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PROTEIN OF YAM TUBERS (*DIOSCOREA ROTUNDATA*)

by

Patricia Janine Harvey

A Thesis Submitted in Accordance with the requirements for the Degree of Doctor of Philosophy in the University of Durham

Botany Department

June 1981

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ABSTRACT

The major proteins of the yam tuber, which were identified as storage proteins by virtue of their abundance (c. 85% of the total protein content), amino acid composition (high in amide content), and location within the tuber, were isolated by ion exchange chromatography and characterised using in particular, polyacrylamide gel techniques.

They consist principally of one size of sub-unit, molecular weight 31,000, N-terminal amino acid glutamine/glutamic acid, of which there are a number of charge isomers; these in general contain one intra-chain disulphide bond. The sub-units associated into polymers depending on the protein concentration, pH and ionic strength of the milieu, and therefore a value for the molecular weight of the native protein(s) is not given. The storage proteins are not glycoproteins, and do not exhibit lectin activity. They are intracellularly located as protein "aggregates" within both cytoplasmic vesicles and cellular protein vacuoles. Their characteristics and other aspects of their biology are compared with those of the storage proteins of the potato tuber.

The first nutritionally limiting amino acids of the tuber protein are the sulphur-containing amino acids although tryptophan was not determined. Comparative amino acid data from several sources, together with the fact that the sub-unit composition of genetically different tubers did not vary greatly, suggests that the potential for breeding for improved protein quality is limited.

The protein content of yam tubers can be adequately evaluated by the Kjeldahl technique, since the non-protein nitrogen content of genetically different tubers grown under different environmental conditions was found to be relatively constant.

Nitrogen content varied considerably with environment, although an investigation of its heritability suggested that the potential for breeding for increased protein content could exist.

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INTRODUCTION

Production Data

The tropical root crops, which consist primarily of yams, cassava, sweet potatoes and cocoyams, feature as major food items throughout the tropical world. Accurate statistics of current production are difficult to obtain because most of the production of these root crops never enters commercial channels. Even so, it is estimated to be in the order of 140 million metric tons per annum which has been calculated as representing the staple food of around 400 million people (Coursey and Haynes, 1970). However, it is only comparatively recently, with a worsening food crisis in the tropical world, that attention has been focussed on food rather than cash crops, and the quantity and quality of these food supplies have now become subjects of serious study. De Vries et al (1967) compared the average world production, in terms of calories edible per hectare and per day of dry matter of yams, cassava, sweet potato and cocoyams, to that of rice, wheat, maize and sorghum. He concluded that the root crops probably have a far higher production capacity than grain crops, that they were far behind in breeding and selection, and that some grain crops, especially rice and maize, were already close to their potential yields.

True yam production (i.e. excluding other root crops which are sometimes grouped together as yams) contributes approximately 25 million metric tons per annum globally. The main area of production is in the West African yam zone which extends from 4^{o} N to about 10^{o} N; and over half the world production of yams comes from Nigeria alone. Other areas producing substantial quantities are the West

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Indies, East Africa, Venezuela, Papua New Guinea, the Philippines, Malagasy and Panama.

Nomenclature

Yams belong to the genus Dioscorea, the largest genus of the monocotyledonous family Dioscoreaceae, formerly placed in the Liliales (Burkill, 1960) but recently established in a separate order, the Dioscoreales (Ayensu, 1972). The family has, however, many features associated with dicotyledons (Purseglove, 1972) and recent studies on some West African species (Lawton and Lawton, 1967) have demonstrated the existence of two cotyledons, one of which showed delayed development.

Botanical and agronomical aspects of yams have been reviewed by Waitt, (1963), Coursey, (1967), Purseglove, (1972), Kaye, (1973) and Onwueme, (1978). Most of the species produce annual storage organs, the tubers, which remain dormant during the dry season when the shoot system dies back. With the onset of the wet season, shoots are produced from the heads of the tubers, which then shrivel away and new tubers are produced. The stems climb by twining, the direction of which is constant for the species and section of the genus.

There are 600 or so species within the genus, around 60 of which produce edible tubers; of these, ten may be considered cultivated. Their growth is restricted to areas with a temperature range of 25-30^{o} C, a dry season of not more than two/four months, a rainfall of at least 1500 mm evenly distributed throughout the remainder of the year, and soils which are deep, friable and fertile.

D.rotundata Poir, the white yam or Guinea yam, is the most important species in West Africa. It is also cultivated in the West Indies and to some extent in East Africa, but not in Asia. The species is a true cultigen, unknown in the wild state except as an escape. It is believed to be of hybrid origin, with D.cayenensis Lam as one of the parents, and the other, a savannah species, either D.praehensilis Benth or D.abyssinica Hochst (Burkill, 1939; Ayensu and Coursey, 1972). The profusion of different cultivars which exist today is believed to have arisen from initial chance hybridisations, and to contain varying proportions of genetic material derived from the two (or possibly more) ancestral species; some may have arisen by somatic mutation.

D.alata L., the asiatic or water yam, of South East Asian origin is the second most important species, and is widely disseminated throughout the tropics. As with D.rotundata, it is a true cultigen.

D.cayenensis Lam, the yellow yam, is the next most important species and occurs in the wild state in West Africa. Its cultivation is mainly confined to West Africa and the West Indies. This species is followed in importance by D.esculenta (Lour.) Burk., the Chinese yam, which originated in Indochina and is widely disseminated. Unlike most of the edible yams, this species produces clusters (up to 20) of small tubers.

Other species include D.dumetorum (Kunth) Pax, the bitter yam whose cultivation is restricted to West Africa, and is used only in times of food scarcity; D.bulbifera L., the aerial yam, which is the sole edible yam species native to both Asia and Africa, and is cultivated largely for its aerial bulbils; D.trifida L., which

originated in the northern part of South America and is the only yam of New World origin to obtain significance as food, and D.japonica (Thumb) which is cultivated extensively in Japan and China.

It should be noted that some workers, for example Miège, (1968), have regarded D.rotundata as possibly a subspecies of D.cayenensis.

The Tubers

The tubers of the edible species have generally been regarded organographically as stem tubers (Burkill, 1960; Njoku, 1963). Recent studies, however, suggest that the tuber is a lateral outgrowth from the hypocotyl region of the plant axis; it lacks scale leaves, buds or "eyes", the growing point is not marked by a terminal bud, and most of the tubers exhibit strong positive geotropism (Martin and Ortiz, 1963; Lawton and Lawton, 1969; Onwueme, 1973). Cultivars of D.rotundata generally produce one or two large tubers which usually weigh 2-5 kg.; in good areas, 10 kg. is not uncommon, and weights of 20-25 kg. have been recorded (Purseglove, 1972). They are normally cylindrical (See Fig. 1) but can assume distorted shapes. The anatomical structure is outlined by Onwueme, 1978.

The proximate chemical composition of yam tubers has been reviewed by Coursey, (1967), and more recently by Martin, (1979), and is summarised in table 1.

By far the largest component of the fresh tuber is water, which accounts for about two thirds of the fresh weight. There are large variations in percentage water content both between species and within species (Coursey, 1967; Baquar and Oke, 1976), and also within a tuber; the tail end has a higher moisture content than the head end (Coursey and Walker, 1960; Ferguson et al, 1980).

Fig.1

Yam tubers (Dioscorea rotundata)

TABLE 1

SUMMARY OF PROXIMATE ANALYSES OF YAM TUBERS (ADAPTED FROM COURSEY, 1967)

Species	Moisture Content %	Carbohydrate %	Fat %	Crude Protein %	Crude Fibre %	Ash %
D.rotundata	58-73	23	0.12	1.09-1.99	0.35-0.79	0.68-2.56
D.cayenensis	83	15	0.05	1.02	0.40	0.53
D.alata	65-73	22-29	0.03-0.27	1.12-2.78	0.65-1.40	0.67-2.06
D.esculenta	67-81	17-25	0.04-0.29	1.29-1.87	0.18-1.51	0.50-1.24
D.dumetorum	79	17	0.28	2.78	0.30	0.72
D.bulbifera	63-67	27-33	0.04	1.12-1.50	0.70-0.73	1.08-1.51
D.trifida	-	38	0.44	2.54	-	-

TABLE 2

CRUDE PROTEIN CONTENT (%N x 6.25) OF YAM TUBERS (DRY WEIGHT BASIS)

Species							Reference
D.rotundata	D.cayenensis	D.alata	D.esculenta	D.dumetorum	D.bulbifera	D.trifida	
4.42	5.44	7.26	7.73	11.73			Oyenuga (1968)
7.28-8.06 av 7.67		6.28-11.22 av 8.33	7.84-13.41 av 9.42		9.90-11.06 av 10.63	7.38	Martin and Thompson (1971)
6.34-8.06 av 7.21		6.56-11.22 av 8.33	7.85-13.41 av 9.42		6.66-11.06 av 9.79	6.69-7.63 av 7.23	Splittstoesser et al, (1973a)
5.13 5.25 5.38	4.19	6.75 8.95				4.38	Francis et al, (1975)
3.8-13.9 av 8.18	4.0	3.8-10.3 av 6.27	5.5-5.9	4.9-14.0 av 8.96	7.9-9.6		Baquar and Oke (1976)

During storage of the dormant tuber, a slight reduction of moisture content by transpiration is reported to occur (Coursey, 1961) and proceeds only slightly more rapidly than the destruction of dry matter by respiration.

Carbohydrate in the form of starch is the chief tuber dry matter component. Amylopectin is the major constituent with amylose representing around 10-28%, but the content of the latter is extremely variable between cultivars of a species (Martin, 1979). Starch grains show a wide variation in size and form according to the species (Martin, 1979). The viscosity of the starches has been summarised by Coursey, (1967); starch from D.rotundata has a high viscosity maximum of 470-690 Brabender units, and a high gel strength, characteristics which enable preparation of the very popular traditional pounded yam dish, fufu.

