observed in the lower region of the N_1S_1 block was caused by interference from the nearest well of the N_1S_4 series which, because of its low molecular weight, diffused rapidly through the gel. The absence of antigenic activity in N_1S_1 was unusual, since precipitin band 1 was formed by a component, the molecular weight of which was of the same order as N_1S_1 .

A similar diffusion study of N_1CM_1 and N_1CM_2 , the two major fractions of N_1 separated by CM-cellulose chromatography, indicated (Plate 8) that N_1CM_1 is devoid of antigenic activity, while N_1CM_2 showed a general precipitin reaction similar to that of N_1S_2 .

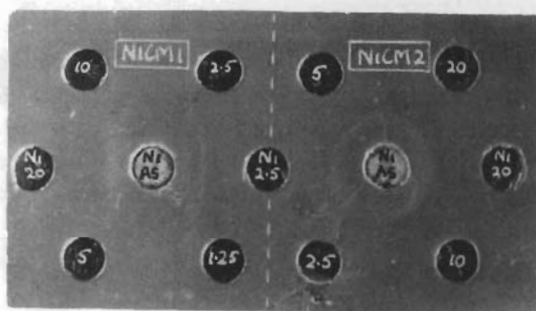


Plate 8 — Double diffusion of fractions N_1CM_1 and N_1CM_2 obtained from separation of N_1 on CM-cellulose (elution profile Fig. 9). Design and figures as for Plate 7.

3.2.2 Quantitative gel diffusion

The results of a study of fraction N_1 by quantitative gel diffusion are presented in Fig. 27, from which it is evident that four major antigenic groups are present in the preparation. The positions of the bands and relative antigenic potencies correspond with the four groups observed in Fig. 26.

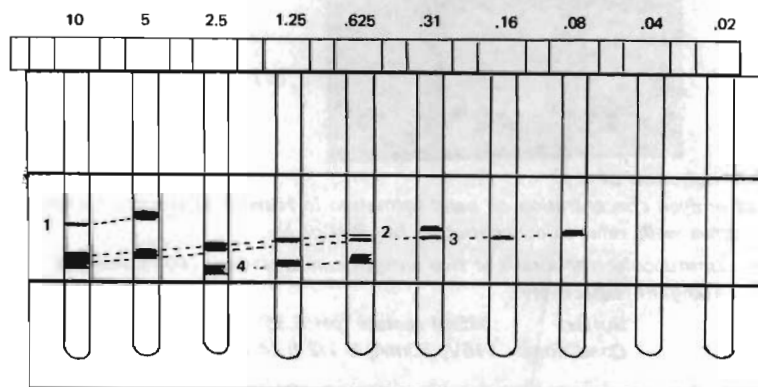


Fig. 27 — Quantitative gel diffusion of N_1 against N_1 antiserum, using serial dilutions of antigen. Antigen concentrations (in mg/ml) are indicated above figure. See section 2.3 for further details.

Diffusion coefficients were calculated for the antigens by measuring the band positions relative to the antigen and antibody menisci. The results are presented in Table 9, from which it appears that

antigen 1, the highest MW species has a diffusion coefficient of 5.2×10^{-7} , antigen 3 is a slightly smaller molecule with $D = 5.6 \times 10^{-7}$, while antigen 2 is considerably smaller with $D = 8.39 \times 10^{-7}$. The coefficient for antigen 4 was not calculated, but would be expected to be considerably higher than the other antigens.

*Table 9 – Calculation of diffusion coefficients from quantitative gel diffusion data
(See section 2.3 for details of calculation).*

Antigen	Xg (mm)	Xb (mm)	Diffusion coeff antigen($\text{cm}^2/\text{sec.}$)	Comments
1	7.687	7.195	5.2×10^{-7}	Approximate
2	8.595	6.365	8.39×10^{-7}	Accurate
3	7.806	7.074	5.6×10^{-7}	Accurate

3.3 Immunoelectrophoresis

As a preliminary to immunoelectrophoretic analysis, the optimal concentration range of antigen was established. Since the sample well is small compared with the normal double diffusion wells, antigen concentration for formation of precipitin bands may be expected to be higher than the corresponding concentrations for double diffusion. The results of such a study, in the 60 to 10mg/ml range, are presented in Plate 9 (a), from which it is evident that there are at least two optimal concentrations – 40 and 10mg/ml.

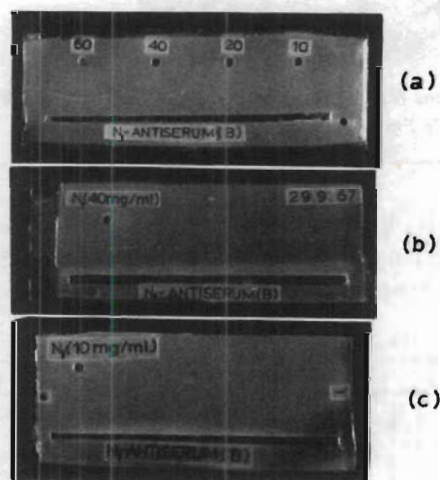


Plate 9 – Immunoelectrophoresis of N₁.

(a) Effect of antigen concentration on band formation in absence of electrophoresis.

Figures above wells refer to concentration (mg/ml) of N₁.

(b) and (c) Immunoelectrophoresis at two antigen concentrations (40 mg/ml and 10mg/ml respectively).

Buffer: .025M acetate, pH 5.35

Conditions: 115V, 30mA, 3 1/2 h (4 slides run simultaneously)

From the study of optimal concentrations, it was concluded that the two bands at 10mg/ml are formed by antigens 2 and 3, while that at 40mg/ml corresponds to antigen 1. Further it is evident that the single bands formed by antigens 1 and 3 are indistinguishable under these conditions since they occur at different antigen concentrations and the conventional tests of identity or non-identity cannot be applied. The difficulty is exemplified by reference to Plate 9 (b) and (c) in which the electrophoretic

phase has been introduced. The two slides (b) and (c) would appear to be identical, yet from the foregoing it is obvious that the two precipitin bands are formed by different antigen-antibody systems.

The studies illustrated in Plate 9 highlight a definite limitation in the immunoelectrophoretic analysis of the NRY toxic system. Because two of the antigens have optima at widely different concentrations, it is not possible to observe them simultaneously. Although the electrophoretic migration of the antigen was not extensive in the slides illustrated, nothing was to be gained by allowing electrophoresis to proceed for longer than the 3½h period reported, since it had already been shown that electrophoresis was incapable of distinguishing between the components of the N₁ fraction.

3.4 Ultracentrifugation

3.4.1 Density gradient ultracentrifugation

Fraction N₁ migrates as a single zone through a sucrose gradient, with a trace of contamination in the high-molecular weight region (Fig. 28 (a) and (b)). After rechromatography on CM-cellulose, however the major component N₁CM₂ shows a higher proportion of the high-MW component (Fig. 28 (c)). N₁CM₃, separated from N₁CM₂ on CM-cellulose, shows an identical profile on the ultracentrifuge, with peaks 1 and 2 occurring in identical relative positions in both samples (Fig. 28 (d)).

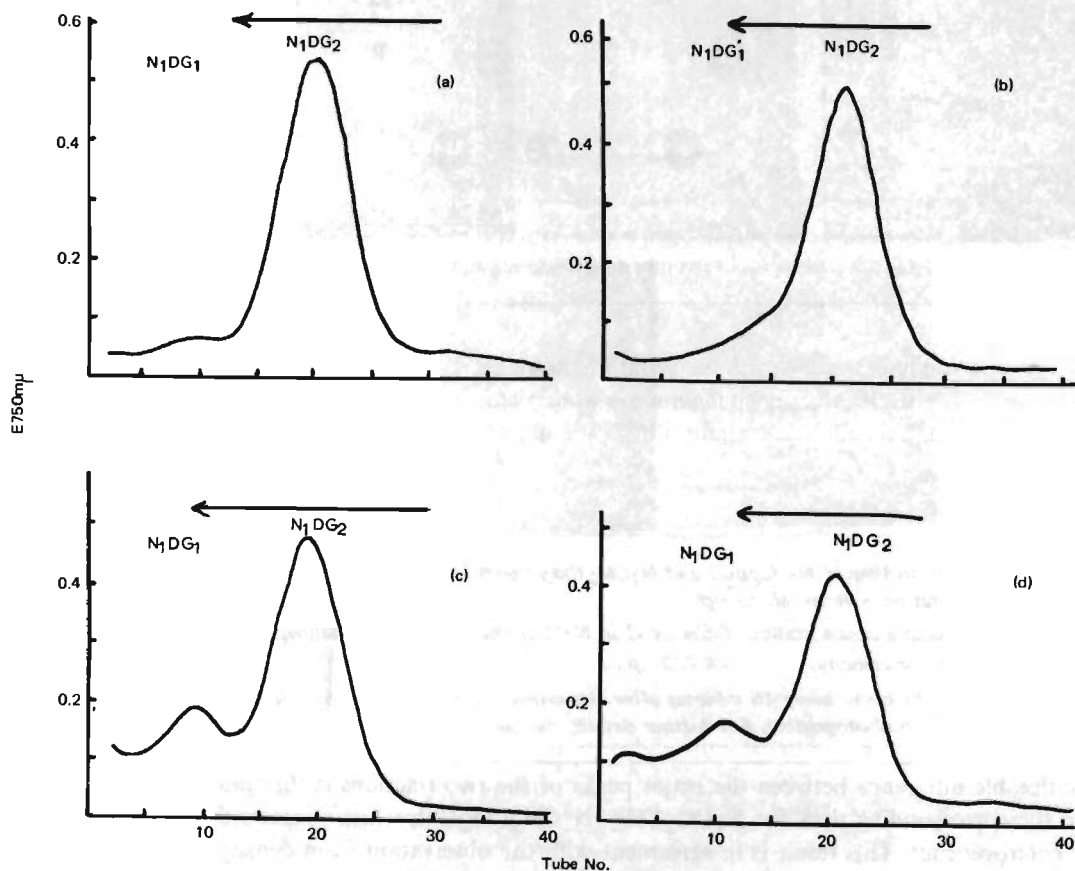


Fig. 28 — Sucrose density gradient ultracentrifugation of NRY fractions:

- | | | |
|-------------|--------------------------------|--------------------------------------|
| (a) and (b) | N ₁ | } prepared on CM-cellulose at pH 5.6 |
| (c) | N ₁ CM ₂ | |
| (d) | N ₁ CM ₃ | |

For further details, see section 2.4.1.

Since the ultracentrifuge was run at 25,000 rpm for 48h, the protein zones were in all probability still moving in the gravitational field. They would thus be subject to separation according to velocity, although it is expected that the rate of sedimentation would be greatly reduced by the increasing viscosity of the sucrose. Protein fraction N_1 under these conditions is almost homogeneous, with a trace of a faster-sedimenting component. Since the CM-cellulose fraction N_1CM_2 and N_1CM_3 have a higher percentage of a similar fast-sedimenting component, these data suggest that chromatography on CM-cellulose, or conditions imposed subsequent to that, predisposes the protein to formation of the contaminant. Since fraction N_1CM_2 and N_1CM_3 were concentrated by Treatment A (as described in section 2.7, Chapter II), these results are in agreement with the observations concerning the conditions which favour formation of the high MW component reported in Chapter III.

3.4.2 Analytical ultracentrifugation

Examination of N_1 in the analytical ultracentrifuge revealed (Plate 10) a profile similar to that observed in the preparative model, with one major and one minor component, with the latter sedimenting more rapidly than the former. Moreover, $N_1CM_2CM_2$ (see Fig. 10) which had been chromatographed twice on CM-cellulose, showed about the same porportion of the rapidly-sedimenting component as N_1 .

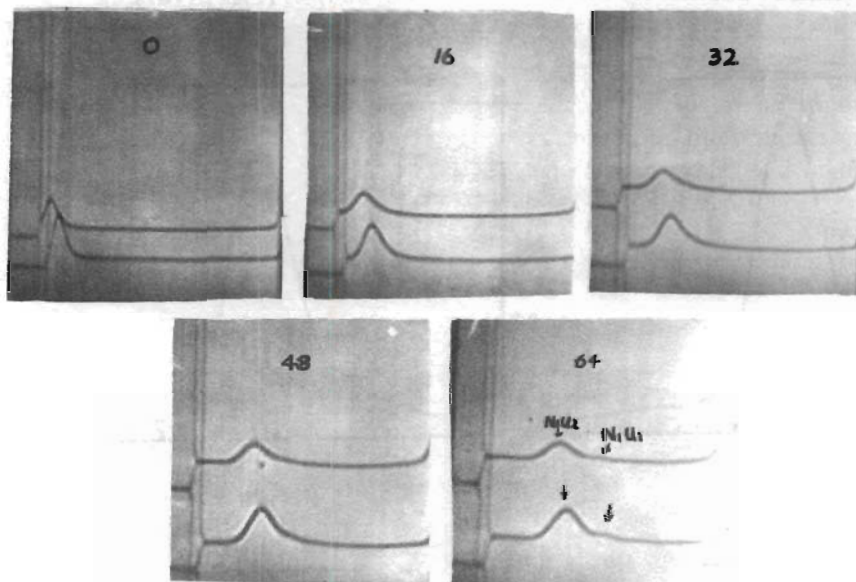


Plate 10 — Schlieren profiles of N_1 (upper) and $N_1CM_2CM_2$ (lower), in Spinco Model E ultracentrifuge. Sedimentation is from left to right.

Sample concentration: 0.5% (w/v) in M/15 phosphate buffered saline, pH 7.1

Rotor velocity: 44,770 r.p.m.

Photographs taken every 16 minutes after attainment of maximum rotor velocity (times indicated on photographs). For further details, see section 2.4.2.

A noticeable difference between the major peaks of the two fractions is that peak N_1U_2 is less sharp than the corresponding peak for $N_1CM_2CM_2$ ($N_1CM_2CM_2U_2$), which suggests that the former is slightly heterogeneous. This result is in agreement with the observation from density gradient centrifugation, that N_1CM_3 , one of the fractions removed by CM-cellulose chromatography, has approximately the same sedimentation velocity as N_1CM_2 . It is probable that the two are insufficiently different to be distinguishable in the analytical ultracentrifuge, but that the slight heterogeneity manifests itself in a broadening of peak N_1U_2 during sedimentation.

Calculation of sedimentation coefficients and molecular weights. The data pertaining to the calculation of sedimentation coefficients (details of which appear in Appendix 3) are presented in Table 10.

Table 10 — Determination of sedimentation coefficients of NRY fractions.

Fraction	log x	t (sec)	$S_{20,w}$ ($\text{sec}^{-1} \times 10^{-13}$)
N_1U_2	.0239	3840	6.575
$N_1CM_2CM_2U_2$.0240	3840	6.548
N_1U_1	.0348	3840	9.495
$N_1CM_2CM_2U_1$.0348	3840	9.495

The data in Table 10 indicate that the two components of the samples studied have the same sedimentation coefficient in either sample. Thus both N_1U_2 and $N_1CM_2CM_2U_2$, the major component in each case, have coefficients of 6.5S, while the minor components (N_1U_1 and $N_1CM_2CM_2U_1$) both have sedimentation coefficients of 9.5S.

Molecular weights were calculated using the diffusion coefficients obtained from quantitative gel diffusion measurements. From a comparison of immunological and ultracentrifugal data, the major antigen (3) was allocated a sedimentation coefficient of 6.5S - that of the major component in the ultracentrifuge. Similarly the weakest antigen (1) was assumed to have a coefficient of 9.5S. Antigen 2, weaker than 3 and of a similar molecular weight, was assumed to have an S-value of 6.5, since this component was not distinguishable from antigen 3 in the ultracentrifuge.

The three parameters applicable to each of the three components in the NRY system, are presented in Table 11. Details regarding the calculation of molecular weight are presented in Appendix 3.

Table 11 — Molecular parameters for three components of N_1 .

Antigen	Sedimentation coefficient $\text{sec}^{-1} \times 10^{-13}$	Diffusion coefficient $\text{cm}^2/\text{sec} \times 10^{-7}$	Molecular weight
1	9.5	5.2	169,000
2	6.5	8.39	75,670
3	6.5	5.60	113,300

Thus the major component of fraction N_1 has an estimated molecular weight of 113,300. The molecular weight of the component which appears to be formed from the major solute, is 169,000 while the third component has an estimated molecular weight of 75,670.

3.5 Amino acid and sugar analysis

The results of amino acid analyses on the crude protein extract (Extract B), DEAE-cellulose fraction N_1 and two rechromatographed fractions N_1CM_2 and N_1S_2 are presented in Table 12. The data are expressed as moles % to facilitate comparison. Extract B and N_1 were calculated as means of three determinations (hydrolysis times 22, 46 and 70h), while data for the two rechromatographed peaks were obtained from single analyses at 22h. Histidine, threonine, serine and tyrosine were found to be subject to gradual destruction during hydrolysis, in agreement with the observations of Hirs, Stein and Moore (78). Consequently, estimates of original concentrations were obtained graphically (see Appendix 4). All other concentrations were calculated as means from the three hydrolysis times.

Table 12 — Amino acid analyses of NRY fractions

AMINO ACID	AMINO ACID COMPOSITION				
	Moles amino acid as percent of total moles				Amino Acid residue wt. g/100g protein
	Extract B	N ₁	N-CM ₂	NS ₂	N ₁
Lys	4.19	3.08	3.01	3.21	2.61
His	2.16	0.66	0.71	0.76	1.12
NH ₃	17.00	15.83	21.81	11.53	
Arg	3.10	2.70	2.84	3.01	2.78
Asp	12.02	13.83	12.59	13.82	10.56
Threo	5.61	6.70	6.21	7.07	4.79
Ser	8.24	8.00	7.98	9.20	5.43
Glut	8.57	6.42	6.21	6.51	5.49
Pro	3.98	3.99	3.72	4.18	2.58
Gly	5.15	7.06	6.74	7.31	2.67
Ala	4.98	5.45	5.32	5.54	2.57
CyS	1.91	0	0	0	
Val	5.94	7.30	6.56	7.63	4.80
Meth	0.47	0	Trace	Trace	
Ilen	4.25	4.33	3.55	4.50	3.25
Len	6.14	7.92	6.56	8.16	5.64
Tyr	2.02	1.49	1.42	1.77	1.77
Phe	4.21	5.22	4.79	5.79	5.10
Total					61.16

A striking feature of the analyses (Table 12), is the complete absence (in the purified preparations) of cysteine, while methionine is present in trace amounts, if at all. Low concentrations of these amino acids in the crude extract were removed during the preparative DEAE-cellulose stage. The crude extract also has higher proportions of histidine and glutamic acid, and less glycine and valine.

The two rechromatographed fractions N₁CM₂ and N₁S₂ have proportions of amino acids almost identical to N₁, the fraction from which they were prepared. This would suggest that anything removed from N₁ in the course of preparing N-CM₂ and NS₂, had the same relative proportions of amino acids. Alternatively, the impurities removed may have contained no amino acids, or a very low proportion.

An estimate of the total recovery of amino acids in terms of the weight of protein hydrolysed, indicates that 61.2% by weight of the protein can be accounted for in terms of amino acid residues.

Analysis of three NRY fractions for neutral sugars indicated (Table 13) that N₁ has a content of some 47.5% by weight of neutral sugars, while the two rechromatographed samples (N₁CM₂ and N₁S₂) have a considerably lower content (9.8% and 12.5% respectively).

Table 13 — Content of neutral sugars in NRY fractions.

Fraction	Mannose equivalents g/100g fraction
N ₁	47.5
N ₁ CM ₂	9.8
N ₁ S ₂	12.5

From the high sugar content of N₁ it can be seen that sugar constitutes one of the major components removed during rechromatography of N₁. The similarity of the amino acid data mentioned previously is thus probably due to the fact that few amino acids are removed from N₁ during rechromatography.

