CHEMICAL, RHEOLOGICAL AND ULTRASTRUCTURAL PROPERTIES
OF A MAJOR ALKALI-SOLUBLE PROTEIN OF RAPESEED

by

THOMAS ALLAN GILL
B.Sc., University of Guelph, 1970
M.Sc., University of Guelph, 1973

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES
DEPARTMENT OF FOOD SCIENCE
UNIVERSITY OF BRITISH COLUMBIA

We accept this thesis as conforming
to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA
September, 1976

© Thomas Allan Gill, 1976
In presenting this thesis in partial fulfilment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the Head of my Department or by his representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Department of Food Science

The University of British Columbia
2075 Wesbrook Place
Vancouver, Canada
V6T 1W5

Date Oct 5, 1976
ABSTRACT

A 12S glycoprotein was isolated from commercial rapeseed meal (*Brassica campestris*) and examined by chemical, microscopical, and rheological methods.

The molecular weight of the protein was estimated to be 129,200 daltons by conventional sedimentation equilibrium ultracentrifugation although the presence of higher molecular weight material was detected in the preparation. The 12S protein was found to be oligomeric, dissociating into low molecular weight fragments in the presence of urea or sodium dodecyl sulfate. The protein aggregate was separated into subunits with apparent molecular weights of approximately 42,000, 37,600, 30,100, 17,400 and 12,200 by SDS gel electrophoresis. Electrophoretic patterns of non-reduced and reduced samples indicated the presence of intermolecular disulfide bonds although the cystine content was low.

The 12S protein contained 12.9% (w/w) carbohydrate and reacted strongly when oxidized and treated with Schiff reagent. PAS-treated SDS gels indicated that most of the carbohydrate was present in one low molecular weight fragment. SDS immunoelectrophoretic analysis suggested that the glycopeptide portion is located on the surface of the complex.

Although the isolate contained a high molecular weight contaminant (17S), immunoelectrophoretic analysis
resulted in the formation of one homogeneous pair of precipitin arcs. This would suggest that the 12S protein self-associates to form aggregates of higher molecular weight. In an attempt to separate the 17S and 12S fractions by gel filtration, a 33.9S protein was isolated, presumably the product of a self-associating system. SDS electrophoretic patterns of the 33.9S and 12S proteins were similar.

Histochemical studies revealed that the 12S glycoprotein was present in some but not all of the cells of the intact rapeseed kernel. Schiff-positive aleurones were distributed randomly throughout the kernels. Transmission electron microscopy of negatively-stained specimens revealed that the protein was morula-shaped with a maximum particle diameter of 120Å.

The 12S rapeseed protein formed gels when dispersions of this material were heated. The rheological and ultrastructural examination of this phenomenon revealed that gel structure depended upon pH and ionic strength but to a lesser degree on low levels of urea or dithiothreitol.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF TABLES</td>
<td>vii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>viii</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>xi</td>
</tr>
<tr>
<td>I  INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>II LITERATURE REVIEW</td>
<td>3</td>
</tr>
<tr>
<td>III EXPERIMENTAL</td>
<td>6</td>
</tr>
<tr>
<td>A. Microstructure and Ultrastructure of the Rapeseed Kernel (\text{Brassica campestris}) and Rapeseed Meal</td>
<td>6</td>
</tr>
<tr>
<td>1. Light microscopy</td>
<td>6</td>
</tr>
<tr>
<td>2. Electron microscopy</td>
<td>7</td>
</tr>
<tr>
<td>B. Recovery of the 12S Protein from Rapeseed Meal</td>
<td>7</td>
</tr>
<tr>
<td>1. Alkali extraction</td>
<td>7</td>
</tr>
<tr>
<td>2. Gel filtration</td>
<td>8</td>
</tr>
<tr>
<td>C. Chemical Characterization of the 12S Rapeseed Globulin</td>
<td>9</td>
</tr>
<tr>
<td>1. Amino acid analysis</td>
<td>9</td>
</tr>
<tr>
<td>2. Determination of the component sugars of the 12S glycoprotein</td>
<td>9</td>
</tr>
<tr>
<td>a) Phenol-sulfuric acid method</td>
<td>10</td>
</tr>
<tr>
<td>b) Sialic acid determination</td>
<td>11</td>
</tr>
<tr>
<td>c) Gas chromatography</td>
<td>12</td>
</tr>
<tr>
<td>d) Hexosamine determination</td>
<td>16</td>
</tr>
<tr>
<td>3. Ultracentrifugation</td>
<td>16</td>
</tr>
<tr>
<td>a) Sedimentation velocity</td>
<td>16</td>
</tr>
<tr>
<td>b) Sedimentation equilibrium</td>
<td>17</td>
</tr>
</tbody>
</table>
4. Disc gel electrophoresis
   a) Davis method
   b) Electrophoresis in urea
   c) Disc immunoelectrophoresis and immunodiffusion
   d) Electrophoresis in the presence of sodium dodecyl sulfate
   e) SDS gel immunoelectrophoresis

5. Gel filtration on Sepharose 6B and examination of a high molecular weight component

D. Microstructural and Ultrastructural Examination of the 12S Glycoprotein
   1. Light microscopy
   2. Transmission electron microscopy
   3. Scanning electron microscopy

E. Rheological Properties of Dispersions and Thermally-Induced Gels of the 12S Glycoprotein
   1. Sample preparation
   2. Rheological characterization

IV RESULTS AND DISCUSSION
   A. Microstructure and Ultrastructure of the Rapeseed Kernel (Brassica campestris) and Rapeseed Meal
   B. Recovery of the 12S Protein from Rapeseed Meal
   C. Chemical Characterization of the 12S Rapeseed Globulin
      1. Amino acid composition
      2. Carbohydrate composition
3. Ultracentrifugation ..................................... 52
4. Disc gel electrophoresis .............................. 56
5. Gel filtration on Sepharose 6B and examination of a high molecular weight component ............................. 68

D. Microstructural and Ultrastructural Examination of the 12S Glycoprotein .......................... 70
  1. Light microscopy ....................................... 70
  2. Transmission electron microscopy .................... 73
  3. Scanning electron microscopy ......................... 77

E. Rheological Properties of Dispersions and Gels of the 12S Glycoprotein .................. 80

V SUMMARY AND CONCLUSIONS ............................... 104
REFERENCES .................................................. 107
APPENDIX I ................................................ 114
APPENDIX II ............................................. 116
APPENDIX III ........................................... 119
APPENDIX IV ........................................... 123
APPENDIX V ........................................... 127
## LIST OF TABLES

<table>
<thead>
<tr>
<th>TABLE</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>MOLECULAR WEIGHT MARKERS FOR SDS ELECTROPHORESIS</td>
</tr>
<tr>
<td>II</td>
<td>AMINO ACID COMPOSITION OF THE 12S GLYCOPROTEIN FROM RAPESEED MEAL</td>
</tr>
<tr>
<td>III</td>
<td>MONOSACCHARIDE AND AMINO ACID COMPOSITION OF THE 12S GLYCOPROTEIN FROM RAPESEED MEAL (B. CAMPESTRIS L. VAR. SPAN)</td>
</tr>
<tr>
<td>IV</td>
<td>POWER-LAW PARAMETERS FOR STEADY SHEAR FLOW BEHAVIOR OF AQUEOUS 12S GLOBULIN DISPERSIONS AT 25°C AND pH 9.2 (EXPERIMENT I)</td>
</tr>
<tr>
<td>V</td>
<td>POWER-LAW PARAMETERS FOR STEADY SHEAR FLOW BEHAVIOR OF 4.5% 12S GLOBULIN GELS AT 23°C (EXPERIMENT II)</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Hydrolysis tube prepared from a 16 x 125 mm culture tube</td>
<td>13</td>
</tr>
<tr>
<td>II</td>
<td>Light micrograph of a section of rapeseed stained by PAS method</td>
<td>34</td>
</tr>
<tr>
<td>III</td>
<td>Light micrograph of rapeseed tissue stained with toluidene blue and showing the protein-rich aleurone grains</td>
<td>34</td>
</tr>
<tr>
<td>IV</td>
<td>Light micrograph of a rapeseed kernel tissue cell rich in PAS-positive aleurone grains</td>
<td>37</td>
</tr>
<tr>
<td>V</td>
<td>Light micrograph of commercial rapeseed meal stained by the PAS procedure</td>
<td>37</td>
</tr>
<tr>
<td>VI</td>
<td>Electron micrograph of rapeseed showing internal cellular structure</td>
<td>38</td>
</tr>
<tr>
<td>VII</td>
<td>Electron micrograph demonstrating the aleurone grain surrounded by a single membrane and lipid bodies</td>
<td>38</td>
</tr>
<tr>
<td>VIII</td>
<td>Flow diagram for the preparation of 12S rapeseed isolate and concentrated dispersions</td>
<td>41</td>
</tr>
<tr>
<td>IX</td>
<td>Elution profile of the gel filtration of crude rapeseed extracts on Sephadex G-100 in 0.1M borate buffer pH 9.2</td>
<td>41</td>
</tr>
<tr>
<td>X</td>
<td>A typical gas chromatogram of the alditol acetate derivatives of the neutral and amino sugars in the 12S glycoprotein</td>
<td>46</td>
</tr>
<tr>
<td>XI</td>
<td>Schlieren patterns of undissociated and dissociated 12S protein extracted from commercial rapeseed meal</td>
<td>53</td>
</tr>
<tr>
<td>XII</td>
<td>Densitometric scan of a disc gel of the 12S glycoprotein isolated from commercial rapeseed meal</td>
<td>60</td>
</tr>
<tr>
<td>XIII</td>
<td>Photograph of the disc immunoelectrophoresis pattern of the 12S rapeseed glycoprotein which was diffused against rooster anti-12S antiserum</td>
<td>60</td>
</tr>
<tr>
<td>XIV</td>
<td>Densitometric scan of an SDS disc gel (12S glycoprotein)</td>
<td>63</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>-----------------------------------------------------------------------------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>XV</td>
<td>Densitometric scans of SDS gels which were run with unreduced dissociated 12S glycoprotein and 12S protein which was reduced with 0.15M 2-mercaptoethanol</td>
<td>63</td>
</tr>
<tr>
<td>XVI</td>
<td>Upper. A photograph of a Schiff-stained SDS gel showing the PAS-positive fragment. Lower. A densitometric scan of a PAS-treated gel showing the position of the carbohydrate-containing band</td>
<td>67</td>
</tr>
<tr>
<td>XVII</td>
<td>The precipitin arcs formed by the PAS-positive fragment of the 12S protein in Ionagar No. 2</td>
<td>67</td>
</tr>
<tr>
<td>XVIII</td>
<td>The elution profile of a concentrate of 12S rapeseed glycoprotein</td>
<td>69</td>
</tr>
<tr>
<td>XIX</td>
<td>Densitometric scans of SDS gels loaded with 12S and 33.9S rapeseed protein fractions prepared by chromatography on Sepharose 6B</td>
<td>69</td>
</tr>
<tr>
<td>XX</td>
<td>Light micrograph of hydrated 12S rapeseed protein isolate oxidized with periodic acid and stained with Schiff’s reagent</td>
<td>71</td>
</tr>
<tr>
<td>XXI</td>
<td>PAS-positive rapeseed protein present as globular masses within the water droplet</td>
<td>71</td>
</tr>
<tr>
<td>XXII</td>
<td>Light micrograph showing the water-protein interface</td>
<td>72</td>
</tr>
<tr>
<td>XXIII</td>
<td>Electron micrograph of a positively-stained section of hydrated isolate</td>
<td>74</td>
</tr>
<tr>
<td>XXIV</td>
<td>Electron micrograph of a positively-stained section of isoelectric precipitate of rapeseed protein alkaline extract</td>
<td>74</td>
</tr>
<tr>
<td>XXV</td>
<td>Electron micrograph of 12S rapeseed glycoprotein (negatively-stained)</td>
<td>76</td>
</tr>
<tr>
<td>XXVI</td>
<td>A higher magnification of the 12S glycoprotein aggregate</td>
<td>76</td>
</tr>
<tr>
<td>XXVII</td>
<td>Scanning electron micrographs of thermally-induced, cryofractured gels formed from 4.5% dispersions of 12S rapeseed glycoprotein</td>
<td>78</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>-----------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>XXVIII</td>
<td>Higher magnifications of gel structure formed from heated 4.5% rapeseed</td>
<td>79</td>
</tr>
<tr>
<td></td>
<td>protein dispersions showing the effect of pH on the 3-dimensional ordering</td>
<td></td>
</tr>
<tr>
<td>XXIX</td>
<td>Rheograms of 12S protein isolate dispersions in 0.01M borate buffer pH 9.2</td>
<td>83</td>
</tr>
<tr>
<td>XXX</td>
<td>Rheograms indicating the effects of heat and PCMB on viscosity of 1% dispersions of 12S protein isolate at pH 9.2</td>
<td>84</td>
</tr>
<tr>
<td>XXXI</td>
<td>Rheograms of pH 9.2 12S protein gels (4.5%) measured under steady shear at 23°C demonstrating the effects of urea, dithiothreitol, NaCl and ageing in the cold</td>
<td>87</td>
</tr>
<tr>
<td>XXXII</td>
<td>Rheograms of 4.5% rapeseed protein gels measured under steady shear at 23°C demonstrating the effects of pH and reductive alkylation</td>
<td>91</td>
</tr>
<tr>
<td>XXXIII</td>
<td>Time profile of a dynamic shear experiment on a viscoelastic material as measured with the Weissenberg Rheogoniometer</td>
<td>96</td>
</tr>
<tr>
<td>XXXIV</td>
<td>Dynamic shear storage moduli as a function of oscillatory frequency for 4.5% rapeseed protein gels at pH 9.2 and 23°C demonstrating the effects of urea, dithiothreitol, NaCl and reductive alkylation</td>
<td>97</td>
</tr>
<tr>
<td>XXXV</td>
<td>Dynamic shear loss moduli as a function of oscillatory frequency for 4.5% rapeseed protein gels at pH 9.2 and 23°C demonstrating the effects of urea, dithiothreitol, NaCl and reductive alkylation</td>
<td>99</td>
</tr>
<tr>
<td>XXXVI</td>
<td>Dynamic shear storage moduli as a function of oscillatory frequency for 4.5% rapeseed protein gels prepared at various pH levels and measured at 23°C</td>
<td>101</td>
</tr>
<tr>
<td>XXXVII</td>
<td>Dynamic shear loss moduli as a function of oscillatory frequency for 4.5% rapeseed protein gels prepared at various pH levels and measured at 23°C</td>
<td>102</td>
</tr>
</tbody>
</table>
ACKNOWLEDGEMENTS

I wish to express my gratitude to Dr. M.A. Tung for his aid and encouragement throughout the course of this study. I would also like to thank Mrs. Pam Gill for her help in numerous activities associated with the electron microscope. I feel that I have been particularly fortunate in choosing a graduate committee whose members have sacrificed not only their time but have also supplied me with numerous pieces of equipment. In particular, I would like to thank Dr. R. Fitzsimmons for furnishing the laboratory animals used in the immunological study.

Last but not least, I would like to dedicate this thesis to my wife Jeanette for her many sacrifices during my years of graduate study and for her work in preparing the manuscript.
Rapeseed (*Brassica napus* and *Brassica campestris*) is the major oilseed crop grown in Canada and this country now leads the world in rapeseed exports (Downey *et al.*, 1974). While at the present time, the seed is crushed for its high oil content (up to 45% on dry basis), the current interest in plant protein sources may make rapeseed attractive for its protein as well since levels of 20% (dry basis N x 5.53) are common. Until recently, the future for rapeseed as an inexpensive protein source for human nutrition appeared doubtful due to toxic factors present in the protein. As a result, the defatted meal which is a by-product of the vegetable oil industry has been used only at low levels as an animal meal supplement. The toxic factors, thiocyanates and isothiocyanates, are formed in the meal by the enzymatic breakdown of glucosinolates which are ordinarily found in the seeds. Several techniques suggested for the detoxification of the protein (Armstrong and Stanley, 1975; Woyewoda and Nakai, 1974; and Ohlson, 1973) as well as recent advances in plant breeding (Agriculture Canada, 1974), have made this material more attractive as a potential ingredient for food manufacture.