Sugars, principally glucose and sucrose, constitute less than 1% of the fresh weight (D.esculenta is a notable exception with 7-11% sugar). The latter sugar appears to be in much greater amount than the former (Fox, cit. Coursey, 1967) and they are believed to be either intermediate products to be stored as starches (Martin, 1979) or derived from the breakdown of starch (Coursey, 1967).

Several studies have been undertaken with respect to the protein content and amino acid composition of yam tubers (see discussion for details). In general, the crude protein content (% N x 6.25) ranges between 5% and 9% of the dry weight; yams are thus more useful as sources of protein than other root crops, particularly cassava. However, wide variations in crude protein content have been reported (Table 2), both between species and

between cultivars within a species. Francis et al (1975) suggested that the production of yams with a relatively high total protein might, therefore, be possible, although Martin and Thompson, (1971a) considered that the variability of protein content was not as broad as that of other important characteristics and should thus not merit a high ranking in a list of breeding objectives.

The distribution of crude protein content has been reported to vary insignificantly between different parts of the peeled tuber (Martin and Thompson, 1971a) although morphologically older regions (upper head and inner portions) appear to have higher quantities than younger regions (Ferguson et al, 1980). The peel, however, which consists of cork cells and an immediate underlying cortex has a higher crude protein content than the rest of the tuber (Oyenuga, 1968; Martin and Thompson, 1971a; Ferguson et al, 1980). It is often utilized as feed for livestock.

The retention of protein during cooking has been reported to be high. Thus Francis et al, (1975) found no apparent loss of total nitrogen from the peeled cooked tuber, although Splittstoesser, (1976) reported a loss of between 25% and 59% of the non-protein amino acids (between 3% and 7.5% of the total amino acid content before cooking).

Amino acid analyses have suggested that the protein of yam is not well balanced nutritionally. The sulphur amino acids have been found in general to be limiting (Miller and Donoso, 1963; Busson, 1965; Oyenuga, 1968; Martin and Thompson, 1972; Francis et al, 1975). Tryptophan is often equally limiting (Splittstoesser and Martin, 1975; Francis et al, 1975), so that the protein of yams cannot be fully utilized unless complemented with foods containing high amounts of these amino acids. Martin and Thompson, (1972),

however, reported that varietal differences in amino acid composition were at least as great as those between species, and that cystine content in particular varied widely among the cultivars examined; these authors suggested that superior varieties less deficient in this amino acid could be selected or bred. Splittstoesser et al, (1973a) used principal component analysis of values of protein and amino acid content for 46 cultivars representing five yam species and suggested, on the basis of their results, that most of the amino acids were inherited independently of each other, so that breeding should indeed allow improvement in the amino acid composition. However, Francis et al, (1975), considered, on the basis of their results which were broadly in accord with those of previous workers, that there was little evidence to provide substantial support to this idea.

Yams are also a source of several vitamins and minerals. Thus yellow fleshed tubers may contain useful amounts of carotene (provitamin A) e.g. tubers of D.cayenensis contain 0.4-1.44 mg/100 g edible portion of carotene (Martin and Ruberté, 1975). However, tubers of D.bulbifera, also very yellow fleshed, contain little or no carotene. The vitamin C content makes a useful contribution to the diet; and yams were widely used for their antiscorbutic properties in the Indian and Pacific oceans in the pre-European era and later by the Portuguese. Vitamin retention during storage, and cooking has been examined by Coursey and Aidoo, (1966); a loss of 20% was observed during four months storage, but only 5% on boiling unpeeled tubers. However, the more common practice of boiling after peeling resulted in a loss of 35%, similar to the loss (40-60%) reported by Oke, (1972). The vitamin B complex is small, in common with other roots and tubers. Calcium is present in

raphides as calcium oxalate, and it is not known to what extent it is available nutritionally, but the amount is probably small (Martin, 1979). There are also small amounts of iron, phosphorus, potassium and magnesium (Winton and Winton, 1935; Platt, 1962; Ferguson et al, 1980). The latter three minerals, together with calcium are distributed in definite gradients between the head and tail of the tuber (Ferguson et al, 1980).

On the negative side, there may be some antiphysiological substances present. Gilbert and Gillman, (1963) observed the development of liver necrosis in rats fed entirely on yam, although the symptoms could have been produced by a deficiency of essential amino acids. Jeffers and Haynes, (1977) found that substitution of a commercial ration with 10% and 20% dehydrated yam had little adverse effect on rats, but at the 30% level of substitution, weight gains were depressed: they concluded, however, that dehydrated yam could serve as a main carbohydrate component of balanced rations but only if supplemented with high quality protein. However, Womack et al, (1977) found that rats which were fed cooked yam as part of a carefully balanced, nutritionally sound diet, often lost weight and in some cases died, but that rats fed uncooked yam remained healthy; also Maranon et al, (1977) found that laying hens which received D.alata or D.esculenta tubers as a part of their carefully balanced diets stopped laying, and in some cases died. When yams were removed from the diet, the hens began to lay eggs again normally.

Although no systematic work has been done to identify any antiphysiological constituents of the food yams, such constituents may be related to those found in the non-edible yams. Some of these contain saponins and sapogenins which have been fairly intensively studied (Martin, 1970; Takeda, 1972; Nakamura et al, 1979;

Tang, 1979; Kakade et al, 1979), interest having stemmed from their application to the pharmaceutical industry. Many of the non-edible yams contain alkaloids, which include dioscorine and dihydro-dioscorine (Willaman et al, 1953); their use as nerve poisons for hunting and fishing purposes is still common. Tannins are also present, the quantity in some species being sufficient to find application in the tanning of hides. Yams may also contain large amounts of oxalic acid.

The Importance of Yam as a Food Crop

(a) Uses:

Yams are primarily marketed as the fresh tuber. The daily ration for those parts of the population of West Africa who habitually consume yam has been estimated to be in the order of 0.5-1 kg. (Coursey, 1965).

The tubers are most commonly peeled, sliced and boiled until tender. The head end which is hard and unpalatable is usually rejected. Losses during culinary preparation amount to between 10 and 25% (Coursey, 1965; Francis et al, 1975). The most popular and traditional form in which yam is eaten in West Africa is as pounded yam, or fufu, prepared by pounding yam to a viscous semi-solid. Yams may also be fried, roasted or baked. A proportion of the yam crop, particularly in the northern forest and semisavannah areas of the West African yam zone is prepared as yam flour, which can be reconstituted with water to prepare a similar but inferior form of fufu. Yam flour and yam flakes have been produced commercially (Rodríguez-Sosa and Gonzales, 1972; Steele and Sammy, 1973; Ayernor, 1976) but the products are expensive and, unlike cassava flour, their preparation for consumption still requires considerable time spent stirring and kneading the meal with boiling water over heat.

Yams may also serve as raw material for a number of different food products such as pastry, boiled fish paste, etc. (Kawakami, 1970). However, the use of yams as a source of industrial starch or alcohol is not widespread because cheaper alternative sources exist; this also applies to their uncommon use as feed for livestock (Oyenuga, 1968).

(b) Role of Yams in the Diet:

Yam cultivation is believed to have developed independently in West Africa and South East Asia around 5000B.P. (Alexander and Coursey, 1969) and is thus of very ancient origin. In the yam zone of West Africa yam was virtually the only staple grown prior to the introduction of cassava, maize and cocoyams in the 16th and 17th Centuries. Many of the associated superstitious and cultural practices have survived (Coursey and Coursey, 1971) and some still play a prominent role in the cultural lives of the people, particularly in the rural areas of West Africa. One of the most important rites in West Africa is the New Yam Festival, which has in some areas reached an accommodation with Christianity. Essentially, no farmer may harvest or consume the new crop of yams until the day of the Festival. The formal inauguration of the new crop is unique to yam, and is indicative of the considerable sentiment attached to them. According to Onwueme, (1978), the urban dweller has retained this attachment for the yam, so that "his idea of a meal the way it was back home is a plateful of pounded yam". This is further evidenced by the large quantities of yam imported by North America, the United Kingdom and other areas for the benefit of the immigrant population (Martin and Thompson, 1971a; Coursey, 1968). Yam has also retained its social supremacy over other crops in so far as the status of a farmer is determined by the quantity of his yam harvest,

irrespective of his production of other crops. Thus, wherever yams are utilized, there is a resistance to supplementing or exchanging them for other staple foods.

However, the production of yam has currently become too costly for the poorer inhabitants of the tropics to afford to purchase them, and the yam is now regarded as a status crop in the countries in which it is grown (Aduayi and Okpon, 1980). There are a number of reasons for high production costs.

Firstly, yam production has a very high labour requirement for land preparation, weeding (40 man days per hectare), staking (60 man days per hectare), harvesting and storage preparation (Onwueme, 1978). In Nigeria, particularly with the advent of oil, many of the younger people have been attracted to the urban areas, leaving farmers without labour and unable to compete with the higher wages of the city for contract labour (Webster, 1979). The yam in its present form does not lend itself easily to mechanisation of some of these processes.

Secondly, small tuber setts result in low yields per sett, so that in order to realise reasonable yields per hectare, large quantities of planting material (2.5metric tons per hectare, or more) have to be utilised. Even so, the ratio of material planted to material harvested is very low, and yields of 12.5 metric tons per hectare are considered good. Furthermore, in contrast with cassava or sweet potato, the planting material is otherwise edible.

Thirdly, the cost of stacking is high, and can represent almost a third of the total production cost (Wholey and Haynes, 1971). Without stacking, a yield reduction in the order of 50% may occur (Enyi, 1970a).