When the sugar content of N₁ is taken into account, the total recovery of amino acids + sugars is 108.6%. While the figure would appear to be too high, the excess is not beyond explanation, for the two analyses were performed on different samples prepared from different batches of beans. It is thus likely that the carbohydrate component of N₁ is almost entirely composed of residues detectable by the Dubois analysis for neutral sugars.

4 DISCUSSION

The application of several criteria of homogeneity to the NRY toxic fractions has indicated that caution is required in the interpretation of data. No single technique provides an unequivocal index of purity, but when the properties of the fractions under all conditions are taken into account, the combined data enable certain conclusions to be drawn.

The electrophoretic analysis of fraction N₁ indicated that two components could be detected. The major component migrated as a single zone over a wide pH range, while the minor component was barely detectable. Both components showed a positive glycoprotein stain. Only one zone could be detected when N₁ was subjected to disc electrophoresis through a polyacrylamide column.

Ion-exchange chromatography of N₁ on CM-cellulose revealed five components. One of these was a non-cationic fraction with a high proportion of carbohydrate. Rechromatography of the major peak (N₁CM₂) resulted in the appearance of a further non-cationic carbohydrate peak, while the Schlieren profile of the rechromatographed major peak (N₁CM₂CM₂) in the ultracentrifuge showed that the carbohydrate peak was still present. Furthermore there was little difference between the Schlieren profiles of N₁CM₂CM₂ and N₁, for both consisted of a single major peak and a smaller, faster-sedimenting peak which corresponded to the non-cationic carbohydrate.

An investigation of the antigenic properties of the NRY fractions revealed a further aspect of the problem. Thus while fraction N₁ consisted of a minimum of four antigen-antibody groups, the fractions separated from N₁ (N₁S₂, N₁S₃, N₁S₄, N₁CM₂) each reacted with all four groups. The single exception was the fast-sedimenting, high carbohydrate component (N₁S₁ and N₁CM₁) which was non-antigenic. Thus even a precipitin band formed by a high MW antigen in N₁, showed a reaction of identity with a low MW fraction N₁S₄. Degeneracy of such an order in immunological studies suggests that the smaller MW species are fragmentation products of the larger and hence bear the same antigenic sites. This is a possibility allowed for by Kabat (79) in a discussion of cases in which several precipitin bands are formed by an apparently homogeneous protein. The results bear out earlier studies concerning the

origin of the various minor components obtained after rechromatography of N_1 .

The observation that fractions N_1CM_1 and N_1S_1 were devoid of antigenic activity was unexpected, since precipitin band 1 in the quantitative gel diffusion (Fig. 27), had been allocated to this fraction. The position of precipitin band 1 in Fig. 27 provides a description of the antigen as a high MW compound, present in low concentration. The description fits perfectly the components N_1CM_1 , N_1S_1 and N_1G_1 , and there are no other components which might be mistaken for it. Since there are no other high MW fractions, it would seem likely that this fraction may be antigenic under certain conditions. Since the antigenic site of band 1 is identical with the other bands (Plate 7), it is possible that some preparations of the high carbohydrate fraction contain a few antigenic groups, whereas in others the groups are absent.

The evidence indicating a fragmentation of the protein molecule naturally raises the question of which antigen can be considered the major or original component. On the basis of antigenic potency in the N_1 system, it would appear the antigen 3 is most likely to be the component from which the others are derived. Furthermore from a comparison of diffusion coefficients, the allocation of precipitin band 3 to the major component of N_1 (N_1S_2 , N_1CM_2 or N_1G_2) would appear to be valid, and would further suggest that antigen 3 is of primary importance;

The fact that the four antigenic components of N_1 showed different optimal concentrations, had implications in the immunoelectrophoretic analysis of N_1 . Since for immunoelectrophoresis, one antigen concentration must of necessity be chosen, it was not possible to achieve optimal conditions for both groups of antigens simultaneously. Since in addition each run is a separate entity, the conventional immunological tests for identity or non-identity could not be applied. Thus, in a system in which all components migrate together electrophoretically, and the antigens form precipitin bands in about the same position relative to the antigen and antibody wells, but at widely different concentrations, immunoelectrophoresis is powerless to differentiate between them. The only possible means of differentiating would be to conduct separate runs at each optimal concentration, then to add back one of the purified antigens to the mixture and repeat the two runs. One of the precipitin bands, corresponding to the added antigen, should move toward the antiserum well, or both antigens might form a band on the same slide as a result of the altered proportions. However, this presupposes a prior differentiation between the two antigens, which would depend on some other criterion. The author considers this to be a major limitation of immunoelectrophoresis, and one which has not been encountered in the literature covering the subject. Publications frequently report purity using immunoelectrophoresis at only one concentration. Such a situation could conceal a heterogeneity similar to that reported in the present chapter.

The quantitative gel diffusion technique provided a useful means of estimating diffusion coefficients for the antigenic components of fraction N_1 . Polson and Deeks (80) assume an error of 5% in the method, which enables the coefficient to be estimated when only small quantities are available and before homogeneity has been established. Molecular weights can be estimated from sedimentation and diffusion coefficients, thus providing a preferable alternative to the Scheraga-Mandelkern (81) treatment, in which molecular weight is calculated from sedimentation and viscosity data. Although viscosity determinations are easily performed, marked deviations would result from the presence of impurities, particularly those of high MW, since viscosity is a weight-average parameter. Viscosity calculations with the NRY system would thus be of little value, because of the presence of the high MW component in unpredictable proportions.

The instability of the toxic protein studied in this investigation shows up a limitation in the immunological criterion as a means of assessing the purity of a preparation. Of necessity the antigen is required to be stable in solution for several days in order to give a single precipitin band. Thus homogeneity in the context of the immunodiffusion test carries the additional condition of stability over relatively long periods.

The foregoing results all led the author to the tentative conclusion that heterogeneity observed in

N_1S_2 and N_1CM_2 might have been due to fragmentation of the molecule, and that these two fractions are homogeneous but unstable compounds. Indeed, N_1 itself was very nearly homogeneous, apart from the component observed in low concentration on electrophoresis, and the non-cationic carbohydrate component. It is difficult to decide which of the fractions most nearly corresponds to the natural form of the toxin – whether the 47% of carbohydrate found in the N_1 fraction can be classified as being a part of the original molecule, or whether it is simply extracted in association with the toxin. Since the carbohydrate peak N_1CM_1 was always obtained from N_1 , whereas in certain cases the similar component was present in only trace amounts when N_1CM_2 or N_1C_2 were rechromatographed, it would appear that the 9-12% of carbohydrate in these fractions is more firmly bound than the carbohydrate in N_1 . The former would thus appear to be a slightly more stable species, and on this basis the rechromatographed peaks (N_1CM_2 , N_1S_2 , N_1G_2) are accepted as being reproducible preparations of the NRY toxin. Undoubtedly in its natural form the toxin would be associated with carbohydrate, and it is evident that further treatment of the above fraction would remove further carbohydrate residues. The isolated fraction thus represent a compromise, in which a certain percentage of associated carbohydrate was of necessity removed during isolation, while a small proportion of residual carbohydrate was retained in a loose association.

The molecular parameters calculated for some of the components are regarded by the author as being provisional. No high degree of accuracy is claimed for either the diffusion coefficient or the sedimentation coefficient. The former is essentially an approximate estimate, since it is obtained by quantitative gel diffusion as opposed to the more exact physical procedures. The sedimentation coefficient was calculated at a single concentration, whereas determination at several concentrations, with extrapolation, to infinite dilution, would give a more accurate figure. The author is also aware that the assumption of an arbitrary value for \bar{V} , the partial specific volume, in the molecular weight calculation, is not recommended for accuracy. Although \bar{V} values for proteins do not differ widely, small differences are multiplied by the position of \bar{V} in the Svedberg equation, for the buoyancy factor $(1 - \bar{V}\rho)$ differs significantly with small variations in \bar{V} .

The assumptions were made, nonetheless, with a view to obtaining a rough estimate of the relative molecular weights of the species in the N_1 protein system. Since the data were only used for comparison within the system, and interpolation between the calculated values (Chapter IV), there was little danger of drawing false conclusions from inaccurate values for any of the parameters. Had more sophisticated facilities been available, this aspect could doubtless have been given more rigorous attention. The author is of the opinion that the present treatment has served a useful purpose within the context of the objectives of this study, and within the limits of the instrumentation available in this laboratory.

The investigations reported in the present thesis have thus indicated that a toxic protein can be isolated from NRY beans in a pure but unstable form. As a final phase in the study of toxicity in NRY beans, the author undertook to examine the role which the toxin might play in the general problem of growth depression, as exhibited by rats fed a raw bean diet. A study of this nature imposed new requirements for large-scale fractionation of legume proteins and for assay of the various factors which are associated with growth depression. A report of the method by which the problem was approached and the significance of the results to the problem of growth depression, is contained in the following chapter.

CHAPTER V

A STUDY OF FACTORS RELATED TO GROWTH DEPRESSION BY THE FEEDING OF ISOLATED NATAL ROUND YELLOW BEAN FRACTIONS

1 INTRODUCTION

The development of research into the nature of growth depression, and the status of current thinking on the subject, have been outlined in the introductory chapter of the present thesis. Brief mention of the most useful recent contributions will however be made at this stage, by way of summarising the present situation.

In recent years it has become increasingly evident that growth depression manifested by rats fed on raw legumes is a complex phenomenon. Initial attempts to attribute the effect to an inactivation of intestinal trypsin or a deficiency of amino acids were found to be inadequate, although the most recent explanation involves both a trypsin inhibitor and an amino acid deficiency as causative factors in growth depression.

The role of the soybean trypsin inhibitor (SBTI) in particular has been clearly defined. A partially purified inhibitor depressed growth when fed with a complete amino acid mixture, under conditions in which food intake and protein availability were not limiting factors (17). In addition the amino acid + SBTI diet caused marked hypertrophy of the pancreas, while the intestinal contents were subject to an increase in the level of trichloroacetic acid-precipitable nitrogen and a massive increase in essential amino acids (17).

Evidence from another quarter is consistent with the concept that amino acid wastage causes growth depression, for penicillin was found to prevent growth depression by raw soybean meal and increase the level of cystine and proteolytic enzymes in the faeces (18). Furthermore, penicillin's beneficial effect could be counteracted by preventing coprophagy (19), suggesting that penicillin, by eliminating bacterial degradation of essential amino acids in the colon, allowed the amino acids to be re-cycled by coprophagy and re-utilized by the animal. Thus it seemed that a hypertrophic pancreas, stimulated to grow and secrete excessive quantities of proteolytic enzymes by the SBTI, was causing growth depression by means of an induced amino acid deficiency.

Although a purified SBTI preparation was not used in the investigations reported above, an earlier report (16) had indicated that a chromatographically pure SBTI stimulated excessive pancreatic secretion. The role of the trypsin inhibitor as a growth depressor thus seemed indisputable, although the direct implication of its trypsin-inhibiting activity remains an open question.

Yet the effect of the SBTI does not account for the whole of growth depression. Rackis (20) calculated that only 30 to 50% of growth depression could be attributed to SBTI. Two isolated haemagglutinins have been reported to depress growth (22, 23), while unpublished observations by the present author suggested that although NRY meal contained little trypsin inhibitor activity, it caused pronounced growth depression and pancreatic hypertrophy. The indications are that both growth depression and pancreatic hypertrophy might prove to be complex effects caused by more than one factor. In addition the role which intraperitoneal toxic activity plays in growth depression remains to be established, for although a toxic factor has been shown to depress growth (23) the factor also showed haemagglutinating activity, and a non-haemagglutinating toxin has yet to be tested by oral administration.

Oral administration of the NRY toxic factor was thus of potential value in illustrating whether toxicity and growth depression are caused by a single factor or whether they are unrelated effects. Since bulk isolation of the pure NRY toxin was not feasible, the problem was approached by feeding limited quantities of fraction N₁ to suckling rats. Since it is to be expected that the suckling animal would be highly susceptible to an oral toxin, this experiment provided a sensitive index of whether the intra-peritoneal toxin exerted physiological activity when administered orally,

The implication of trypsin inhibitors, haemagglutinins and toxins in the general problem of growth depression required a more radical departure from the isolation procedures thus far reported. Such a study imposes a requirement for large quantities of relatively pure fractions which differ significantly in the above activities and are suitable for feeding to rats over extended periods. Satisfactory biochemical separations, and feeding trials, are not however usually found together. The reason is that yields from the former are incompatible with the requirements of the latter. Thus the demonstrably pure factor seldom reaches the feeding trial, and the well-planned feeding experiment is usually compromised by inclusion of a partially purified active extract. While fractional precipitation is the principle most easily adapted to large-scale separations, the resolving properties are usually limited by solubility differences of the components in a mixture. Little success was experienced by the present author when ammonium sulphate was used for the fractional precipitation of NRY proteins (49), although in certain cases a more satisfactory purification has been reported (27).

The use of a novel agent for fractional precipitation was reported by Polson, Potgieter, Largier, Mears and Joubert (82), who precipitated serum proteins with the synthetic polymer polyethylene glycol (PEG). A striking feature of the PEG procedure was its selectivity, for a single precipitation of gamma-globulin resulted in a preparation which was homogeneous in the analytical ultracentrifuge. By contrast, ammonium sulphate-precipitated gamma globulin was considerably less pure. Polyethylene glycol has also been used to purify agarose (83) and gelatin (43) although the latter report appeared long after the present investigations had begun.

On the basis of the successful results reported with PEG as a fractional precipitant, the author decided to investigate its possible application to the NRY protein system. Since there was no existing literature regarding the use of PEG in plant protein extracts, all conditions had to be developed *de novo*. Small-scale exploratory studies were thus used initially to establish suitable conditions for precipitation. Because of the number of variables associated with a study such as this, an exhaustive examination of each to determine optimal conditions was not possible. Certain choices were quite arbitrary. The only justification for their use was that they appeared to give a satisfactory result.

The present chapter describes the method by which polyethylene glycol was used to precipitate proteins from NRY meal. Results of exploratory studies, and certain difficulties which were encountered are also discussed. PEG-precipitation was then applied to the bulk preparation of fractions from NRY bean meal, and the isolated fractions were fed to rats to determine their growth-depressing activity. Assays of the fractions for *in vitro* and *in vivo* activities (haemagglutinating, trypsin-inhibiting and toxic activities) provided a means of assessing their possible implication in growth depression. In the final trial, assays for pancreatic hypertrophy were included with a view to establishing whether hypertrophy and growth depression are necessarily two aspects of the same effect, or whether either could occur in the absence of the other.

The results of these investigations throw light on the factors associated with growth depression in NRY beans and provide a basis for assessing current assumptions concerning growth depression in general.

2. ASSAY METHODS

2.1 Protein Concentration of Solutions

The microbiuret method described by Bailey (84) was used to estimate the protein concentration of protein solutions reported in this chapter. The Folin-Ciocalteu procedure, used elsewhere in this investigation for the same purpose, was unsuitable because polyethylene glycol precipitates the Folin-Ciocalteu colour reagent.

The microbiuret procedure was as follows: Benedict's reagent (sodium citrate, 173g; sodium carbonate, 100g; dissolve in distilled water with warming; copper sulphate, 17.3g in 100ml of distilled water; distilled water to 1L) (0.2ml) was added to a test tube containing 0.1ml of sample protein (0.1 to 2mg of protein) and 4ml of 3% sodium hydroxide. Protein concentration was estimated from the

extinction of the solution at 330m μ (Zeiss PMQ II spectrophotometer) at least 15 min after addition of Benedict's reagent.

2.2 Protein content of rations

The protein content of rations was estimated by semi-micro Kjeldahl digestion with micro-distillation using the Markham steam distillation unit.

Solid samples (80mg) were weighed on rice paper and introduced with stainless steel forceps into semi-micro Kjeldahl digestion flasks. Concentrated A.R. sulphuric acid (5ml), two catalyst tablets (1g of Na₂SO₄ and 0.1g of Hg per tablet) and a few pumice boiling stones were added to the flask, which was then boiled on an electrical heater. Boiling was continued for 90 min after the solution became clear; thereafter the flask was allowed to cool. The flask contents were dissolved in a small volume of distilled water and transferred quantitatively into a 100ml volumetric flask. A film of grease applied to the lip of the digestion flask aided quantitative transfer, and several rinses with distilled water ensured complete removal of the digest. Distilled water was added to the volumetric flask to 100ml and the contents were well mixed.

Aliquots (10ml) of the digest were removed for steam distillation. Nitrogen was released as ammonia by the addition of 16ml of sodium hydroxide-sodium thiosulphate solution (259g of NaOH + 25g of Na₂S₂O₃ · 5H₂O; dissolve and add distilled water to 500ml) and passage of steam through the alkaline solution for 5 min. Released ammonia was trapped in saturated boric acid solution (20ml) containing 1 part in 50 of mixed indicator (5 parts 0.2% bromocresyl green in ethanol and 1 part 0.2% methyl red in ethanol).

Ammonia in the distillate was estimated by titration to the original indicator colour with .01N hydrochloric acid (standardised against .02N borax standard using methyl red indicator). A reagent blank and nitrogen standard (3.7731g of (NH₄)₂SO₄ in 100ml; contains 8mgN/ml) were included with each batch of analyses

2.3 Haemagglutinating activity

Haemagglutinating activity was estimated by the procedure outlined in Chapter II, section 2.8.2.

2.4 Intraperitoneal toxicity

As described in Chapter II, section 2.8.1, intraperitoneal toxicity was tested in mice (bodyweight 25 \pm 2g) by a single intraperitoneal injection of the sample suspended in sterile saline (0.9% NaCl). Graded doses were administered to facilitate comparison of fractions with respect to potency.

2.5 Trypsin inhibitor activity

N-benzoyl-L-arginine ethyl ester (BAEE) was used for the assay of trypsin activity and trypsin inhibition. The widely-used assay, based on a study by Schwert and Takenaka (85) and adapted from a method described in a manufacturer's catalogue (Seravac Laboratories, Cape Town), was conducted according to the following procedure.

A stock solution of BAEE (Seravac Laboratories, Cape Town), (8.6mg in .05M-borate buffer, pH 8.0, to 100ml), was prepared each day. Trypsin (twice-crystallized, salt free; Seravac Laboratories, Cape town), (40mg/ml in .001N-HCl + .02M-CaCl₂), was prepared not more than a few hours before use. The reaction was carried out in 1cm quartz cuvettes in the cell compartment of a Zeiss DMR 21 recording spectrophotometer. The cell compartment was maintained at 25 $^{\circ}$ by a flow of water through the hollow jacket from a thermostatically controlled water bath. Components in the reaction and blank cuvettes were as follows:

Blank cuvette: 3ml of BAEE + 0.2ml (.001N-HCl + .02M-CaCl₂)

Trypsin calibration cuvette 3ml of BAEE + 0.02 - 0.1ml of trypsin + 0.18 - 0.1ml (.001N-HCl + .02M-CaCl₂)

Trypsin inhibitor cuvette 3ml of BAEE + 0.2ml of trypsin: test solution (1:1)

Each trypsin solution was calibrated by incubating five aliquots of the enzyme (between 0.02 and

0.1ml) with BAEE and appropriate volumes of .001N-HCl to maintain a total volume of 3.2ml. For each aliquot the change of extinction with time (ΔE_{min}) was recorded directly by the spectrophotometer, and a calibration curve was drawn from the data, relating the slope of the line to weight of enzyme (μg).