In order to utilize a protein isolate or concentrate effectively, both physical and chemical characterization of the individual proteins are necessary. Numerous studies of this nature have been reported in the literature concerning
the constitutive proteins of soybean, for example, the most recent by Badley et al. (1975). The rapeseed protein selected for this study was the 12S glycoprotein described by Bhatty et al. (1968), Finlayson et al. (1969), and Goding et al. (1970). This protein, isolated by previous investigation, was extracted from seed rather than commercial meal and constituted approximately 21% of the salt-soluble nitrogen. The purpose of the present study was to develop a method for the isolation of the 12S globulin from meal in sufficient quantities to permit chemical, physical, and ultrastructural characterization which would provide an insight into possible functional qualities of this oilseed globulin.
II LITERATURE REVIEW

Bhatty et al. (1968) extracted the proteins of rapeseed with 0.01M sodium pyrophosphate (pH 7.0) and separated them into 9 chromatographically different fractions. Most of the total rapeseed nitrogen was found in 2 fractions, a neutral high molecular weight protein and a basic 1.7S fraction with a molecular weight of 13,800. The larger protein had an observed sedimentation coefficient of 12S in 0.1M borate buffer (pH 8.6). The 12S protein was also recovered in a 10% NaCl extract of rapeseed and represented 21% of the nitrogen recovered. This water-insoluble protein was chromatographically and electrophoretically pure at alkaline pH's but dissociated into subfractions at pH values below 3.5 and in urea solutions. In addition to the 3S and 7S fragments which appeared in the ultracentrifuge patterns even at alkaline pH's, a 17S component was detected in the 12S globulin and was considered a contaminant.

In another study, Finlayson et al. (1969) reported differences in the amino acid compositions of the 12S proteins isolated from different species and varieties of rapeseed. Although the method of recovery was the same as that reported by Bhatty et al. (1968), the 12S isolate in the second study contained almost 2% more nitrogen than in the first study although no apparent reason for this phenomenon was given. There were considerable differences in cystine and methionine
contents noted between species, and cultivars within the same species. It was concluded that because obvious differences did occur in structurally-important amino acids, the 12S proteins from the different species probably had different structures although similar sedimentation coefficients were observed.

Further characterization of the 12S rapeseed globulin was reported by Goding et al. (1970). A different technique for the isolation of the protein was outlined in their study. The new procedure involved the extraction of protein in a ball mill using 0.1M borate buffer (pH 9.2) containing 1M sodium chloride. The insoluble residue and lipid were separated from the soluble material by centrifugation and the 12S protein enriched by high speed centrifugation at 110,000 x g. Final purification was carried out by gel chromatography on Sephadex G-100. The 12S protein was dissociated and chromatographed at pH 2.8 in 2M urea. Four fractions were collected from dissociated 12S proteins prepared from *B. napus* L. var. Target and *B. campestris* L. var. Echo. Cationic gel electrophoresis in 6M urea resulted in the separation of 7 components from the dissociated complex. It was concluded that cystine was present intramolecularly rather than intermolecularly since mercaptoethanol did not effect electrophoretic or chromatographic separation. The major component of the 12S complex, a 2.7S protein, was composed of 2 polypeptide chains and contained most of the carbohydrate.
The 12S glycoprotein complex contained 0.15 - 0.20% galactosamine and 1 - 1.5% glucose and arabinose.

Similar studies have been reported on other members of the genus *Brassica*. MacKenzie and Blakely (1972) reported the recovery of the 12S globulins from *B. juncea*, *B. nigra* and *B. hirta*. Besides purification procedures described by previous authors, the 12S fraction was chromatographed on Sephadex G-200 (superfine). Species differences in amino acid composition of both the 12S and 1.8S proteins were analyzed by a multivariate statistical technique. MacKenzie (1975) reported that the impurities separated from the 12S protein in the 1972 study had amino acid compositions which were indistinguishable from the major component. The author suggested that such "impurities" actually represented products of a self-associating system. According to MacKenzie, mercaptoethanol did effect the dissociation of the 12S complex isolated from *B. juncea* although no effect was observed at levels lower than 0.1M. The dissociated, reduced 12S protein of *B. juncea* was separated into five acidic, five basic and one neutral component by preparative isoelectric focussing. The basic components were present in much greater amounts than the acidic components although amino acid analyses of the basic fractions indicated that aspartic and glutamic acids were present in greater abundance than basic amino acids, suggesting that the former acids were presumably in the amide forms of asparagine and glutamine.
III EXPERIMENTAL

A. Microstructure and Ultrastructure of the Rapeseed Kernel (Brassica campestris) and Rapeseed Meal.

1. Light microscopy

The objective of this study was to elucidate the microstructure of the rapeseed kernel, to locate the protein-rich components and to observe the effects of commercial solvent extraction and drying on the meal. Intact seeds and meal were fixed for 1 h in 2.5% glutaraldehyde in Sorensen's phosphate buffer (Sorensen, 1909) pH 7.0. The seeds were split while immersed in the fixative. Samples were dehydrated in a series of ethanol solutions (30, 50, 70, 95, 100%), each change lasting for 10 min. After 2 or 3 changes of 100% ethanol, samples were infiltrated and embedded in Spurr's epon (Spurr, 1969). Thick (1 µm) sections were cut on a Reichert Om U3 ultramicrotome and heat fixed to glass slides. Slides were placed in saturated ethanolic-KOH for 20 min to dissolve the embedding material and rinsed exhaustively, first with tap water and then with distilled water. Slides were stained with Schiff's reagent (Sheehan and Hrapchak, 1973) for 15 min in order to examine any periodic acid Schiff (PAS) positive material. The slides were rinsed three times in 2% sodium metabisulfite, each rinse lasting for about 2 min, and then counterstained with hematoxylin. Thick sections of seeds were
also stained for proteinaceous material with toluidene blue for approximately 1 min and then rinsed with tap water. Prepared slides receiving the above treatments were examined using a Wild M20 microscope equipped with a 35 mm camera system.

2. Electron microscopy

The preparative methods for electron microscopical study of ultrathin sections of seed kernels were identical to those described for light microscopy except that glutaraldehyde fixation was followed by a 60 min fixation with 1% OsO₄ in pH 7.0 phosphate buffer. Silver sections were cut with a Reichert Om U3 ultramicrotome using freshly broken glass knives. Sections were mounted on uncoated 300 mesh copper grids and stained with uranyl acetate (Watson, 1958) and lead citrate (Reynolds, 1963). Micrographs were taken on an AEI Corinth 275 transmission electron microscope operating at a 60 kV accelerating voltage.

B. Recovery of the 12S Protein from Rapeseed Meal.

1. Alkali extraction

Commercial defatted rapeseed meal (Brassica campestris) was extracted for 12 h at 4°C with 0.1M borate buffer pH 9.2 (1 part meal: 9 parts buffer). Extraction was carried out on a Fisher Thermix magnetic stirrer with the speed adjusted
such that the slurry was moving slowly enough to prevent foam formation on the surface. The crude extract was centrifuged at 20,000 x g for 30 min at 4°C in a Sorvall RC2-B refrigerated centrifuge. The supernatant was filtered through Whatman number 1 filter paper and applied to a Sephadex G-100 column.

2. Gel filtration

Fifty ml aliquots of crude extract were applied to an 82 x 5 cm Sephadex G-100 column and eluted with 0.1M borate buffer pH 9.2 at 2 ml/min. The first peak contained the 12S fraction and was dialyzed against water at 4°C for 72 h. The brown color characteristic of alkaline extracts of rape-seed was removed completely after this time, leaving a white water-insoluble dispersion in the dialysis bag. The dispersion was then either pervaporated to a desired concentration for rheological characterization or cryogenically frozen and lyophilized.

The void volume of the column was determined with Blue Dextran 2,000 (Pharmacia Fine Chemicals). Elution patterns were observed as absorbance at 280 nm. Protein contents of meal, crude extracts and 12S fractions were determined by the rapid micro Kjeldahl method of Concon and Soltess (1973), (%N x 6.25).

The 12S fraction was identified as such in 0.1M borate buffer pH 9.2 by sedimentation velocity ultracentrifugation
as described in Section III.3.a.

C. Chemical Characterization of the 12S Rapeseed Globulin.

1. Amino acid analysis

Samples of freeze-dried 12S isolate were derivatized with 4-vinylpyridine by a method described by Cavins et al. (1972). The authors found that the S- β-(4-pyridylethyl)-L-cysteine formed from the selective alkylation of cystine and cysteine residues, was stable under the conditions of acid hydrolysis. The alkylated protein was hydrolyzed with p-toluenesulfonic acid in glass ampoules which were heat sealed under a vacuum of 20 to 30 μm Hg. Samples were hydrolyzed for 24, 25, 26, 27, 28 and 36 h at 110°C. Hydrolysis in p-toluenesulfonic acid was suggested by Liu and Chang (1971) as a means of protecting tryptophan from hydrolytic destruction. Samples and standards were analyzed on a Hitachi Model KLA-3B amino acid analyzer utilizing a single column elution system. Details of the sample preparation are described in Appendix I.

2. Determination of the component sugars of the 12S glycoprotein

It would be reasonable to expect that a large amount of carbohydrate chemically bound to protein molecules would
have a pronounced effect on the physical behavior of the macromolecules. Glycoproteins generally exhibit lower partial specific volumes than proteins containing no carbohydrate (Gibbons, 1966). The anomalous behavior of glycoproteins in sodium dodecyl sulfate electrophoresis becomes more pronounced as the carbohydrate content for a given glycoprotein increases (Segrest and Jackson, 1972). It is believed that carbohydrate side chains tend to radiate outward from the amino acid backbone in some glycoproteins (Morawiecki, 1964 and Winzler, 1969). As a result of its hydrophilic nature, the carbohydrate portion of the macromolecular complex is often responsible for the immunological identity of the structure. Such molecules would be expected to show a relatively high degree of protein-protein interaction in solution as well as apparent viscosities which are highly shear dependent (non-Newtonian). A detailed analysis of the sugar components of the 12S glycoprotein was undertaken in order to aid in the explanation of chemical and physical properties.

a) Phenol-sulfuric acid method.

Initially, an approximate carbohydrate content was determined by the phenol-sulfuric acid method of Dubois et al. (1956). Since the identification of the carbohydrate component was not carried out at this time, the content was estimated from a standard curve for glucose.
b) Sialic acid determination.

A preliminary examination of electrophoretic mobility in urea led to the suspicion that an acid carbohydrate may be present on the surface of the 12S complex occluding a basic amino acid backbone. The presence of sialic acid was suspected because the undissociated complex migrated toward the anode, however, the dissociated fragments appeared to be neutral or basic in nature. Sialic acid content was determined by the method of Warren (1959). Acid hydrolysis was carried out in 16 x 125 mm screw cap test tubes. Samples of the 12S protein and of bovine thyroglobulin (Sigma) weighing between 10 and 20 mg were dissolved in 10 ml 0.1N H₂SO₄. Hydrolysis was carried out for 1 h at 80°C in a water bath. Tubes were cooled and 0.2 ml of each sample were mixed with 0.1 ml of solution containing 0.2M sodium metaperiodate in 9M phosphoric acid. After 20 min at ambient temperature, 1 ml of sodium arsenite solution (10% sodium arsenite in a solution of 0.5M sodium sulfate and 0.1N H₂SO₄) was added and the tube shaken until the yellow color disappeared. Three ml of a thio-barbituric acid solution (0.6% thiobarbituric acid in 0.5M sodium sulfate) were added and the sample tubes placed in a boiling water bath for 15 min. During this time, the chromogen developed (a complex between β-formyl pyruvic acid produced from sialic acid oxidation and thiobarbituric acid) in samples containing sialic acid. Tubes were cooled and 4.3 ml cyclo-hexanone were added and shaken to extract the chromogen. The
tubes were then centrifuged at 1,000 x g for 2 min and the solvent layer read at 549 nm in a Beckman DB spectrophotometer. A standard curve was prepared for standards containing between 0 and 20 μg sialic acid (Sigma).

c) Gas chromatography.

Analyses for total neutral and amino sugars were carried out using a modification of the procedure described by Porter (1975). The major advantages to gas-liquid chromatographic analysis of the sugars as their alditol acetate derivatives were that both neutral and amino sugars could be analyzed simultaneously and that multiple peaks for each sugar, as a result of anomeration and ring isomerization, were avoided. The method described by Porter utilized an hydrolysis procedure in which the sugars were liberated from the protein in the presence of a cation exchange resin and the amino sugars released from the resin through a nitrous acid deamination reaction. Because several modifications of the procedure were incorporated, details of the technique are described.

i) Hydrolysis. Samples weighing between 2 and 3 mg were placed in hydrolysis tubes consisting of 16 x 125 mm screw cap culture tubes which had been drawn out to approximately 20 mm in length (Figure I). A 3 mm hole was drilled in the center of each cap and the cardboard cap liners replaced with silicon rubber septa. To each vial was added 100 μl of a
FIGURE I: Hydrolysis tube prepared from a 16 x 125 mm culture tube.

40% (w/v) suspension of AG 50W-X2 (H⁺ form) 200-400 mesh ion exchange resin (Bio-Rad Laboratories) in 0.02N HCl, followed by 100 μl distilled H₂O. The vials were placed in a 100 ±2°C oven for 40 h. In order to assess completeness of hydrolysis, two samples were hydrolyzed for an extra 12 h. In initial runs, 0.4 μmoles of myoinositol were added to each vial in a 50 μl injection as an internal standard. It soon became apparent that inositol was also present in the sample, therefore, xylose was substituted as an internal standard in sub-
sequent runs.

ii) Derivatization. The samples and standards were deaminated with the addition of 20 μl of a freshly prepared 5.5M solution of NaN₂O₂. After intermittent vortexing for a period of 30 min, 200 μl of a 40% aqueous suspension of AG 50W-X2 (H⁺ form) resin were added and intermittent vortexing continued for 30 min. The liberated sugars were then separated from the protein components by quantitatively transferring the samples to ion exchange columns prepared from Pasteur pipets plugged with glass wool and filled with 0.4 ml of a 40% aqueous suspension of AG 50W-X2. The pipets were arranged such that each pipet would drain into a second pipet filled with 1 ml of a 20% aqueous suspension of 200-400 mesh AG 1-X8 (Cl⁻ form prepared from formate form). Hydrolysis tubes were rinsed three times with 0.4 ml distilled water and transferred to the columns. The columns were then washed with 0.8 ml of 50% methanol in water and the effluents collected in test tubes. The sugar solutions were dried under a stream of nitrogen and reduction carried out by the addition of 100 μl distilled water and 100 μl 0.22M NaBH₄. After 1 h, excess borohydride was decomposed with the addition of 40 μl glacial acetic acid and the samples dried under nitrogen. Borate was volatilized with the addition of four 200 μl portions of methanol-concentrated HCl (1,000:1) with concentration to dryness after each addition. Acetylation of the sugars was carried out with the addition of 100 μl pyridine.
and 100 μl acetic anhydride. The tubes were sealed with rubber stoppers wrapped in Teflon tape, placed in a 100°C oven for 15 min, vortexed, and heated for an additional 15 min.

iii) **Analysis.** Portions (5 μl) of the samples and standards were chromatographed on a Tracor MT220 gas chromatograph equipped with 1/4 in x 6 ft dual silanized glass columns packed with 3% ECNSS-M on 100/120 mesh Gas Chrom Q (Applied Science Laboratories) as suggested by Laine et al. (1972). The injection port temperature was 230°C and the flame ionization detector was 280°C. Oven temperature was programmed from 150°C to 190°C at 1°C min⁻¹. The nitrogen carrier gas was adjusted to 40 ml min⁻¹ and all samples were applied via on-column injection. Alditol acetate derivatives were identified by retention times as compared to standards prepared from reagent grade sugars. The fact that xylose eluted at exactly the same time as glucosamine precluded the use of xylose as an internal standard. Standard curves relating the peak areas to sugar concentrations were prepared and samples were chromatographed on the same day as the standards. Standards were analyzed in quadruplicate and three concentrations of standard mixtures run in duplicate, both before and after samples had been run. Peak areas were integrated with an electronic planimeter (Numonics Corp.).
d) Hexosamine determination.