Fourthly, yams unlike cassava or sweet potato, have a high soil fertility requirement which was met in the past with the practice of shifting agriculture combined with long periods of bush fallow. Many farmers, particularly in West Africa, have continued to practise bush fallow agriculture but with increasing pressure for land, the fallow period has become progressively shorter with a concomitant decrease in the average fertility of the farmed plots and lowered yields. Fertilizers and crop protection chemicals are rarely used. In addition, although yams may survive periods of drought, yields are invariably sacrificed particularly if drought occurs during the fourteenth and twentieth week after emergence, the period of vigorous tuber bulking (Oyolu, 1961).

Fifthly, most of the yam production is under peasant cultivation, and under these conditions yields are generally not in excess of nine metric tons per hectare. Enyi (1970b) has summarised aspects of this form of agronomic practice which are responsible for the low yields in Africa. Under more advanced agronomic situations and using the same unimproved species, yields in the order of 22 to 23 metric tons per hectare and higher have been obtained (Enyi, 1970b; Coursey and Haynes, 1970).

Finally, yam tubers are generally stored fresh; in this form storage losses due to rotting and respiration may amount to 40% over a period of six months (Coursey, 1961).

These factors combined have lead to the steady replacement of yam by, in particular, cassava, which is also consumed essentially for carbohydrate content, but which requires no staking, is more tolerant of weeds and neglect by the farmer, has a low fertility requirement, can be propagated by means of the non-edible

stem portion, and is stored and marketed primarily in a dried processed form (meal). The displacement of yam by cassava has been accelerated over the last 20 to 30 years by the rapid trend towards urbanisation and concomitant evolution of an urban proletariat living at or below subsistence level, creating a higher demand for the cheapest food.

On a nutritional basis, however, cassava is considerably inferior as a source of protein (Martin and Thompson, 1971b; Splittstoesser and Rhodes, 1973; Splittstoesser et al, 1973b; Martin and Splittstoesser, 1975; Jeffers and Haynes, 1977). The Net Dietary Protein calories per cent (N.Dp.Cal %) of cassava has been estimated by Payne (1969) to be in the order of 0.9, whereas that of yam was estimated as 4.6, comparable to that of maize, 4.7. The world "protein gap" may have been exaggerated in the past i.e. a lack of food rather than a lack of protein per se is the major problem, but in those areas with a root crop such as cassava emerging as a primary staple, there may often be a requirement for protein per se in the diet. Thus much of the malnutrition reported in, for example, the urban areas of West Africa may be associated with the replacement in the main of the relatively high protein yam by low protein-containing cassava. This highlights the need to improve the yield and/or protein content of yam.

Yam production may be increased substantially with improved agrononic management (qv), but considerable further improvement should be possible with a systematic selection of existing lines, and by breeding. Although the present day yam cultivars have undergone selection by man over thousands of years this has not been in any way systematic (Coursey and Martin, 1970; Coursey, 1976).

The collection of existing lines is now in progress by various centres around the world, notably at the International Institute of Tropical Agriculture (I.I.T.A.) in Ibadan, Nigeria, and the U.S. Department of Agriculture in Puerto Rico. Even so, breeding of yam is considerably hampered, because over the centuries yams have been vegetatively propagated, and most of today's cultivars are high polyploids which, in general, have lost their ability for efficient sexual expression. Thus the three commercially most important species of yam have irregular flowering. D.cayenensis produces only male flowers; D.alata rarely produces any flowers, and most cultivars of D.rotundata never flower, although there are a few which do. Breeding amongst these latter, however, is complicated by the fact that they are truly dioecious, flower production is not assured and seed production even less so. Results from I.I.T.A. (1972) indicate that flower abortion may range from 38% to 94%, whilst ovule abortion may range from 70% to 80%. It is estimated that only five or six filled seeds can be obtained on average from each female flowering plant. Besides the relative scarcity of well formed seeds, germination is preceded by a dormancy period of three to four months. Accordingly, it was until recently widely accepted that the seeds of D.rotundata were not viable. However, in 1973 knowing that there was a dormancy period and the fact that large numbers of the seeds lacked well developed embryos, seeds of this species have now been successfully germinated on a large scale basis at I.I.T.A. (Sadik, 1976; Sadik and Okereke, 1975). A breeding programme was established in 1975, and since then more than 70,000 biotypes have been grown from seed in breeding populations both at I.I.T.A. and in several off-site locations representing different cultural and ecological conditions.

The short term objectives of this breeding programme are to select clones from segregating seedling populations grown in different ecological zones for all the conventional attributes of high yield, disease and pest resistance, storability, quality i.e. palatability, poundability and protein content, and also to select for varieties which will fit into traditional cropping systems. A population improvement scheme has been initiated to generate seed populations containing high frequencies of these desirable characteristics (Wilson, 1978). Long term objectives are to select cultivars which lower the labour requirements e.g. those whose tubers are shallow setting, oval or round and regular in shape, tough skinned and produce several smaller tubers per plant rather than one large one so that partial or complete mechanical harvesting is feasible, as well as to select for cultivars which are adapted to no-stake conditions, and furthermore, to reduce the amount of plant material required for propagation by e.g. the production of uniform populations of true seed.

Although these are the primary breeding aims of the I.I.T.A. programme in Nigeria, it is essential, in view of the important nutritional role of yams, that the protein content and quality of potential new cultivars should be monitored, lest, in establishing desirable agronomic features these aspects suffer. Furthermore, in 1976, when 100 biotypes from the programme were analysed for total nitrogen (crude protein content) some high nitrogen varieties (2.35% N or 14.7% crude protein content) were identified (I.I.T.A. in-house report, 1976). Thus, whilst the major object is not to increase protein content, this aspect should be borne in mind.

The aims of the present study with respect to the I.I.T.A.'s breeding programme were:

(i) to assist with the selection of clones with a high nitrogen content;

(ii) to examine the variability of nitrogen content between tubers of a plant in relation to that between plants of a clone;

(iii) to examine the variability of nitrogen content between seedling derived plants vegetatively propagated from one year to the next;

(iv) to ascertain whether there are any strong phenotypic correlations of protein content with other characteristics of the plant and tuber;

(v) to estimate the heritability of nitrogen content.

The screening of large amounts of material for protein content and quality, although deemed necessary, is a considerable task, and therefore it was felt important to evaluate the protein situation in the yam, concurrently with the examination of plant material from the breeding programme. In this respect, since major proteins would be largely responsible for the protein characteristics of the yam meal, were there few or many of these proteins present? Information of this nature might, for example, be utilised to make the task of screening for quality more realistic. Furthermore, information on the character of the proteins may well relate to their storage stability, and perhaps more importantly, may have application with regard to the processing of yam, for example, as a dried product for more convenient storage, etc.

Whilst cereal and legume grain proteins have been investigated fairly intensively, apart from some proteins of potato, little is known about the major proteins of root and tuber storage organs.

To this end, the approach adopted in this work was

(1) to ascertain whether there were one or several major proteins in the tubers of the species D.rotundata;

(2) to acquire information as regards the general biology of the proteins in the tuber, for example, the location of the proteins at the subcellular level;

(3) to characterise the major tuber protein(s).

The timely development over the past few decades of a number of experimentally relatively simple techniques for the purification and characterisation of proteins considerably facilitated this task. Chromatography on cellulose-based ion exchangers (Sober and Peterson, 1954; 1956); molecular sieve chromatography on both artificially cross-linked dextrans (Porath and Flodin, 1959) and polyacrylamide gels (Hjerten and Mosbach, 1962); adsorption chromatography on hydroxylapatite (Swingle et al, 1951), zone electrophoresis on polyacrylamide gels (Davis, 1964; Raymond and Weintraub, 1959), as well as fully automated means for amino acid analysis on ion exchange resins (Moore et al, 1958; Spackman et al, 1958) are the major examples of these techniques which have been applied in this work.

Perhaps of widest application in this study has been the use of polyacrylamide gel (PAG), principally as an electrophoretic matrix but also as a stationary phase in molecular sieve chromatography. PAG is distinguished by its chemical stability and inertia, transparency, stability to pH and temperature variations, insolubility in most solvents, analytical purity of its components and absence of adsorption effects. Furthermore, with respect to its application in zone electrophoresis, electro-osmotic effects are absent, and in particular it is possible to control its broadly variable structure

to produce a wide range of average pore size with a high degree of reproducibility.

Exploitation of this latter property, combined with the development of the physical chemical theory of the movement of molecules through gels (Ferguson, 1964; Morris, 1966; Rodbard and Chrambach, 1970) has provided a powerful and rapid means of differentiating the heterogeneity of native yam proteins based on their charge from that based on size differences, (Hedrick and Smith, 1968; Rodbard and Chrambach, 1971; Morris and Morris, 1971). Furthermore, sub-unit structures have been assessed, and the molecular weight of sub-units determined by combining the use of sodium dodecyl sulphate (SDS) with zone electrophoresis on polyacrylamide gels (Shapiro et al, 1967; Weber and Osborne, 1969; Laemmli, 1970): even greater resolution was afforded in this respect by the use of multiphasic (discontinuous) buffer systems (Ornstein, 1964; Chrambach et al, 1976). Also, PAG prepared together with synthetically produced ampholytes (Vesterberg and Svensson, 1966) provided a rapid and extremely sensitive analytical means for the fractionation of charge isomers, and estimation of isoelectric points (Awdeh et al, 1968; Dale and Latner, 1968; Wrigley, 1968).

MATERIALS

I. BIOLOGICAL MATERIALS

Yam samples of the species Dioscorea rotundata were supplied by the International Institute of Tropical Agriculture, Ibadan, Nigeria. The variety Nwapoko, and bio-lines "Yam 2", R16-214-127, R16-215-58, R16-215-101 and R16-215-134 were used for protein analysis. Flours from five populations of plants were screened for nitrogen content; the composition of each population is supplied in table 3.