For estimation of trypsin-inhibitor activity, 1ml of each test protein (0.2mg/ml in .001N-HCl + .02M- CaCl_2) was incubated with an equal volume of trypsin solution. The mixture was swirled and incubated at 25° for 10 min, after which 0.2ml aliquots were removed for assay of residual trypsin activity. From the slope of the line obtained with each test fraction, and the trypsin standard curve, a weight of residual (or active) trypsin was determined. This gave a weight of inhibited trypsin (by difference) and as estimate of specific activity ($\frac{\mu\text{g inhibited}}{\mu\text{g inhibitor}}$) for each test protein.

2.6 Growth studies

Diets containing test fractions were fed to weanling rats (40 - 45g) of the Wistar strain, housed in groups of three under conditions of constant temperature (22°) and humidity (50%), and with controlled lighting. Rations and water were supplied *ad libitum*; rats were weighed individually and food pots replenished daily. Group weights were matched on the first day so that variation within one experiment was approximately 1g. Both males and females were used in the first feeding trial, while the second trial consisted only of females, although no sex differences had been observed in the first instance.

Basal rations were constituted according to two schedules. The first, used in conjunction with precipitation procedure 1 was based on a stock ration fed routinely to the colony with alterations to the proportions of certain components to accommodate the NRY meal without altering the total protein content of the diet. The second schedule, used with precipitation procedure 2 was made up from purified components of which casein served as the protein source. The composition of diets used in the two experiments is summarised in Tables 14 and 15.

Table 14 – Composition of diets used in conjunction with precipitation procedure 1

Constant components consisted of the following (g/100g diet): skim milk solids, 8; full cream solids, 8; carcass meal, 4; lucerne, 3; peanut oil meal, 6; Fermivite, 2.5; bone meal, 1; salt, 1; lime, 0.5.

Variable components described below (g/100g diet).

Component	Stock Diet	NRY diets	
		Raw 15%	Heated 30%
Maize Meal	58	44.5	31
Fish Meal	8	6.5	5
Heated NRY Meal	0	0.0	30
Raw NRY Meal	0	15.0	0

From the data in Table 14 it is evident that for the 30% NRY ration, 30g of NRY meal (approx. protein content 15%) replaced 3g of fish meal (approx. 70% protein) and 27g of maize meal (9% protein), thus maintaining a total protein content equivalent to the stock ration (19.6%).

'Heated' NRY meal was prepared by spreading the raw meal on stainless steel trays (maximum depth of meal: 5cm) and heating in an autoclave at 15lb./in² for 30 min.

The '30% heated' diet was used as a basal diet for testing the isolated fractions which were incorporated in quantities to be reported in the relevant 'Experimental' sections. The purpose of adding the isolated fractions to the heated NRY diet was to study the activity of each fraction in the presence of all other NRY proteins, but in the absence of other activities.

Diets used with precipitation procedure 2 contained casein as the protein source, while the remaining dietary constituents were added in purified form. The casein ration reported by Rackis (20) was used as a basis for the present study. When NRY proteins were included in the ration, casein, cellulose and cerelose were omitted in proportions which would maintain a constant protein content. Estimates of protein content were made by the semi-micro Kjeldahl technique using a factor of 6.25 for conversion of nitrogen to protein.

The basal casein diet (Table 15) contained 11.5% casein, which gave a total dietary protein content of 7.6%. Inclusion of 15% NRY meal for raw and heated rations was made at the expense of casein, cerelose and cellulose, on the basis of the calculated protein contents of the NRY meal and casein. The batch of NRY meal used in the D series contained 18.9% protein, so that 15g NRY meal replaced 3.2g casein (87.9% protein). Cellulose (2g) and cerelose (9.9g) were omitted to make up a total weight of 15g replaced by NRY meal, and the protein content of the constituted ration was measured at 8%.

Table 15 – Composition of diets used in conjunction with precipitation procedure 2

Constant components consisted of the following (g/100g diet): corn starch, 25.4; salt mix* (4.0; vitamin mix* 1.0; maize oil, 4.0; cod liver oil + vitamin E (1% w/w of c.l.o.), 1.0; choline chloride (100% w/v), 0.3. Diet 'RES 2' contained 9/10 of weights of all constant components.

Variable components are described below (g/100g diet).

Component	Casein diet	NRY meal diets		NRY fraction diets		
		Raw	Heated	Fo	RES 1	RES 2
Casein	11.5	8.3	8.3	5.78	9.2	16.0
Cerelose	50.8	41.0	41.0	41.0	41.0	37.9
Raw NRY meal	0	15	0	0	0	0
Heated NRY meal	0	0	15	14.5	4.5	4.2
NRY fraction (Fo or RES)	0	0	0	3.0	9.6	8.9
Protein content mg/100mg diet (N x 6.25)	7.6	8.1	7.9	6.3	6.9	15.1

Composition described in Appendix 5

NRY fractions were added to the 'heated' diet in proportion to their yield from the meal. Diets of different composition were made up for the two fractions Fo and RES, preparation of which will be described in a subsequent section. These were added to the diet in such quantity that disturbance of the nutritive balance would have occurred unless adjustments were made to the proportions of other components. Thus the weights of casein and NRY meal were altered to accommodate Fo and RES (76.6% and 12.4% protein respectively) and maintain a constant protein content in the diet.

Evaluation of growth depression. The degree to which a diet depressed growth was expressed in terms of a Growth Depression Index (GDI). This factor is obtained as the difference in group body weight between rats fed a test diet and those receiving the heated control. A positive GDI indicates that the test group weighs less than the control (i.e. Growth Depression), while a negative GDI indicates the reverse (Growth Stimulation relative to the heated control).

2.7 Estimation of pancreas weights

At the conclusion of the feeding phase of an experiment, rats were killed by ether anaesthesia and the pancreases dissected out. The tissue was rinsed briefly in cold physiological saline and placed

on a petri dish containing 2ml of distilled water. Under a dissecting microscope, fat, mesenteries, blood vessels and the pancreatic duct were removed. Pancreatic tissue and the accompanying fluid were transferred to a small weighed aluminium foil cup, and water was removed by freeze-drying. The freeze-dried cups were transferred to a desiccator containing CaCl_2 for 2 hours and weighed.

Immediately after dissection, rat carcasses were placed in an oven at 110° for 72h. At the end of this period they were cooled and weighed to provide dry weight data.

3. EXPERIMENTAL PROCEDURE AND RESULTS

3.1. Oral administration of toxic fraction N_1 to suckling rats

As mentioned previously, the objective of the following experiment was to ascertain whether the toxic fraction N_1 would cause an adverse physiological response when administered orally. Tube-feeding of suckling rats was used as an alternative to the conventional feeding trials since the latter require inordinate quantities of purified materials.

Two litters of ten rats each were used, and the animals were two weeks old at the start of the experiment. Each litter was treated as an independent experiment, although the two were conducted simultaneously. The young in each litter were divided into five pairs, weighed and force-fed graded levels of fraction N_1 in 0.1ml of physiological saline. The solution was force-fed directly into the stomach by means of a 1ml hypodermic syringe fitted with a short length of 1/16" teflon tubing. Doses of 2, 4, 6 and 8mg per rat were fed, while the fifth group (8mg, boiled at 100° for 5 min) served as a control. After administration of the toxin the rats were returned to the dam and apart from regular weighing were given no further treatment.

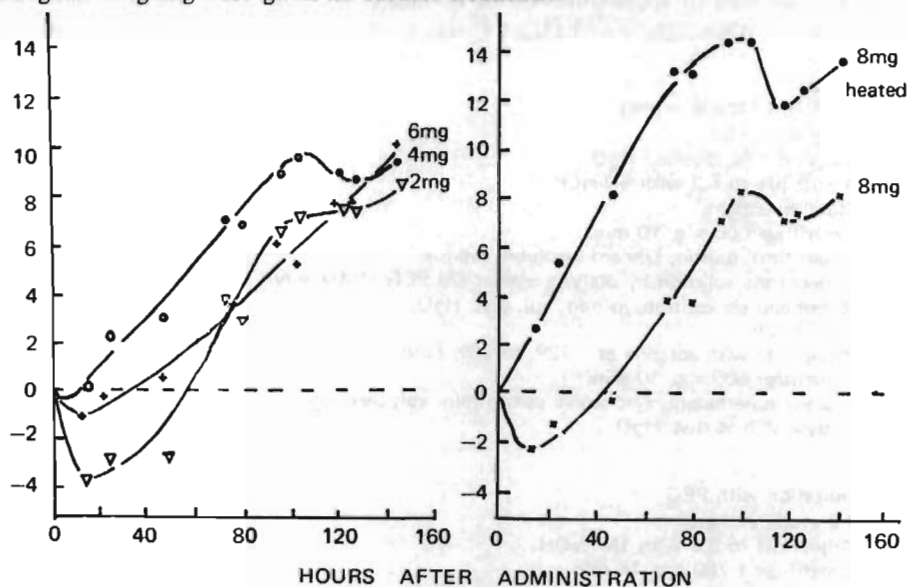


Fig.29 — Weight gains of suckling rats (± 18 g body weight) fed graded doses of toxin N_1 . Each point is a mean value obtained from four rats.

The results of this experiment (Fig. 29) indicate that the factor which is toxic when injected intraperitoneally, is non-toxic upon oral administration to suckling rats. Although the rats did not succumb to the levels of toxin used (2 to 8mg per 18g rat), their growth was checked for between 20 and 60h when compared with a heated control. The raw protein is thus not entirely innocuous, but the effect is less pronounced than when the same factor is injected intraperitoneally into 25g mice ($\text{LD}_{50} = 2.7\text{mg}$) and 60 - 70g rats (7mg produced 80% mortality). A further observation which may be made from the data in Fig. 29 is that the response does not appear to increase in proportion to

the weight of protein administered. Thus the lowest level (2mg) produced the most marked effect on growth. On the other hand the remaining three levels induced progressively greater growth retardation. No explanation could be found for this response, which was observed in both litters.

3.2 Exploratory experiments on use of polyethylene glycol for fractional precipitation.

Before PEG could be used in the NRY system it was necessary to establish that it would effectively precipitate proteins without adversely affecting their physiological activity. In addition a method was required whereby the precipitated protein could be separated from the PEG and recovered in a suitable form.

The toxic fraction N_1 was used as a marker for the exploratory experiments, and its antigenic activity as an index of undestroyed integrity. Thus it was possible to ascertain that the 20M variety of PEG (Union Carbide Corporation) precipitated fraction N_1 without destroying its antigenic activity, and that the protein could be recovered by adding acetone to 70% (v/v), under which conditions the protein was precipitated while the PEG remained soluble.

3.3 Precipitation procedure 1

Since precipitation of NRY proteins appeared feasible, the procedure was applied on a relatively large scale to an extract of NRY meal.

The precipitation schedule is summarised in Fig.30. Polyethylene glycol solution (50% w/v in 0.2M-sodium acetate buffer, pH 5.5) was added to an extract of NRY meal in increments of 0.5% (w/v), to a total concentration of 7.5%. The protein concentration of the extract, estimated by the microbiuret assay, was adjusted to approximately 4% by addition of 0.2M-sodium acetate buffer, pH5.5. The extinction of a 4% solution of Extract C was used as a standard for determination of the required dilution.

1. Preparation of crude extract

620g NRY meal
Suspend 1.9L distilled H_2O
Adjust pH to 4.2 with 5N-HCl
Stand overnight
Centrifuge 600 x g, 10 min.
Strain thro' muslin, Discard insoluble residue.
Concentrate solution by dialysis against 6M PEG (100% w/v)
Resuspend concentrate in min. vol. dist. H_2O .
Chill to 2°.
Precipitate with acetone at -12°, to 70% (v/v)
Centrifuge 600 x g, 10 min.
Discard supernatant. Redissolve ppt in min. vol. dist. H_2O .
Dialyse 20h vs dist. H_2O .

2. Precipitation with PEG

1L crude extract
Adjust pH to 5.5 with 1N-NaOH
Centrifuge 1,700 x g, 15 min.
Discard ppt.
Determine protein content of supernatant (microbiuret)
Dilute with equal volume of 0.2 M-acetate buffer, pH 5.5
X { Equilibrate in water bath to 30°.
Add 1 volume of 100 of 50% (w/v) PEG in acetate buffer
Stand 30 min, 30°.
Centrifuge 1,700 x g, 15 min.
Retain precipitate (F_1)
Repeat cycle 'X'
Retain precipitate (F_2).
etc. for total of 15 cycles, to F_{15}
Retain supernatant (S)

Fig. 30 — Preparation of crude NRY extract, and fractionation with polyethylene glycol (procedure 1).

Treatment of precipitates F₁ to F₁₅ and supernatant S. Precipitates F₁ to F₁₅ were dissolved in a minimum volume of distilled water, and together with the supernatant S, were chilled to 20°. Acetone (−150) was added to 70% (w/v) to precipitate protein, and 1ml of sodium chloride solution (30%) was added to each suspension to promote coagulation. After standing at −150 for 2h the suspensions were centrifuged (1,700 x g, 10 min) and the precipitates redissolved in a minimum volume of distilled water, chilled and precipitated a second time as before. The redissolved precipitates were dialysed overnight against distilled water, and freeze-dried.

The fractions were added to a ration containing 30% heated NRY meal, the composition of which is described in Table 14. The weight of each fraction added to the ration was in proportion to its yield from the original meal, since it was of interest to estimate the proportion of the effect attributable to each fraction. A weight of fraction equivalent to 15g NRY meal was calculated. It was increased by an arbitrary factor of four to account for losses in activity during the precipitation procedure, or distribution of a single factor over several fractions. The duration of the feeding experiment was 10 days.

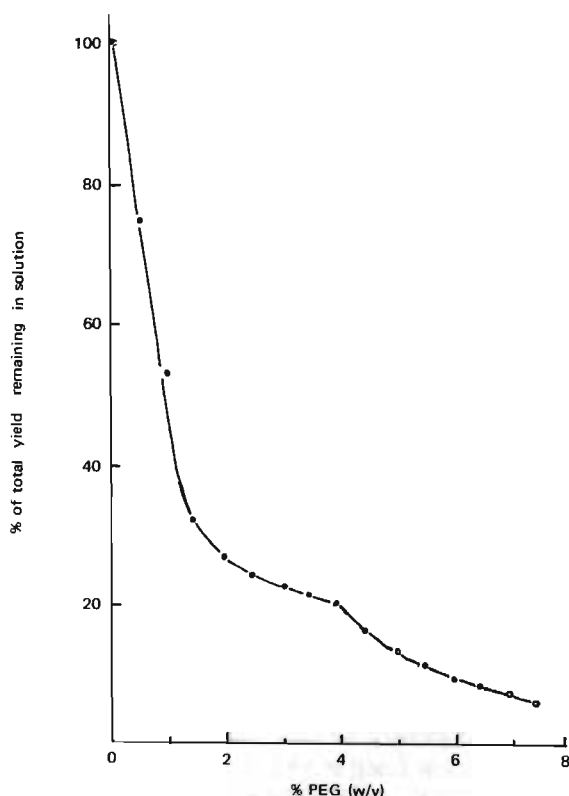


Fig. 31 — Precipitation curve of NRY extract; procedure 1.

A total of 24.08g of material was recovered by PEG precipitation of the extract obtained originally from 620g of meal, representing a yield of 3.9% of total weight of meal (Table 16). Although PEG is not an electrolyte, precipitation followed the typical logarithmic pattern describing a change in solubility with increasing concentration of an electrolyte (Fig. 31). The shape of the curve suggests that it is compounded from the curves of a number of species, which fall into two distinct categories. Precipitation of the first group occurs between 0 and 4% PEG. Beyond 4%, proteins of the second group begin to precipitate, following a similar pattern. At 7.5% PEG, some 6% of total yield remained in stable suspension; this was precipitated by addition of cold acetone.

Assays on fractions F₁ to F₁₅ and S, both for *in vitro* effects (trypsin inhibition, haemagglutina-

tion) and the *in vivo* responses (toxicity, growth depression) reveal a definite separation of activities (Table 16). Trypsin inhibitor activity was uniformly low, showing a slight maximum in fraction 11-13. Although the last three analyses were lost in this study, a subsequent precipitation, covering the same range, showed that activities did not increase for F₁₄, F₁₅ or S.

Table 16 – *In vivo* and *in vitro* activities of fractions prepared by PEG precipitation of NRY extract (procedure 1) Fractions added to 30% heated NRY diet. Included are 15% raw and 30% heated NRY diets (see Table 14 for constitution of diets).

Feed efficiency = gm weight gain/gm food consumed

Growth depression index = body weight of '30% heated' group – body weight of test group

Fraction	Yield g/100g meal	Wt. added to diet g/100g	Feed efficiency	Growth Depression Index 910 days)	G.D.I. per g active factor	Trypsin inhibitor activity specific activity	HG activity HU/mg	Toxicity 5mg per mouse
F ₁	.98	1.17	.37	– 1.0	–	.032	165	0/5
F ₂	.85	1.01	.16	56.1	55.5	.032	535	0/5
F ₃	.80	.96	.35	15.7	16.3	.021	360	0/4
F ₄	.20	.24	.35	14.9	62.1	.032	600	0/5
F ₅	.12	.15	.41	– 8.5	–	.044	390	0/5
F ₆	.06	.07	.40	–12.3	–	.044	390	0/5
F ₇	.05	.05	.40	– 5.5	–	.067	415	0/5
F ₈	.04	.05	.26	1.2	24.0	.055	590	0/5
F ₉	.14	.17	.22	42.9	252.3	.090	1975	3/5
F ₁₀	.09	.11	.40	– 1.4	–	.070	2300	3/5
F ₁₁	.11	.13	.32	15.8	121.5	.090	3350	0/5
F ₁₂	.05	.05	.44	– 7.5	–	.101	1850	0/5
F ₁₃	.05	.05	.42	0.9	18.0	.101	1195	0/5
F ₁₄	.04	.05	.44	–10.5	–	–	1260	0/3
F ₁₅	.04	.05	.45	0.9	18.0	–	1600	1/5
S	.24	.29	.22	53.0	182.7	–	1500	0/5
'Raw 15%'	–	15.00	–.07	89.4	5.96	–	–	–
'Heated 30%'	–	30.00	.40	–	–	–	–	–

Haemagglutinating (HG) activity was distributed through all the fractions, with maximum activity concentrated in F₁₁ and adjacent fraction also showing high activity. Of a total of 2.6×10^6 units in all fractions, 36% are located in F₉ to F₁₂ and a further 20% in F₁₃ to S. Toxicity and HG activity occur together in some fractions, although the strongest haemagglutinin, F₁₁ is non-toxic and maximum toxicity is found in F₉ to F₁₀.

Growth-depressing activity is found mainly in three fractions – F₂, F₉ and S, for 75% of total growth depression by all fractions is located in these three. While one growth-depressor (F₉) is also toxic and shows moderately high HG activity, a second (S) is non-toxic and shows less HG activity. The most potent growth depressor on a per milligram basis is F₉, which is toxic, but the adjacent F₁₀, also toxic, does not depress growth.

The feed efficiency data in Table 10 indicate that growth depression is accompanied by a decreased feed efficiency. The failure to gain weight is thus not caused by a reduced food intake, as might be induced by an unpalatability of the diet. The lowered feed efficiency indicates that growth

depression is a true physiological response which operates at the level of feed utilization.

A closer examination of the activities in F₉, F₁₀ and F₁₁ provides a useful substantiation for the earlier comment that the solubility curve was probably compounded of a number of individual curves. By reference to Fig. 31 it is seen that F₉ was the first fraction in the second phase of precipitation (i.e. above 4% PEG); It would thus be anticipated that F₉ and subsequent fractions would show a sharp change in activity due to the precipitation of a new group of proteins. This was in fact the case, for toxicity, HG activity and growth depression all showed a marked increase at this point. In F₁₀, however, growth depression was no longer evident although both toxicity and HG activity remained at a high level. F₁₁ showed another change, for toxicity had diminished relative to F₁₀, growth depression was also at a low level, while HG activity reached a maximum.