Since glucosamine could not be resolved from xylose by gas chromatography, and since no other amino sugars were detected (galactosamine and mannosamine were resolvable on the 3% ECNSS-M column), the colorimetric method of Elson and Morgan (1933) was used to detect glucosamine. Hydrolysis of the glycoprotein was carried out as described for gas chromatography and separation of the amino sugars from the neutral sugars was carried out according to the ion exchange method of Boas (1953) in which the amino sugars were adsorbed to a cation exchange column and subsequently eluted with 2N HCl. The only modification of this procedure was the substitution of AG 50W-X2 (Bio-Rad Laboratories) for Dowex 50-X4 resin. The Elson-Morgan reaction and the hydrolysis procedure are outlined in detail in Appendix II.

3. Ultracentrifugation

a) Sedimentation velocity.

A sample of approximately 1% 12S protein in 0.1M borate buffer pH 9.2 was run at 55,000 rpm in a Beckman L2-65B ultracentrifuge equipped with a 2-place An-D analytical rotor. The mobility of the protein was observed through a schlieren optical system and sedimentation patterns recorded photographically. Similar runs were performed in 0.1M phosphate
buffers pH 7.0 adjusted to 5M and 6M urea and 0.1% 2-mercaptoethanol.

b) Sedimentation equilibrium.

Nitrogen determinations were performed upon a stock solution of 12S protein in 0.1M borate buffer, pH 9.2. Serial dilutions of the stock solution were prepared in the same buffer and the absorbance readings at 280 nm were plotted against protein concentrations (%N x 6.25). Equilibrium runs were performed at 7,800 rpm and 20°C in the L2-65B ultracentrifuge equipped with a 4-place An-F analytical rotor and a Beckman Prep UV Scanner. The observed molecular weights for six concentrations were calculated and an $M_0$ (molecular weight at infinite dilution) determined from a least squares fit of the $M$ vs. %N data.

4. Disc gel electrophoresis

a) Davis method.

In order to assess the degree of electrophoretic homogeneity of the isolate, a solution of the freeze-dried material was subjected to polyacrylamide disc gel electrophoresis by the method of Davis (1962). This system stacked at pH 8.9 and ran at pH 9.5 on 7% acrylamide gels. Bromphenol blue was used as a tracking dye in the upper electrode buffer.
Details of the gel preparation are given in Appendix III. All gels were run in a Pharmacia gel electrophoresis apparatus Model GE-4 at 5 mA per tube. Gels were stained with 0.25% Coomassie blue dissolved in a mixture of 5% methanol and 7% acetic acid for a period of 8 - 12 h. Gels were destained in a Pharmacia gel destainer, Model GD-4 with a mixture of 5% methanol in 7% acetic acid. Gels were scanned in a Transidyne TG 2980 automatic scanning densitometer at 550 nm.

b) Electrophoresis in urea.

The electrophoretic buffer system of Weber and Osborn (1969) was modified by the incorporation of 5M deionized urea and 0.1% 2-mercaptoethanol into gels and electrode buffer to gain some information concerning the overall charge of the urea-denatured fragments of the 12S agglomerate. Both anionic and cationic runs were performed. An electrophoretic system of Reisfeld and Small (1966) which ran at pH 9.4 in the presence of 10M urea was also used to observe the electrophoretic behavior of the denatured 12S protein.

c) Disc immunoelectrophoresis and immunodiffusion.

Immunoelectrophoresis and immunodiffusion have been used to study the taxonomy of several of the species of the genus Brassica (Vaughan et al., 1966; Vaughan and Waite, 1967; and Vaughan and Denford, 1968). A technique termed "disc
immunoelectrophoresis" by Catsimpoolas and Meyer (1968), and Catsimpoolas et al. (1968) was used to establish the homogeneity of the 11S soybean protein and to follow changes in reserve proteins in germinating soybeans. Disc immunoelectrophoresis was undertaken in the present study to establish the immunological identity of the 12S rapeseed protein.

i) Preparation of antisera. Two species were selected for the production of precipitating antibody. In a preliminary experiment, 18 white mice were injected with a crude extract of whole rapeseed protein (WRE). WRE was prepared by the overnight extraction of commercial rapeseed meal with 0.1M borate buffer pH 9.2. The filtered extract was stirred at 4°C and the proteins precipitated with 98 g granular ammonium sulfate added over a period of 30 min. The slurry was centrifuged at 5,000 x g for 30 min, and the pellet was resuspended in a small amount of water and placed in a dialysis bag. The sample was dialyzed against cold running water for 72 h and then 0.01M sodium phosphate buffer for 48 h at 4°C. Protein concentration was determined by micro Kjeldahl analysis (%N x 6.25). A 1.30% protein suspension was mixed 1:1 with Freund's complete adjuvant and 0.1 ml of the mixture administered intraperitoneally. Two subsequent injections of the same size were given at one week intervals. After a rest period of 4 weeks, the animals were given a booster injection of 0.1 ml and bled by cardiac puncture 2 weeks later. The blood was pooled and allowed to clot at room temperature for approximately 1 h and then refrigerated
overnight. The clots were carefully cut and separated from the serum by centrifugation.

In a later experiment, 2 Single-Comb White Leghorn roosters were injected with a 1:1 mixture of Freund's complete adjuvant and WRE (0.90% protein) prepared as before. In addition, an aqueous sample of 12S protein (0.54% protein) was prepared in the usual manner and dialyzed against 0.01M sodium phosphate buffer, pH 7.6, and administered 1:1 in Freund's complete adjuvant to 2 Leghorn roosters. The four roosters were given 1 ml antigen on the first week, 2 ml on the second week and 3 ml on the third, by intraperitoneal injection. A bird that had received the 12S antigen died during a trial bleeding. The three remaining roosters were boosted with 5 ml intraperitoneal injections after a 30 day rest period and, again, 1 week later. After 2 weeks, the birds were bled by cardiac puncture and the serum collected.

ii) Electrophoresis and diffusion. Samples of the 12S isolate were electrophoresed by the method of Davis (1962) on 4% acrylamide gels (Appendix III). Gels were loaded with 130 to 400 mg protein and after running were ejected from their tubes and placed in disposable Petri dishes. Each dish was filled with enough agar to cover each gel. The agar solution was composed of 1% Ionagar No. 2 (Oxoid) in pH 8.6 sodium diethylbarbiturate-sodium acetate buffer, ionic strength 0.05. The agar contained 0.02% sodium azide as a preservative. After solidification, trenches were cut a few mm deep,
running parallel to the gel columns but separated at a distance of approximately 1 cm and filled carefully with the mouse anti-WRE antiserum. The reactants were allowed to diffuse at room temperature in a glass desiccator containing a small amount of water. Gels containing the same antigen loads were electrophoresed along with the above gels and stained with Amido black 10B in order to locate the protein band. Antigen (12S protein) was also diffused against anti-WRE by the Ouchterlony (1958) method utilizing the above agar solution.

Similar procedures were carried out with the rooster anti-WRE and the anti-12S antisera. WRE was first dialized against electrode buffer and immunoelectrophoresed against rooster anti-WRE serum. Lyophilized 12S protein was dissolved directly in electrode buffer and immunoelectrophoresed against rooster anti-WRE serum. Protein loads ranged from 50 to 200 µg per gel for the 12S isolate and 20 to 80 µg for the WRE. Control gels were stained with Coomassie blue, which was found to be more sensitive and yielded superior resolution as compared to Amido black.

Double diffusion of the anti-12S antiserum was carried out against a wide range of concentrations of the six component sugars of the 12S glycoprotein (Section C.2.) in order to test the possibility that any of the sugars would be immunodeterminants. Aqueous solutions containing 1%, 0.1% and 0.01% of each sugar were diffused against precipitating antibody on agar plates.
d) Electrophoresis in the presence of sodium dodecyl sulfate.

Sodium dodecyl sulfate (SDS) gel electrophoresis was carried out in a buffer system described by Neville (1971) which produced improved resolution compared with the method of Weber and Osborn (1969). The stock solutions were prepared as in Appendix IV. Reagent grade sodium lauryl sulfate (Fisher Scientific Co.) was recrystallized three times in absolute ethanol. Urea solutions were deionized to a conductivity of 5 μmhos or less, however, no differences in electrophoretic patterns were observed between samples dissociated with non-deionized urea and those treated with deionized urea. Acrylamide gels were tested for resolving power at 7%, 10% and 15% acrylamide concentrations, the best results observed with the 10% gels. Samples of 12S protein and standard molecular weight markers were prepared as described in Appendix IV and heated in a boiling water bath for 2 to 5 min to ensure complete denaturation and to prevent proteolysis due to impurities or any inherent proteolytic activity of the sample itself (Weber, et al., 1972). The discontinuous buffer system of Neville (1972) stacked at pH 8.64 and ran at pH 9.50. Gels were run at 2 mA/tube. Temperature was held constant at about 15°C by circulating tap water through the countercurrent heat exchanger in the base of the unit. At the end of each run, the position of the bromphenol blue tracking dye in the gel was marked with a hypodermic needle containing drafting ink. Since it was
suspected that one of the fragments of the 12S oligomer migrated with the tracking dye, a run was performed in which the bromphenol blue was omitted from the upper electrode buffer and some of the sample gels. Gels were stained and destained as described in Section C.4.a.

In order to identify the carbohydrate-containing components in SDS gels, a modification of a procedure described by Glossmann and Neville (1971) was employed. These authors reported that for several glycoproteins (such as ovomucoid and $\alpha_1$-glycoprotein) the Coomassie-stained bands represent minor contaminants, whereas PAS-reactive bands represented the true molecular subunits. Many carbohydrate-rich glycoproteins stained only faintly with Coomassie blue. To avoid any possibility of artifactual staining with Coomassie blue, the glycoprotein molecular weight markers (Table I) were located by both Coomassie blue and Schiff reagent. The PAS staining procedure was performed as follows. Gels were washed in the GD-4 destainer with 40% methanol in 7% acetic acid overnight (2 changes) in order to remove bound and unbound SDS. All gels, except controls, were oxidized for 1 h in 1% periodic acid in 7% acetic acid in the dark and transferred to a 0.5% sodium arsenite in 5% acetic acid (Fairbanks et al., 1971) for 1 h. Three 20 min changes of 0.1% sodium arsenite in 5% acetic acid were followed by a 20 min wash in 5% acetic acid. All gels, including controls, were stained with Schiff reagent (Appendix IV) overnight at 4°C in the dark. The unreacted Schiff reagent was removed with several rinses of
1% sodium metabisulfite in 0.1N HCl. All gels were subsequently stored in the dark at 4°C in the metabisulfite solution.

Gels were scanned in a Transidyne TG2980 automatic scanning densitometer at 550 nm for Coomassie blue gels and 560 nm for PAS gels. Relative mobilities compared to the bromphenol blue tracking dye were calculated from the densitometer traces. Regression lines were fitted for the relation: \( \log \text{MW} = a + bR_f \) where MW, a, b, and \( R_f \) represent subunit molecular weight, intercept, slope and relative mobility, respectively. Apparent molecular weights of the subunits of the 12S glycoprotein were estimated from the regression equation derived from the mobility of molecular weight markers (Table I).

### TABLE I: MOLECULAR WEIGHT MARKERS FOR SDS ELECTROPHORESIS

<table>
<thead>
<tr>
<th>Marker</th>
<th>Source</th>
<th>% Carbohydrate</th>
<th>Subunit MW</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine thyroglobulin</td>
<td>Sigma</td>
<td>8.5</td>
<td>335,000</td>
</tr>
<tr>
<td>Conalbumin</td>
<td>NBC</td>
<td>2.5</td>
<td>86,180</td>
</tr>
<tr>
<td>Ovalbumin</td>
<td>ICN</td>
<td>3.2</td>
<td>43,500</td>
</tr>
<tr>
<td>RNase (Bovine pancreatic)</td>
<td>Sigma</td>
<td>11.3</td>
<td>12,640</td>
</tr>
</tbody>
</table>

1 from Sober (1970)
e) SDS gel immunoelectrophoresis.

The electrophoretic separation of the 12S glycoprotein fragments remained unchanged if SDS was eliminated from running and stacking gels thus presenting the possibility of running gels with reduced levels of SDS. Although immunoelectrophoresis is not normally carried out in the presence of SDS due to the induction of conformational changes in both antigen and antibody, it has been found that glycoproteins (particularly those containing sialic acid) bind substantially less SDS than proteins containing no carbohydrate (Segrest and Jackson, 1972).

Immune SDS gels were run by the method of Glossmann and Neville (1971) as previously described. Gels were loaded with samples ranging from 10 to 400 μg of the dissociated 12S isolate (Appendix IV). After electrophoresing for approximately 1.5 h at 2 mA per gel, the positions of the bromphenol blue tracking dye were marked and the gels embedded in Ionagar No. 2. Trenches were cut and filled with rooster anti-12S antiserum and the reactants allowed to diffuse as previously described. After the precipitin bands had formed, the Petri dishes were washed for several days in saline and stained with Amido black (Grabar and Burtin, 1964). Results were recorded photographically.
5. Gel filtration on Sepharose 6B and examination of a high molecular weight component

High speed ultracentrifugation and disc gel electrophoresis (Sections IV.C.3. and IV.C.4.) indicated the presence of a high molecular weight component which sedimented at 17.4S in 0.1M borate buffer pH 9.2. Although the component could be detected by disc gel electrophoresis, it did not appear as a separate precipitin arc in disc immunoelectrophoresis (Section IV.C.4.) or immunodiffusion experiments.

Such observations suggested the possible presence of a 12S dimer rather than a protein impurity. In order to investigate this possibility, a 12S peak was collected from the Sephadex G-100 column (Section III.B.2.) and concentrated in an ultrafiltration apparatus equipped with a PM-30 filter (molecular weight exclusion>30,000 daltons). An aliquot of the 12S material was applied to a 26 x 790 mm column packed with Sepharose 6B (Pharmacia) in 0.1M borate pH 9.2. The column was run at 2 ml min⁻¹ and two well-defined peaks collected. The UV-absorbing peaks were water dialyzed and lyophilized. The lyophilized material was subjected to SDS gel electrophoresis as described in Section III.C.4.d.
D. Microstructural and Ultrastructural Examination of the 12S Glycoprotein.

1. **Light microscopy**

Aqueous pastes of the 12S fraction were fixed with 2.5% glutaraldehyde in phosphate buffer at pH 7.0 for 1 h. Dehydration, embedding, sectioning, and Schiff staining were carried out as described for the seeds and meal. Slides were examined and the images recorded with a 35 mm camera system.

2. **Transmission electron microscopy**

Samples of the 12S isolate were prepared for ultrastructural examination by the above procedure except that glutaraldehyde fixation was followed by a 60 min fixation with 1% OsO$_4$ in pH 7.0 phosphate buffer. Samples of an isoelectrically precipitated rapeseed isolate prepared by Keshavarz (1974) were included in the study so that comparisons between the two isolates could be made. All sections were mounted on 300 mesh copper grids and stained with uranyl acetate (Watson, 1958) and lead citrate (Reynolds, 1963). Grids were examined in an AEI Corinth 275 transmission electron microscope operating at 60 kV.
An attempt was made to examine the ultrastructural detail of the isolate treated with an electron-dense stain which would react specifically with the carbohydrate moieties of the protein. Ultrathin sections of glutaraldehyde-fixed isolate were mounted on 300 mesh gold grids, oxidized with periodic acid and stained with silver methanamine as described by Rambourg (1967). This reagent is believed to react with periodate-oxidized sections in a manner analogous to Schiff reagent; however, due to its reaction with copper, gold or platinum grids are necessary.

A negative staining technique, similar to that described by Haschemeyer and Myers (1972) was employed for the examination of the structure of the 12S agglomerate. Copper grids (400 mesh) were washed thoroughly with acetone, dried, and dropped onto a floating layer of collodion. Filter paper was used to pick up the collodion film which supported the grids. After air drying, the collodion-coated grids were placed in a Balzers Micro BA3 high vacuum coating unit and coated with carbon. Immediately before use, the collodion was removed from the grids with a 1.5 min dip in acetone. A drop of protein solution (approximately 0.001% w/v in 0.1M borate buffer pH 9.2) was placed on each grid and the excess removed by touching the edge of the grid with a torn piece of filter paper after 4.5 min. A drop of 0.5% aqueous uranyl acetate (freshly dissolved) was placed on each grid for 2.5 min and the excess liquid removed as before. After air drying,
the negatively-stained grids were examined on a Zeiss 10 transmission electron microscope with an accelerating voltage of 60 kV and images recorded at magnifications from 40,000 to 60,000 diameters.