II. CHEMICALS AND REAGENTS

Chemicals and reagents, apart from those listed below were obtained from British Drug House (BDH) Limited, Poole, Dorset and were of analytical grade when necessary.

Beckman Instruments Inc., Fullerton, California
- Cellulose Acetate Membranes

Biorad Laboratories Limited, Richmond, California
- Bio-Gel HT (hydroxylapatite)
- Bio-Gel P-4
- Bio-Gel P-150
- Bio-Gel P-300

Calbiochem, San Diego, California
- Ferritin B grade (equine spleen)

Combithek Bochringer, Mannheim, West Germany
- Aldolase
- Catalase

Cannings, W. E., Avonmouth Way, Avonmouth, Bristol, England
- Uncoated cellophane for drying gels

EMScope Laboratories Limited, London/Ashford, Kent, England
- Gluteraldehyde
- Osmium Tetroxide
- Sodium Cacodylate
- ER 4206
- DER 736
- NSA
- S-1

Gurr, High Wycombe, Bucks., England
- Procion Brilliant Blue

Hopkin and Williams Limited, Essex, England
Glycerol
Repelcote Water Repellent

Koch-Light Laboratories Limited, Colnbrook, Bucks., England
Dimethyl Sulphoxide
Ninhydrin
Trichloroacetic Acid
L-arginine
L-lysine

LKB Instruments Limited, South Croydon, Surrey, England
"Ampholine" carrier ampholytes pH 3.5-10

Medical International, 49 Queen Victoria Street, London
Visking dialysis tubing

Miles-Seravac, Maidenhead, Berks.
Chymotrypsinogen A
Ovalbumin, 5x crystallized

Pharmacia Fine Chemicals, Uppsala, Sweden
Dextran Blue 2000
Pharmalyte carrier ampholytes pH 3-10
Sephadex G-100, Fine and Superfine grades
Sephadex G-75, Fine

Pierce, Rockford, Illinois 61105, U.S.A.
Guanidine Hydrochloride
Polyamide sheets. These were also obtained from

Cheng Chin Trading Company Limited, Tapei, Taiwan, through
BDH Chemicals Limited

Rathburn Chemicals, Walkerburn, Peebleshire
Pyridine

Sigma London Chemical Company Limited
Benzoyl-DL-arginine p-nitroanilide hydrochloride (BAPNA)
Bovine serum albumin
Carboxypeptidase A (Bovine Pancreas) Type I
Concanavalin A Grade IV (Jack Bean)
Coomassie Brilliant Blue R250
Cytochrome-c Type III (Horse heart)
Dansyl hydrazine
Dansyl chloride
Digitonin
Fetuin
γ -Globulin Fraction II (Bovine)
L-glutamic acid
Hemoglobin
β -lactoglobulin
Myoglobin Type I (Skeletal Muscle)
Ovalbumin Grade V
Polyvinylpolypyrrolidone (PVP)
Soy bean Trypsin Inhibitor Type I-s
Sucrose
Thyroglobulin Type I (Bovine)
Transferrin, Fe free (Human, Siderophilin)
Trypsin Type III
Trizma Base (Tris)

Thompson and Cappa, Runcorn, Cheshire
Kjeltabs

Whatman Biochemicals Limited, Maidstone, Kent
Cellulose Powder
Diethylaminoethyl (DEAE) cellulose, DE-52

Worthington Biochemical Corporation, Freehold, New Jersey, U.S.A.
Lysozyme

Zoology Department, University of Durham
Rabbit erythrocytes

TABLE 3

COMPOSITION OF PLANT POPULATIONS (D.ROTUNDATA)

Population Number	Composition and Background Information
1	Plants of the variety Nwapoko, a non-flowering clone.
2	Plants of two clones, W387-3 and W387-4 each originating from seed of the same female plant W387 which was sown in 1976.
3	Nine clones, each clone originating from seed sown in 1976.
4	Eighty one clones which comprise five half-sib families and which represent the second vegetative progeny of an unselected population derived from seed sown in 1976. The seeds were harvested from local cultivars growing in farmers' fields and are thus from unimproved populations.
5	Five full sib families of plants together with parent clones. The sibs are first vegetative progeny.

METHODS

I. PREPARATION OF YAM MEAL

A. Unprocessed Yam Meal:

The yam meals from varieties Nwapoko and Yam 2 were prepared at the International Institute of Tropical Agriculture (I.I.T.A.) and airfreighted to Durham, whilst those from the seedling-derived varieties were prepared at Durham.

Tubers were peeled, cut into thin slices (1 cm x 1 cm), frozen and stored at -20°c. They were subsequently lyophilised then ground in a Wiley Mill (I.I.T.A.) or a Junke and Kunkel water-cooled mill (Durham) to pass a 423 µm sieve.

B. Cooked Yam Meal:

Freeze-dried cubes of tuber flesh were weighed (0.7-0.8 g dry basis for each experiment) and immersed in boiling deionised water (1:15 $^{w}/v$) contained in porcelain crucibles. Lids were fitted and boiling continued for 45 minutes until the cubes were tender, as tested with a blunt needle. The cooking liquid was quantitatively transferred from each crucible. Both liquids and cooked meals were lyophilised, and the weight of material determined. Samples were analysed for moisture content (qv IV.A), nitrogen content (qv IV.B) and amino acids (qv IV.E). (All equipment was washed with chromic acid prior to use).

II. EXTRACTION

A. Extraction with 70% $^{v}/v$ Ethanol:

In order to determine the optimum extraction conditions, duplicate samples of meal were weighed in cellulose thimbles and extracted with 70% $^{v}/v$ ethanol (1:50 $^{w}/v$) on Soxhlet apparatus. Extracts taken after 1h, a further 5h, a further 3h and a further 11h of extraction were rotary evaporated to dryness, dissolved in

0.5 ml distilled water and 10 µl aliquots analysed by thin layer chromatography (qv VII.A). Extracts taken after 6h and two further 3h periods of extraction were rotary evaporated and analysed for total nitrogen content (qv IV.B). The ethanol-extracted meals were dried in a thermostated vacuum oven at 60°c to constant weight to determine their moisture contents, correction being made for the moisture content of the cellulose cups, and then analysed for total nitrogen content.

On the basis of results obtained from preliminary experiments, the following extraction conditions were used. Yam meals were extracted for 19h in 70% $^{v}/_{v}$ ethanol in a weight:volume ratio of 1:100.

B. Extraction of Protein:

(1) Efficiency of Extraction:

Three different extractants were employed:

(a) 0.05 M sodium borate buffer pH 8.3

(b) 1 M sodium phosphate buffer pH 8.3

(c) deionised water containing c. 2×10^{-5} M sodium hydroxide, pH 8.3

Yam meals were stirred for 15h at 4°c in 20 ml of each extractant at ratios of 1:10 $^{w}/_{v}$, 1:20 $^{w}/_{v}$, 1:50 $^{w}/_{v}$ and 1:100 $^{w}/_{v}$. The extracts were clarified by centrifugation at 2400 g for 30 minutes. Aliquots from each extract were dried under vacuum in Kjeldahl flasks, and the residues were dried in a thermostatic vacuum oven to constant weight.

(2) Addition of Polyvinylpyrrolidone (PVP) 2 - Mercaptoethanol (2-ME) and Cellulose to Extraction Media:

The composition of different extraction media tested were:

(a) 0.05 M sodium borate pH 8.3, 5% $^{w}/_{v}$ PVP, 10mM 2-ME

(b) 0.05 M Tris-hydrochloride pH 8.3, 5% $^{w}/_{v}$ PVP, 10mM 2-ME

(c) 0.05 M Tris-hydrochloride pH 7.2, 5% $^{w}/_{v}$ PVP, 10mM 2-ME

(d) 0.05 M Tris-hydrochloride pH 8.3, 36% $^{w}/_{v}$ cellulose, 10mM 2-ME

Extraction media of the same composition but without the additions of PVP, 2-ME and cellulose were tested simultaneously.

Extracts were prepared using each of the pre-cooled extraction media (qv II.B.1, meal weight : volume ratio 1:20). They were then dialysed against several changes of distilled water for 24h at 4°c, and then lyophilised in pre-weighed flasks. The weights of lyophilisates were recorded, and the effects of the various additions to the extraction media upon the weight of lyophilisate obtained tested by a one-way analysis of variance.

(3) Routine Extraction Procedure:

As a result of the above experiments the following two extractants were used in all subsequent experiments reported in the text:

(a) Tris-hydrochloride, 0.05 M pH 8.3, containing 10mM 2-ME (Tris buffer)

(b) Sodium borate, 0.05 M pH 8.3 (Borate buffer).

Yam meals were stirred in extractants at weight: volume ratios of 1:20, or 1:18 for 2h or overnight (15h) at 4°c. The extracts were clarified by centrifugation at 2400 g for 30 minutes (4°c) and then, after removal of the residue, by further centrifugation at 30,000 g for 30 minutes (4°c).

III. FRACTIONATION OF EXTRACTED PROTEINS; PURIFICATION OF THE STORAGE PROTEINS

A. Molecular Sieve Chromatography:

Columns of Sephadex G100 Fine (84 x 1.5 cm and 110 x 2.5 cm) and of Sephadex G100 Superfine (52 x 1.5 cm) were prepared in borate buffer (0.05 M, pH 8.3), and operated essentially by the

method of Andrews (1964). Dextran Blue 2000 was used to detect any irregularities of packing and to determine the void volume of each column.