The three distinct maxima in this group of fractions suggest therefore that the activities might be caused by three separate factors, though the data do not permit extensive speculation on this point. The low level of trypsin inhibiting activity in all fractions suggests that this activity plays little part in the growth depression caused by NRY bean fractions

3.4 Precipitation procedure 2

The preceding study had demonstrated the feasibility of precipitating physiologically active proteins with polyethylene glycol. Several changes in methodology were indicated as a result of the experience gained in the first trial, while further modifications were required to increase the volume of NRY extract separated. Although several precipitation trials were conducted between those reported as procedures 1 and 2, each represented a small alteration in precipitation conditions and only the final trial will be reported since it incorporates all changes. A summary of the extraction data and total yields from the intervening trials is presented in Appendix 5, while a discussion of the reasons for introducing changes in methodology is presented below.

Extraction conditions. The initial extraction of material from NRY meal was made at pH 7.0 in procedure 2, instead of pH 4.2 as used in the previous trial. The change was primarily the result of an alteration in the pH of precipitation, which is discussed in a subsequent section.

In order to reduce the length of time required for the initial extraction stage, the suspended meal was maintained at room temperature for four hours (as opposed to 15 hours) before the insoluble residue was removed.

During the 40h dialysis against distilled water a dark precipitate formed in the extract. The precipitate probably consists of globulins, which become insoluble as the ionic strength of the extract reaches a low level. The precipitate was removed by centrifugation and freeze-dried. It was designated the symbol F₀ and studied with the other fractions. The unextracted residue from the meal (known as RES) was air-dried, ground and studied for its possible effect on rat growth.

Precipitation pH. One of the difficulties encountered with the fractions prepared in procedure 1 and subsequent unreported trials, was the low solubility of the precipitates. Since they all retained biological activity, denaturation, if it occurred, could not have been too extensive. Yet all fractions were difficult to resuspend after freeze-drying and required homogenisation with a Dounce-type glass homogeniser.

Various aspects of the precipitation process were changed in an attempt to improve the precipitates. Finally it was observed that when precipitation with PEG was conducted in phosphate buffer at pH 7.0 instead of acetate, pH 5.5, the fractions were easily resuspended after freeze-drying. Thus the precipitation pH was changed from 5.5. to 7.0, and pH was maintained with 0.2M-phosphate buffer.

Addition of polyethylene glycol. Since it was the intention to add PEG up to 20% (w/v) of solution, addition as a concentrated solution was no longer feasible. Furthermore, quantitative transfer of the highly viscous PEG solution introduced a systematic error in the "percent PEG in solution" figure. Consequently, PEG was finely ground in a Wiley mill and added to the reaction vessel as a dry powder.

The final concentration of PEG was increased, since it appeared from procedure 1 that some 6% of total yield remained unprecipitated by 7.5% PEG. Since in addition it was desirable to reduce the number of fractions to be handled, the increment of PEG was increased from 0.5 to 2.0%. In procedure 2, PEG was added in five increments of 2% and one final increment of 8% (F6).

Fraction S of procedure 1 was not obtained in procedure 2 because of the greater volume of solution to be handled. It was noticed that when the protein solution contained more than 10% of PEG, addition of acetone failed to precipitate the protein. Protein could be recovered by diluting out the solution with distilled water and then adding acetone. However with 5 litres of solution containing 18% of PEG, it was not feasible to dilute three times (to reduce PEG concentration to 6%) and then add acetone to 70%. The supernatant fraction was not used therefore for further study. It was considered that at 18% PEG only a small proportion of protein would remain in solution, and that this could be neglected without adversely affecting the experiment.

Equilibration conditions. The period during which the protein solution was held at constant temperature, following addition of PEG and prior to centrifugation, was known as the equilibration period. The equilibration conditions were changed from 30 min at 30° to 45 min at 20°. No specific reason could be given for the change – it was merely felt that a lower temperature would minimise the chance of undesirable alterations to proteins occurring. The longer equilibration time was necessitated by the change to solid PEG, which required 5 - 10 min to dissolve.

Centrifugation of PEG precipitates. To facilitate the handling of volumes greater than 2.8L (the limit of the International bucket-type centrifuge used in the previous procedure) and the application of greater gravitational forces, a continuous-flow centrifuge was used. The Cepa centrifuge (Carl Padberg, Lahr/Baden) has a rotating cylinder with inlet and outlet ports at opposite ends. The cylinder can be rotated at up to 35,000 rpm and flow rates of 9L per hour are obtainable.

When rotating, the cylinder (internal diameter 4.5cm) retains a volume of 210ml against the inner wall. This volume is in the form of a cylinder, the walls of which are 0.34cm thick. This is the greatest distance which a precipitating particle has to move before reaching the wall. At 9L per hour, any 1ml of solution spends about 1.4 min in the cylinder, under a centrifugal force of about 20,000 x g (at 25,000 rpm). By contrast, the International centrifuge applied a force of 1,700 x g to the solution for 20 min, and migration distance was about 10cm. Centrifugation was thus considerably more efficient with the continuous flow apparatus, the high capacity of which made it possible to separate large quantities of extract.

Diets. Diets described in Table 15 were used for the feeding phase of procedure 2. The lower protein content of these rations (7.6 - 9%) was better suited to a study of growth depression, for, since 8% of good quality protein is just adequate for normal growth, any deleterious physiological effect would be easily detected.

The feeding phase of procedure 2 included rations containing the unextracted residue (RES) and the dialysis-precipitate (F0). As explained under section 2.5, these factors represented such high proportions of the original meal, that they could not be added to the normal 'heated NRY' ration as were the PEG fractions. The proportions of casein and NRY meal were adjusted so that when F0 and RES were added in the required quantity, the protein content remained within the range of the other rations. This adjustment did have one disadvantage, however. It meant that the comparison would be influenced by protein quality. The author had attempted to avoid the danger of a protein quality effect being confused with active growth depression, by including small quantities of fractions in the normal, adequate ration. By removing casein and adding RES in its place, a depression of growth could be expected purely on the basis of a difference in protein quality. In spite of this disadvantage the casein-replacement approach was used, as no suitable alternative was available. The only other possibility was to use an amino acid mixture as a basis for the diet instead of casein, and adjust the amino acid content of each diet to compensate for deficiencies in the added fractions. However the difficulties of such an approach excluded it as a practicable alternative to the casein replacement method in the

present study. In this connection the high-protein RES ration (RES 2) provided evidence of the effect of RES in the presence of adequate protein of good quality.

Addition of fractions to diets. Two conflicting objectives governed the decision regarding the quantity of each fraction to be added to the ration. The first objective, that of ascertaining the percentage of total growth depression induced by the raw meal which was contained in each fraction, required that the fractions be added at levels which approximated their level in the bean meal. The second objective was to determine the effects of the fractions relative to one another and relative to other assayed activities. To this end it was important to ensure that the effects were pronounced, so that differences would be more obvious and comparisons less hazardous. Consequently it would be desirable that the quantity of each fraction in the diet be fairly large in order to amplify the effects.

The first objective would be difficult to realise in practice, since fractionation of the crude meal into a number of components involves inevitable losses, partial denaturation, and separation of activity between several fractions. Decisions regarding the level in the original meal would thus be subject to arbitrary estimates of the extent of these effects. Moreover the estimation of activities relative to the original meal was of relatively little consequence in the present study.

The second objective was however considered to be more important in the context of factors

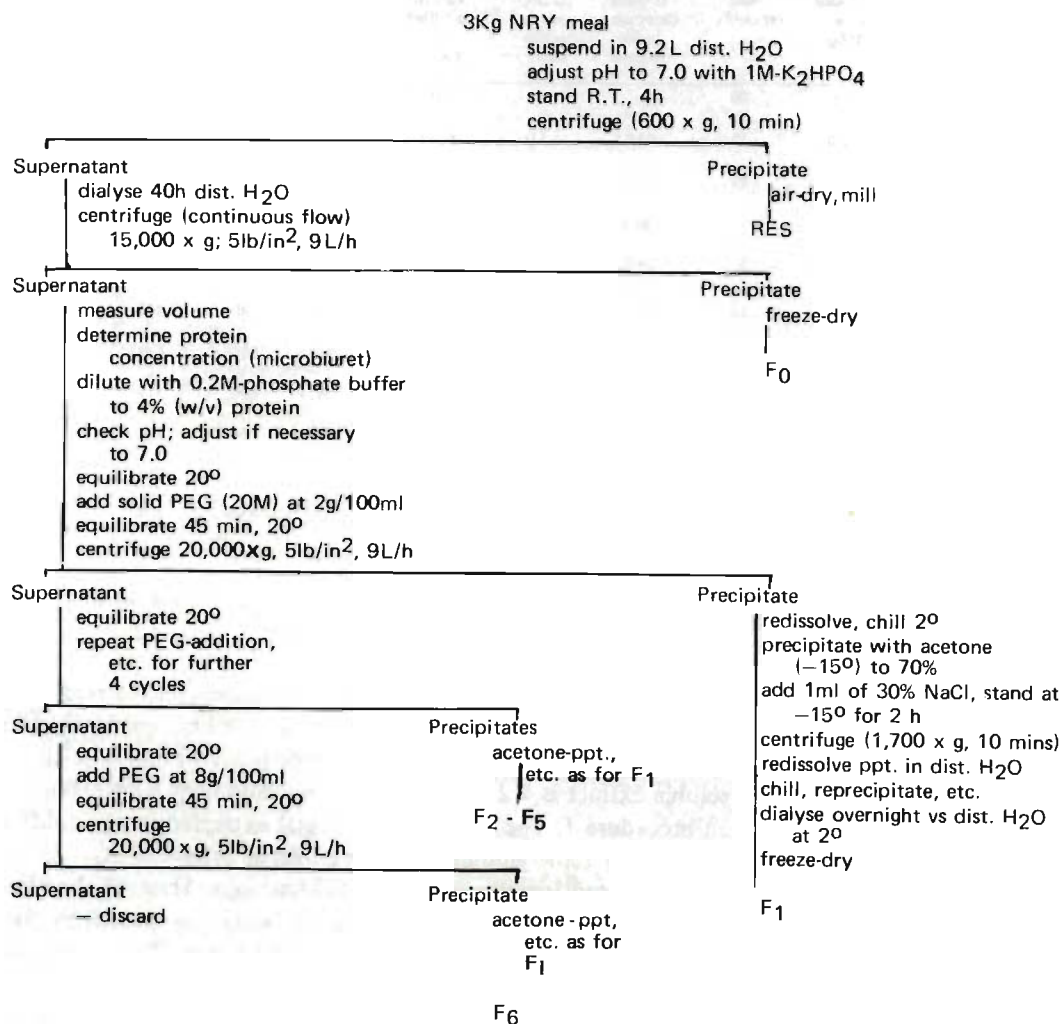


Fig. 32 — Preparation of crude NRY extract and fractionation with polyethylene glycol (procedure 2).

related to growth depression. Fractions were thus added in large quantities so that their physiological effects would be amplified. The weight of fractions F₀ to F₆ to be included in the diet was calculated on the basis of theoretical yield from 15g of NRY meal, with a ten-fold increase to ensure substantial physiological responses. The ten-fold increase was not applied to the unextracted residue RES, since it represented a high proportion of the original meal and had not been subjected to extensive treatment during preparation

Details concerning extraction and precipitation procedure 2 are summarised in Fig. 32, which incorporates the changes which have been discussed in the foregoing sections.

Fractions F₀ to F₆ were assayed for trypsin inhibiting, haemagglutinating and toxic activity. They were fed to rats in the casein-NRY rations, in conjunction with groups fed raw NRY meal, heated meal, casein diet, and the unextracted residue RES. The latter was fed both in a low-protein diet (RES 1) and in a casein-supplemented diet (RES 2).

Table 17 - *In vivo* and *in vitro* activities of fraction prepared by PEG precipitation of NRY extract (procedure 2)
Fractions added to 15% heated NRY diet, with exception of F₀, RES 1 and RES 2 (see Table 16 for explanation of parameters. For individual pancreatic weights, see Appendix 5.

Fraction, diet	Yield g/100g meal	Wt. added to diet g/100g	Feed efficiency	Growth Depression Index 14 days	G.D.I. per g active factor	Pancreas weight g/100g body wt. mean \pm S.E. mean	Trypsin inhibitor activity BAEE units/mg	HG activity HU/mg	Toxicity LD ₅₀ (mg)
Heated 15%	—	15.0	.20	—	—	.3856 \pm .015	—	—	—
Raw 15%	—	15.0	-.22	107.6*	7.0	.6851 \pm .058	.025	—	—
Casein	—	11.5	.24	— 16.2	—	.3920 \pm .016	—	—	—
RES 1	64.1	9.6	-.17	90.9	9.5	.5726 \pm .039	—	—	—
RES 2	64.1	9.6	.31	— 34.5	—	.5320 \pm .022	—	—	—
F ₀	2.0	3.0	— .21	99.2*	33.2	.6400 \pm .066	.040	315	20.0
F ₁	0.2	0.3	.03	63.7	212.5	.6086 \pm .048	.060	633	14.2
F ₂	0.45	0.68	.11	46.8	69.0	.6123 \pm .052	.020	792	10.5
F ₃	1.1	1.65	.03	65.1	39.0	.7085 \pm .039	.040	404	8.9
F ₄	0.21	0.32	.19	14.6	45.5	.5139 \pm .010	.040	275	7.6
F ₅	0.17	0.26	.28	— 33.7	—	.4458 \pm .026	.030	224	6.6
F ₆	0.08	0.11	.14	33.0	289.5	.5239 \pm .023	.040	163	6.0

* After 10 days on a 15% ration, Raw and F₀ animals had lost weight so severely that the ration was changed to 10% of NRY meal and the equivalent of F₀.

The results of the extraction experiment, including *in vitro* and *in vivo* assays on the fractions, are summarised in Table 17. Data for yield of fractions indicate that the 4 to 6% PEG increment (F₃) produces a high yield relative to the other fractions, about 50% of PEG-precipitated protein being recovered in F₃. The total yield of soluble extract is 4.21g/100g meal, compared with 3.88g/100g obtained under the conditions used in procedure 1. The distribution of total extracted material differs considerably in the two trials, for in procedure 1 the globulin fraction (isolated prior to PEG-precipitation in procedure 2 as F₀) is precipitated together with the PEG-fractions. Thus, of the total 3.88g recovered in procedure 1, 3.64g was PEG-precipitated (the remainder being recovered from the PEG-supernatant by acetone precipitation). In procedure 2, 2.0g was isolated prior to PEG-precipitation as F₀, while the remaining 2.21g was precipitated by between 0 and 18% PEG.

The low yield of total recoverable material in procedure 2 (68.3%) is primarily due to the loss of a portion of the unextracted residue (consisting mainly of starch) as a consequence of bacterial action

during air-drying. In a previous experiment (see Appendix 5), the yield of RES was 93.5% with a total recovery of 97%. It is logical to assume that the weight of extracted residue would have been of the same order in procedure 2.

The Growth Depression Index in Table 17 provides an estimate of the relative contribution of each fraction to growth depression. Activity was observed in all fractions except F₅, which stimulated growth slightly compared with the heated meal ration.

Maximum growth-depressing activity was found in F₀, which was slightly greater in effect than RES. F₁ and F₃ were also active as growth depressors, while F₂ and F₆ showed a lesser contribution. The most potent depressor on a "per gram" basis was F₆ which, with F₁, was some 4 times more potent than any other fraction.

Marked pancreatic hypertrophy was observed in all groups which suffered growth depression. The relative activities of the fractions with regard to pancreatic hypertrophy were similar, though not identical, to the relative order of growth depressing activities. The orders of activities were as follows:

Pancreatic hypertrophy: F₃ > F₀ > F₂ > F₁ > RES > F₆ > F₄ > F₅

Growth depression: F₀ > RES > F₃ > F₁ > F₂ > F₆ > F₄ > F₅

The displacement of RES to a high order of activity on the growth depression scale is almost certainly due to the protein quality effect mentioned previously, while the position of F₀ as being more active than F₃ may be due to the same effect. It is thus to be expected that the growth depression induced by RES and to a lesser extent F₀ is a combination of an active physiological effect (associated with pancreatic hypertrophy) and a growth response to an inadequate supply of good quality protein in the diet. While the above explanation is in accordance with the available data, verification would have to await further study.

Pronounced enlargement of the pancreas was observed in the F₃ group, which showed an 84% increase in mean dry weight of pancreas per 100g body weight compared with the heated NRY meal control. In this respect F₃ was unique. Rats fed fractions F₂, F₁, F₀ and RES 1 were all in the same range and showed an average increase of 59%. The size of pancreas relative to body weight in rats fed F₄ and F₆ was on average 35% greater than that of the controls, while the group fed F₅ showed a small 16% increase. The high-protein RES group (RES 2) had pancreas weights relative to body weight similar to F₄ and F₆. This group, together with the F₅ group, demonstrate that provided adequate supplies of high quality protein are available, the animal gains weight normally in spite of having an enlarged pancreas. This is in agreement with other reports on the effects of pancreatic hypertrophy.

The results of the present study provide support for the hypothesis that growth depression and pancreatic hypertrophy are intimately related. Although a correlation does not necessarily imply a causal relationship, available evidence from other sources suggests that a hypertrophic pancreas causes growth depression. The present data are therefore in agreement with existing evidence, and substantiate the current theory regarding the mechanism of growth depression. In addition the present study suggests that in NRY beans, the whole of growth depression is associated with pancreatic hypertrophy, since no fraction depressed growth without causing hypertrophy.

Toxicity was noted in all fractions precipitated by PEG, although not all fractions were equally potent. A gradual increase in potency was observed with increasing PEG concentration. Thus F₁ was least toxic of the fractions precipitated by PEG (LD₅₀ = 14.2mg), and F₆ was most toxic (LD₅₀ = 6.0mg), while F₂ to F₅ increased gradually between the two extremes. F₀, the dialysis precipitate, was non-toxic at 20mg per mouse.

A comparison of the toxicity and growth depression data indicates that, while the most toxic fraction (F₆) was also the most potent growth depressor (GDI/g = 289.5), the next most toxic fraction (F₅) did not depress growth, while the second most potent growth depressor (F₁) was only mildly toxic (LD₅₀ = 14.2mg). A comparison of the order of potency reveals that the two effects are unlikely to be related by a common mechanism:—

Toxicity (LD ₅₀)	F ₆ >	F ₅ >	F ₄ >	F ₃ >	F ₂ >	F ₁ >	F ₀
Growth depressing potency (GDI/g)	F ₆ >	F ₁ >	F ₂ >	F ₄ >	F ₃ >	F ₀ >	F ₅

The recovery of haemagglutinating activity from fractions F₀ to F₆ is markedly lower in the second trial than the first. Thus whereas a total of 2.9×10^6 HU were recovered in the first trial, the second experiment yielded 1.5×10^6 units, or just over half of the recovery in the first trial. Although differences between fractions were not pronounced, F₂ showed a maximum of activity compared with the others. No explanation was at hand for the decreased recovery of haemagglutinin units, for none of the alterations to technique in procedure 2 would appear to be potentially deleterious to the haemagglutinins.

The second precipitation procedure provided evidence which indicated that trypsin inhibiting activity is a minor factor in NRY meal, and that it is unlikely to be related in any way to pancreatic hypertrophy or growth depression. Thus the raw NRY meal and all PEG-precipitated fractions showed specific activities between .020 and .060, or approximately 5% of the activity of a purified trypsin inhibitor. The NRY bean system differs in this respect from soybeans, for the soybean trypsin inhibitor has been shown to be a potent stimulator of pancreatic hypertrophy.