3. **Scanning electron microscopy**

Gels were prepared as described in Section III.E.1. with 4.5% protein dispersions adjusted to various pH levels. Pieces of gel were immersed in liquid nitrogen and cryo-fractured. The frozen fragments were immediately transferred into a 2.5% glutaraldehyde solution buffered to pH 7.0 and fixed for 2 h. The gels were subsequently dehydrated in ethanol solutions of increasing strength (30, 50, 70, 95 and 100%) and transferred into ascending concentrations of propylene oxide in ethanol (1:1, 2:1, 3:0). The specimens were then dried in a critical-point dryer, glued to aluminum specimen stubs and coated with a gold-palladium alloy in a sputter coating device (Technics Inc.). The gels were examined in an ETEC Autoscan scanning electron microscope at 20 kV and images recorded on Polaroid Positive/Negative 4 x 5 in film.
E. Rheological Properties of Dispersions and Thermally-Induced Gels of the 12S Glycoprotein.

1. Sample preparation

In an initial study, samples of the 12S protein were collected from a Sephadex G-100 column and dialyzed against running water for at least 72 h. The contents of the dialysis bags were concentrated by pervaporation and subsequently dialyzed against 0.01M borate buffer, pH 9.2. Protein levels of the concentrates were determined by micro Kjeldahl analysis (%N x 6.25). This procedure was chosen instead of rehydration of the dried isolate because the lyophilized product appeared to lose some of its solubility.

The steady shear flow behavior of concentrates ranging from 1 to 5.4% protein was examined over a wide range of shear rates. The effect of heating upon the viscometric properties of the dispersions was also investigated. Heated samples were prepared by placing 2 ml of each sample into a 12 x 125 mm test tube which was then loosely stoppered. The tubes were weighed and placed in a Litton microwave oven (frequency, 2450 MHz) for approximately 1 sec to heat until boiling. Samples were removed immediately and cooled in an ice water bath for about 5 min. In order to determine the possible contribution of sulfhydryl-disulfide interchange on thermally-induced gelation, a sample of 1% protein was reacted with 0.08 mM p-mercuribenzoate (PCMB) before heating.
Additional experiments were designed to characterize the gelation phenomenon exhibited by dispersions of the 12S glycoprotein. Samples were concentrated to approximately 5% protein (w/v) by pervaporation and subsequently dialyzed against Britton and Robinson-type universal buffers (Dawson et al., 1969) of pH 2, 4, 6, 8 and 10. The final concentration of each dispersion was adjusted to 4.5% (%N x 6.25) with buffer. A sample of protein dispersed in 0.01M borate buffer pH 9.2 was also prepared as previously described. Aliquots of this dispersion were adjusted to 0, 0.5, and 1.0M sodium chloride, 1M urea and 0.15M dithiothreitol in order to observe the effects of varying ionic strength, the presence of a dissociating agent, and a reducing agent, respectively.

A formaldehyde treatment described by Means and Feeney (1968, 1971) was used for the selective reductive alkylation of the ε-amino groups of lysine. A protein suspension (10 mg/ml) was prepared in 0.2M borate buffer, pH 9.0, and 0.5 mg/ml NaBH₄ added at 0°C. Aqueous formaldehyde (37%) was added in five increments totalling 0.5 μl/ml suspension over a period of 30 min. The suspension was dialyzed against water to remove the excess formaldehyde and pervaporated to approximately 5% protein (w/v), then dialyzed against 0.01M borate pH 9.2. After dialysis, the final concentration was adjusted to 4.5% protein (%N x 6.25) with borate buffer in preparation for rheological characterization. Samples of water-dialyzed, derivatized protein as
well as underivatized 12S protein were lyophilized and the ε-amino groups determined by the trinitrobenzenesulfonic acid (TNBS) method of Eklund (1976).

All samples in the second experiment were gelled in stoppered 12 x 125 mm test tubes by heating in boiling water for 5 min and cooling in ice water for 2 min.

2. Rheological characterization

Steady shear flow properties of the dispersions in the initial experiment were evaluated over more than three decades of shear rate with a Model R.18 Weissenberg Rheogoniometer (Sangamo Controls Ltd., 1971) utilizing 10 cm diameter 0.25° cone/plate fixtures.

In experiments designed to test the effect of pH and various additives on gelation, the gels were tested under steady shear to measure flow behavior and dynamic conditions of small amplitude oscillatory shear in order to determine viscoelastic properties. Both 10 cm diameter 0.25° and 5 cm diameter 2° cone/plate fixtures were employed. The shear strain input signals, the resultant shear stress output signals and the phase difference between the two sinusoidally varying functions were monitored with a Tronotec Model 703A digital analyzer (Tronotec Inc., Franklin, N.J.) in the dynamic shear experiments. Because these tests required relatively large quantities of the protein, only one run was performed for most treatments.
IV RESULTS AND DISCUSSION

A. Microstructure and Ultrastructure of the Rapeseed Kernel \((Brassica campestris)\) and Rapeseed Meal.

The study of the microstructure and ultrastructure of the rapeseed kernel was initiated to elucidate the cellular organization and localization of the proteinaceous materials. The intact seed is about 1 to 2 mm in diameter, spherical and black or reddish brown depending upon the variety. A section through a rapeseed kernel as observed with the light microscope is illustrated in Figure II. The section demonstrates the fact that rapeseed, unlike many other seeds, contains almost no endosperm. Instead, the major protein and lipid-containing tissues are found in the cotyledons \((C)\) which are the embryonic leaves and the meristematic tissue \((M)\), from which the radicle (root), hypocotyl (stem) and epicotyl (bud) will develop. The endosperm, which usually serves a storage function to provide energy during germination and early stages of development is restricted to a single layer of cells \((E)\) located beneath the epidermis. The thick cell walls and seed coat contribute to the high crude fiber content (up to 11.5\% on dry basis). The tissue section illustrated in Figure II was stained by the PAS method and it is apparent that no areas of concentration of Schiff-reactive material exist.
FIGURE II: Light micrograph of a section of rapeseed stained by PAS method. Note cotyledons (C), meristematic tissue (M) and endosperm (E).

FIGURE III: Light micrograph of rapeseed tissue stained with toluidene blue and showing the protein-rich aleurone grains (A). Note the lighter staining and fragmentation of the aleurones in an area of rapid cell division (arrows).
Figure III is a photomicrograph of a section of a rapeseed kernel which was stained with toluidene blue. The major proteins of rapeseed (and most other oilseeds) are located in subcellular packages called aleurone grains (A) that are considered to have a storage function supplying the seedling with organic nitrogen rather than structural or catalytic functions (Appelqvist and Ohlson, 1972; Dieckert and Dieckert, 1976). The term "aleurone" was first used by Hartig (1855) who isolated protein bodies from various oilseeds by a nonaqueous technique. Since the defatted particles resembled flour, he named them "aleuron" after the Greek word for flour. In several sections it was observed that rapidly dividing cells contained many small and poorly-defined aleurones (arrows) as opposed to the typical globular inclusions in non-dividing tissue. Cells about to divide or undergoing cytokinesis contain aleurones which stain weakly for protein, presumably due to the utilization of the aleurone protein for the formation of new tissue.

Available experimental evidence indicates that the "aleurins" or aleurone proteins are synthesized on the rough endoplasmic reticulum and then transported by way of the lumina of the endoplasmic reticulum to the dictyosomes (Dieckert and Dieckert, 1976), where the protein is concentrated into droplets. The membrane-bounded protein droplets migrate through the cytoplasm to the aleurone vacuoles and pass through the vacuolar membrane by a process of membrane
fusion. Finally, the mature aleurone grains form by dehydration of the aleurone vacuoles.

Since the 12S glycoprotein is the major rapeseed protein and is highly reactive to Schiff reagent, it would be reasonable to expect that those aleurones containing large quantities of this protein would themselves be PAS-positive. Figure IV illustrates that there are indeed aleurones which contain high levels of Schiff-reactive material suggesting that for some reason, the 12S glycoprotein is synthesized by some cells but not others. The PAS-positive aleurones were distributed randomly throughout the tissue.

A micrograph of rapeseed meal stained with Schiff reagent (Figure V) illustrates that both fragmented (FA) and intact aleurones (A) are present. Fragmented cell walls (CW) appear to comprise a sizeable portion of the meal.

Figure VI is an electron micrograph of rapeseed showing the internal structure of the cell. The mature rapeseed, like many other oilseeds, is remarkably free of cytoplasmic organelles, the major cellular components being vacuolated lipid (L) and aleurone grains (A). Note also the thick cell wall (C), nucleus (N) as well as the presence of globoid bodies (G) within the aleurone grains. Dieckert et al. (1962) found peanut globoids contained phytic acid, potassium, magnesium and copper. Similar results were found for cotton (Lui and Atschul, 1967) and cucurbita (Lott, 1975).
FIGURE IV: Light micrograph of a rapeseed kernel tissue cell rich in PAS-positive aleurone grains (A). The PAS-rich cells appeared to be evenly distributed throughout the entire seed.

FIGURE V: Light micrograph of commercial rapeseed meal stained by the PAS procedure. Note that both fragmented (FA) and intact (A) PAS-positive aleurones are present.
FIGURE VI: Electron micrograph of rapeseed showing internal cellular structure. Note the thick cell wall (C), aleurone grains (A), nucleus (N) and lipid vacuoles or spherosomes (L).

FIGURE VII: Electron micrograph demonstrating the aleurone grain (A) surrounded by a single membrane (M) and lipid bodies (L).
Similar to other oilseeds, the aleurone grains of rapeseed are surrounded by a unit membrane (M in Figure VII). Lipid bodies (L) constitute a major portion of the cytoplasm. The importance of effective milling should be emphasized if meal is to be used for the purpose of protein recovery. Optimum protein extraction would not likely be achieved as long as the aleurone membrane was intact. Figure V has illustrated that in some cases, commercial milling is inadequate to fragment all aleurones.

B. Recovery of the 12S Protein from Rapeseed Meal.

The solvent-extracted meal is a by-product of the vegetable oil industry. It has traditionally been used as fertilizer or as an animal feed supplement. The current study was carried out on this highly denatured material because it is likely that rapeseed will always be cultivated primarily as a source of oil and that secondary protein production will utilize the inexpensive meal. Although the terms "rapeseed protein" and "12S rapeseed protein" are referred to in the text, it is not intended that the reader confuse this material obtained from a highly denatured source with that obtained from the intact rapeseed kernel.

The purification procedure was a modification of that described by Goding et al. (1970). In this procedure,
a high-speed centrifugation step was included in order to concentrate the high molecular weight components. This step was eliminated in the present study for the following reason. Ultracentrifugation indicated that the major contaminant of the 12S glycoprotein was determined to be of a higher molecular weight (17S), thus high speed centrifugation would tend to enrich the 17S contaminant. Since gel filtration rather than protein extraction was a rate-limiting step in recovery, NaCl was eliminated from the extraction buffer described by Goding et al. The nitrogen recoveries for extraction and purification of the 12S isolate are shown in Figure VIII.

The commercial defatted meal contained 5.5% nitrogen and yielded a crude alkaline extract containing 0.22% nitrogen of which approximately 50% appeared in the first peak when subjected to gel filtration (Figure IX). The column void volume was approximately 500 ml so that the 12S peak was at least partially excluded by the column. Further purification to remove the 17S or other contaminants was not attempted for practical purposes. The freeze-dried isolate produced by this procedure was fluffy and white, contained 12.5% nitrogen and although quite bland, possessed a slightly nutty aftertaste. The term "isolate" may perhaps be confusing when applied to the rapeseed 12S glycoprotein since it has been suggested by Burrows et al. (1972) that this term be restricted to a preparation containing a minimum of 90%
FIGURE VIII: Flow diagram for the preparation of 12S rapeseed isolate and concentrated dispersions.

FIGURE IX: Elution profile of the gel filtration of crude rapeseed extracts on Sephadex G-100 in 0.1M borate buffer pH 9.2. Fractions were collected in 10 ml fractions.
PREPARATION OF 12 S ISOLATE

5.5% N DEFATTED MEAL

6.3% N SIFTING (60 MESH)

EXTRACTION

CENTRIFUGATION

0.22% N SUPERNATE

GEL FILTRATION

12 S RECOVERY 50% OF TOTAL N APPLIED TO COLUMN DIALYSIS vs H2O

PERVAPORATION OR LYOPHILIZATION ISOLATE

DIALYSIS OF CONCENTRATE

(0.01M BORATE, pH 9.2)

Absorbance at 280 nm.

Tube No.
protein (N x 6.25) on a moisture-free basis when applied to manufactured soybean products. However, the Kjeldahl factors for glycoproteins may be higher than 6.25, particularly when a significant level of neutral sugar is present. Thus, the nitrogen content of a protein does not necessarily reflect the degree of purity in a preparation.

C. Chemical Characterization of the 12S Rapeseed Globulin.

1. Amino acid composition

The results of the amino acid analysis are tabulated in Table II. Five samples representing five hydrolysis times (24 to 36 h) were analyzed and the nitrogen recoveries calculated. The data in Table II represents a 27 h hydrolysis in which the nitrogen recovery was 94%. Moore and Stein (1960) have suggested that data for serine, threonine, tryptophan and half cystine be extrapolated to zero hydrolysis time, however, these values were found to maximize at different times. The total nitrogen recoveries were 68, 78, 82 and 87% for the 24, 36, 25 and 28 h hydrolysis times, respectively. Results from the runs in which lower recoveries were observed are listed in Appendix V.

The 12S protein would appear to be a typical oil-seed aleurin (Dieckert and Dieckert, 1976) in that relatively
<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>g Residue per 16g N recovered</th>
<th>mM per 16g N recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid</td>
<td>9.56</td>
<td>83.0</td>
</tr>
<tr>
<td>Threonine</td>
<td>3.82</td>
<td>37.7</td>
</tr>
<tr>
<td>Serine</td>
<td>4.91</td>
<td>56.4</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>20.9</td>
<td>162</td>
</tr>
<tr>
<td>Proline</td>
<td>4.20</td>
<td>43.3</td>
</tr>
<tr>
<td>Glycine</td>
<td>4.65</td>
<td>81.4</td>
</tr>
<tr>
<td>Alanine</td>
<td>3.87</td>
<td>54.4</td>
</tr>
<tr>
<td>Valine</td>
<td>3.86</td>
<td>38.9</td>
</tr>
<tr>
<td>Methionine</td>
<td>1.88</td>
<td>14.3</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>3.60</td>
<td>31.8</td>
</tr>
<tr>
<td>Leucine</td>
<td>7.42</td>
<td>65.6</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>3.13</td>
<td>19.2</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>4.93</td>
<td>33.5</td>
</tr>
<tr>
<td>Ammonia</td>
<td>2.68</td>
<td>158</td>
</tr>
<tr>
<td>Lysine</td>
<td>2.96</td>
<td>23.1</td>
</tr>
<tr>
<td>Histidine</td>
<td>2.87</td>
<td>20.9</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Arginine</td>
<td>6.03</td>
<td>38.6</td>
</tr>
<tr>
<td>Pyridylethyl-L-cystine</td>
<td>Trace</td>
<td>Trace</td>
</tr>
<tr>
<td>Total recovery (%N)</td>
<td>94</td>
<td></td>
</tr>
</tbody>
</table>
high levels of glutamic acid, aspartic acid and arginine are present. The amino acid composition of the aleurins is usually biased toward the more readily utilizable acids—arginine, glutamic acid (glutamine) or aspartic acid (asparagine). Other notable features of the 12S amino acid profile are the absence of tryptophan and the scarcity of 1/2 cystine and methionine. These trends were observed by Finlayson et al. (1969) and Goding et al. (1970) for a 12S globulin extracted from Brassica campestris and by MacKenzie and Blakely (1972) for a similar protein extracted from B. nigra, B. juncea and B. hirta. Goding et al. (1970) reported 0.145 and 0.05 mM of 1/2 cystine and tryptophan, respectively, per gram 12S protein isolated from B. campes-tris compared to means of 0.002 and 0 mM of these amino acids found per gram isolate in the present study. Although precautions were taken to protect these labile amino acids, degradation during acid hydrolysis may account for the differences in composition.