Extracted proteins were lyophilised after extensive dialysis against distilled water, then resolubilised (7 mg/ml) in borate buffer (0.05 M, pH 8.3). After brief ultrasonication the solutions were filtered twice through millipore filters (G^F/C and G^F/F), then layered onto the top of each column. Elution was effected with the same buffer (borate). Flow rates of 3 $ml/h/cm^2$ were maintained by gravity flow. Eluants were monitored continuously at 280 nm and 260 nm, and collected in fractions (2 ml). Selected fractions were pooled, dialysed extensively against distilled water and lyophilised before further analysis.

B. Ammonium Sulphate Precipitation:

The extracted proteins were lyophilised after extensive dialysis against distilled water, and then resolubilised (10 mg/ml) in 10 ml borate buffer (0.05 M, pH 8.3) before being fractionated by ammonium sulphate precipitation.

Protein solutions were saturated, over ice, with ammonium sulphate in steps of 10%, up to 100% saturation. The initial pH values of solutions were maintained with 1 N sodium hydroxide.

Precipitated proteins were separated by centrifugation at 30,000 g for 1h. They were resolubilised in borate buffer, and then lyophilised after extensive dialysis against distilled water.

C. Dialysis against Acetate Buffer, pH 5.0:

Protein solutions were dialysed against several changes of sodium acetate buffer (0.02 M, pH 5.0) for 70h (4^oc). Acetate-insoluble and -soluble protein fractions were separated by

by centrifugation at 30,000 g (4^{o}c) for 1 h. The former fractions were resolubilised in either borate (0.05 M, pH 8.3) or Tris (0.05 M) buffer with the addition of sodium chloride (0.15 M); all the fractions were then dialysed extensively against several changes of distilled water before further analysis.

D. Quantitative Evaluation of Fractionation by Ammonium Sulphate Precipitation, and Dialysis against Acetate Buffer, pH 5.0:

The following method was carried out at 0-4^{o}c, and appropriate precautions taken to ensure a maximum recovery of each fraction.

An extract was prepared in 120 ml borate buffer as described in Section II.B.3. (meal weight: volume ratio 1:20; 2 h extraction). Meal residues from both centrifugation steps were pooled, dialysed extensively against several changes of distilled water and lyophilised. A 10 ml aliquot of the clarified extract (control) was similarly treated.

The extract was brought to 50% saturation of ammonium sulphate (qv IIIB), and after separation of precipitated protein, brought to 100% saturation of ammonium sulphate. Precipitated protein was similarly separated from solution.

Both of the protein precipitates were separately solubilised in borate buffer, then further fractionated by dialysis against acetate buffer (qv IIIc). The 100% saturated solution remaining, was ultrafiltered over ice, then similarly dialysed against acetate buffer.

The resultant fractions were lyophilised (three days) and their dry weight contents determined. Protein content was determined by the Lowry method (qv IV.D.1) using borate buffer (0.05 M pH 8.3) containing 1% $^{w}/_{v}$ SDS as sample solvent.

E. Ion Exchange Chromatography:

Columns (12.5 x 2.5 cm) of DE-52 Cellulose were packed and equilibriated using the method described in Whatman publication 607L which entailed equilibriation of the resin with concentrated buffer (0.2M Tris-hydrochloride pH 8.3) followed by re-equilibriation with the starting buffer (0.05 M Tris-hydrochloride pH 8.3).

In preliminary experiments, extracts prepared in 140 ml Tris buffer (qv II.B.3, meal weight: volume ratio, 1:20) were loaded onto the top of columns at pumped flow rates of 8 ml/hr/cm^2. Elution commenced with the starting buffer (400-500 ml), and continued with linear sodium chloride gradients (0-1 M) in the same buffer (400 ml). Eluants were monitored continuously at 280 nm and fractions collected. Gradient densities were measured by refractometry using a Bellingham and Stansley refractometer, and the corresponding salt concentrations determined by reference to calibration curves which related refractive index with salt concentration (example in fig. 2).

On the basis of results obtained from preliminary experiments, the following modifications were made to enable large scale preparation of the storage protein. Extracts were prepared using 450 ml Tris buffer (meal weight: volume ratios 1:18). Elution of adsorbed material was effected firstly with a step (480 ml) of 0.15M sodium chloride in starting buffer, then a linear gradient (400 ml) of sodium chloride (0.15M-0.45M) and finally a step of 1M sodium chloride. Fractions which contained protein represented by the first peak recorded after elution with the step of 0.15 M sodium chloride were used for further characterisation of the storage proteins.

F. Sucrose Density Gradient Centrifugation:

Peeled tuber flesh (40 g) was finely lacerated with a razor over ice into 50 ml pre-chilled phosphate buffer (0.05 M pH 7.5

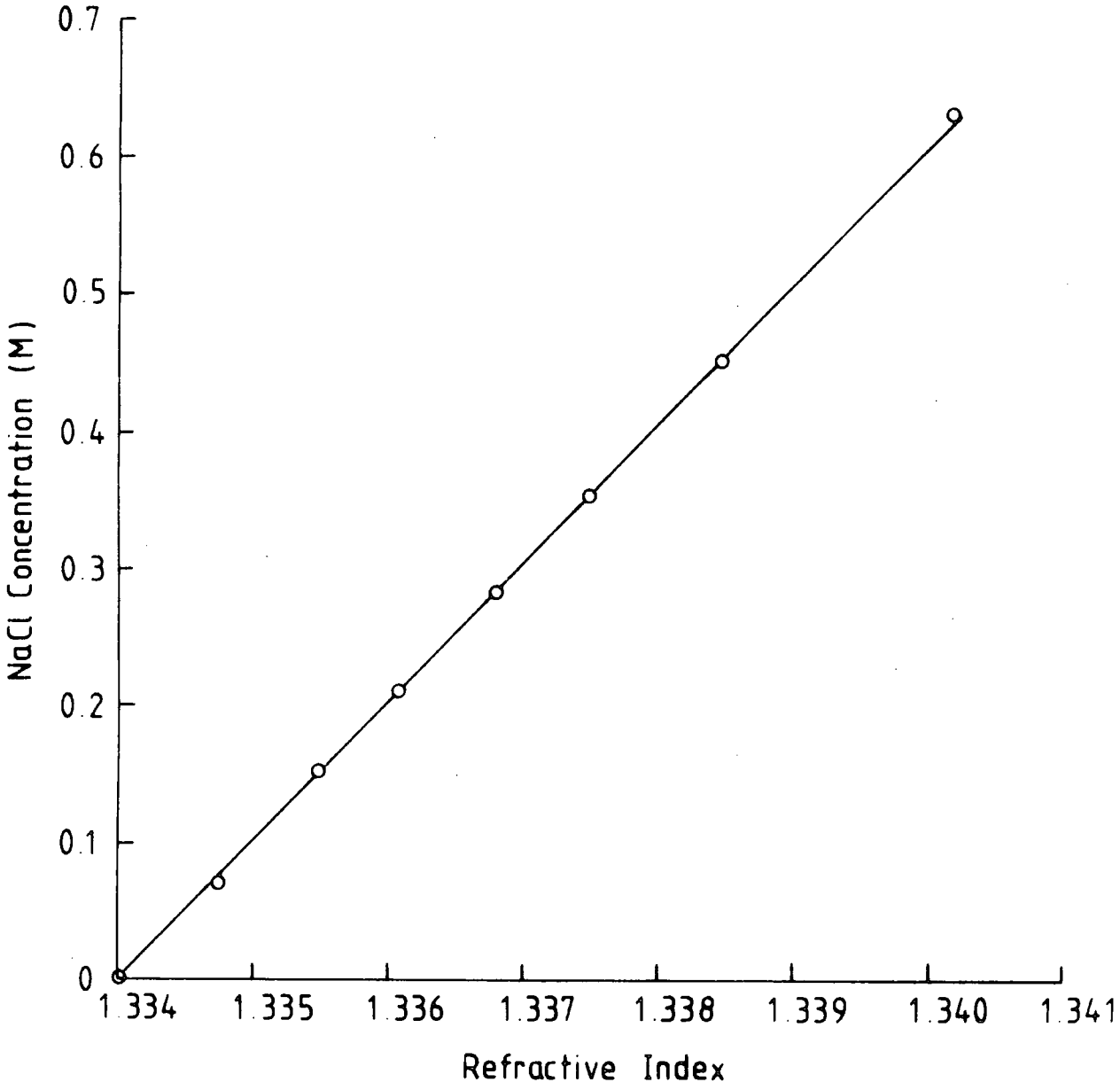


Fig. 2

Calibration curve relating values of refractive index with the concentration of NaCl in 0.05M Tris HCl buffer pH 8.3.

containing 0.5M sucrose) and gently stirred for 10 minutes using a pestle and mortar. The slurry was strained through gauze into pre-chilled centrifuge tubes, then re-extracted. The solution was bench centrifuged twice at 1000 g for 2 minutes ($4^{o}c$); after each centrifugation step the pelleted material was discarded. The remaining supernatant was centrifuged twice at 12,000 g for 30 minutes, and the ensuing pelleted material from each centrifugation step gently resuspended in 2 ml of the same buffer. Aliquots (0.25 ml, 0.5 ml and 2 ml) were layered on to stepped gradients of sucrose solution (30% $^{w}/v$, 70% $^{w}/v$ and 90% $^{w}/v$ sucrose in 0.1M phosphate buffer pH 7.5) prepared in disposal centrifuge tubes, and overlayered with the same buffer containing 15% $^{w}/v$ sucrose. These were then centrifuged at 25,000 g for 1h using an MSE Prepspin 65 centrifuge. The resultant separated fractions were collected through the gradient with a fine syringe for further analysis.