4 DISCUSSION.

The NRY proteins differ in certain respects from legume proteins reported hitherto. Indeed it is by virtue of these differences that some contribution has been made to the field of physiological activities and anti-nutritional effects. For while pancreatic hypertrophy in rats fed soybeans was shown to be caused by a trypsin inhibitor, hypertrophy is stimulated by an NRY fraction showing negligible trypsin inhibitor activity. And while Phaseolotoxin A, a toxin isolated from another cultivar of *Phaseolus vulgaris* (23), depressed growth when fed to rats, the NRY toxin did not appear to play an important role in growth depression.

The results of the present study have re-emphasized the need for caution in interpreting an association of two measured activities as implying a causal relation between one and the other. Thus even though a trypsin inhibitor causes pancreatic hypertrophy and an intraperitoneal toxin depresses growth, an assumption of causality between the activity of a factor and an effect is, on the basis of these results alone, not justified.

An analogous situation has developed over the dual activity of the kidney bean phytohaemagglutinin (50), for the question of whether HG and mitogenic activity were due to the same protein has been the subject of some controversy. Thus whereas Borjeson, Bouveng, Gardell, Norden and Thunell (86) had shown that with a certain isolation procedure the one activity could be abolished while the other was retained, further evidence (50) indicated that a demonstrably pure protein exhibited both properties. The present opinion is that while a single molecule may be responsible for both effects, two active sites are probably involved and hence no causal link between haemagglutination and mitogenicity is warranted.

The next stage in the study of factors and effects in the NRY system would be to obtain pure preparations of toxin and pancreatic hypertrophy-stimulating factor and to establish the limits and extent of the effects which each factor induces. Information of such a nature would contribute substantially to an understanding of the mechanisms by which the physiological responses are induced.

Naturally the requirements for such an isolation are immense, since the assay for activity requires such quantities of material, and preparative procedures would have to be adapted to an unusually large scale. Sambeth, Nesheim and Serafin (87) attempted to overcome this problem by conducting an ion-exchange separation on a Buchner funnel using discontinuous buffer changes. However, as previously discussed in the present thesis, preparative ion exchange chromatography is limited in its separative potential. Alternatively, Cantrell (88) used ion-exchange columns of more conventional dimensions, but tube-fed the fractions and studied short-term physiological changes. The second approach raises

certain questions regarding the significance of any observed changes, to a normal feeding regime.

While the PEG-precipitation procedure did not provide pure NRY fractions, the method yielded large quantities of a material which could well be applied to a subsequent separative method. Preparative electrophoresis, as previously mentioned, might well be used to purify certain of the fractions prepared by PEG-precipitation. Alternatively, molecular exclusion could be used on a large scale, since with the ready availability and ease of preparation of 'tanned' gelatin the operation of large molecular exclusion columns has become a distinct possibility.

While the contribution of the studies reported in the present chapter have been limited by a lack of resolution between PEG-precipitated fractions, certain common assumptions concerning growth depression have been confirmed and others questioned. In addition the approach has indicated that further progress may be achieved by purification of fractions showing maxima of different activities, while the application of a similar procedure to a different legume variety would further contribute to an understanding of the nature of growth depression and related effects.

GENERAL DISCUSSION

The isolation of a factor responsible for toxicity in NRY beans fulfilled the primary objective of the present investigation. While several difficulties were encountered in the establishment of homogeneity of the final fraction, evidence from molecular exclusion and immunological studies indicated that a pure but unstable product had been obtained. An assessment of the significance of toxicity and certain other activities in relation to growth depression, constituted a second aspect of the investigation. The development of a procedure for fractionating NRY meal with polyethylene glycol enabled the relative activities of fractions to be compared and related to the growth depression which occurred when the same fractions were fed to rats. These two aspects of the study will be treated separately in the ensuing discussion, which for reasons of clarity will be divided into several categories.

The nature and properties of the NRY toxin

The toxin was isolated in a form which suggested a loose association between a protein moiety and a small proportion of carbohydrate. Although the toxic fraction was subject to changes which resulted in loss of the major toxic component, the major component could be isolated reproducibly in a form which showed a consistent molecular weight. Thus the protein-carbohydrate association, though weak, would seem to have a measure of specificity, and the weakly-bound carbohydrate would appear to be an integral part of the natural toxic molecule. Furthermore, the extent to which carbohydrate could be removed from the protein was not determined, and hence it is not known whether all carbohydrate is removable under mild conditions, or whether a small residue remains covalently bound to the protein. Thus the NRY protein may or may not be a glycoprotein, and classification as a glycoprotein in the exact sense of the definition could only be claimed once the covalent link had been proved by the isolation of a glycopeptide.

The isolation of a toxin from NRY beans has added to an already extensive list of protein-carbohydrate complexes having an important biological function or producing some effect on living tissue. Glycoproteins of animal origin have been extensively studied, and the protein-carbohydrate bond is found to vary from a strong covalent to a loose salt-bridge association. They show a wide range of functions from the specialised mechanical role of connective tissue, cartilage, blood vessels and synovial fluids, to the specific function of the blood-group factors. Very few enzymes are classified as glycoproteins (89, 90).

Plant glycoproteins, on the other hand, are comparatively unexplored (39). While carbohydrate and protein have been observed in association in plant extracts (91), little information is available on the nature of the linkage in specific proteins. In 1965 Pusztai (36) isolated from kidney beans a glycoprotein of no specified activity, which contained mannose and glucosamine, and in 1966 soybean haemagglutinin was found by Lis, Sharon and Katchalski (39) to be a glycoprotein with a polysaccharide unit covalently linked to the protein in a manner similar to some animal glycoproteins. Recently a haemagglutinin from the wax bean was found to be similarly constituted, although the polysaccharide is more complex than soybean haemagglutinin (54).

The manner in which protein and carbohydrate are associated is a matter of some interest in the study of glycoproteins and related compounds. The NRY toxin has revealed an unusual feature in that apart from the ready loss of polysaccharide from the protein, a subsequent polymerisation occurs which results in the formation of a compound the molecular weight of which is greater than the parent compound. This effect would appear to be unique, for no mention of an analogous situation has been found in the literature. Nevertheless two observations from the literature suggest that a change of this nature may have occurred without being noticed, for Takahashi *et al.* (52) reported that uncharged, protein-free carbohydrate was eluted from the wax bean haemagglutinin during chromatography on DEAE- and CM-cellulose. The fraction, similar in origin and properties to N₁CM₁ in the present study, was not studied further, and since the purified haemagglutinin was homogeneous in the ultracentrifuge,

no subsequent polymerisation would seem to have occurred. Dechary and Altshul (92) reported a small contaminant which persisted during purification of the peanut protein arachin. The ion-exchange elution profile reported by these workers showed a minor response at the void volume of the column, similar to the response produced by N₁CM₁ in the present study. Since no assay for carbohydrate was included, the peak appeared insignificant and received no further attention. The polymerisation effect is thus possibly not confined to the NRY protein system, but apparently has not been detected by other workers.

At first sight the spontaneous formation of a macromolecule from smaller units would seem unlikely. Whether the high MW fraction N₁G₁ is formed by polymerisation of small polysaccharide units alone, or by addition of units to the intact toxin, the requirements for polymerisation are the same. Studies on certain protein-protein interactions are pertinent to the present discussion. Lauffer (93) describes two examples of spontaneous endothermic polymerisation in proteins – in the insulin monomer and the so-called A protein from tobacco mosaic virus. In each case the spontaneous, endothermic nature of the reaction is explained on the basis of a release of bound water by the monomers during polymerisation. The entropy increase which accompanies release of bound water results in a negative enthalpy function in the fundamental thermodynamic equation and hence a spontaneous endothermic reaction. Release of bound water has been demonstrated in polymerising A-protein particles, and similar endothermic phenomena are reported to be widespread in biological processes.

An advantage of the 'bound water' theory of polymerisation is that no attractive forces need be postulated. Attractive energy is provided by the entropy change resulting from release of bound water and if the change is sufficiently large, polymerisation could occur against the repulsive force of two molecules bearing a like charge (93). Thus a polymerisation of neutral or slightly charged polysaccharide chains with the toxic molecule is not altogether unlikely, although the author is aware of the speculative nature of any proposed mechanism.

Further aspects of the protein-carbohydrate association may be deduced, albeit tentatively, from a study of the composition and properties of the NRY toxin. Thus the amino acid analysis data, indicating a high content of aspartic acid, serine and threonine, suggest that covalent bonding is probable, for these are the amino acids most likely to serve as sites, for the attachment of carbohydrate residues. In addition glucosamine, the presence of which was detected in the NRY toxin, would be expected to play a role in protein-carbohydrate bonding. In this connection it is interesting to note that an N-acetyl glucosamine linkage has been demonstrated between the β -carboxyl of aspartic acid and N-acetyl glucosamine in egg albumin (94), while Tsugita and Akabori (95) showed that an O-glycosidic link between serine and a hexose is likely in β -amylase. In general, however, information regarding specific linkages in glycoproteins is limited.

Bonding of a less permanent nature is suggested by the ready dissociation of the toxin N₁ under mild conditions. Bonds broken under these conditions are likely to be weak salt bridges or hydrogen bonds, or alternatively they might be electrostatic bonds formed between a strongly acidic polysaccharide and a weakly basic protein. Since sialic acid is frequently encountered in glycoproteins and the acidic chondroitin sulphate is another common constituent, many glycoproteins have a strongly acidic polysaccharide moiety. This frequently results in a low pI for the protein, and electrostatic attraction is promoted.

Electrostatic attraction would not appear to be a major factor in the association between protein and carbohydrate in the NRY toxin. While the amino acid composition of N₁ suggests a weakly acidic nett charge (acidic amino acids constitute 20% of total moles; basics make up 6%), the estimated pI is about 8. The acidity of aspartic and glutamic acids might therefore be suppressed by combination with a sugar amine, to give a more basic nett change. Whatever the reason for the high pI, the situation is inconsistent with the postulate of a strongly acidic polysaccharide bound to a weakly basic protein. It thus seems more likely that neither the carbohydrate nor the protein are strongly charged, but that

salt-bridges are formed between a weakly acidic protein moiety and weakly basic amino-sugars. An assumption inherent in the discussion of the preceding two paragraphs is that the observed dissociation or fragmentation of the toxin involves a separation between protein and carbohydrate. An alternative explanation could be that the protein consists of sub-units. While the latter possibility cannot be excluded, it is rendered unlikely by the fact that no evidence was obtained for intermediate-MW subunits following fragmentation, while the high proportion of carbohydrate released as a consequence of fragmentation suggested that the postulate of a carbohydrate-protein separation was preferable. The terms 'carbohydrate' and 'protein' do not imply the exclusiveness of composition, for it is entirely possible that the carbohydrate moiety contains amino acid residual and the protein moiety contains residual amounts of carbohydrate.

The question of homogeneity arises in the present study, as it does in any isolation of a protein. Certain aspects of the NRY-toxin properties however, indicate that homogeneity in this case is not easily defined.

The difficulties of establishing homogeneity of the toxin by molecular exclusion and ultracentrifugal criteria have been discussed in Chapter IV. In addition, however, heterogeneity of a more subtle order was noted within preparations. It was observed that the width of the peak N₁G₂ (N₁ on 'tanned' gelatin — Fig. 14) was considerably greater than the width of a tryptophan sample under the same conditions. Tryptophan, being fully retarded by the column, would be subjected to all the effects of mechanical non-ideality which cause zone spreading. Consequently the greater width of the protein zone is in all probability an indication of polydispersity with respect to molecular weight. Thus NG₂ possibly consists of a range of molecular weights, insufficiently different to be distinguishable as separate peaks, and distributed around a mean value. In addition, occasional differences were observed in the mean elution volume of NG₂ with different treatments, although this produced no apparent difference in toxicity. It thus appeared that the toxin could exist in more than one form, and that a measure of heterogeneity was to be found within any one preparation.

The problem of micro-heterogeneity has been discussed by Haurowitz (96) and Gibbons (97). Both suggest that many proteins may exist as a population of closely related, but not identical, individuals. In such a case, rigorous definition of homogeneity is not possible. Polydispersity may be genetic in origin, or it may arise from conditions applied during the isolation. As might be imagined, differentiation between these two alternatives poses one of the more profound problems in protein chemistry. Cole (98) discusses the difficulties associated with discovering how a natural molecule exists *in vivo*, and the limitations of present techniques in enabling this to be achieved. Precisely this situation is encountered with the NRY toxin, for although certain differences in molecular weight of the toxin could be attributed to isolation treatments, the polydispersity within one preparation remains unexplained. It may be an inherent property of this protein, or alternatively it may be an effect which is superimposed by the isolation procedure.

Comparison of the NRY toxin with other related proteins

A survey of the recent literature reveals that a number of toxic proteins or polypeptides have been isolated. Of these, four are of reptile or insect origin, three are produced by micro-organisms, and four have been isolated from beans (Table 18). They vary widely, both as regards molecular weight and potency. The snake and scorpion toxins are relatively small (MW 6,822 to 18,000), while the bacterial toxins are larger, varying in size from the staphylococcal α -toxin (MW 30,000) to the α -toxin of *Pasteurella pestis* (MW 240,000). The bean toxins, on the other hand are fairly uniform, with the exception of ricin, the castor bean toxin. While a MW of 60,000 has been calculated for ricin, it has

Table 18 — Molecular weight and potency of certain naturally-occurring toxins

Source	Molecular Weight (daltons)	Potency (LD50 for mice, unless stated to contrary)	Reference
A Reptiles and Insects			
<i>Vipera palestinae</i> venom (neurotoxin)	11,600	10mg/kg	99
Formosan cobra venom	11,000	55 µg/kg	100
Scorpions	11,000 — 18,000	—	101
<i>Androctonus australis hector</i> (scorpion)	I 6822 II 7249	19 µg/kg 10 µg/kg	102
B Bacteria			
<i>Clostridium botulinum</i>	150,000	3.5×10^{-4} µg/kg	103
<i>Pasteurella pestis</i> (bubonic plague)	A: 240,000 B: 120,000	50-200 µg/kg	104
<i>Staphylococcus aureus</i> (or <i>pyogenes</i>)	α-toxin: 30,000	50 µg/kg	105, 106
C Beans			
<i>Ricinus communis</i> L (castor bean) (castor bean)	60,000	80 µg/kg (rat)	107, 108
<i>Glycine max</i> (soybean)	110,000	50mg/kg (rat)	21, 39
<i>Phaseolus vulgaris</i> (black bean)	130,000	50mg/kg	27, 109
<i>Phaseolus vulgaris</i> 113,000 (NRY bean)	113,000	80mg/kg	Present thesis

been suggested that the molecule exists as a dimer, in which case the MW would be 30,000. Thus ricin differs markedly from the other bean toxins with respect to molecular weight.

Wide variations are observed in the weight of material required to produce toxicity. Botulinum toxin is renowned for its potency, and Das Gupta and Boroff (103) report that a solution having an extinction of 1.0 contains 7×10^6 minimum lethal doses for mice (giving an estimated LD₅₀ of 3.5×10^{-4} mg/kg). When compared with other toxins on the basis of potency, the botulinum toxin is unique. Next in decreasing order of potency are the scorpion and cobra venoms which, together with the two remaining bacterial toxins and the castor bean toxin, are toxic in the range 10 to 200 mg/kg. Ricin is uniquely potent among plant toxins, for the *Phaseolus vulgaris* and *Glycine max* toxins are some 1,000 units less toxic (LD₅₀: 50 to 100 mg/kg). The toxin isolated from *Vipera palestinae* is slightly more potent than the bean toxins (with the exception of ricin) but with an LD₅₀ of 10 mg/kg is considerably less potent than the other snake and scorpion toxins.

Of all the naturally-occurring toxic substances, the botulinum toxin is probably the most enigmatic. In spite of its molecular size it gains entry into the body through the intestinal wall, for a

toxic substance corresponding in size with the toxin was found in the lymph draining the intestinal wall of orally intoxicated rats (110). The protein is thus either absorbed intact or in the form of smaller units which recombine after absorption. Electron microscope studies (111) suggest that the toxin is localised at the myoneural junctions, and intoxicated animals were found to be incapable of transmitting a nervous impulse across the junction. The mechanism of toxicity thus appears to be a direct and specific interference in a nervous pathway.

While the toxicity of legume proteins is in no way comparable to botulinum toxicity, studies on the latter suggest that proteins are able to exert physiological effects as intact species even after oral ingestion. The fact that both toxicity and growth depression in legumes can be eliminated by heating, likewise suggests that these effects are a property of the intact protein. A valuable contribution to this field will be made when an active unit of the growth depressor is isolated and its mode of absorption determined.

A comparison of the NRY toxin with proteins purified from related legumes reveals a close similarity in composition and molecular parameters (Table 19). The NRY toxin is compared with four haemagglutinins isolated from cultivars of *Phaseolus vulgaris*, and from soybeans, these being the only other proteins isolated from legumes related to the NRY bean. Two of the haemagglutinins were reported to be toxic, a third was non-toxic, while the fourth was not tested for toxicity.

A remarkable similarity is observed, particularly with respect to amino acid composition and molecular weight. As would be expected, differences within the *Phaseolus vulgaris* species are (apart from occasional aberrations) less than the inter-genus differences between *Phaseolus vulgaris* and *Glycine max*. Nonetheless these latter differences are remarkably small when the genetic differences are taken into account. The NRY toxin differs from other *Phaseolus vulgaris* proteins principally in its iso-electric point and molecular weight, though refinements in the method of determining either parameter for the NRY toxin might indicate that the differences were less than suggested by the present provisional estimates. The isolation of a fraction showing high haemagglutinating activity from NRY beans would add further interesting data to a comparison such as presented in Table 19.

The problem of multiple physiological activities associated with single preparations of legume protein

The NRY toxin differs from other legume toxins reported in Table 19 in that it exhibits only mild HG activity. Due to difficulties in obtaining comparative haemagglutination data it is not possible to express the difference in quantitative terms, but it is estimated that the soybean and black bean proteins are 50 and 10 times more active respectively than the NRY toxic fraction N₁S₂. Thus it would appear that in the NRY toxin, the property of agglutinating erythrocytes is not a prerequisite for toxic activity.

The fact that other isolated toxins are potent haemagglutinins has tended to promote the hypothesis that a causal relationship might exist between observed activity (HG activity) and physiological effect (intraperitoneal toxicity). The observation that soybean and black bean haemagglutinin were active as growth depressors (22, 23) extended the association still further to include growth depression as a manifestation of the haemagglutinin's activity. The trend of thought is epitomised in a speculative suggestion by Jaffé (23) that the growth-depressing activity of the haemagglutinin might result from a specific reaction with the intestinal mucosa similar in mechanism to the agglutination of erythrocytes. While other authors have been less specific, the hypothesis has been encountered elsewhere in the literature (29) and a certain degree of confusion was observed in the use of the term 'toxicity' by Liener in two recent reviews (112, 113).

Observations on the NRY toxin suggest that the terms haemagglutination, toxicity and growth depression should be used exclusively to describe three distinct phenomena, and that greater caution should be exercised in ascribing the cause of toxicity or growth depression to the activity of a haemagglutinin. It is stressed that the issue only arises when a causal role is attributed to a factor. The mere association of two effects because both are induced by the same homogeneous protein is perfectly valid, but the formulation of a causal hypothesis on the basis of this association is potentially misleading.

Table 19 – Composition and molecular parameters of four isolated haemagglutinins and NRY toxin

Amino acid data are presented as 'residues per molecule'. Data for Black bean were only obtainable as 'moles %'. These were normalised for comparison by increasing ten-fold. Figures in parentheses for Natal Round Yellow and Soybean have been calculated as 'residues per 130,000 daltons', to eliminate differences due to molecular weight.