2. Carbohydrate composition

Goding et al. (1970) reported the presence of 0.15 to 0.20% galactosamine and 1.0 to 1.5% glucose and arabinose, with the major neutral sugar being glucose. These results were reported for the 12S globulin isolated from B. campestris L. var. Echo and B. napus L. var. Target. The 12S protein was separated into four chromatographically different
components at pH 2.8 in the presence of 2M urea by the same authors. A fragment containing 20% of the total nitrogen (2.7S) also contained 0.5% of its nitrogen as galactosamine and 4 to 5% reducing sugar. The 2.7S fragment did not reduce to smaller subunits in the presence of 0.01M mercaptoethanol.

The present investigation was initiated in order to obtain similar data on the 12S glycoprotein isolated from commercial rapeseed meal (B. campestris L. var. Span). Results of the phenol-sulphuric acid determination of total sugar yielded an estimated 8% on the basis of a standard curve for glucose. It should be emphasized that because various sugars yield different results, this method cannot be considered quantitative.

Results of the sialic acid determination (N-acetyl neuraminic acid) indicated the absence of this material in the isolate although its presence was suspected because of electrophoretic behavior.

The gas chromatographic analysis of both neutral and amino sugars indicated the presence of 6 components. A typical gas chromatogram of the monosaccharide derivatives (alditol acetates) is illustrated in Figure X. The peaks identified by retention times as compared to those of authentic sugars were (a) arabinose, (c) mannose, (d) galactose, (e) glucose, and (f) inositol. A peak (b), emerging shortly after arabinose, was found to have a retention time
FIGURE X: A typical gas chromatogram of the alditol acetate derivatives of the neutral and amino sugars in the 12S glycoprotein.

a) arabinose
b) unidentified
c) mannose
d) galactose
e) glucose
f) inositol
equal to that of both glucosamine and xylose. No such other coincidences in retention times were observed on the ECNSS-M column for a wide range of standard sugars tested. Since glucosamine and xylose were not resolvable, glucosamine content was quantitated colorimetrically by the Elson-Morgan reaction (1933). Results of the sugar analyses are tabulated for the freeze-dried 12S isolate in Table III along with amino acid content (from Table II), assuming 100% recovery. A calculation of theoretical partial specific volume (Vp) (Schachman, 1957) utilizing monosaccharide partial specific volumes cited by Gibbons (1966) is included in Table III. The total sugar content of the 12S glycoprotein was found to be 12.9% while the remaining 87.1% was presumed to be amino acids. This figure would appear reasonable since the lyophilized isolate contained 12.5% nitrogen. An oven-dried sample of the isolate contained slightly more nitrogen (13.4%) indicating that a small amount of moisture could not be removed by conventional freeze drying. It is evident from these data that there are large varietal differences or differences between seed and meal. Although amino acid profiles are similar, the carbohydrate and nitrogen contents differ considerably from those reported by Goding et al. (1970) for the 12S protein isolated from the seeds of B. campestris L. var. Echo and B. napus L. var. Target. Instead, the 12S protein isolated from commercial meal more closely resembles "fraction A" isolated from B. nigra, B. juncea and B. hirta by MacKenzie and Blakely (1972) and from B. juncea
<table>
<thead>
<tr>
<th>Component</th>
<th>% (w/w)</th>
<th>Partial specific volume (Vi)</th>
<th>% (w/w) x Vi</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arabinose</td>
<td>7.24 ±0.16</td>
<td>0.613</td>
<td>4.44</td>
</tr>
<tr>
<td>Glucosamine</td>
<td>0.29 ±0.042</td>
<td>0.666</td>
<td>0.193</td>
</tr>
<tr>
<td>Mannose</td>
<td>0.340±0.030</td>
<td>0.613</td>
<td>0.208</td>
</tr>
<tr>
<td>Galactose</td>
<td>3.32 ±0.12</td>
<td>0.613</td>
<td>2.04</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.870±0.019</td>
<td>0.613</td>
<td>0.533</td>
</tr>
<tr>
<td>Inositol</td>
<td>0.840±0.077</td>
<td>0.613</td>
<td>0.515</td>
</tr>
<tr>
<td>Sialic acid</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>9.40</td>
<td>0.59</td>
<td>5.55</td>
</tr>
<tr>
<td>Threonine</td>
<td>3.75</td>
<td>0.70</td>
<td>2.63</td>
</tr>
<tr>
<td>Serine</td>
<td>4.83</td>
<td>0.63</td>
<td>3.04</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>20.6</td>
<td>0.66</td>
<td>13.6</td>
</tr>
<tr>
<td>Proline</td>
<td>4.13</td>
<td>0.76</td>
<td>3.14</td>
</tr>
<tr>
<td>Glycine</td>
<td>4.57</td>
<td>0.64</td>
<td>2.92</td>
</tr>
</tbody>
</table>

Cont'd.
<table>
<thead>
<tr>
<th>Component</th>
<th>% (w/w)</th>
<th>Partial specific volume (Vi)</th>
<th>% (w/w) x Vi</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>3.80</td>
<td>0.74</td>
<td>2.81</td>
</tr>
<tr>
<td>Valine</td>
<td>3.79</td>
<td>0.86</td>
<td>3.26</td>
</tr>
<tr>
<td>Methionine</td>
<td>1.85</td>
<td>0.75</td>
<td>1.39</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>3.54</td>
<td>0.90</td>
<td>3.19</td>
</tr>
<tr>
<td>Leucine</td>
<td>7.29</td>
<td>0.90</td>
<td>6.56</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>3.08</td>
<td>0.71</td>
<td>2.19</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>4.85</td>
<td>0.77</td>
<td>3.73</td>
</tr>
<tr>
<td>Lysine</td>
<td>2.91</td>
<td>0.82</td>
<td>2.39</td>
</tr>
<tr>
<td>Histidine</td>
<td>2.82</td>
<td>0.67</td>
<td>1.89</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arginine</td>
<td>5.93</td>
<td>0.70</td>
<td>4.15</td>
</tr>
<tr>
<td>Pyridylethyl-L-cystine</td>
<td>Trace</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

... Cont'd.
TABLE III:  Continued

Theoretical partial specific volume (see Schachman, 1957)

\[ V_p = \frac{\sum \% \text{ (w/w)} \times V_i}{\sum \% \text{ (w/w)}} = 0.704 \]

* All monosaccharide data except glucosamine reported as a mean of 4 determinations ± standard error of the mean. Glucosamine reported as a mean of 6 determinations ± standard error of the mean.

+ Amino acid values calculated from those of Table II assuming 100% recovery.
by MacKenzie (1975). The nitrogen content of this fraction (containing mainly 12S protein) was 12.7, 13.7 and 14.9% for B. nigra, B. juncea and B. hirta, respectively. Despite the similarity in amino acid and nitrogen content between the 12S protein isolated from rapeseed meal and that isolated by the above authors, large discrepancies remain in the carbohydrate contents. A major factor in this discrepancy could be the accuracy of the phenol-sulfuric acid method used by MacKenzie and Blakely (1972) for this material. Only slightly more than 50% of the true sugar content was detected with this technique in the present study. The differences in carbohydrate content of the 12S aggregates could also be explained by the effects of commercial fat extraction. It has been reported that the content of reducing sugars and available lysine in commercial meal drops appreciably due to the high temperatures employed (Josefsson, 1972). This phenomenon was explained as a result of the Maillard reaction (Maillard, 1912) in which reducing sugars and basic amino acids form condensation products via Schiff base formation. However, if the 12S aggregate in the present study were being extensively modified through non-enzymatic browning of the meal, such effects would be expected to be reflected in the sedimentation characteristics and in the homogeneity of the preparation. The implications of Josefsson's theory are discussed further in Section IV.C.4.
The two most notable features of the carbohydrate composition are the relatively large amounts of arabinose and the presence of inositol. Although there is a lack of detailed information on carbohydrate components of rapeseed, Appelqvist (1972) has cited the presence of sugars in aqueous extracts of white mustard. The sugars of white mustard were predominantly arabinose, galactose and glucuronic acid and were discovered in both hot and cold water-soluble fractions. The presence of inositol in the 12S protein may possibly be explained as follows. Phytic acid, a phosphate ester of myoinositol, has been isolated from the globoid bodies of the aleurone grains of various oilseeds (Dieckert et al., 1962; Lui and Altschul, 1967; Lott, 1975). Rapeseed aleurones would be expected to have a similar composition, thus there would be a possibility for inositol incorporation into the major aleurin proteins.

3. Ultracentrifugation

The 12S glycoprotein isolated from meal was examined by sedimentation velocity ultracentrifugation. The schlieren pattern for the protein dissolved in extraction buffer is shown in Figure XI.A. The major and minor components had observed sedimentation coefficients (s_{20}, Buffer) of 12.45 and 17.25S. It is also apparent from this photograph that a small quantity of lower molecular weight material exists in the 12S fraction. Figure XI.B. demonstrates the effect
FIGURE XI: Schlieren patterns of undissociated and dissociated 12S protein extracted from commercial rapeseed meal. (A) Pattern after 17.25 min at 55,000 rpm (0.1M borate buffer pH 9.2). (B) Pattern after 82.5 min at 55,250 rpm (0.1M phosphate buffer pH 7.0 + 5M urea and 0.1% 2-mercaptoethanol).
(C) Pattern after 321 min at 55,100 rpm (0.1M phosphate buffer pH 7.0 + 6M urea and 0.1% 2-mercaptoethanol).
of 5M urea and 2-mercaptoethanol on the 12S aggregate. The photograph illustrates the presence of at least 3 distinct fragments: a high molecular weight fragment ($S_{obs} = 11.55$), presumably undissociated material, and two smaller fragments whose uncorrected sedimentation coefficients were 6.3S and 0.8S. The degree of dissociation would appear dependent upon urea concentration. Figure XI.C. shows that only one low molecular weight peak is present in 6M urea and 0.1% 2-mercaptoethanol. The uncorrected sedimentation coefficient for this peak was 0.64S. Results of sedimentation velocity runs are similar to those published by Bhatt et al. (1968) and Goding et al. (1970) for rapeseed globulins as well as those published by MacKenzie and Blakely (1972) for fraction A extracted from *B. juncea*.

According to previous studies and the present data, the 12S fractions extracted from both seeds and meal are heterogeneous. It is difficult at the present time to ascertain whether the higher and lower molecular weight components of the 12S protein isolates are minor contaminants or products of a system which readily dissociates and self-associates, depending upon pH and temperature. The major contaminant in all previous studies of rapeseed 12S globulins has been a 17S component. This component was not observed in the urea-dissociated protein at pH 7.0. MacKenzie and Blakely (1972) also reported the presence of higher molecular weight components in the 12S isolates produced from *B. juncea*,
B. hirta and B. nigra and separated the 12S component of B. juncea from the higher molecular weight contaminants. The nitrogen content of the protein increased from 13.7% to 16-17% during the purification procedure on Sephadex G-200 superfine. In a subsequent publication, however, MacKenzie (1975) stated that the amino acid composition of the high molecular weight contaminant was indistinguishable from that of the purified 12S fraction which suggests that the 15S protein in B. juncea is merely a dimer of the 12S component. These two points would appear contradictory, however, were not discussed by the author(s). The value of the 13.7% nitrogen for the "unpurified" 12S isolate of MacKenzie corresponds much more closely to the value obtained for the 12S isolate extracted from rapeseed meal in the present study.

Ultracentrifugation in urea as well as results to be discussed in Sections IV.C.4. and IV.C.5. suggest that the 17S and lower molecular weight "contaminants" are the products of self-association and fragmentation of the 12S glycoprotein of rapeseed meal. Dieckert and Dieckert (1976) have suggested that the evolutionary significance of the aleurins, often being multi-subunit self-associating proteins, is that such assemblies reduce the internal osmotic pressure of the cell. Molecular weight determinations of such systems are tedious because of the difficulties encountered in assessing the purity of a preparation. The 12S protein aggregate in the present study was obviously heterogeneous by sedimentation velocity ultracentrifugation (schlieren optics) and yielded
curvilinear plots of $\log c$ vs. $r^2$ ($c = \text{concentration at any point in the cell, } r = \text{distance from center of rotation}$) in equilibrium runs. Conventional sedimentation equilibrium ultracentrifugation yielded a weight average molecular weight of 129,000 daltons when six molecular weights were extrapolated to zero concentration.

4. **Disc gel electrophoresis**

The proteins of the 12S aggregate of rapeseed are oligomeric. Dissociation of the complex may be brought about by the use of urea or solutions at pH values below 3.5 (Goding et al., 1970). Assessment of electrophoretic homogeneity of the intact complex is, therefore, necessary in an anionic system and at alkaline pH's. However, complete assurance of electrophoretic homogeneity cannot be obtained at only one pH level. In addition to the problems inherent in the evaluation of homogeneity of oligomeric proteins which tend to dissociate and self-associate, other problems involved with the electrophoresis of glycoproteins have been well documented. "Microheterogeneity represents that variation in the carbohydrate groups of glycoproteins produced by partial substitution of sugar residues on a basically similar core structure" (Montgomery, 1972). For example, the $\alpha_1$-glycoprotein was deemed monodisperse by all conventional methods including immunological characterization (Schmid et al., 1962), however, separated into 7 variants during starch gel
electrophoresis. Similar phenomenon have been observed in human plasma β₁-glycoprotein (Labat et al., 1969) and fetuin (Oshiro and Eylar, 1968). Montgomery (1972) stated that the microheterogeneities found in so many glycoproteins may represent oligosaccharides in varying stages of completion, or resulting from transglycosylations that do not have an absolute specificity. One would expect that the microheterogeneities could be possible in a system which has been heat denatured in the presence of reducing sugars. Heterogeneities of this type could occur by means of non-enzymatic browning reactions in the defatted meal. The degree of such differences in carbohydrate composition resulting from Maillard condensation could be expected to depend upon the types of reducing sugars present in the meal.

The lyophilized 12S isolate was subjected to the disc electrophoretic method of Davis (1962). Figure XII illustrates a densitometric scan of a typical gel stained with Coomassie blue. At pH 9.5, the protein migrates only slowly and is clearly separated into a major component, and a slower moving minor component, presumably in the 17S protein described in the previous section. Preliminary studies utilizing Amido black did not demonstrate a separation between these two components. Electrophoresis at pH 7.0 in the presence of 5M urea and 0.1% 2-mercaptoethanol resulted in a broad band which migrated slowly toward the anode as well as a second fragment which was either neutral or basic in
nature. When run cationically under the same conditions, only one band was evident and this component remained close to the origin. In 10M urea and at alkaline pH, the 12S protein did not appear to enter the running gel when electrophoresed anionically. MacKenzie (1975) reported that the major dissociated fragments of the 12S protein isolated from *B. juncea* were basic, with the largest proportion of the protein possessing an isoelectric point of 9.15. If the protein in the present study was similar, the electrophoretic behavior could possibly suggest that the surface of the 12S aggregate occludes a highly basic amino acid backbone. An explanation for the apparent charge occlusion could be that the carbohydrate portion is located on the surface of the structure. The amino acid profile reported in Section IV.C.1. suggests that the 12S rapeseed protein would be acidic because of the presence of large quantities of both glutamic and aspartic acids. From the amino acid profiles of the various isoelectrically-separated components of *B. juncea*, MacKenzie (1975) concluded that aspartic and glutamic acids must be present largely as the amides asparagine and glutamine. It may be reasonable to expect similar results from the fragments of the 12S globulin isolated from rapeseed meal since the amino acid profiles are similar, although investigation by means of isoelectric focussing would be necessary to confirm such speculation.

Since the 12S protein undergoes dissociation, and because of the possible confusion of subunit structure with
the presence of contaminants, immunochemical methods of characterization were undertaken. Immunodiffusion and disc immunoelectrophoresis were carried out with precipitating antibody produced from two different species. Figure XIII shows that the 12S protein prepared from commercial rapeseed meal was immunologically homogeneous. This photograph demonstrates the results of an experiment in which a wide range of concentrations of the 12S isolate were electrophoresed and the disc gels subsequently diffused against rooster anti-12S antiserum. Although two components were clearly present in the disc gels, close examination of the precipitin arcs (even in gels containing up to 400 µg protein) failed to reveal the presence of a contaminant. Similar results were obtained with mouse anti-12S antiserum. Diffusion of disc gels on which whole rapeseed extract (WRE) had been electrophoresed against rooster anti-12S antiserum resulted in the formation of only one pair of precipitin arcs. Double diffusion experiments revealed identical results. If a foreign 17S contaminant were present in the isolate, it would likely have been detected by immunoelectrophoresis since 12S and 17S components were first separated on the basis of electrophoretic mobility. Such evidence strongly suggests that the immunochemically homogeneous preparation of 12S glycoprotein in the present study self-associates probably to form dimers which are immunologically identical to the monomer aggregate.