Samples which were examined by electron microscopy were fixed with glutaraldehyde (2% $^{v}/v$ final concentration) over ice, then diluted by half with 0.1M phosphate buffer pH 7.5. They were centrifuged at 10,000g for 10 minutes, and the pellets resuspended in the same buffer. This process was repeated four times, and the final pellets then post fixed in 1% osmium tetroxide for 12h. These were then alcohol dehydrated and embedded using the procedure described in Section VIII.A.

IV. ANALYTICAL CHEMICAL METHODS

A. Determination of Moisture Content:

Weighed samples were dried in porcelain crucibles to constant weight at $105^{o}c$, allowed to cool over silica gel in an evacuated dessicator for 15-30 minutes, and then re-weighed to determine their moisture content. The procedure was the same when estimating the moisture content of small samples (100-200 mg) except that these

were weighed in paper cups of constant size for which a correction was made for the moisture content, determined to be 3.820 ± 0.108 % (8) of the weight of paper.

B. Determination of Total Nitrogen Content:

The total nitrogen content was determined as the indophenol blue complex on an aliquot of solution after digestion by a microKjeldahl technique, using the method of Varley (1966) as modified by Evans and Boulter (1974) with a further modification by Evans (unpublished results) whereby samples were weighed in N-free paper cups and digested, each with one "Kjeltab", 5 ml 95% $^{v}/v$ N-free sulphuric acid - 5% $^{v}/v$ phosphoric acid, and 3 ml hydrogen peroxide. A typical calibration curve constructed using digested tyrosine, from which nitrogen values were obtained, is given in fig. 3.

C. Determination of Total Sulphur Content:

Total sulphur was estimated using the method of Mottershead (1971) and Evans and Boulter (1974), modified for automatic analysis using a McFarlane Robson Carlo Erba Flow Photometer system.

D. Determination of Protein Content:

(1) Lowry Method:

Trichloroacetic acid (TCA) was added, over ice, to protein solutions to a final concentration of 10% $^{w}/v$. After one hour, precipitated proteins were separated from the supernatants by bench centrifugation, then washed twice with pre-cooled 5% $^{w}/v$ TCA, then twice with pre-cooled absolute ethanol and finally dried overnight at room temperature. The precipitates were dissolved in 1M sodium hydroxide and after about one hour, diluted to give a solution of protein in 0.2M sodium hydroxide. The solutions were then analysed for protein content using the method of Lowry *et al*,

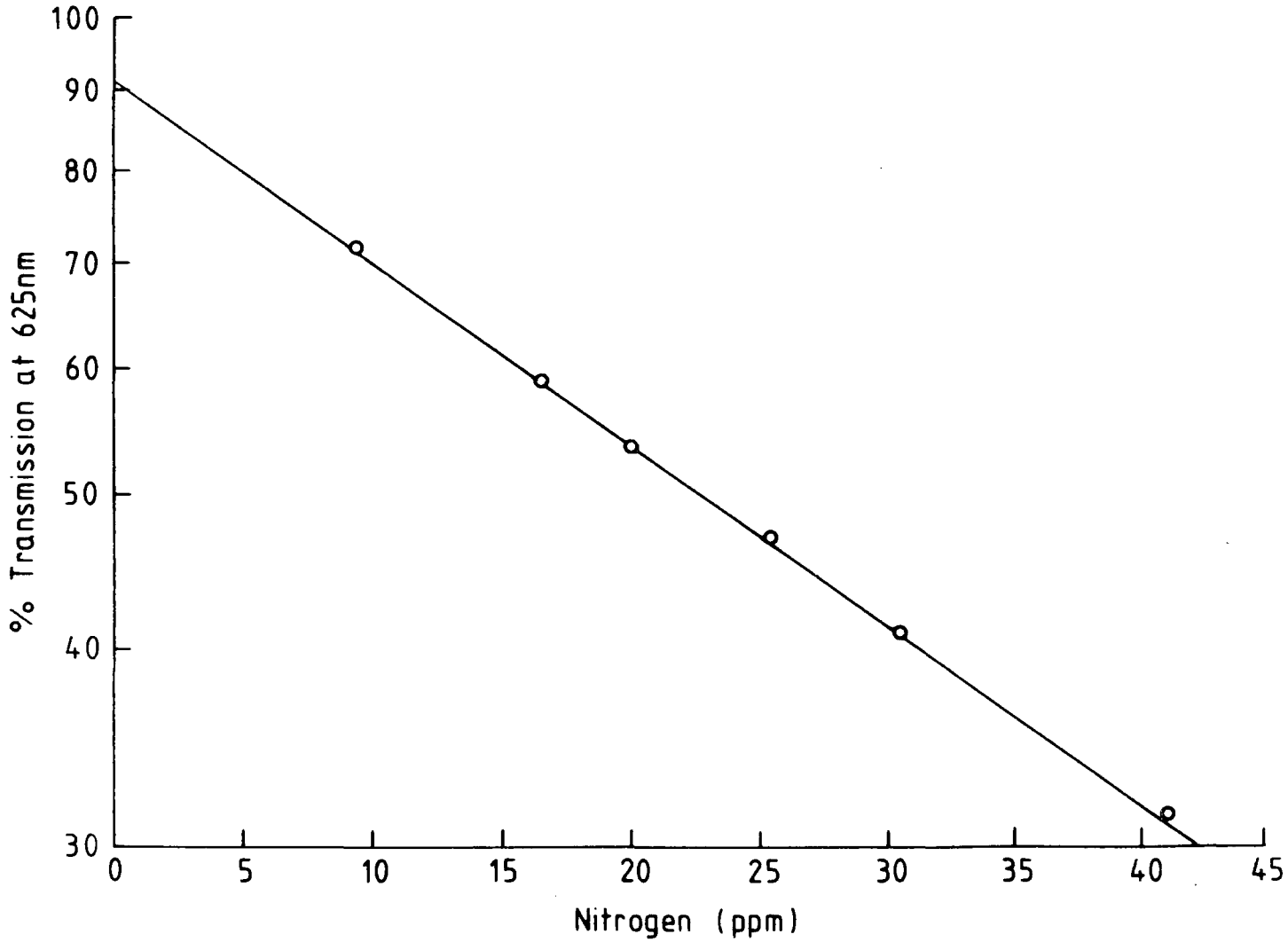


Fig. 3 Calibration curve for the estimation of nitrogen content

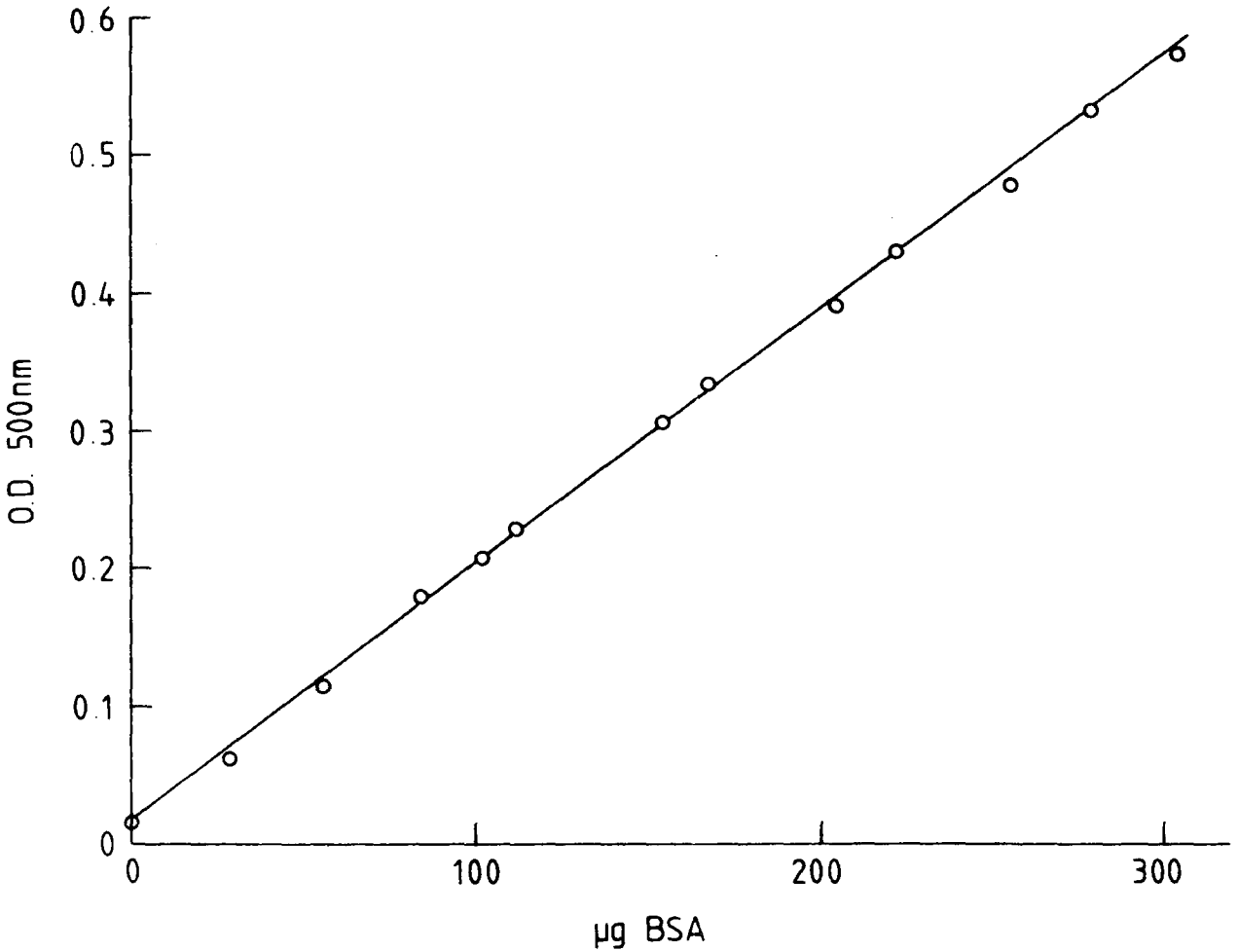


Fig. 4

Calibration curve for the estimation of protein content

(1951). Calibration curves (example in fig. 4) were constructed to relate concentration of a standard protein, bovine serum albumin (BSA), with absorption at 500 nm; a new curve was constructed each time an analysis was carried out.