Botanical Name	Phaseolus Vulgaris				Glycine max
Common Name	Red Kidney Bean	Black Bean	Wax Bean	Natal Round Yellow Bean	Soybean
Reference	25, 50	25, 35, 109	52, 54	<i>Present thesis</i>	38, 39
Toxicity	No	Yes	Not tested	Yes	Yes
S _{20,w}	6.5	5.9	5.4	5.5	6.0
MW	128,000	126 - 130,000	121 - 132,000	113,000	110,000
pI	6.5	4.9	5.5	8.2	—
Carbohydrate %	3.4	5.7	10.4	11	4.5
Amino acids:					
Lys	56.2	46.1	36.2	24.3 (27.9)	40 (47.2)
His	11.1	9.2	8.9	10.5 (12.0)	14 (16.5)
Arg	27.3	31.9	30.0	22.4 (25.8)	18 (21.2)
Asp	155.4	136.0	152.0	119.8 (137.8)	97 (114.5)
Thr	93.0	85.5	83.7	63.1 (72.6)	50 (59.0)
Ser	110.8	103.7	115.0	85.0 (97.7)	76 (89.7)
Glu	68.3	68.1	67.7	54.8 (63.0)	52 (61.4)
Pro	14.1	48.3	40.8	35.6 (40.9)	47 (55.5)
Gly	75.5	98.3	76.5	69.5 (79.9)	40 (47.2)
Ala	68.2	68.0	62.1	51.2 (58.9)	61 (72.0)
Lys	2.0	5.1	—	—	—
Val	84.7	67.5	80.3	64.6 (74.3)	45 (53.1)
Meth	1.4	1.0	—	—	6 (7.1)
Ileu	52.8	75.9	43.3	37.6 (43.2)	38 (44.8)
Leu	95.0	40.1	86.0	65.3 (75.1)	63 (74.3)
Tyr	20.0	27.7	25.1	13.6 (15.6)	16 (18.9)
Phe	60.8	59.0	62.0	43.9 (50.5)	37 (43.7)
Try	17.0	20.7	18.9	—	18 (21.2)
NH ₃	—	—	—	118.3 (136.0)	80 (94.4)

Data concerning the relation between trypsin inhibitor activity and pancreatic hypertrophy have been discussed in Chapter V and are mentioned here only as a further example of the same situation, for although the trypsin inhibitor has been ascribed a causal role in pancreatic hypertrophy, the NRY proteins with a negligible level of inhibitor activity, induced marked pancreatic hypertrophy. In addition, studies on the dual activity of the kidney bean phytohaemagglutinin, also reported in the previous chapter, indicate that mitogenic and haemagglutinating activities, while both caused by the same homogeneous protein, are in all probability due to separate active sites.

It is interesting that toxicity and haemagglutination have been encountered together in several cases. Thus Ishiguro, Takahashi, Hayashi and Funatsu (114) differentiated between factors causing toxicity and haemagglutination in the castor bean (*Ricinus sanguineus* L.), while Das Gupta and Boroff (103) separated toxicity from haemagglutination in crystalline *Clostridium botulinum* Type A toxin – a preparation which had long been considered homogeneous.

Application of a quantitative technique for estimating HG activity has shown that a number of different fractions isolated from NRY meal are able to agglutinate erythrocytes. Thus although the HG activity of the toxin N₁ is considerably less than that of fraction L (from DEAE-cellulose column) (49), the activity is reproducibly associated with the pure toxin. This result suggests that the distribution of haemagglutinating activity over three DEAE-cellulose fractions (49) is not the result of trailing or contamination, but is due to distinct factors exhibiting different levels of agglutinating activity. Multiplicity of haemagglutinins has been reported by Ishiguro *et al.* (114), Lis, Friedman, Sharon and Katchalski (115) and Jaffé and Hannig (35) in three different legume genera.

These findings, in the author's opinion, suggest that haemagglutination is not a specific property of a particular protein. The term 'haemagglutinin' is thus not ideal, since it implies a uniqueness of activity and makes no allowance for the range of activity observed. It would appear that the property of agglutinating erythrocytes depends on a particular configuration of the protein, which may be present in varying degrees in different proteins. This suggests a degree of degeneracy in the proteins, which in turn has bearing on the mechanism by which these proteins are synthesized.

No discussion of physiologically active legume proteins would be complete without a consideration of the more philosophical aspect of the problem. Why, for instance, should the storage proteins of a bean have the property of specifically agglutinating erythrocytes, or inhibiting trypsin, or stimulating pancreatic hypertrophy? Any attempt to introduce an evolutionary explanation would be subject to criticism on the grounds that the survival value of these specific activities is difficult to establish, since they are unrelated to any natural consumer which might have governed the course of their development. Furthermore, their properties do not appear to have any specific function in the plant, although evidence of some function may be forthcoming in the future. For the present, the most plausible explanation could seem to be that the observed activities are the result of a fortuitous combination of structural factors.

These considerations, together with the degeneracy in legume proteins discussed previously, suggest to the author that it is unlikely that plant storage proteins are specified by the synthesizing cell with complete precision. Gibbons (97) has suggested that such proteins may be gene-controlled only in the sense that the biosynthesis of the enzymes involved in their synthesis is gene-controlled. The blood-group substances are cited as an example of such a system, and it is not unlikely that certain plant storage proteins are synthesized by a similar mechanism. Many features of the protein systems observed in the course of the present investigation, such as the general lack of definition between the proteins extracted from NRY beans, the overlapping of activities in adjacent fractions, the multiplicity of effects, and the similarity of proteins from different cultivars and genera, suggest an agreement with the above explanation of storage protein synthesis.

Conclusion

The pressing need for protein to feed the rapidly expanding world population needs no further emphasis. Since legumes are such an eminently suitable source of protein, elimination of the anti-nutritional factors would contribute significantly to the world supply of edible protein. Extensive heat treatment is costly and a high-protein bean which requires only a minimum of cooking would provide a long-term solution to the global nutritional problem. Since climatic conditions vary so widely, the most productive local varieties in an area would need to be used as a basis for the development of more suitable plants. Subsequent progress would depend on a combination of exacting biochemical studies of the undesirable factors present in the seeds, with a breeding programme designed to eliminate them without detracting from the virility of local plants. Progress in this direction depends on the prior establishment of exact causal relationships between active proteins and the effects which they produce, and the development of sensitive accurate tests to serve as a basis for genetic trials.

Consistent emphasis has therefore been laid in the present investigation on the establishment of purity of the toxic factor. Difficulties were encountered in the interpretation of data regarding the homogeneity of the toxin, although these were explainable and in fact contributed to an understanding of the properties of the NRY toxin. An appreciation of the limitations inherent in each of the many criteria of purity currently in use was found to be necessary for a reliable interpretation of data, and the need to apply as many criteria as possible was emphasized.

An assessment of the significance of toxicity and haemagglutination, trypsin inhibition and pancreatic hypertrophy in the general context of growth depression was made on the basis of the relative activities of fractions separated from NRY meal. Since the fractions were isolated in large quantity they were fed to rats and the extent to which the fractions depressed growth was also determined. The properties of the NRY proteins were such that the general applicability of certain common assumptions regarding the role of the trypsin inhibitor in pancreatic hypertrophy and the significance of the intraperitoneal toxin in growth depression has been questioned. The study has pointed to the dangers which arise when one activity is used as an index of another, and the need for the establishment of homogeneity by rigorous criteria when the effects of a pure protein are to be studied.

Further progress must lie in the direction of purification. The final picture of growth depression will only emerge when all factors have been purified and their physiological significance investigated. While requirements of the growth depression assay impose immense demands on the methods of separating proteins, indications are that certain available procedures and recently developed media will enable the demands to be met.

SUMMARY

The incentive for isolating a toxin from a local cultivar of *Phaseolus vulgaris* beans was provided by the potent growth-depressing effect produced by the raw bean, and the pivotal role which legumes are destined to play in human and animal nutrition. Since a water-soluble extract of the bean was found to be toxic to rats when injected intraperitoneally, it was of interest to isolate the toxin, not only with a view to studying its properties but also to determine its possible role in the growth-depression phenomenon.

A crude toxic product was obtained from Natal round yellow (NRY) bean meal by aqueous extraction, salting-out with ammonium sulphate and acetone-precipitation of the redissolved precipitable material. The toxic extract ($LD_{50} = 5\text{mg}/25\text{g}$ mouse) was further purified by anion exchange chromatography on DEAE-cellulose. One fraction, N_1 , was toxic ($LD_{50} = 3\text{mg}$) and was also mildly active as a haemagglutinin, although maximal haemagglutinating (HG) activity had been observed in another DEAE-cellulose fraction.

Rechromatography of N_1 on CM-cellulose, Sephadex G-200 or 'tanned' gelatin resulted in a major toxic component (N_1CM_2 , N_1S_2 or N_1G_2) with slightly increased potency over N_1 ($LD_{50} = 2\text{mg}$). Amino acid analyses revealed little difference in composition between N_1 and N_1S_2 , but the neutral sugar content decreased from 47% by weight in N_1 to about 10% by weight in the rechromatographed fractions. It thus appeared that the principal result of rechromatography was the removal of a high-carbohydrate compound.

When fractions N_1CM_2 or N_1G_2 were rechromatographed under identical conditions, the elution profile indicated a non-toxic component similar to that removed from the toxin in the previous chromatographic separation. Furthermore, the major rechromatographed fraction from CM-cellulose ($N_1CM_2CM_2$), on examination in the analytical ultracentrifuge, showed a profile almost identical to N_1 . Two components were evident in each profile — a major solute ($S_{20,w} = 6.5S$) and a faster-sedimenting, minor component ($S_{20,w} = 9.5S$). Diffusion coefficients calculated by a quantitative immunological technique enabled molecular weights to be estimated provisionally as 113,000 and 169,000 respectively. Finally it was found that the bulk of the high-carbohydrate material removed during rechromatography of N_1 was identical with the fast-sedimenting minor component (MW 169,000) detected in the ultracentrifuge.

The evidence indicated that the high MW, high carbohydrate fraction was originally part of the smaller, major toxic component and was in some manner derived from it. Further study of the stability properties of fraction N_1 indicated that the proportion of high MW component (N_1G_1) was reduced by rechromatography of N_1 on a gel exclusion column under certain conditions, but that subsequent rechromatography of the major fraction (N_1G_2) showed a high proportion of N_1G_1 at the expense of N_1G_2 . Several additional peaks were observed at elution volumes between N_1G_1 and N_1G_2 , and their molecular weights were found to increase in regular increments of 13,000 from 113,000 to 169,000.

The conclusion that the high MW component was being formed from the lower MW toxin thus seemed inescapable. No means could be found of preventing the change, and since the express aim of the study was to isolate the toxin in natural form, no attempt was made to promote fragmentation in order to derive a stable, toxic breakdown product. It was concluded nevertheless that while preparations N_1S_2 , N_1G_2 and N_1CM_2 remained heterogeneous in the conventional sense, they in all probability represented a pure but unstable compound.

Evidence from electrophoresis substantiated this conclusion, for N_1S_2 and N_1G_2 migrated as a single zone. Immunological studies indicated that at least four distinct antigens were present in N_1 . However when the four fractions isolated from N_1 by exclusion chromatography were tested for antigenic activity, it was discovered that with the exception of N_1S_1 , all fractions showed reactions of identity with each of the precipitin bands formed by N_1 . Thus the different fractions shared a

common antigenic site, which further suggested that the smaller components were fragmentation products of the major component. Thus the immunological data were consistent with the suggestion that heterogeneity in fractions obtained from N₁ was due to the unstable properties of the toxin, and there was reasonable justification for the conclusion that a pure toxin had been isolated. Association with carbohydrate suggested that the toxin was a glycoprotein, although further information would be necessary for this to be established with certainty.

The toxic protein, with an approximate molecular weight of 113,000, is similar to a toxin isolated from another cultivar of *Phaseolus vulgaris* (black beans) and a toxin from soybeans. While the soybean and black bean toxins also showed a high level of haemagglutinating activity the NRY toxin was only mildly active as a haemagglutinin.

A second aspect of the investigation involved an assessment of the role of the toxic factor in the phenomenon of growth-depression. Studies using the purified toxin were limited to the oral administration of small quantities to suckling rats. The raw toxin was found to check normal growth when compared with a heated control, although the effects were less pronounced than those which followed intra-peritoneal administration. Limitations in the quantity of available material precluded further dietary studies on the purified toxin.

In an alternative approach, fractional precipitation with polyethylene glycol was used to prepare NRY fractions in large quantity. The fractions were tested for efficacy as growth-depressors by feeding to weanling rats, and for toxicity, HG activity and trypsin inhibiting activity. Pancreas weights of test animals were examined so that the degree to which pancreatic hypertrophy accompanied growth depression could be ascertained. The object of this study was to develop a means of assessing which of the measured activities were most likely to be important factors in growth depression.

While growth depression caused by NRY beans was invariably associated with a hypertrophic condition of the pancreas, no such association was established between growth depression and toxicity, HG activity or trypsin inhibiting activity. Thus a fraction showing potent toxicity was not necessarily a potent depressor of growth, and HG activity was also not invariably associated with growth depression. Since trypsin inhibitor activity was uniformly low in both NRY meal and fractions, direct implication of the inhibitor in growth depression is unlikely.

By virtue of certain features of the NRY bean system, the results of the present study contribute to the current discussion on growth depression. For whereas pancreatic hypertrophy on soybeans was shown to be caused by a purified trypsin inhibitor, a more pronounced hypertrophy is produced by an NRY fraction showing negligible trypsin inhibitor activity. Similarly, toxicity and HG activity frequently occur in the same protein in other legumes, and are associated with growth depression, while in the NRY system it would appear that they need not necessarily be associated. The main conclusion from this exploratory study has been that increased emphasis should be directed toward bulk purification of active factors, particularly from different legume varieties, so that the full physiological significance of each activity may be determined in relation to the growth-depression syndrome.

CALCULATION OF LD₅₀ AND PRESENTATION OF TOXICITY DATA1 CALCULATION OF LD₅₀ according to the method of Litchfield and Wilcoxon (40).

The method of LD₅₀ calculation involves the establishment of a straight line relationship between the weight of toxin administered and the percentage of deaths which result, when graded doses of a toxin are injected into groups of mice. To this end, the percentage effect is plotted against 'dose per mouse' on log probability paper (Codex Book Co., Inc., Norwood, Massachussets). A straight line is fitted to the data and tested for closeness of fit by $(\text{Chi})^2$, which also provides a test for heterogeneity within the data. From the straight line a value for the LD₅₀ is determined, and in addition a slope function which is used to calculate confidence limits for the LD₅₀, for 19/20 probability.

Two preparations are compared by first testing the dose-effect lines for parallelism. Provided that they do not deviate significantly from parallelism, the data are compared on the basis of a potency ratio which is tested against an expected error ratio. Finally, confidence limits may be calculated for the potency ratio.

The method provides an acceptable statistical treatment of toxicity data, while the calculations are relatively simple. Furthermore it provides a means of utilizing data for 0 or 100% effects, which are not usually amenable to statistical analysis.

As an example of an LD₅₀ calculation, the comparison of Extracts B and C (Chapter II) will be described in detail below, although for a complete description the reader is referred to the original publication.

1.1 Fitting of straight line to toxicity data for Extracts B and C.

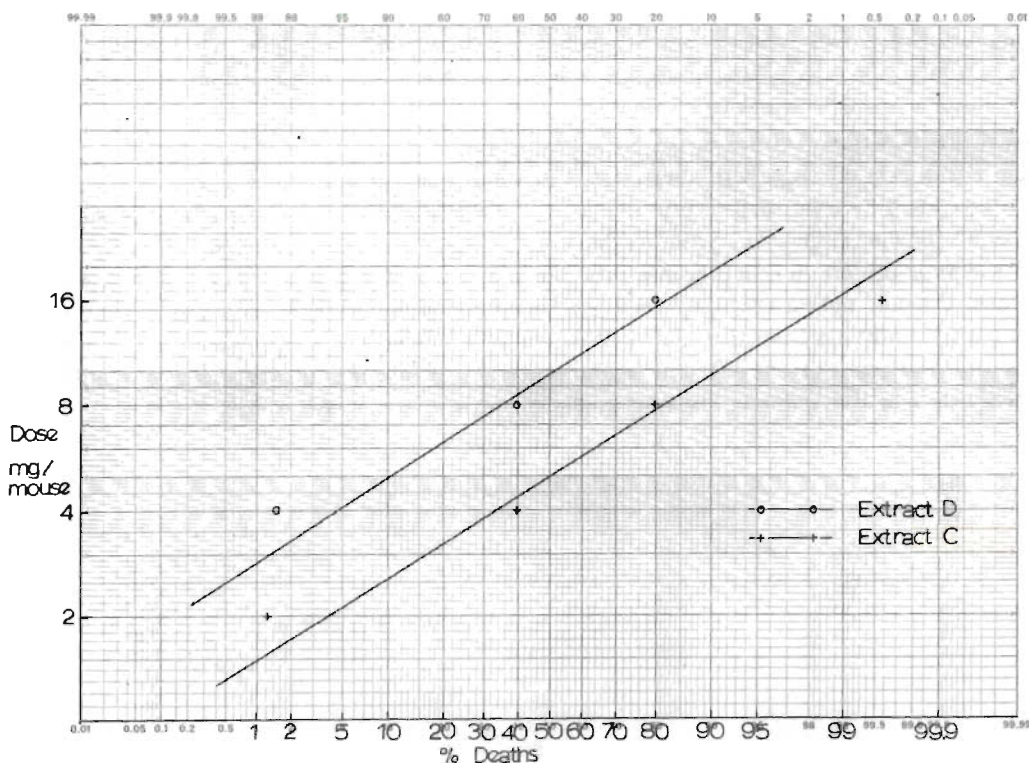


Fig. 33 — Log probability plot of 'dose' against 'effect' for NRY Extracts C and D.

Extract	Dose mg/mouse	Dead/ tested	Observed % dead	Expected* % dead	Observed - expected	Contribution to (Chi) ²
B	16	4/5	80	82.5	2.5	.015
	8	2/5	40	35.0	5.0	.010
	4	0	0 (1.5)	4.5	3.0	.020
	2	0	0	—	—	—
						.045
C	16	5/5	100 (99.6)	98.8	0.8	.005
	8	4/5	80	82.0	2.0	.002
	4	2/5	40	34.0	6.0	.016
	2	0	0 (1.3)	4.0	2.7	.020
						.043

* Determined from graphs, Fig. 33.

	Extract B	Extract C
Average animals/dose	5	5
Contribution to (Chi) ²	0.225	0.215
Degrees of freedom (n)	1	2
(Chi) ² for n deg. freedom [(Chi) ² _t]	3.840	5.990

Since (Chi)² < (Chi)²_t for both extracts, data are not significantly heterogeneous, and lines are sufficiently well fitted (see Fig. 33).

1.2 Calculation of LD₅₀ and confidence limits.

	Extract B	Extract C
LD ₈₄	16.4	8.3
LD ₅₀	9.8	5.0
LD ₁₆	5.8	2.9
Slope function $S = \frac{LD_{84} - LD_{16}}{LD_{50}} =$	1.68	1.69
N ¹	10	10

	Extract B	Extract C
f LD ₅₀	1.68 ^{2.77/10}	1.69 ^{2.77/10}
	1.59	1.60

Confidence limits for 19/20 probability:—

Upper: LD ₅₀ × f LD ₅₀	15.58	8.0
Lower: LD ₅₀ / f LD ₅₀	6.16	3.12

LD₅₀ + confidence limits: 9.8 (6.2 to 15.6) 5.0 (3.1 to 8.0)

1.3 Comparison of data

1.3.1. Test for parallelism

$$\text{Slope ratio SR} = S_1/S_2 = 1.69/1.68 = 1.006$$

$$f \text{ SR} = 1.44$$

Since $\text{SR} < f \text{ SR}$, lines may be considered parallel within experimental error.