Since glycoproteins in aqueous solution are often oriented such that the carbohydrate moieties are located on
FIGURE XII: Densitometric scan of a disc gel of the 12S glycoprotein isolated from commercial rapeseed meal. Electrophoresis carried out at pH 9.5, indicated that two components were separated near the origin, presumably the 12S and 17S proteins. (t) marks the position of the bromphenol blue tracking dye.

FIGURE XIII: Photograph of the disc immunoelectrophoresis pattern of the 12S rapeseed glycoprotein which was diffused against rooster anti-12S antiserum. (t) marks the position of the bromphenol blue tracking dye.
the surface of the molecule, antibodies are sometimes formed against this portion rather than the protein portion located in the interior of the molecule. A set of double diffusion plates were prepared in which the precipitating anti-12S antibody was diffused against various concentrations of the component sugars of the 12S protein. No precipitin arcs formed after one week and the plates were discarded. These results suggest that antibody production was either directed toward di-or polysaccharides or that the synthesis of antibody was not directed against the carbohydrate moieties.

The accuracy of the molecular weight determination of proteins by electrophoresis in sodium dodecyl sulfate depends upon two factors: proteins must bind a constant amount of SDS resulting in a constant charge to mass ratio, and the proteins which are reacted with SDS must assume a rod-like conformation such that the lengths of the structures are proportional to their polypeptide chain lengths (Segrest and Jackson, 1972). The anomalous behavior of glycoproteins on SDS gels has been well documented (Segrest and Jackson, 1972; Anderson et al, 1974; and Voyles and Moskowitz, 1974). This effect is believed to be due to the decreased binding of SDS to the carbohydrate portion of the molecule and often results in slower electrophoretic migration and thus overestimates of molecular weight. The degree of molecular weight anomaly is generally a linear function of its carbohydrate content, however, non-sialoproteins bind less SDS than those containing sialic acid (Segrest and Jackson, 1972). In
oligomeric glycoproteins in which the carbohydrate is present in some but not all polypeptide fragments, the non-glycoprotein subunits would be expected to behave normally.

The discontinuous buffer system of Glossmann and Neville (1971) was used for the polyacrylamide gel electrophoresis of the 12S glycoprotein. Figure XIV is a densitometric scan of an SDS gel on which the reduced, dissociated 12S aggregate was electrophoresed. Apparent molecular weights of the separable 12S fragments were 37,300±400, 30,100±1,100, 17,400±400 and 12,200±1,000 where the limits represent the standard errors of the estimates for four determinations. The smallest subunit travelled with the bromphenol blue tracking dye although its presence was confirmed from experiments in which no tracking dye was used. In one of the runs, the component with the highest molecular weight was clearly separated into two components with apparent molecular weights of 42,000 and 37,600 daltons.

Figure XV (upper) illustrates a densitometric scan of the dissociated 12S complex which was not reduced with 2-mercaptoethanol. The major component (a) of the unreduced protein has an apparent molecular weight of 60 to 70 thousand daltons, although minor peaks which correspond to the reduced fragments (b, c and d) were observed. A high molecular weight component (e) was observed which may correspond to a small amount of unfragmented 12S protein. The lower portion of Figure XV demonstrates the effect of a
FIGURE XIV: Densitometric scan of an SDS disc gel (12S glycoprotein). The apparent molecular weight scale was determined with the standard proteins bovine thyroglobulin, conalbumin, ovalbumin and RNase.

FIGURE XV: Densitometric scans of SDS gels which were run with unreduced dissociated 12S glycoprotein (upper) and 12S protein which was reduced with 0.15M 2-mercaptoethanol (lower).
reducing agent on the fragmentation of the 12S aggregate. Although only trace amounts of cystine were recovered in the amino acid analyses, it is evident that intermolecular disulfide bonds are present. Similar results were observed when 0.15M 2-mercaptoethanol was substituted with dithiothreitol. Goding et al. (1970) reported that 0.02M 2-mercaptoethanol had no effect on extraction chromatography or electrophoresis of the 12S globulin prepared from rapeseed, however, MacKenzie (1975) reported that 0.1M mercaptoethanol did contribute to the dissociation of the *B. juncea* 12S aggregate, although 0.05M mercaptoethanol had no effect. Thus, it is possible that the effects reported in the earlier study resulted from an insufficient level of disulfide-reducing reagent.

A method similar to that of Glossmann and Neville (1971) was used to detect the presence of carbohydrate-containing fragments in SDS gels. The technique involves the total removal of both bound and unbound SDS from the protein and gel before staining with Schiff's reagent. Figure XVI illustrates a typical densitometric scan. The only PAS-positive fragment (g) resulting from the dissociation of the 12S aggregate migrated with the tracking dye (t) which was marked with drafting ink before periodate oxidation and Schiff staining. A control gel which was not oxidized with periodic acid did not react. Band (g) was broader than the lowest molecular weight fragment (12,200 daltons) observed with Coomassie blue staining technique and, in some cases
(particularly gels with large sample loads), appeared to precede the tracking dye slightly. One explanation for this phenomenon could be that the fragment containing the carbohydrate contains little protein. Glossmann and Neville (1971) reported that many true glycoproteins react only faintly with Coomassie blue, and molecular weight determinations by this method were sometimes inaccurate since the Coomassie blue bands occasionally represented non-glycoprotein impurities in a preparation. Since peak (g) contains all the PAS-reactive material, it may be possible that this fragment possesses a molecular weight lower than 12,000 daltons.

On the other hand, the Schiff-reactive fragment may indeed be a part of the low molecular weight peak detected with Coomassie blue. Since this fragment must contain a major proportion of its weight as carbohydrate, Coomassie blue may not react with the entire fragment but only a specific end of the glycoprotein complex. Isolation of the glycopeptide (s) perhaps by isoelectric focussing, may resolve this question.

An experiment designed to test the hypothesis that the carbohydrate-containing fragment is located on the surface of the 12S aggregate was performed by the use of SDS gel immunoelectrophoresis. SDS gels on which the dissociated 12S protein had been run were subsequently diffused against rooster anti-12S precipitating antibody. A technique was described in Section III.4.e. in which the
level of chemical denaturant in the gel was reduced.
Conformational changes induced by SDS in either antigen or IgG could certainly lead to a loss in the ability of the antigen-antibody complex to form. After 4 or 5 days at room temperature, dense, white precipitates formed around the PAS-positive band in all of the plates. Sample loads on the gels ranged from 10 to 400 µg 12S protein which had received extensive denaturation in hot SDS. Upon extensive washing in saline, a technique commonly used to remove unreacted antibody (Grabar and Burtin, 1964), the arcs disappeared from some of the plates. The plates which had received the highest antigen loads retained their original pattern. Figure XVII is a photograph of such a plate which was stained with Amido black to increase the contrast of the bands. The bands in the SDS immune gels precipitated nearer to the agar-acrylamide interface than in ordinary immune gels and are atypical since they appeared somewhat feathery, possibly due to the partial dissolution of the precipitin arcs by the SDS. The white precipitate formed in the same place in all gels. This is not to say, however, that other fragments were not immunoresponsive since the antigen reaction with precipitating antibody would be expected to be reduced in the presence of the dissociating agents, urea and SDS. It is understandable, however, that if any fragment were expected to complex with antibody, it would be a fragment which bound a minimum of SDS, namely, the carbohydrate-containing moiety. This experiment, although not conclusive, suggests that the
FIGURE XVI: Upper: a photograph of a Schiff-stained SDS gel showing the PAS-positive fragment which electrophoresed slightly ahead of the tracking dye, compared with a control gel (c) which received no periodic acid treatment.
Lower: a densitometric scan of a PAS-treated gel showing the position of the carbohydrate-containing band (g) and the tracking dye (t).

FIGURE XVII: The precipitin arcs formed by the PAS-positive fragment of the 12S protein in Ionagar No. 2. A densitometric scan of a Coomassie blue-stained SDS gel is superimposed on the plate so that the locations of the fragments could be marked. Note the "feathery" appearance of the arcs in the presence of SDS (compare with Figure XIII).
carbohydrate-containing fragment is situated on the surface of the 12S aggregate. Perhaps confirmatory evidence could have been obtained if the experiment was carried out in the absence of SDS. Such a separation may have been achieved on polyacrylamide gradient gels, however, such an experiment was beyond the scope of the present study.

5. Gel filtration on Sepharose 6B and examination of a high molecular weight component

Since elution of rapeseed protein on Sephadex G-100 would not successfully separate the 12S protein from the 17S protein (Section IV.C.3.), a 12S peak from such a run was applied to a Sepharose 6B column. The elution profile of the sample in 0.1M borate buffer pH 9.2 is shown in Figure XVIII. Two peaks were observed and collected. The first peak (a) was lyophilized and analyzed by both sedimentation velocity ultracentrifugation and SDS gel electrophoresis. It possessed a sedimentation coefficient \( s_{20 \text{ Buffer}} \) of 33.9S in 0.1M borate buffer and was evidently an aggregate of the original 12S fraction since no such material had previously been observed in the ultracentrifuge. No 17S material was detected from the elution profile. The presence of the newly formed aggregated material may possibly be explained by the ultrafiltration step following chromatography on Sephadex G-100 and in which the 12S sample was constantly stirred under pressure for 2 days. SDS electrophoresis was used to examine
FIGURE XVIII: The elution profile of a concentrate of 12S rapeseed glycoprotein. The sample was chromatographed on a 26 x 70 mm column packed with Sepharose 6B. Elution buffer was 0.1M borate, pH 9.2 run at 2 ml per min. Peaks (a) and (b) were collected in 3 ml fractions and lyophilized for further characterization.

FIGURE XIX: Densitometric scans of SDS gels loaded with 12S (upper) and 33.9S (lower) rapeseed protein fractions prepared by chromatography on Sepharose 6B. The location of the tracking dye (t) was marked with drafting ink.
both peaks (a) and (b) in order to confirm that the 33.9S peak was an aggregate of 12S material or a mixed aggregate of 12S and 17S fractions. Figure XIX illustrates two densitometer tracings: 12S (upper) and 33.9S (lower). Both tracings appear to represent the same major fragments although the electrophoretic pattern of the 33.9S protein is not as well-defined as that of the 12S protein. This may be due to the incomplete dissociation of the larger agglomerate. Although this experiment does not add to the evidence that the 17S protein is a dimer of the 12S aggregate, it does indicate that the 12S protein self-associates. MacKenzie (1975) has shown that the 12S protein of *B. juncea* self-associates when stored in the cold. It has also been observed in the present study that solutions of the 12S rapeseed isolate become turbid at 0°C.

D. Microstructural and Ultrastructural Examination of the 12S Glycoprotein.

1. Light microscopy

Light micrographs of sections stained by the PAS technique (Figures XX, XXI, and XXII) indicate that the isolate contains an appreciable amount of PAS-reactive carbohydrate. The protein lacked wettability at pH 7.0 and large pockets, believed to contain water before dehydration, are indicated in the micrographs as "w". In many cases, globules
FIGURE XX: Light micrograph of hydrated 12S rapeseed protein isolate oxidized with periodic acid and stained with Schiff's reagent. Slightly more reactivity appears near the water droplet (w)-protein interface (arrow).

FIGURE XXI: PAS-positive rapeseed protein present as globular masses within the water droplet (w). Increased PAS reactivity occurs at the water-protein interface (arrow).
FIGURE XXII: Light micrograph showing the water-protein interface (arrow).
of protein were located within these pockets as shown in Figure XXI. For some reason, the PAS reaction appeared more intense at the protein-water interfaces (arrows). One explanation for this could be that the carbohydrate moiety is more hydrophilic and thus is attracted to the protein-water interface.

2. Transmission electron microscopy

Figure XXIII is an electron micrograph of the hydrated isolate which had been positively stained with uranyl acetate and lead citrate. It demonstrates areas of protein agglomeration (A). The 12S aggregate exhibited minimum solubility between pH 3.5 and 7.2. It is likely that the isoelectric point of the undissociated complex is located within this range, although it may be that the isoelectric points of the major fragments of the dissociated complex are somewhat higher (Section IV.C.4.). Since the protein represented in Figure XXIII was fixed at pH 7.0, it is possible that the tendency to clump was due to the absence of overall electrostatic repulsion. The photomicrograph may be compared with Figure XXIV which demonstrates the ultrastructure of an isoelectrically precipitated protein produced by Keshavarz (1974). Fibrous structures composed of spherical particles 4 - 5 nm in diameter present in isoelectrically precipitated alkaline extract of commercial meal were not present in the 12S aggregate.
FIGURE XXIII: Electron micrograph of a positively-stained (lead citrate, uranyl acetate) section of hydrated isolate fixed at pH 7 in 2.5% glutaraldehyde followed by 1% OsO₄. The protein appears highly agglomerated (A), perhaps as a result of the pH of fixation.

FIGURE XXIV: Electron micrograph of a positively-stained section of isoelectric precipitate of rapeseed protein alkaline extract. Note fibrous structures which were not found in the 12S isolate (Figure XXIII).
Transmission electron microscopy of the unfixed isolate applied to carbon films is demonstrated in Figures XXV and XXVI. The specimens were negatively stained with aqueous uranyl acetate. Figure XXV illustrates a field of view in which several hundred of the 12S particles are distributed. The 12S aggregate visualized by this procedure would appear to be a much more complicated structure than the 11S protein of soybean which has been examined with a similar technique by Badley et al. (1975). The soybean globulin is an oligomer composed of 12 subunits packed into two identical hexagons placed one upon the other with a maximum particle diameter of 110Å. The rapeseed glycoprotein would appear to be a morula-like structure composed of more than 12 subunits and much more irregularly shaped than the soybean protein. The 12S rapeseed protein has a maximum diameter of 120Å and is more or less spherical. However, caution should be exercised in making conclusions concerning molecular size and shape from such micrographs. It is possible that conformational changes take place when a macromolecule is transferred from solution to its dehydrated state. The recent work of MacKenzie (1975) on the subunit structure of the 12S globulin isolated from *B. juncea* showed that at least 11 different fractions could be obtained from isoelectric focusing experiments whereas only 6 distinct subunits have been isolated from glycinin (11S soybean protein) by a similar procedure (Catsimpoolas et al., 1971). An isoelectric focusing study was not included in the examination of rapeseed, however, in
FIGURE XXV: Electron micrograph of 12S rapeseed glycoprotein. The specimen was negatively stained and supported on a carbon film.

FIGURE XXVI: A higher magnification of the 12S glycoprotein aggregate. Mean particle diameter $\approx 120\AA$. 
view of the ultrastructural complexity and evidence reported by MacKenzie (1975) on a related species, it would not be surprising to find the 12S glycoprotein to be more chemically heterogeneous than the 11S soybean globulin.

The stain of Rambourg (1967) used to detect carbohydrate at the ultrastructural level was found to be non-specific in its reactivity as compared to Schiff reagent used in the light microscopy of rapeseed protein.

3. **Scanning electron microscopy**

The 12S rapeseed glycoprotein readily self-associates upon heating in pH's of 4 and above. Thermally induced gelation was observed in dispersions at 4.5% protein concentration and measurable thickening occurred at the 1% protein level. This phenomenon will be more thoroughly discussed in Section IV.E.

In order to examine the effects of varying pH on the ultrastructural properties of the thermally induced gels, cryofractured samples of the gels were examined by means of scanning electron microscopy (SEM). Figures XXVII and XXVIII represent gels which were prepared at pH's of 4 - 10 in Britton and Robinson type universal buffers (Dawson et al., 1969). Gels A, B, C, and D were prepared at pH 4, 6, 8, and 10, respectively. At pH 4, the 12S protein was extremely insoluble and a thermally-induced gel matrix was unstable.
FIGURE XXVII: Scanning electron micrographs of thermally-induced, cryofractured gels formed from 4.5% dispersions of 12S rapeseed glycoprotein.

A - pH 4.0
B - pH 6.0
C - pH 8.0
D - pH 10.0
FIGURE XXVIII: Higher magnifications of gel structure formed from heated 4.5% rapeseed protein dispersions showing the effect of pH on the 3-dimensional ordering.