A modification of the technique entailed solution of lyophilised protein fractions in 0.05M sodium borate buffer which contained 1% $^{w}/_{v}$ SDS; solutions of the standard, BSA, were prepared similarly, and protein content determined as before.

(2) Absorption at 280 nm:

A relative measure of protein content was determined by recording the E^{280}_{1cm} value of clear protein solutions at 280 nm.

E. Amino Acid Analysis:

Samples in 6N HCl were hydrolysed in vacuo at 105°c for 22h, 48, and 72h using the method of Moore and Stein (1963). The amino acid compositions of the hydrolysates were analysed on a Locarte automatic loading amino acid analyser. Cysteine (½ cystine) was determined after performic acid oxidation as cysteic acid (Moore, 1963).

F. N-Terminal Amino Acid Analysis:

N-Terminal amino acids of non-dissociated protein were determined by the dansylation procedure of Gros and Labousse, (1969): those of protein sub-units were determined after performic acid oxidation and treatment with SDS by the method of Gray, (1972).

G. Carbohydrate Analysis:

(1) Periodic Acid - Schiff Staining Technique:

Electropherograms (protein samples 500 µg) were prepared under dissociating conditions (qv V.B.2(i), T = 17.6%, C = 0.45%) and treated using the Periodic acid-Schiff (PAS) staining technique of

Zacharius et al, (1969) as modified by Glossmann and Neville, (1971) with the following further modifications: Gel slabs were initially washed for 20h in 10% $^{w}/_{v}$ TCA, and 10% $^{w}/_{v}$ sulphosalicylic acid with several changes and then in 40% $^{v}/_{v}$ methanol and 7% $^{v}/_{v}$ glacial acetic acid for 2h, again with several changes. They were stored in 1% $^{v}/_{v}$ acetic acid and the relative mobilities (qv V.B.4) of positively stained sub-units determined.

Control strips from each electropherogram (100 µg protein) were stained with Coomassie Blue (qv V.B4). After final equilibriation in 1% $^{v}/_{v}$ acetic acid, the relative mobilities of the sub-units were determined and compared with those determined for the carbohydrate-positive sub-units.

PAS-treated slabs were also subsequently stained with Coomassie Blue for a direct localisation of carbohydrate containing sub-units.

The glycoproteins ovalbumin and fetuin were used as positive controls for the method.

(2) Fluorescent Method using Dansyl Hydrazine:

Electropherograms (protein samples 250 µg) prepared under dissociating conditions (qv V.B2(i), T = 17.6%, C = 0.45%) were treated using the fluorescent method of Eckhardt et al, (1976). The relative mobilities of carbohydrate-positive sub-units were determined (qv V.B4) and compared with those determined from control strips stained with Coomassie Blue (qv V.B4). Treated slabs were subsequently stained with Coomassie Blue for an unambiguous identification of carbohydrate-positive sub-units.

The glycoprotein ovalbumin was used as a positive control for the method, and the omission of the oxidation step with periodic acid served as a negative control.

(3) Gas Liquid Chromatography:

The storage proteins were prepared by ion exchange chromatography (qv III.E), dialysed extensively against several changes of distilled water and lyophilised. The lyophilisate was analysed for carbohydrate content according to the method of Clamp et al, (1971). Ovalbumin and bovine serum albumin were used as positive and negative controls respectively.

The lyophilisate was subsequently dissolved in water, and, over ice, TCA was added to a final concentration of 11% $^{w}/v$. After one hour, precipitated protein was separated from the supernatant by bench centrifugation and washed three times with 10% $^{w}/v$ TCA, then once with a 50:50 solution of 10% TCA and ethanol. The protein was suspended in 100% ethanol; this solvent was evaporated from the protein and the latter then lyophilised before analysis for carbohydrate content using the same method as before.

H. Determination of Saponin Content:

Yam meal (45 mg/ml (d.b.)) and a lyophilised protein extract (46 mg/ml (d.b.)) were assayed for saponin content using the haemolytic method of Segal et al, (1974). Rabbit erythrocytes were washed (Lis and Sharon, 1972) and prepared as a 2% suspension in 0.006M sodium phosphate buffer, pH 7.4, containing 0.9% $^{w}/v$ sodium chloride. A calibration curve was constructed (fig. 5) to relate % haemolysis with the concentration of a positive saponin control (digitonin). The optical densities were read at 540 nm against the appropriate blank, and 50% haemolysis determined.

I. Determination of Trypsin Inhibitor Content:

Assays for trypsin inhibitor content in yam meal, and the purified storage protein preparation were carried out by measuring the extent of inhibition of the trypsin catalysed hydrolysis of benzoyl-DL-arginine p-nitroanilide hydrochloride (BAPNA) at 410 nm.

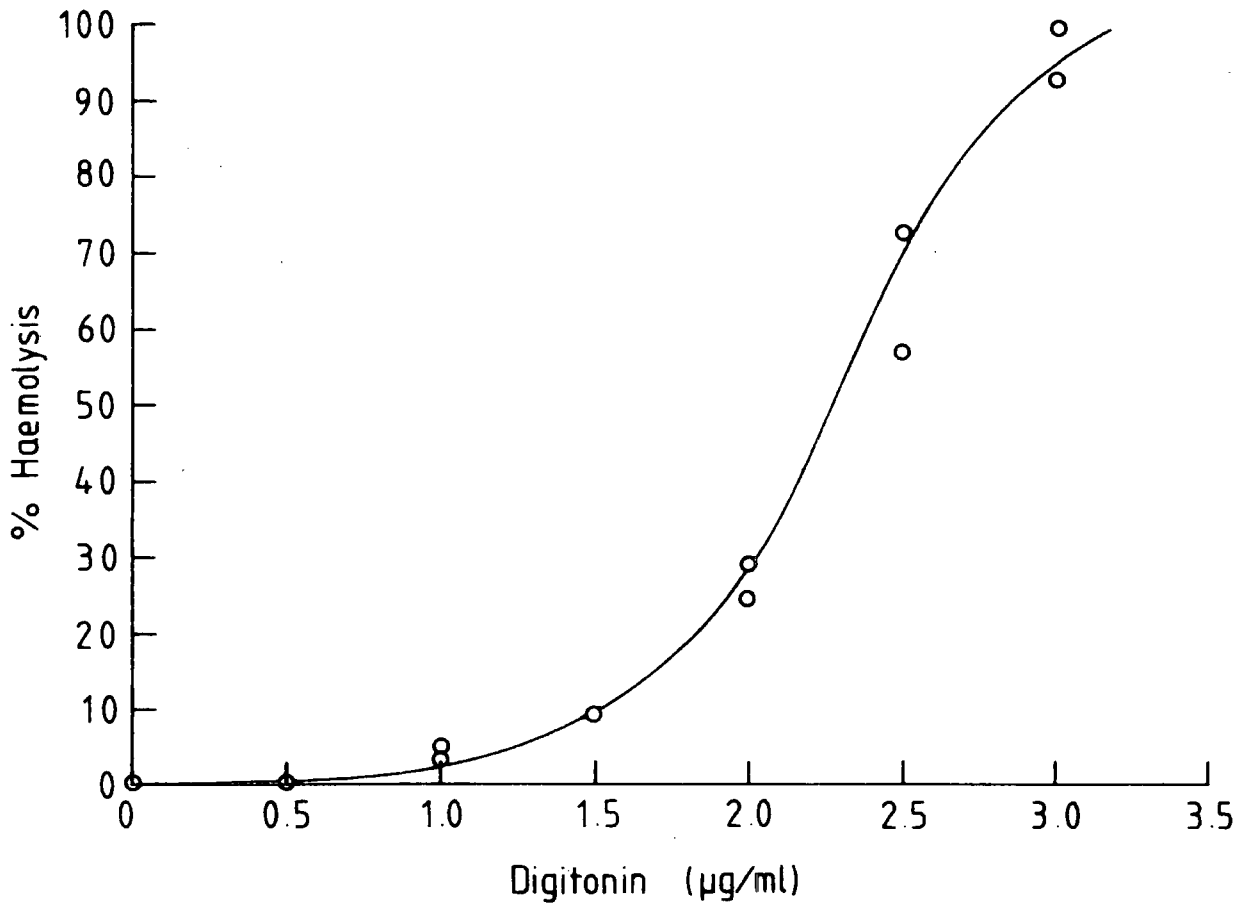


Fig. 5

Calibration curve for the estimation of saponin content

The hydrolysis reaction was performed according to the method of Erlanger et al, (1961). Soya bean trypsin inhibitor and cowpea trypsin inhibitor were used as positive controls for inhibitory activity.

J. Lectin Analysis:

(1) Diffusion against Glycoproteins in Plates of Agarose:

For each experiment, six wells surrounding a central well were cut out of agarose gel (1% $^{w}/v$ in 0.1M Tris-hydrochloride, 0.2M sodium chloride pH 7.5) using a 6 shooter immunodiffusion template.

The central wells were filled with 20 µl of the storage protein preparation (2 mg/ml). The outer wells were each separately filled with solutions of the glycoproteins ovalbumin, thyroglobulin and fetuin, and the lectin concanavalin A (Con A), each serially diluted to cover the concentration range 0.25 mg/ml to 8 mg/ml. Replicate plates were stained after 18h, 24h, 28h, 32h, 40h, 48h, 69h and 96h of diffusion.