1.3.2 Potency ratio (PR)

$$\text{PR} = \text{LD}_{50 1} / \text{LD}_{50 2} = 9.8/5 = 1.96$$

$$f \text{ PR} = 1.93$$

Since $\text{PR} > f \text{ PR}$, the two preparations differ significantly in potency.

Confidence limits for 19/20 probability:

$$\text{Upper: PR} \times f \text{ PR} = 1.96 \times 1.93 = 3.784$$

$$\text{Lower: PR} / f \text{ PR} = 1.96 / 1.93 = 1.016$$

$$\text{PR} + \text{confidence limits} \dots \dots \dots 1.96 (1.02 \text{ to } 3.78)$$

2 PRESENTATION OF TOXICITY DATA

Table 20 - Toxicity data for NRY extracts and column fractions (Chapter II)

Extract / Fraction	Dose mg/mouse									LD ₅₀
	20	16	10	8	5	4	3	2	1	
	Total dead/Total injected									
B		4/5		2/5		0/5		0/5		9.8
C		5/5		4/5		2/5		0/5		5.0
D	6/6		6/6	5/6	3/6	2/6				5.0
N ₁ (Extract C)					5/5	5/6	4/6	1/6		2.7
N ₁ (Extract D)					4/4	4/6	3/6	0/6		3.2
N ₁ CM ₁						0/2	0/3			
N ₁ CM ₂					4/4		4/5	3/9	1/6	2.25
N ₁ CM ₃						0/2				
N ₁ S ₁							0/3			
N ₁ S ₂						3/3	3/4	0/9	1/9	2.0
N ₁ G ₁							0/3			
N ₁ G ₂							2/3	2/2		
N ₁ G ₃						0/2				

Table 21 – Toxicity data for fractions obtained by PEG-precipitation (Chapter V, procedure 2)

Fraction	20	Dose mg/mouse					LD ₅₀
		15	10	8	5	4	
		Total dead/Total injected					
F ₀	0/6						
F ₁	4/6	3/6	2/6	0/6	2/12	0/6	14.2
F ₂	5/6	6/6	1/6	1/6	2/12	0/6	10.8
F ₃	6/6	4/6	4/6	2/6	0/12	1/6	8.9
F ₄	4/6	4/6	4/6	4/6	0/12	1/6	7.6
F ₅	6/6	3/6	4/6	4/6	1/12	2/6	6.6
F ₆	5/6	6/6	4/6	5/6	1/6	0/6	6.0

Toxicity data for NRY extracts and purified preparations are presented in Table 20, while similar data for NRY fractions prepared by PEG-precipitation are presented in Table 21.

DIFFICULTIES ASSOCIATED WITH THE ESTIMATION OF HAEMAGGLUTINATING ACTIVITY

1 INTRODUCTION.

The quantitative determination of haemagglutinating activity was an important, though secondary aspect of the studies reported in the present thesis. As previously described, unanswered questions concerning the physiological activities encountered in legumes included the possible toxic and growth-depressing activities of the haemagglutinins. A quantitative estimate of haemagglutinating activity was thus of importance, not only during the isolation of the toxic factor, but also as an additional assay in the experiments involving feeding of PEG-precipitated fractions from NRY meal.

It has long been known that many plant extracts, particularly those of the *Leguminosae*, exhibit the property of agglutinating erythrocytes *in vitro*. Though widely distributed, and extensively studied, the phytohaemagglutinins remain a source of interest because no specific function has been described for them in the plant. The origin and significance of an effect as specific as erythrocyte agglutination is thus of considerable interest. The phytohaemagglutinin of a cultivar of *Phaseolus vulgaris* (red kidney bean) has found widespread use as an initiator of mitosis in leucocytes, cells normally incapable of division (50). The capacity of reversing the normal control of cell division in leucocytes has naturally suggested parallels with carcinogenic agents or factors initiating differentiation.

The method of estimating agglutinating activity has undergone little refinement as a result of studies on the phytohaemagglutinins. In most cases, activity or potency of the factor is estimated by addition of a standard suspension of washed erythrocytes to serial dilutions of the haemagglutinin, with visual estimation of the dilution at which agglutination fails to occur. In 1955, however, Liener (33) published a photometric method for estimating the degree of agglutination, thereby extending the usefulness of the haemagglutination phenomenon and providing a quantitative measure of activity in terms of haemagglutinin units. More recently Drescher (116) has described a photometric 'haemaggregation' assay for nucleic acids, in which the agglutination phenomenon is quantitated in a manner similar to Liener's.

Haemagglutinating activity was estimated in the present study by a procedure based on that described by Liener. Several modifications were introduced as a result of difficulties encountered in the use of Liener's method in its original form. It is the purpose of this appendix to outline the difficulties and limitations associated with the estimation of haemagglutinating activity, and to indicate reasons for the choice of particular conditions in preference to those described in the original publication.

In addition, certain difficulties were encountered in the evaluation of haemagglutination data. As a result, an alternative method of evaluation was developed by the author, and data are presented in this appendix which facilitate a comparison of the original method with the proposed alternative.

2 EXPERIMENTAL PROCEDURE.

The basic procedure for preparing erythrocytes and conducting the assay has been described in Chapter II and will receive no further comment, apart from those aspects which were subject to modification.

The results to be described in section 3.1.2 of this appendix were calculated according to the method published by Liener (33), further description of which will be found in section 3.2. Slight modifications to the original procedure, which affected the definition of haemagglutinating activity and the treatment of a standard, are discussed in section 3.1.1.

Trypsin activity was determined by the model substrate method described in Chapter V.

3 RESULTS AND DISCUSSION.

3.1 Difficulties associated with methodology.

3.1.1. Time of settling.

The definition of haemagglutinating activity described by Liener included a condition in which the erythrocytes were allowed to settle for 2½ hours. Studies in the author's laboratory indicated at the outset that a settling time of 2½ hours could not be used. At the end of this time all erythrocytes, whether agglutinated or not, had settled below the 1cm window through which extinction values were determined.

No explanation could be found for this difference. While the original publication does not specify whether the diameter of the colorimeter tube (10mm) refers to an internal or an external measurement, and the position of the 1cm square window is given from the top of the adaptor instead of the bottom, it is unlikely that small differences in either of these two measurements would account for the anomaly. In the present study the window was positioned as low as possible without encountering the curvature of the test tube base, so that extinction readings were taken from the lowest position of the suspension.

The change in extinction of an erythrocyte suspension in the absence of haemagglutinin (Fig. 34) indicated that the optimal time for reading the extinction would be at about 90 minutes. The extinction of a suspension which had settled for longer than 120 min decreased sharply as the meniscus of sedimenting erythrocytes passed the upper limit of the window. Thus 90 min was taken as an optimal settling time.

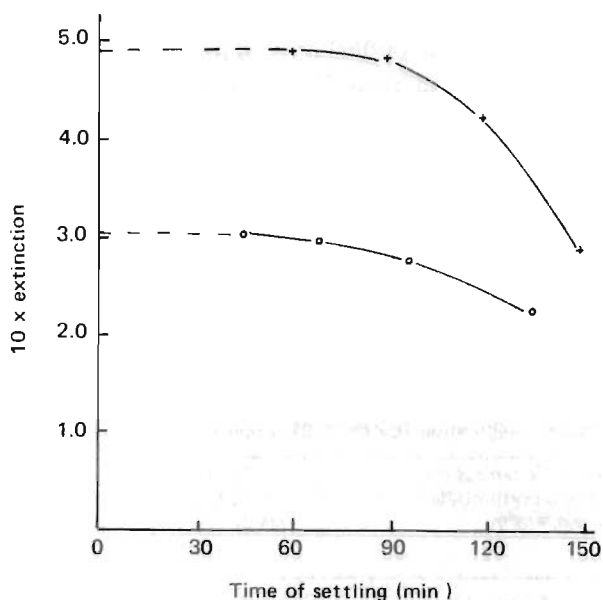


Fig. 34 — Settling properties of a washed, trypsinated erythrocyte suspension in the absence of haemagglutinin.

- + — + — 100% cells in suspension
— o — o — 50% cells in suspension

Observations of the change in extinction with time on the '50% cells in suspension' tube, indicated a slow decrease in extinction (see Fig. 34). Since E_{50} plays an important role in the calculation of activity, it was advisable that allowance be made for normal settling of unagglutinated cells from the erythrocyte suspension. Thus whereas the E_{50} used by Liener was obtained from freshly suspended erythrocytes, E_{50} in the present study was measured on duplicate tubes which had settled for the same time as the tubes containing haemagglutinin.

3.1.2 Concentration of trypsin used for sensitisation.

Prior to incubation with the haemagglutinin, erythrocytes are sensitised by digestion with trypsin for 1 hour, after which residual enzyme is removed by washing.

Difficulties were encountered in obtaining a trypsin preparation similar to that used in the original publication. Liener used a 1% Bacto-trypsin solution (Difco Laboratories, Inc., Detroit, Michigan). Since the solution is unstable and is required to be kept under refrigeration, importation for routine analyses was not practicable. The present author decided therefore to study the effect of various concentrations of a locally-available trypsin preparation on the haemagglutinating activity of a protein sample. Trypsin activity of the crude preparation was determined, and for comparison, a highly purified trypsin preparation was also tested. The object of the study was to derive a level of trypsin activity which would effect optimal sensitisation and which would provide a means of standardising procedures between laboratories.

The results of several experiments designed to study the effect of trypsin concentration on the determination of HG activity reveal that the situation is a complex one. The activity of NRY fraction L (obtained by DEAE-cellulose chromatography, Fig. 5) which had been shown (49) to have a high HG activity, increased as the concentration of enzyme in the sensitisation step was increased (Table 22). A trypsin concentration was reached, however, above which the erythrocytes were extensively haemolysed and spontaneously agglutinated after 60 minutes of digestion. Thus 100mg of trypsin (either crude or pure) per 100ml erythrocyte suspension was the maximal concentration which did not haemolyse the cells. Although not indicated in Table 22, lower concentrations of pure trypsin resulted in a lower HG activity for fraction L. In addition, a control experiment indicated that increasing concentrations of trypsin had no effect on the settling of erythrocytes in the absence of the haemagglutinin. It is immediately evident from Table 22 that there is a marked disparity between trypsin activity and HU/mg when the crude and pure trypsin preparations are compared. Pure trypsin at 100mg/ml erythrocyte suspension, though supplying some 30 times more trypsin units to the suspension than the crude preparation, resulted in about 1/3 of the activity in the haemagglutinin. It

Table 22 — The effect of different enzyme treatments during the sensitisation phase, on the activity of NRY fraction L

Erythrocytes digested for 1 hour at 37°, at concentrations indicated in body of table. After digestion, erythrocytes washed and mixed with haemagglutinin as previously described.

Crude trypsin: an unspecified crude preparation supplied by British Drug Houses, Poole, England.

Pure trypsin: a 3 x crystallized preparation (Batch No. 230) supplied by Seravac Labs., Cape Town.

Pure α -chymotrypsin: a 3 x crystallized preparation (Batch 35B) supplied by Seravac Labs., Cape Town.

Enzyme	Enzyme concentration mg/100ml erythrocyte suspension	Trypsin Activity BAEE units/100ml erythrocyte suspension $\times 10^{-3}$	Haemagglutinating Activity HU/mg
Crude trypsin	1	0.25	82
Crude trypsin	10	2.5	834
Crude trypsin	100	25.0	2105
Pure trypsin	100	860.0	684
Pure chymotrypsin	100	—	450
Pure trypsin + pure chymotrypsin	50 + 50	430	885

appeared therefore that some factor other than trypsin, present in the crude preparation but absent in the pure, might also be necessary for complete sensitisation. Chymotrypsin was tested for efficacy in this respect, since this is a likely contaminant of the crude trypsin preparation. While chymotrypsin is slightly effective (Table 22) either alone or in combination with pure trypsin, the combined activity did not reach that of an equivalent concentration of crude trypsin.

Table 23 — The effect of pre-treatment of trypsin on sensitisation of erythrocytes to the activity of NRY fraction L. Enzymes treated as indicated prior to incubation, or initial incubation followed by secondary treatment, after which erythrocytes were washed and incubated with fraction L, as previously described.

Different preparations of stock erythrocytes used in Experiment 1 and 2

Purified soybean trypsin inhibitor obtained from Sigma Chemical Co.

Experiment	Initial digestion					Secondary digestion				Haemagglutinating activity HU/mg
	Trypsin preparation	Trypsin concentration mg/100ml erythrocyte suspension	Inhibitor concentration mg/100ml erythrocyte suspension	Trypsin activity BAE units 100ml erythrocyte suspension $\times 10^{-3}$	Time of digestion min.	Trypsin preparation	Trypsin concentration mg/100ml erythrocyte suspension	Inhibitor concentration mg/100ml erythrocyte suspension	Time of digestion min.	
1	Crude	10	—	2.3	60	—	—	—	—	914
	Crude inhibited	10	4.0	0.05	60	—	—	—	—	100
	Crude boiled (5 min.)	10	—	3.6	60	—	—	—	—	982
	Crude boiled + pure	10 crude + 10 pure	—	21.4	60	—	—	—	—	1224
2	Crude	10	—	—	60	—	—	—	—	2544
	Crude	10	—	—	10	—	—	—	—	2070
	Crude inhibited	10	0.6	—	60	—	—	—	—	653
	Crude inhibited	10	0.6	—	10	—	—	—	—	471
	Crude inhibited	10	2.0	—	60	—	—	—	—	237
	Crude inhibited	10	2.0	—	10	—	—	—	—	242
	Pure	10	—	—	60	Crude inhibited	10	2	5	3748
	Pure	10	—	—	10	Crude inhibited	10	2	5	5067
	Crude inhibited	10	2.0	—	60	pure	10	—	5	3235
	Crude inhibited	10	2.0	—	10	pure	10	—	5	2192

Further experiments indicated (Table 23, Experiment 1) that inhibition of trypsin activity prior to incubation with erythrocytes markedly reduced the sensitising effect of crude trypsin. Furthermore, reduction of the sensitising effect appeared to parallel the degree of trypsin inhibition (Table 23, Experiment 2) for the addition of a higher proportion of inhibitor resulted in a lower value for HG activity. Thus trypsin activity as such appeared to be a primary requirement for sensitisation. When crude trypsin was boiled (Experiment 1) the trypsin activity increased slightly, as did the HG activity of fraction L. Since the heat-denaturation of trypsin is reversible, its activity would be expected to remain constant, although the slight increase is not explainable using the present data. Since boiling of the crude preparation did not reduce its efficacy in sensitising the erythrocytes, it would appear that the second factor required for sensitisation is heat stable. As might be expected, sensitisation with a mixture of crude and boiled trypsin resulted in an activity slightly higher than that of the control (crude, untreated trypsin).

When the initial 60-minute incubation with pure trypsin was followed by a brief secondary incubation with the inhibited, crude preparation (Table 23, Experiment 2) activity of the haemagglutinin was increased to well above that of the control. A similar stimulation was noted when the order was reversed — i.e. when a 60-minute incubation with inhibited, crude enzyme was followed by a 5-minute

treatment with pure trypsin. The effect was identical whether the initial digestion time was 60 minutes or 10 minutes.

In all of these experiments, enzyme treatments had no effect on the settling of erythrocytes in the absence of haemagglutinin.

The results presented in Table 23 substantiate the earlier suggestion that a second factor is necessary for sensitisation of erythrocytes by trypsin. In addition, while trypsin activity is necessary, it would appear unlikely that a proteolytic digestion is involved since reduction of the digestion time from 60 min to 10 min did not diminish sensitisation.

It was not possible on the basis of the above experiments to define with certainty the components required for sensitisation. Since an analysis of the haemagglutination reaction was not in accord with the major objectives of the present study, the author decided to use the trypsin preparation which gave maximal sensitisation without requiring two digestion periods or mixing of crude and pure preparations. Consequently crude trypsin at 100mg/ml erythrocyte suspension was used for all subsequent analyses.

The experiments described in this section emphasise the hazards of relating haemagglutination data from different laboratories. Different preparations of trypsin and different erythrocyte suspensions result in different activity values, as is evident from certain of the data presented in the preceding tables. The equalisation of differences by the inclusion of internal controls is thus a necessity, while a valuable contribution towards standardisation of the technique would be made by an analysis of the exact requirements for optimal sensitisation.

3.2 Difficulties associated with evaluation of data.

As indicated previously, the method of evaluating data described by Liener was based on a definition of one haemagglutinating unit (H.U.) as "... that level of test solution which causes 50% of the standard cell suspension to sediment in 2½h under the conditions described ...". While the "level" is somewhat ambiguous, it can be seen from the formula upon which the calculation is based:

$$\log x = \log A + \frac{(E_{50} - RA)}{(RB - RA)} \log 2$$

where A = reciprocal of dilution in tube A (nearest tube having an extinction less than E₅₀)
 RA = extinction of tube A
 RB = extinction of tube B (nearest tube having an extinction greater than E₅₀),

that the primary determinant is A, the reciprocal of dilution in the tube the extinction of which is less than E₅₀. The remaining term in the equation is an algebraic interpolation to allow for the differences of extinction between A and E₅₀, relative to the difference between A and B (the next highest tube). Values for x give the HU/ml, which are converted to HU/mg according to the concentration of the sample.

Thus while it may not be immediately evident from the equation, haemagglutinin units are related directly to the reciprocal of dilution and inversely to the concentration of haemagglutinin. Furthermore the calculation is based on three extinction values – E₅₀, RA and RB. Of these, E₅₀ and RA are the most important, since they determine which tube will in fact be tube A, and hence which will be the dilution on which the calculation is based.

Certain aspects of this method do not commend it as routine procedure for calculating haemagglutinating activity. Apart from the lengthy calculations involved, the dependence on three extinction readings is not desirable, even though these are means of duplicate determinations. Unlike a colorimetric assay, the extinction values in the present assay are subject to deviation from a number of external sources – accidental disturbance of cells, adhesion of cells to tube walls, etc. Thus slight deviations in extinction values are not uncommon and these would be carried through the calculation without any means of detecting them or reducing their effect.

In the alternative evaluation procedure developed by the author, an attempt was made to use a graphical illustration of the decreasing extinction values of each colorimeter tube. By reading the extinction at more than one time, and plotting extinction values against time, the time of coincidence between each tube and E_{50} could be determined by interpolation. The procedure is based on an inverse relationship between weight of haemagglutinin and the time required to sediment 50% of cells (i.e. for extinction to coincide with E_{50}). Obviously, the smaller the weight of haemagglutinin and the shorter the time required the more active the haemagglutinin.

Graphical estimation of a time of intercept between haemagglutinin tubes and the E_{50} tubes reduces the effect of spurious extinction values. Each intercept depends on four readings, and more than one intercept is frequently obtained for each sample. Furthermore visual presentation of results enable stray deviations to be detected with ease.

In the alternative evaluation method, 'time of intercept' becomes a variable. The original method involved a constant time (90 or 150 min) while extinction (relative to E_{50}) was a variable. Thus one of the requirements in the development of the graphical procedure, was to devise a means of relating differences in 'time of intercept'. Since activity is defined (see Chapter II) as the reciprocal of a weight which produces coincidence with E_{50} at 90 minutes, it was necessary to relate times of intercept at less than or greater than 90 minutes, to haemagglutinin units at 90 minutes.