A - pH 4.0
B - pH 6.0
C - pH 8.0
D - pH 10.0
It remained as a gel only for a few minutes and subsequently reverted back to a sol form. The pH 4.0 gel (Figures XXVII.A. and XXVIII.A.) appears as an amorphous mass. Although highly aggregated at this pH (presumably near its isoelectric point), very little space is provided between the particles of protein for the entrapment of water. At pH 6.0, the beginning of a 3-dimensional structure is evident (Figures XXVII.B. and XXVIII.B.). Many more cavities of smaller size are observed and thus the gel more effectively immobilizes water. Figures XXVII.C. and XXVIII.C. as well as XXVII.D. and XXVIII.D. illustrate a progression in 3-dimensional ordering and a decrease in pore size as the pH of the protein is raised from 8 to 10. An increase in intermolecular cross-linking is evident at high pH values although large differences in elastic properties were not observed in gels at pH 6 or higher (Section IV.E.). Part of the reason for this phenomenon could be that insolubility of the protein at pH 6 resulted in anomalously high gel strengths.

E. Rheological Properties of Dispersions and Gels of the 12S Glycoprotein.

Gelation phenomena of globular proteins are poorly understood, although numerous studies concerning fibrous proteins appear in literature. Ferry (1948) proposed that thermally induced gelation of globular proteins occurs by way of a two-stage process. The native (corpuscular) protein
becomes denatured and assumes a more or less extended and irregular form. The denatured protein often exhibits reduced solubility and is more hydrophobic than the native precursor. Under a specific range of pH and ionic strength, a gel network is formed by the association of polypeptide chains which interact along the entire molecular length. These bonds may be covalent, electrostatic, hydrophobic or hydrogen. In a recent article, Tombs (1974) suggested that globular proteins form gels as a result of aggregation of the denatured protein to form strands followed by the interaction of the strands to form a gel mesh. Although complete characterization of the forces involved in the 12S globulin gels was beyond the scope of the present study, the experiments described gave valuable information concerning the possibilities for thermally-induced interaction.

Rheological measurement has been particularly useful in the examination of protein interaction during gel formation. The effects of heating on soybean gel formation have been assessed by Circle et al. (1964), Wolf (1970), Catsimpoolas and Meyer (1970), and Catsimpoolas and Meyer (1971a and b). The globulin fraction of soybean whose major component is an 11S oligomeric protein will gel at 8% concentration or above (Catsimpoolas and Meyer, 1970). Hermansson (1975) criticized the earlier methods of rheological evaluation since most of the data consisted of single point measurements and gave no indication of flow behavior
at various rates of shear. In addition, the term "apparent viscosity" has been misused in several studies since this term applies to a value obtained when a non-Newtonian fluid is subjected to a constant rate of shear. The instrument used in such studies (Brookfield Syncro-Lectric Viscometer) was equipped with disc-like spindles which rotate in a solution and in which the rate of shear varies continuously across the surface of the disc. Such an instrument would be useful for the measurement of Newtonian liquids in which viscosity is independent of shear rate but cannot provide useful information in terms of flow behavior of more complex rheological systems.

The results of a preliminary experiment involving the steady shear measurements of dispersions and gels at different concentrations are demonstrated in Figures XXIX and XXX. All dispersions displayed shear-thinning flow behavior characteristic of pseudoplastic power-law fluids, that is, apparent viscosity ($\eta$, poise) decreased with increasing shear rate ($\dot{\gamma}$, sec$^{-1}$) according to the relation:

$$\eta = m \dot{\gamma}^{n-1}$$

where $m$ is the consistency index (dyne sec$^n$ cm$^{-2}$) and $n$ is the flow behavior index (dimensionless). The power law parameters for the steady shear flow behavior of the various rapeseed protein dispersions at 25°C are summarized in Table IV. Significant increases in viscosity were observed when samples containing as little as 1% protein were heated. Gelation due to microwave heating was obtained readily at
FIGURE XXIX: Rheograms of 12S protein isolate dispersions in 0.01M borate buffer pH 9.2. Heated samples were prepared in a microwave oven.

- □ 1.0% unheated
- △ 2.7% heated
- ○ 5.4% unheated
- ■ 5.4% heated
FIGURE XXX: Rheograms indicating the effects of heat and PCMB on viscosity of 1% dispersions of 12S protein isolate at pH 9.2.

○ unheated + PCMB

△ heated

□ heated + PCMB
(EXPERIMENT I)

<table>
<thead>
<tr>
<th>Dispersion</th>
<th>Consistency coef., $\text{dyne sec}^n \text{ cm}^{-2}$</th>
<th>Flow behavior index</th>
<th>Coefficient of determination</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0% Unheated</td>
<td>0.670</td>
<td>0.21</td>
<td>0.96</td>
</tr>
<tr>
<td>2.7% Heated</td>
<td>6.82</td>
<td>0.11</td>
<td>0.99</td>
</tr>
<tr>
<td>5.4% Unheated</td>
<td>4.37</td>
<td>0.23</td>
<td>0.99</td>
</tr>
<tr>
<td>5.4% Heated</td>
<td>69.6</td>
<td>0.17</td>
<td>0.99</td>
</tr>
<tr>
<td>1.0% Heated</td>
<td>1.09</td>
<td>0.20</td>
<td>0.98</td>
</tr>
<tr>
<td>1.0% Heated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ PCMB</td>
<td>1.20</td>
<td>0.21</td>
<td>0.98</td>
</tr>
<tr>
<td>1.0% Unheated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ PCMB</td>
<td>0.315</td>
<td>0.073</td>
<td>0.99</td>
</tr>
</tbody>
</table>
the 5.4% protein level. Water loss due to this rapid method of heating as determined by weighing was not detectable. Treatment with the sulphydryl blocking reagent, PCMB, failed to result in a decrease in viscosity after heating (Figure XXX).

The effects of varying heating time were not investigated due to the difficulty with which samples of the 12S isolate were obtained. In a second experiment, however, samples were heated much more slowly. All gels in the second study were prepared in sealed test tubes which were placed in boiling water for 5 min. Figure XXXI demonstrates the effect of various additives on the steady shear flow behavior of pH 9.2 gels. Perhaps the most notable feature of the rheogram is that samples of 4.5% protein gelled on different days yielded different flow properties. The gel which was formed from a dispersion stored for 4 days at 4°C, had a consistency coefficient of $10^4$ dyne sec cm$^{-2}$, whereas the consistency coefficient of a gel produced 4 days earlier from the same stock dispersion was only $16.3$ dyne sec cm$^{-2}$. A possible explanation for this phenomenon could be that the protein self-associates in the cold to form higher molecular weight complexes which in turn produce firmer gels. The temperature dependent self-association of the 12S protein of *B. juncea* has been reported by MacKenzie (1975). Because of this phenomenon, it is difficult to make conclusions concerning the effects of additives on thermally-induced intermolecular bonding.
FIGURE XXXI: Rheograms of pH 9.2 12S protein gels (4.5%) measured under steady shear at 23°C. Samples were prepared by placing in 100°C water bath for 5 min.

- no additives
- + 1M urea
- + .15M dithiothreitol
- + .5M NaCl
- + 1M NaCl
- △ no additives but aged 4 days at 4°C
The effects of 1M urea and 0.15M dithiothreitol on gel formation were not as dramatic as those of varying ionic strength and pH. The interacting effect of aging the dispersions in the cold makes the assessment of contributions of hydrogen and disulfide bonding difficult, however, neither of the reagents were capable of preventing gelation when added at the stated levels. Gels containing urea or dithiothreitol possessed higher consistency coefficients than the pH 9.2 gel containing no additives but exhibited lower apparent viscosities (at the shear rates tested) than the gel prepared from an "aged" dispersion. Unlike gelatin gels which are believed to be primarily cross-linked with hydrogen bonds (Bello and Vinograd, 1958), the rapeseed protein gels remained relatively unaffected. Refrigerated gelatin was found to gel at the 4.5% protein level in the presence of 1M urea, however, reverted to a sol when warmed to 22°C. Similar reversion was not observed for gelatin in the absence of urea. The pH 9.2 rapeseed protein gels were observed to be thermally irreversible, at least up to 100°C although measurements of gel strength were not taken at elevated temperatures. Attempts to dissolve the rapeseed protein gels in 8M urea were unsuccessful although slow disruption of the gel took place when the temperature of the dissociating agent approached 100°C. Such observations suggest that although hydrogen bonding may be present, it is not likely to be a major factor involved in the formation of intermolecular cross-links during the gelation of the 12S rapeseed protein.
The effect of the disulfide reducing agent, dithiothreitol, upon gelation was not dramatic. Although tempting, the validity of concluding the absence of sulfhydryl-disulfide interchange may be questionable. Catsimpoolas and Meyer (1970) found that while low concentrations of mercaptoethanol (0.1%) inhibited the gelation of soy protein, high concentrations (10%) actually enhanced it. The same authors reported that the addition of 0.1% N-ethylmaleimide (a sulfhydryl blocking reagent) to soybean dispersions had no effect on gelation. Although the existence of intermolecular disulfide bonding has been demonstrated in the 12S glycoprotein complex (Section IV.C.4.), it is difficult to imagine that the small amount of cystine reported in the amino acid profile could reflect such a highly cross-linked network within the gel.

Figure XXXI also illustrates the effect of increased ionic strength on the apparent viscosity of the gels. Gels which were adjusted to 0.5M and 1.0M NaCl demonstrated dramatically higher consistency coefficients (396 and 472 dyne sec$^n$ cm$^{-2}$, respectively) and slightly higher flow behavior indices than gels with no additives. These data are listed in Table V and contrast the results published for soybean isolates (Catsimpoolas and Meyer, 1970, and Hermansson, 1972). It was postulated in these studies that lowering the ionic strength of a soybean protein dispersion resulted in strong intermolecular forces of replusion, resulting in destabilization of the quaternary structures of the major globulins.
If ionic bonds were of major importance in rapeseed protein gels, increased ionic strength would be expected to result in reduced gel strength due to competition of the ions for the interacting functional groups of the protein. However, it may be that the 12S aggregate becomes much more soluble at high ionic strength, allowing more effective overlapping of functional groups. No experiments were performed to further investigate the effect of ions on gelation.

In the previous section, the effects of varying pH on the ultrastructure of rapeseed gels were discussed. Unfortunately, rheological data on all of the gels were not obtained, however, some of the results are presented in Figure XXXII and Table V. The highest apparent viscosities were obtained from gels prepared at pH 10. Although not shown on the graph, a gel formed at pH 4 was unstable and quickly reverted to a sol form. No gel formation was observed at pH 2 although the 12S glycoprotein was much more soluble at pH 2 than at any of the other pH levels. The gel strengths as reflected by the rheograms indicate little difference between pH 6, 8 and 9.2 gels. The data from Table V suggests that the pH 6.0 gel was firmer than both pH 9.2 and pH 8.0 gels. This result does not appear logical and is not consistent with the ultrastructural evidence presented in Section IV.D.3. It is possible that the high apparent viscosities observed for the pH 6.0 gel resulted from protein insolubility rather than an increase in structural integrity. Catsimpoolas and Meyer (1970) reported that the gelation of
FIGURE XXXII: Rheograms of 4.5% rapeseed protein gels measured under steady shear at 23°C. pH adjustments were made by dialysis against Britton-Robinson universal buffers.
- pH 10
- pH 8
- pH 6
- pH 2
- modified with NaBH₄ and formaldehyde
- "aged" 12S protein, pH 9.2
TABLE V: POWER-LAW PARAMETERS FOR STEADY SHEAR FLOW BEHAVIOR OF 4.5% 12S GLOBULIN GELS AT 23°C.
(EXPERIMENT II)

<table>
<thead>
<tr>
<th>Gel</th>
<th>Consistency coef., dyne sec^n cm^-2</th>
<th>Flow behavior index</th>
<th>Coefficient of determination</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 9.2</td>
<td>16.3</td>
<td>0.30</td>
<td>0.99</td>
</tr>
<tr>
<td>pH 9.2 &quot;aged&quot;*</td>
<td>104</td>
<td>0.25</td>
<td>0.99</td>
</tr>
<tr>
<td>pH 9.2 + 1M urea</td>
<td>54.1</td>
<td>0.15</td>
<td>0.99</td>
</tr>
<tr>
<td>pH 9.2 + 0.15M DTT</td>
<td>71.1</td>
<td>0.26</td>
<td>0.99</td>
</tr>
<tr>
<td>pH 9.2 + 0.5M NaCl</td>
<td>396</td>
<td>0.36</td>
<td>0.99</td>
</tr>
<tr>
<td>pH 9.2 + 1M NaCl</td>
<td>472</td>
<td>0.38</td>
<td>0.99</td>
</tr>
<tr>
<td>pH 10</td>
<td>300</td>
<td>0.13</td>
<td>0.99</td>
</tr>
<tr>
<td>pH 8.0</td>
<td>70.8</td>
<td>0.20</td>
<td>0.99</td>
</tr>
<tr>
<td>pH 6.0</td>
<td>133</td>
<td>0.28</td>
<td>0.99</td>
</tr>
<tr>
<td>pH 2.0</td>
<td>0.109</td>
<td>0.89</td>
<td>0.99</td>
</tr>
<tr>
<td>pH 9.2 &quot;modified&quot;**</td>
<td>8.35</td>
<td>0.43</td>
<td>0.99</td>
</tr>
</tbody>
</table>

... Cont'd.
TABLE V: Continued

* pH 9.2 "aged" gel refers to a sample prepared from a 4.5% rapeseed protein dispersion which had been stored for 4 days at 4°C.

** pH 9.2 "modified" gel refers to a sample prepared from a 4.5% rapeseed protein dispersion which had been reduced and alkylated by the technique of Means and Feeney (1971).
soybean globulins was pH-dependent, gel strengths increasing with corresponding increases in pH below pH 10. The effect of increasing pH was explained partially by an increased solubility and molecular expansion due to an increase in net negative charge (Hermansson, 1972).

In order to determine the possible involvement of lysine in some form of cross-linking reaction, a sample of the 12S protein was reduced with low levels of sodium borohydride and subsequently treated with formaldehyde in order to methylate the free ε-amino groups (Means and Feeney, 1971). The 12S protein (4.5% protein) modified by this procedure did not gel to form a solid self-supporting matrix (Figure XXXII). When the content of free ε-amino groups of lysine was determined by the method of Eklund (1976), no differences were detected between modified and unmodified 12S protein, indicating that modification of some other functional group essential for gelation had taken place in preference to ε-amino. No further experimentation was carried out in order to determine the identity of this functional group. It may be that the crosslinking reactions involve carbohydrate as well as amino acid functional groups, however, more research is required to establish such a relationship.

The steady shear rheological data are commonly used for the characterization of gelatinous semi-solids. Gels, however, behave neither in a purely viscous nor purely
elastic manner. A viscoelastic material is one which simultaneously exhibits both elastic and viscous responses to applied stress. This complex rheological behavior is displayed by all polymeric materials (Wohl, 1968). The dynamic response which is a manifestation of the viscoelastic material properties was measured for rapeseed gels in the Weissenberg Rheogoniometer. This instrument measures the shear stress resulting in a material which is subjected to a sinusoidally varying shear strain. The sample is located in a gap between a circular flat plate and a cone of equal diameter, providing a small angle between the cone and plate. The applied strain measured in the driving mechanism of the lower platen and the resulting stress transferred by the sample to the upper platen are transduced as electrical signals which can be monitored. A typical input strain wave and output stress wave is shown in Figure XXXIII. In this diagram, the shear stress and strain signals are separated by a phase difference, $\phi$, the tangent of which is a direct measurement of the ratio of energy lost as heat due to viscous flow and the energy stored due to elastic deformation. The elastic and viscous components may be separated and expressed as the dynamic shear moduli, $G'$ and $G''$, which describe the viscoelastic behavior of a material. $G'$ is the storage modulus which is a measure of the energy stored and recovered in a cycle of deformation, and $G''$ is the loss modulus which is associated with dissipative effects. Both moduli may be expected to depend on the oscillatory frequency
FIGURE XXXIII: Time profile of a dynamic shear experiment on a viscoelastic material as measured with the Weissenberg Rheogoniometer. The shear stress (σ) and shear strain (γ) waves are shown separated by the phase angle φ.

FIGURES XXXIV and XXXV illustrate the effects of frequency of oscillation on the storage and loss moduli, respectively, for the pH 9.2 gels containing various additives. The high degree of cross-linking in all gels, particularly those prepared in high salt concentrations, is indicated by the relatively constant values of the storage modulus over the range of oscillatory frequency. Even at low frequencies
FIGURE XXXIV: Dynamic shear storage moduli as a function of oscillatory frequency for 4.5% rapeseed protein gels at pH 9.2 and 23°C.

- no additives
- + 1M urea
- + 0.15M dithiothreitol
- ▲ + 0.5M NaCl
- ○ + 1M NaCl
  △ modified by reductive alkylation
(long relaxation times), the gels prepared with NaCl retain a constant level of stored energy. The gels with dithiothreitol and urea appear to store less energy in oscillatory shear than the pH 9.2 gel with no additives. The shapes of these three curves are similar, with a small inflection evident at low frequency. This phenomenon is sometimes due to entanglement coupling (Ferry, 1970) in which extended linear fragments interact in a specific frequency range such that an increase in elastic behavior is observed. This effect is less pronounced in the 0.5M NaCl sample and not detectable in the 1M NaCl gel. The modified sample which did not form a solid self-supporting matrix demonstrates a much greater dependence on frequency of oscillation. At high frequency, the liquid demonstrates a plateau or region of more typical elastic behavior. At lower frequencies, the uncross-linked liquid also shows the characteristic maxima perhaps due to entanglement coupling.

Figure XXXV illustrates the effect of frequency on the energy dissipated or lost as heat per cycle of sinusoidal deformation. Gels containing NaCl, urea, dithiothreitol and no additives exhibited only small increases in dissipated energy with increasing frequency of oscillation. The only major effect on the viscoelastic properties of the 12S gel at pH 9.2 was the reductive alkylation procedure. Although the addition of NaCl increased the absolute strength of the pH 9.2 gel system, its effects on the rubberlike elasticity were minimal.
FIGURE XXXV: Dynamic shear loss moduli as a function of oscillatory frequency for 4.5% rapeseed protein gels at pH 9.2 and 23°C.

- no additives
- + 1M urea
- + 0.15M dithiothreitol
- + 0.5M NaCl
- + 1M NaCl
- modified by reductive alkylation
Figures XXXVI and XXXVII demonstrate the effect of oscillatory frequency on storage and loss moduli of samples at different pH's. Gels prepared at pH 6, 9.2 and 10 indicated only small changes in both storage and loss moduli over a relatively wide range of frequency. The relative values of storage and loss moduli for the pH 6 gel compared with pH 9.2 and 10 are not in agreement with the steady shear data reported in Figure XXXII since under steady shear, the pH 6 gel exhibited lower apparent viscosities than the pH 10 gel. It may be that a significant amount of structure was lost during steady shear and would account for the lower observed apparent viscosities in the steady shear experiments. It may also be that since the pH 6 gel had a much "lumpier" texture than the gels at higher pH, highly elastic localized aggregates could have been formed due to electrostatic forces in the pH 6 gel. Such aggregates could have been responsible for the highly elastic recoveries illustrated in Figure XXXVI. Upon steady shearing, however, the aggregates may have been able to move with respect to one another since each aggregate would not necessarily be cross-linked with others. Thus, under conditions of high coulombic attraction and minimum solubility, the moduli observed need not necessarily reflect the true gel strength. Scanning electron micrographs presented in Section IV.D.3. would tend to support this explanation and suggest that an increase in three dimensional gel structure occurs with increases in pH rather than a maximum observable structure at pH 6.
FIGURE XXXVI: Dynamic shear storage moduli as a function of oscillatory frequency for 4.5% rapeseed protein gels prepared at various pH levels and measured at 23°C.

- pH 2
- pH 9.2
- pH 10
- pH 6
FIGURE XXXVII: Dynamic shear loss moduli as a function of oscillatory frequency for 4.5% rapeseed protein gels prepared at various pH levels and measured at 23°C.

- pH 2
- pH 9.2
- pH 10
- pH 6
Other problems exist with the method used for rheological characterization of gel structure. Since it was necessary to cut the gels several times and then force them into a narrow gap by compression, it is likely that much of the three dimensional structure was destroyed before rheological measurements could be taken. This problem could perhaps be eliminated if the gel were formed in a narrow gap between the platens of the rheometer. Such an experiment was not undertaken in the present study but should be considered for future studies of gelation phenomena.
V SUMMARY AND CONCLUSIONS

The 12S glycoprotein extracted from commercial rapeseed meal (*B. campestris* L. var. Span) was recovered by gel filtration and characterized by chemical, microstructural and rheological methods. The aggregate was not a sialoprotein; however, it contained arabinose, galactose, glucose, inositol glucosamine and mannose and strongly reacted when oxidized and exposed to Schiff reagent. Microstructural evidence suggested that the 12S aleurin was located within some but not all of the cells. The PAS-positive aleurone grains were distributed randomly throughout the meristematic tissues and cotyledons which comprise a large proportion of the seed. The aleurone grains of rapeseed contain globoid bodies which suggest the presence of phytic acid. This observation may be related to the presence of inositol in the protein aggregate. It may be that this sugar is added to the protein backbone some time after protein synthesis is completed or perhaps could be complexed with the protein by way of a Maillard condensation reaction in the meal during lipid removal. It is also possible that the differences observed between this and previous reports of carbohydrate composition of 12S glycoprotein recovered from seed could have resulted from non-enzymatic browning reactions.

The amino acid profile of the 12S globulin was dominated by the acidic amino acids glutamic and aspartic. However, it may be that the acidic components are largely
present in their amide forms. There was a scarcity of the sulfur-containing amino acids 1/2 cystine and methionine although the 12S agglomerate would appear at least partially held together with intermolecular disulfide bonds. Tryptophan was not detected from a ρ-toluenesulfonic acid hydrolyzate of the protein.

The protein aggregate is morula-like and has a maximum particle diameter of 120Å as determined from electron-micrographs of negatively-stained specimens. The complex yielded weight average molecular weight of 129,200 daltons as determined by conventional sedimentation equilibrium ultracentrifugation. The 12S isolate appeared heterogeneous according to polyacrylamide gel electrophoresis and ultracentrifugal methods, however, was immunologically homogeneous as determined by disc immunoelectrophoresis and immunodiffusion experiments. Since the 12S protein has been observed to self-associate over time at low temperature, the available evidence suggests that the high molecular protein fractions observed in preparations of this material are self-association products.

The 12S protein dissociated into five components of lower molecular weight in the presence of urea, SDS and mercaptoethanol. The fragments had apparent molecular weights of approximately 42,000, 37,600, 30,100, 17,400 and 12,200. SDS electrophoresis revealed that only one fragment contained all of the the Schiff-reactive material and migrated with or
slightly ahead of the smallest subunit detectable with Coomassie blue. The disc immunoelectrophoresis of SDS-treated protein against anti-12S antisera suggests that the glycopeptide portion of the complex is located on the surface and is perhaps the major immunodeterminant group.

Dispersions of the 12S glycoprotein form gels upon heating. Gelation was observed at the 4.5% protein level and increases in apparent viscosity were detected at the 1% protein level. The gel strengths were effected by changes in pH and sodium chloride concentrations, the strongest gels being formed at high pH and ionic strength. Urea, mercaptoethanol and p-mercuribenzoate had little effect on thermally-induced polymerization. Gel formation in this system is obviously a complex phenomenon which may involve covalent, ionic, hydrophobic and hydrogen bonding. The presence of such a high level of carbohydrate (12.9%) may also suggest the possibility of protein-carbohydrate interaction during gel formation.
REFERENCES


APPENDIX I
Sample Preparation for
Amino Acid Analysis

Cysteine alkylation. One hundred mg of isolate were dissolved in 10 ml buffer:

- 0.4 g Tris
- 19 mg KCl
- 0.25 ml 0.01% EDTA solution
- 12.1 g urea
  - dilute to about 20 ml and adjust pH to 7.5 with nitric acid and further dilute to 25 ml.

The sample was reduced with 0.1 ml 2-mercaptoethanol and stirred for 16 h under a nitrogen atmosphere. Derivitization was accomplished with the addition of 0.15 ml 4-vinylpyridine (freshly distilled) and stirred for 2 h under nitrogen. The pH was adjusted to 3 and the sample dialyzed against 2 changes of 0.01N acetic acid followed by exhaustive dialysis against several changes of distilled water. The sample was subsequently frozen and lyophilized.

Hydrolysis in p-toluenesulfonic acid. The hydrolysis mixture was prepared by dissolving 2.85 g p-toluenesulfonic acid and 10 mg 3-(2-aminoethyl) indole in 2.5 ml distilled water. This mixture was placed in a boiling water bath and diluted to 5 ml. Each 2.5 mg 12S isolate was dissolved in 1 ml of the hydrolysis mixture, placed in an ampoule, and frozen in an acetone-dry ice bath. Each ampoule was then evacuated to 20 to 30 μm Hg and heat sealed.
APPENDIX II
Hexosamine Determination -
Elson-Morgan Reaction

Samples weighing between 1.5 and 5.5 mg were placed in hydrolysis vials and hydrolyzed as described in the experimental section III.C.2.c. The contents of each vial (sample and 200-400 mesh AG 50W-X2 resin) were transferred to a 17 x 155 mm test tube with a ground glass joint and each vial rinsed several times with distilled water, the washings transferred each time to the test tube. The contents of each tube were evaporated to dryness at 50°C in a rotary evaporator in order to remove the HCl used in hydrolysis. Ion exchange columns prepared from 10 ml pipets were plugged at the tip with glass wool and filled with 7.5 ml of an aqueous suspension (1:1;w/v) of AG 50W-X2 (200-400 mesh, H⁺ form). The dried hydrolyzate was transferred in each case to a column with water and the neutral sugars eluted from the column with 15 ml of distilled water, and the effluent discarded. The hexosamines were then eluted with 10 ml 2N HCl, the eluate collected in a 50 ml round bottom flask and the contents of the flask were evaporated to dryness as before. Two ml of water were added to each evaporation flask and the dried material dissolved. One-ml aliquots of the dissolved samples were then placed in 16 x 125 mm screw-capped culture tubes using caps with Teflon liners. One ml of 2% (v/v) 2,4-pentanedione (freshly distilled) in 1N aqueous sodium carbonate was added to each tube. The tubes were sealed and placed in a 90°C water bath for 45 min and cooled. Four ml of 95%
ethanol were added and the contents were thoroughly mixed. One ml of a ρ-dimethylaminobenzaldehyde (PDMAB) solution (677.5 mg PDMAB dissolved in 25 ml of 1:1 ethanol-concentrated HCl) was mixed into each sample and after 1 h the absorbance readings of samples and standards were taken at 540 nm. A standard curve of $A_{540}$ vs glucosamine concentration was prepared and a best-fitting straight line determined by the least squares method. The concentration of glucosamine in each sample was determined from the regression equation:

$$\mu M \text{ glucosamine} = 0.000135 + (0.449)(A_{540})$$
APPENDIX III
Stock Solutions (polyacrylamide gel electrophoresis)

Upper reservoir buffer: pH 8.5

3.0 g Tris
14.4 g glycine
- dilute to 1:1 with distilled water and use 1:10 dilution of stock solution for buffer.

Lower reservoir buffer: same as above

A  Running gel buffer: pH 8.9, running pH 9.5

24 ml 1N HCl
18.1 g Tris
0.12 ml N,N',N''-tetramethylethylene diamine (Temed)
- dilute to 100 ml with distilled water

B  Stacking gel buffer: pH 6.7, running pH 8.9

5.98 g Tris
0.46 ml Temed
- adjust pH to 6.7 with 1N HCl and dilute to 100 ml with distilled water

C  Running acrylamide (7% gels):

28.0 g acrylamide
0.74 g N,N'-methylenbisacrylamide (Bis)
- dilute to 100 ml with distilled water

C' Running acrylamide (4% gels):

16.0 g acrylamide
0.74 g Bis
- dilute to 100 ml with distilled water
D  Stacking acrylamide:
   20.0 g acrylamide
   5.0 g Bis
   - dilute to 100 ml with distilled water

E  Riboflavin:
   4.0 mg
   - dilute to 100 ml with distilled water

F  Sucrose:
   40.0 g
   - dilute to 100 ml with distilled water

G  Ammonium persulfate catalyst:
   0.14 g
   - dilute to 100 ml with distilled water

H  0.005% Bromphenol blue tracking dye

Running gel preparation:
   1 part stock solution A + 1 part stock
   solution B + 2 parts catalyst G, overlay with
   water and allow 30 min to gel.

Stacking and sample gel preparation:
   1 part stock solution B
   1 part stock solution D
   1 part stock solution E
   4 parts stock solution F
   1 part distilled water
- remove water from top of running gel and add stacking gel followed by overlay
- photopolymerize with fluorescent light
APPENDIX IV
Stock Solutions (SDS gel electrophoresis)

Upper reservoir buffer: pH 8.64
- 0.04M boric acid
- 0.041M Tris
- 0.1% SDS

Lower reservoir buffer: pH 9.18, running pH 9.50
- 0.031M HCl
- 0.42M Tris

A  Running gel buffer: pH 9.18, running pH 9.50
- 0.12M HCl
- 1.7M Tris
- 0.12% N,N,N',N'-tetramethylenediamine (Temed)

B  Stacking gel buffer: pH 6.1, running pH 8.64
- 0.21M H₂SO₄
- 0.43M Tris

C  Running acrylamide (10% gels):
- 3.60 g N,N'-methylenebisacrylamide (Bis)
- 36.4 g acrylamide
  - dilute to 100 ml with distilled water

D  Stacking acrylamide (3.2% gels)
- 1.60 g Bis
- 23.4 g acrylamide
  - dilute to 100 ml with distilled water
E  Riboflavin:
   4 mg
   - dilute to 100 ml with distilled water

F  Sucrose:
   40 g
   - dilute to 100 ml with distilled water

G  Ammonium persulfate catalyst:
   0.14 g
   - dilute to 100 ml with distilled water

H  Schiff reagent:
   Dissolve 16 g potassium metabisulfite in 2 l H₂O
   and add 21 ml concentrated HCl. Add 8 g basic
   fuchsin and stir slowly for 2 h. Let solution
   stand for 2 h and decolorize with a small amount
   of Darco G60 charcoal filter.

Running gel preparation:
   1 part stock solution A
   1 part stock solution B
   2 parts catalyst G
   This mixture may be made 0.1% in SDS or the SDS
   may be omitted without any change in the electro-
   phoretic pattern.
Stacking and sample gel preparation:
1 part stock solution B
1 part stock solution D
1 part stock solution E
4 parts stock solution F
1 part distilled water
- add 0.1% SDS and expose to fluorescent light to polymerize

Sample preparation:
10 mg of each protein was dissolved in 0.2 ml buffer B and 1.8 ml 8M urea, the final mixture containing 1% SDS and 0.14M 2-mercaptoethanol or dithiothreitol. No difference in the electrophoretic pattern could be detected when the sample solution was adjusted to 10% SDS or when dithiothreitol was substituted for 2-mercaptoethanol. Best results were obtained when 10 to 50 µg of protein were applied to each gel.
Amino Acid Analysis Results*  
*All values reported in g amino acid residue per 16 g N recovered.

Using Various Hydrolysis Times for the 12S Glycoprotein

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Hydrolysis Time at 110°C (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>10.2</td>
</tr>
<tr>
<td>Threonine</td>
<td>4.15</td>
</tr>
<tr>
<td>Serine</td>
<td>4.54</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>19.3</td>
</tr>
<tr>
<td>Proline</td>
<td>4.89</td>
</tr>
<tr>
<td>Glycine</td>
<td>5.02</td>
</tr>
<tr>
<td>Alanine</td>
<td>4.21</td>
</tr>
<tr>
<td>Valine</td>
<td>4.29</td>
</tr>
<tr>
<td>Methionine</td>
<td>2.09</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>3.95</td>
</tr>
<tr>
<td>Leucine</td>
<td>8.10</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>3.17</td>
</tr>
<tr>
<td>Ammonia</td>
<td>2.35</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>5.01</td>
</tr>
<tr>
<td>Lysine</td>
<td>3.00</td>
</tr>
<tr>
<td>Histidine</td>
<td>2.97</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>0</td>
</tr>
<tr>
<td>Arginine</td>
<td>5.93</td>
</tr>
<tr>
<td>Pyridylethyl-L-cystine</td>
<td>0</td>
</tr>
<tr>
<td>Total recovery (%N)</td>
<td>68</td>
</tr>
</tbody>
</table>