A study of the settling properties of the erythrocyte suspension revealed that the pattern of sedimentation is a complex one which is not easily explained by a mathematical relationship. Thus while at certain time periods the rate of sedimentation is exponential in character, deviation from this form becomes more pronounced at other times. It did not appear possible therefore to relate 'time of coincidence', 'weight of haemagglutinin' and 'HG activity' mathematically, without encountering considerable difficulties. Consequently the author used an empirical alternative to allow for 'time' as a variable in the settling phenomenon. The principle was based on the fact that tubes containing serial dilutions of the same haemagglutinin would have $E = E_{50}$ at different times. Thus weight of haemagglutinin and time of coincidence would be expected to coincide at one value for haemagglutinating activity. This principle was applied to 16 fractions which had been isolated from NRY meal by PEG fractionation (Chapter V, Procedure 1) and to the fraction L which was used as a haemagglutinin standard in the previous section. Ranging in activity from 184 to 3,555 HU/mg (determined by the original method), they provided a means of establishing the feasibility of this approach over the range of activities normally encountered.

After many possible combinations had been tested, a suitable relationship was established. Expressed in nomogram form, 'time' and 'weight of haemagglutinin' appear on parallel scales with directions opposed to give the reciprocal effect. Both scales are logarithmic, although they are not identical. The distances between scales, and the allotment of values on each scale were performed by empirical testing of values from the fractions mentioned previously. Haemagglutinin units are marked off the 'HU' scale from the 'weight of haemagglutinin' scale using the standard time of intercept of 90 minutes. The completed nomogram is presented in Fig. 4.

Comparison of algebraic and graphical methods

The haemagglutinating activity of seventeen NRY protein fractions, calculated by both algebraic and graphical methods, is presented in Table 24. It is evident that with two exceptions the activities are almost identical. Certain differences between the methods, however, became apparent upon closer examination of the data from two fractions, 11 and 13. The activity of Fraction 11 shows little difference whether calculated by the algebraic or graphical methods (2,299 and 2,200 HU/mg respectively). Fraction 13, however, is one of the exceptions in which the activities differ (2,128 and 1350 HU/mg respectively).

Data from which the activity of Fractions 11 and 13 is calculated by the algebraic method, are presented in Table 25. In addition the graphical method of presenting extinction data (Fig. 35) and the data from which activity is calculated (Table 26) enabled comparison to be made between the two evaluation procedures.

Table 24 – Haemagglutinating activity of fractions isolated from NRY meal by polyethylene glycol fractionation (Fractions 1 - 15 and S) and by DEAE-cellulose chromatography (Fraction L)

Activity calculated by algebraic and graphical methods.

Fraction	Haemagglutinating activity (HU/mg)	
	Algebraic Method	Graphical Method
1	184	165
2	667	650
3	441	450
4	590	600
5	465	440
6	499	455
7	436	415
8	658	640
9	2082	1975
10	1804	2300
11	2299	2200
12	1729	1850
13	2128	1350
14	1343	1320
15	1485	1600
S	1482	1500
L	3555	3600

Table 25 – Data used in calculation of haemagglutinating activity of fractions 11 and 13, using algebraic method
Extinction figures represent 10 x true extinction value.

Fraction	A	E50	RA	RB	Activity HU/mg
11	64	3.90	2.91	4.08	2299
13	64	3.68	3.57	3.72	2128

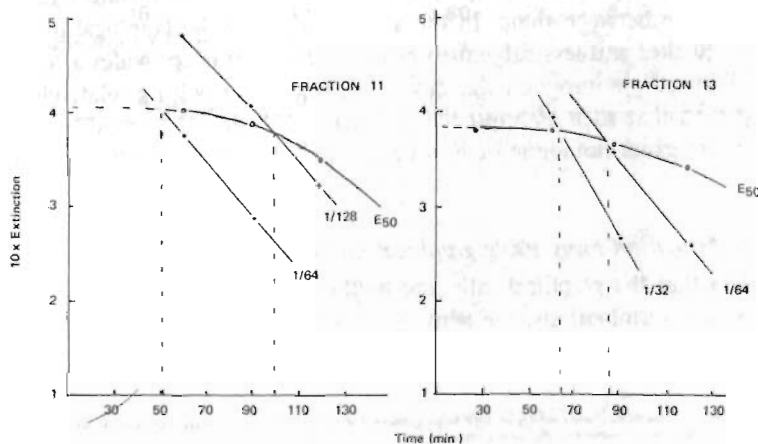


Fig. 35 - Graphical presentation of extinction data for fractions 11 and 13.

Table 26 - Data used in calculation of haemagglutinating activity of fractions 11 and 13, using graphical procedure

Fraction	Initial concentration mg/ml	Dilution	Wt. of fraction in tube μ g	Time of intercept min.	Activity HU/mg	Mean Activity HU/mg
11	0.5	1/640	0.78	52	4,500	
11	0.5	1/1280	0.39	101	2,200	2,200
13	0.5	1/320	1.56	64	1,040	1,040
13	0.5	1/640	0.78	87	1,350	1,350

It is evident from the data that whereas the algebraic method does not distinguish between Fractions 11 and 13 in terms of activity, they are markedly different when the calculation is made graphically. From Table 25 it can be seen that while the fractions have the same 'A'-value, the difference between the extinction of tube A and E₅₀ is smaller in 13 than in 11. Similarly the RB - RA factor is

smaller in Fraction 13 than in 11. Thus the factor $\frac{E_{50} - RA}{RB - RA}$ becomes $\frac{0.99}{1.17}$ in fraction 11 and $\frac{0.11}{0.15}$ in

Fraction 13. Thus although the E₅₀ - RA difference is considerably greater in Fraction 11 than in Fraction 13, the difference is all but eliminated by a low RB value in Fraction 13, which reduces the RB - RA factor.

The graphical presentation in Fig. 35 illustrates the difference between the two fractions. Coincidence with E₅₀ occurs in the 1/64 dilution at 52 min (Fraction 11) and 87 min (Fraction 13). Thus in terms of the graphical method the two fractions are markedly different in potency. Since the algebraic method depends not on consecutive extinction readings from the same tube, but on simultaneous readings from different tubes, the proximity of the next highest dilution to E₅₀ is able to modify the calculated activity. In the graphical method, such a reading would only play a part in the

calculation if a subsequent reading from the same tube were lower than E₅₀, in which case an intercept would be obtained.

Although in general it is difficult to compare the two methods, the previous example has illustrated the basic difference between them. In the author's opinion the graphical procedure, apart from being considerably quicker and less subject to errors in calculation, provides a more realistic basis for estimating activity. While the nomogram was developed empirically with a relatively small number of samples, it has demonstrated that such an approach is feasible. Should other conditions justify the investment of time, a more exact nomogram could be computed mathematically from a larger body of data.

Repeatability of haemagglutination assay using graphical method of evaluation

In order to establish that the graphical intercept method of estimating haemagglutinating activity was repeatable over several determinations, the activity of fraction L was determined in six replicates on each of two days.

Table 27 — Haemagglutinating activity of NRY haemagglutinin Fraction L as determined in six replicates on each of two days, using graphical method of evaluation

Day	Determination	Wt. of sample in tube ug	Time of intercept min	HU/rng	Mean HU/rng
1	1	1.2500	44	3500	3750
		0.3125	84	3750	
	2	1.2500	50	2300	3750
		0.3125	84	3750	
	3	0.6250	59	4200	3550
		0.3125	86	3550	
		0.1562	130	2550	
	4	0.6250	58	4500	3225
		0.3125	82	4100	
		0.1562	129	2350	
	5	0.6250	66	3050	3100
		0.3125	92	3100	
		0.1562	122	2900	
	6	0.6250	60	4000	3600
		0.3125	85	3600	
2	1	1.2500	58	1600	2350
		0.625	71	2500	
		0.3125	109	2200	
	2	1.2500	61	1480	2700
		0.6250	75	2150	
		0.3125	96	2700	
		0.1562	123	2800	
	3	0.6250	72	2450	2950
		0.3125	94	2950	
		0.1562	123	2800	
	4	0.6250	72	2450	2525
		0.3125	98	2600	
	5	0.6250	73	2400	2400
		0.3125	103	2400	
	6	0.6250	68	2800	2800
		0.3125	95	2800	

The results, showing weight of sample required to produce equivalence with E₅₀, time of intercept and HU calculated from the nomogram, are presented in Table 27. The results from Day 1 to

Day 2 are not comparable since the stock solution of Fraction L used on Day 2 had been stored for 24 hours, with consequent loss of activity. Thus the values for Day 2 do not represent the true specific activity of the haemagglutinin; they do however provide an illustration of the variation of readings and the method of calculation in a slightly lower HU range than that used for Day 1.

The data in Table 27 enable two observations to be made:

1. Intercepts at different times by dilutions of the same sample are brought into a fairly narrow range by means of the nomogram. Although deviations with extremes of time can be considerable (e.g. determination 3 of Day 1 — 59 min gave 4,220 HU/mg., while 130 min. gave 2,550 HU/mg), the mean activities calculated as described in Chapter II, do not deviate excessively from each other.
2. There is an element of variability inherent in the system, even within determinations on the same day and with the same erythrocyte suspension. Thus on Day 1 the time of coincidence with E₅₀ for the sample tube containing .3135 μ g of material, varies between 82 and 92 minutes.

The graphical procedure would therefore appear to be a satisfactory means of calculating activity, for in spite of variation in settling rate between replicates, the mean values for activity were within reasonable limits.

APPENDIX 3

CALCULATION OF SEDIMENTATION VELOCITIES

1 PLOT OF LOG X vs T.

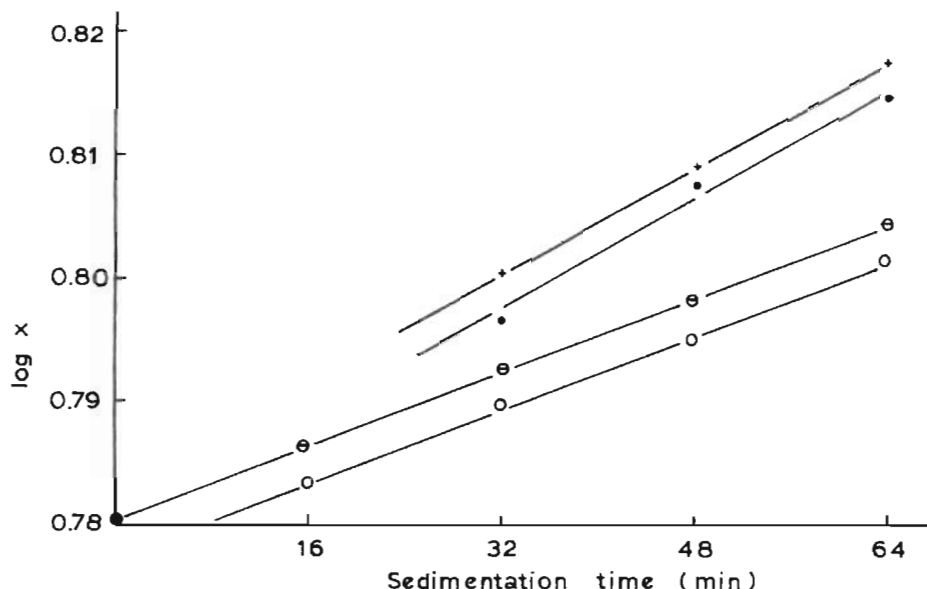


Fig. 36 - Sedimentation velocity plot of peaks obtained from fractions N_1 and $N_1CM_2CM_2$ in analytical ultracentrifuge.

For conditions, see Plate 10.

	$\Delta \log x$
- • - • - N_1U_10358
- o - o - N_1U_20239
- + - + - $N_1CM_2CM_2U_1$0339
- e - e - $N_1CM_2CM_2U_2$0240

2 CALCULATION OF SEDIMENTATION COEFFICIENTS

Example: $N_1CM_2CM_2U_2$

$$S = \frac{2.303 \times .0240}{64 \times 60 \times (2\pi \frac{44770}{60})^2} = 6.548 \times 10^{-13}$$

NOTE: - The minor component (U_1) was present in each case in such small proportion that measurement of distances from the peak maximum proved difficult. Consequently the two estimates of $\Delta \log x$ were combined to provide a mean value from which a sedimentation coefficient was calculated.

APPENDIX 4

ESTIMATION OF AMINO ACID DESTRUCTION IN FRACTION N₁ DURING HYDROLYSIS

Amino acid concentrations at three hydrolysis times are presented in Table 28. The data indicate that losses occurred mainly in threonine, serine and tyrosine, in agreement with the observations of Hirs *et al.* (78) on ribonuclease. Histidine was also found to decrease as hydrolysis proceeded, although Hirs *et al.* reported no similar loss.

Table 28 — Concentration of amino acids in fraction N₁ at three hydrolysis times.

To facilitate comparison, concentrations of basic amino acids were normalized to the mean lysine concentration (2.98), while acidics and neutrals were normalized to the mean glycine concentration (3.51).

Amino Acid	Concentration (g AA/100g protein)		
	22h	46h	70h
Lys	2.98	2.98	2.98
His	1.03	0.90	0.52
NH ₃	1.77	1.94	1.57
Arg	3.60	2.88	2.90
Asp	12.19	12.12	12.30
Threo	5.48	5.23	5.15
Ser	6.08	5.58	5.10
Glut	6.25	6.26	6.27
Pro	2.85	3.06	3.24
Gly	3.51	3.51	3.51
Ala	3.19	3.22	3.24
Cys	0.00	0.00	0.00
Val	5.60	5.73	5.68
Meth	0.00	0.00	0.00
Ileu	3.77	3.76	3.77
Leu	6.84	6.92	5.96
Tyr	1.88	1.80	1.71
Phe	5.75	5.74	5.68

The initial concentrations of the four amino acids were estimated by graphical extrapolation of the concentrations to zero time. The data in Fig. 38 illustrate the relative rates of destruction, from which it is evident that serine was most rapidly destroyed, being reduced by some 22% of the expected original concentration in 70h. Threonine was destroyed by 8.8% and tyrosine by 13%. Although histidine was destroyed less rapidly than serine, the nett destruction after 70h amounted to 59% of the initial histidine concentration.

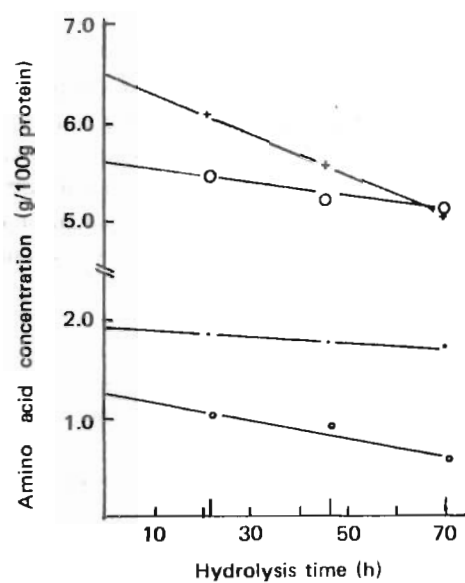


Fig. 37 – Decrease of concentration with time of hydrolysis for histidine (—●—●—); serine (—+—+—); threonine (—○—○—); and tyrosine (—•—•—). Data from Table 28.

SUPPLEMENTARY DATA ASSOCIATED WITH CHAPTER V

1 COMPOSITION OF VITAMIN AND SALT MIXTURES USED IN CASEIN DIETS.

Composition of the vitamin mixture (in g/100g) was as follows:

Thiamine, 0.2; Riboflavin, 0.2; Niacin, 1.0; Ca-pantothenate, 0.8; Pyridoxal-HCl, 0.1; Folic acid, 0.008; Menadione, 0.02; Biotin, 0.004; Mannitol trituration, 0.8; Inositol, 4.0; Ascorbic acid, 2.0; Sucrose, 90.87.

The salt mixture consisted of the following (in g/100g):

CaCO₃, 29.29; CaHPO₄ · 2H₂O, 0.43; KH₂PO₄, 34.31; NaCl, 25.06; MgSO₄ · 7H₂O, 9.98; Fe(C₆H₂O₇) · 6H₂O, 0.62; CuSO₄ · 5H₂O, 0.16; MnSO₄ · H₂O, 0.12; ZnSO₄ · 7H₂O, 0.33; KI, 0.00067; (NH₄)₆ Mo₇O₂₄ · 4H₂O.

2 SUMMARY OF EXTRACTION AND YIELD DATA FOR PEG-PRECIIPITATION EXPERIMENTS.

Data concerning the PEG-precipitation experiments conducted in the present investigation, are summarised in Table 29. The two experiments reported as procedure 1 and procedure 2 appear as the first and last entries respectively in Table 29.

3 INDIVIDUAL PANCREAS WEIGHTS.

The individual pancreas weights from which means were calculated for Table 17 are presented in Table 30.

Notes on pancreas dissection technique.

A feature of the dissection technique used in the present study was that the small amount of water in which the pancreas was dissected, was retained and freeze-dried together with the pancreatic tissue. Thus all soluble proteins were retained, and contributed to pancreatic weight. When compared with a previous technique in which the pancreas was dissected in about 10ml of distilled water, only a small proportion of which was retained for freeze-drying, the pancreas weights presented above were higher, and subject to greater variation. A possible reason for the greater variation is that the water in which the pancreas was dissected, was not controlled exactly. Thus if soluble proteins are to be included in the estimate of pancreas weight, it is advisable that meticulous attention be paid to recovery of the dissection fluid.

Table 29 — Summary of extraction and yield data for PEG-precipitation experiments

In 'Centrifuge' column, I refers to International, C to Cepa centrifuge (details discussed in Chapter V).

EXTRACTION DATA									YIELD g/100g MEAL					REMARKS
Date	Extraction pH	Precipitation pH	Equilibration temp. (°)	PEG concentration range (%)	Dialysis to ppt globulins	Centrifuge	Supernatant recovered	Insoluble residue	Globulins (F ₀)	PEG- precipitated material	Total extracted material	Total yield		
8/5/67 Proce- dure 1	4.2	5.5	30	0 - 7.5	No	I	Yes	—	—	3.88	3.88	3.88	PEG added as 50% solution in buffer	
25/7/67	4.2	5.5	20	0 - 10	No	I	Yes	—	—	5.10	5.10	5.10	Used same stock extract as previous experiment	
12/9/67	4.2	5.5	20	0 - 20	Yes (96h)	C	No	—	2.4	1.03	3.43	3.43		
8/12/67	4.2	5.5	20	0 - 12	Yes (48h)	C	No	93.5	1.2	2.38	3.58	97.08		
26/7/68 Proce- dure 2	7.0	7.0	20	0 - 18	Yes (40h)	C	No	64.1*	2.0	2.21	4.21	68.31	Extraction time reduced from 16 to 4h. Precipitates easily suspended	

*Starch-containing portion of unextracted residue destroyed by fermentation and hence discarded.

Table 30 — Individual pancreas weights from feeding trial conducted in conjunction with PEG precipitation procedure 2

Diet/ Fraction	Wt. of pancreas g/100g body wt.	Diet/ Fraction	Wt. of pancreas g/100g body wt.
Heated	.4153	F ₁	.6323
	.4468		.6782
	.3754		.5153
	.3564	F ₂	.5820
	.3596		.5404
	.3599		.7144
Raw	.7129	F ₃	.6722
	.5818		.6661
	.7606		.7873
Cas	.3927	F ₄	.4975
	.4198		.5318
	.3636		.5124
RES 1	.5095	F ₅	.3941
	.6427		.4813
	.5657		.4621
RES 2	.5712	F ₆	.4989
	.5300		.5695
	.4947		.5033
F ₀	.7655		
	.4310		
	.7623		
	.5420		
	.6994		

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