Before staining, the agarose slabs were turned out onto agarose coated glass plates, and dried between filter papers. They were washed twice in solutions of 0.1M sodium chloride, rinsed in water, then dried firstly between filter papers then under a hot air stream. They were stained with Coomassie Blue R.250 (0.5% $^{w}/v$) in 45% $^{v}/v$ ethanol, 10% $^{v}/v$ glacial acetic acid, and destained by simple diffusion in the same solvent.

(2) Haemagglutination Assay:

Haemagglutination assays were carried out using rabbit erythrocytes, washed and prepared as a 2% $^{v}/v$ suspension in phosphate buffer (0.05M pH7, containing 0.15M sodium chloride) (Lis and Sharon, 1972). The assays were also performed using erythrocytes which had been treated with trypsin according to the method of Lis and Sharon, (1973).

Different preparations of the storage protein (10 mg/ml) and the lectin Con A (1 mg/ml), were dissolved in the same buffer and serially diluted into microtitration plates. An equal volume (50 µl) of the erythrocyte suspension was added. After one hour the plates were examined; agglutination was assessed by the failure of erythrocytes to settle.

K. Polymerisation of Protein with Glutaraldehyde:

The storage protein was polymerised in the presence of 0.0625 M Tris-hydrochloride buffer pH 6.8 by using 0.05% $^{v}/_{v}$ glutaraldehyde for the protein at a concentration of 50 mg/ml (other conditions as in Payne, 1973).

L. Denaturation of Protein followed by S-Carboxymethylation:

A solution of the storage protein was denatured with guanidine hydrochloride, then alkylated with iodoacetic acid using the method of Weber et al, (1972).

V. ELECTROPHORETIC METHODS

A. Cellulose Acetate Membrane Electrophoresis:

(1) Routine Analysis:

Electrophoresis was conducted using 0.05M sodium phosphate buffer pH7 in a Beckman microzone apparatus. Sample aliquots (0.25 µl, concentration 10 mg/ml) were loaded centrally on the membrane. A constant current of 5 mA/membrane for 12 minutes was maintained. The membranes were stained with 0.025% $^{w}/_{v}$ Coomassie Blue in 25% $^{v}/_{v}$ methanol, 7% $^{v}/_{v}$ glacial acetic acid for 2 hours then destained by simple diffusion in solvent of the same composition.

(2) Estimation of Isoelectric Point of the Storage Proteins:

Universal Buffer (Johnson and Lindsey, 1939) was prepared at pH values of 4, 5, 6, 7 and 8 and diluted where necessary so that a voltage of value 300 was recorded for 5 mA current at the start of electrophoresis. Aliquots (0.25 µl) of β-lactoglobulin (control), and

the storage protein preparation (10 mg/ml) were loaded at three different starting positions on the membrane for each electrophoretic run, at either the anode or cathode ends. A constant current of 5 mA was maintained for 12 minutes exactly. The separatedbands were visualised as described above.

B. Polyacrylamide Gel Electrophoresis:

(1) Preparation of Gels and Electrophoretic Procedure:

Slabs of polyacrylamide gel (PAG) were prepared as described by Ames, (1974), using 1 mm thick perspex spacers sealed with a high vacuum grease between glass plates (20 x 18.4 x 0.4 cm).

Rods of PAG were prepared using the method described by Davis, (1964) as outlined by Payne, (1976) and Smith, (1976). Sample gels were omitted, and stacking gels (1 cm depth) were polymerised on top of separating gels. Pyrex tubing (i.d. 0.5 cm or 0.25 cm depending on use) cut by triangular file to appropriate lengths was siliconised in either 5% dimethyldichlorosilane in chloroform (Smith, 1976) or repelcote, prior to use, and washed following a procedure of Vesterberg, (1972).

The systems used are outlined in sections V B.2 and V B.3. Gel concentrations are described by the notation of Hjerten, (1962). Acrylamide solutions were filtered (Whatman No. 1) prior to use; and degassed together with buffer solutions on a water suction pump for four minutes at a regulated pressure of 25 mm Hg, with magnetic stirring. Concentrations of initiator (Tetraethylmethylenediamine (TEMED)) and catalyst (ammonium persulphate or riboflavin) were selected by prior experiment to give complete polymerisation of the separating and stacking gels within 15 minutes and 30 minutes respectively. Polymerised separating gels stored satisfactorily for 2/3 days covered with buffer of the same composition, but stacking gels were prepared just prior to use.

Routine polyacrylamide gel electrophoresis (PAGE) was carried out at room temperature using a constant current power source. Gel slabs were electrophoresed on a commercially available apparatus (Raven Scientific Limited) described originally by Reid and Bieleski, (1968), modified by Studier, (1973), whilst rod gels were electrophoresed on apparatus similar to that described by Davis, (1964). When a more quantitative approach to electrophoresis was required (construction of Ferguson plots, qv V.E), the rod gel apparatus was modified so that the rod gels were completely immersed in magnetically stirred buffer which was maintained at constant temperature with the use of a thermostatically regulated water bath.

Buffers in the upper electrode chamber contained a 0.5 ml aliquot of either 0.001 % $^{w}/_{v}$ bromophenol blue (alkaline buffer systems) or 0.005% $^{w}/_{v}$ methyl green (acid buffer systems). On termination of electrophoresis, the positions of these dyes were marked, either with India ink (slab gels) or by the insertion of fine copper wire. Alternatively, rod gels were sliced at this position. In this way the mobility of the tracking dye could be measured after the gels had been stained (qv V.B4).

(2) Dissociating (SDS) Systems:

(i) Discontinuous (multiphasic) buffer systems:

The systems used (table 4) were based on that of Laemmli, (1970). Slab gels were electrophoresed at 17 mA for the stacking gel, and 25 mA for the separating gel, whilst rod gels were electrophoresed using 8 mA/cm^2 for the stacking gel, 21 mA/cm^2 for the separating gel.

(ii) Continuous Buffer System - (Shapiro et al, 1967):

Slab gels (T = 7.7% $^{w}/_{v}$, C = 2.6% $^{w}/_{w}$) were electrophoresed using a constant current of 60 mA overnight. Samples were incubated for two minutes at 100°c in 0.01M sodium phosphate buffer pH7,

TABLE 4. Final Concentration of Reagents used for Dissociating (SDS) Polyacrylamide Gel Electrophoresis (Discontinuous Buffer Systems)

	Sample Buffer	Stacking Gel	Separating Gel				Reservoir Buffer
acrylamide[a] $\%T^{w}/v$	-	3.0	17.6	10.0	10.2	10.4	-
$\%C^{w}/w$		1.42	0.45	0.88	1.77	3.33	
Tris (M)	0.0625[b]	0.125[b]	0.380[b]	0.375[b]	0.375[b]	0.375[b]	0.025
Glycine (M)	-	-	-	-	-	-	0.188
Ammonium persulphate($\%^{w}/v$)	-	0.025	0.025	0.025	0.025	0.025	-
TEMED($\%^{v}/v$)	-	0.05	0.03	0.03	0.03	0.03	-
SDS ($\%^{w}/v$)	$\pm$ 2	0.1	0.1	0.1	0.1	0.1	0.1
2-ME($\%^{v}/v$)	$\pm$5	-	-	-	-	-	-
Urea (M)	$\pm$6	-	-	-	-	-	-
Glycerol ($\%^{v}/v$)	10	-	-	-	-	-	-
Final pH value	6.8	6.8	8.8	8.8	8.8	8.8	8.3

[a] Nomenclature according to Hjerten (1962) where T = total weight of monomer (acrylamide + bis) per 100 ml. solvent.
C = amount of bis as a $\%^{w}/w$ of the total amount of monomer

[b] Titrated to final pH value with hydrochloric acid.

containing 5% $^{v}/_{v}$ 2-ME, 2% $^{w}/_{v}$ SDS and 10% $^{v}/_{v}$ glycerol prior to electrophoresis.

(3) Non-dissociating Systems:

(i) Alkaline Buffer Systems:

System A was based on that of Davis, (1964), modified by the ommission of sucrose to the separating gel. System B was that of Rodbard and Chrambach, (1971) for separation at pH 9.45, ionic strength 0.0158. Slab gels were electrophoresed at 20 mA (constant current), rod gels at 10 mA/cm^2.

(ii) Acid Buffer Systems:

The systems used were modifications of the original system of Reisfield et al, (1962):

System A: described by Maurer, (1971).

System B: described by Rodbard and Chrambach, (1971) for separation at pH 3.51, ionic strength 0.0206.

System C: described by Rodbard and Chrambach, (1971) for separation at pH 3.51, ionic strength 0.0021.

Slab gels were electrophoresed at 20 mA (constant current), rod gels at 10 mA/cm^2.

(4) Detection of Separated Proteins and Measurement of Mobility:

Separated proteins were stained with either:-

(a) Coomassie Brilliant Blue R.250, 0.025% $^{w}/_{v}$, (overnight) or 0.25% $^{w}/_{v}$ (2h) in 50% $^{v}/_{v}$ methanol (slab gels) or 25% $^{v}/_{v}$ methanol (rod gels), and 9% $^{v}/_{v}$ glacial acetic acid.

(b) Procion Brilliant Blue RS, 1% $^{w}/_{v}$ in 25% $^{v}/_{v}$ methanol and 7% $^{v}/_{v}$ glacial acetic acid (for 2h),

or, for larger protein samples (100 µg and greater), they were detected after precipitation with TCA (15% $^{w}/_{v}$) in distilled water.

Gels were destained by simple diffusion in the respective solvents.

Densitometric profiles of separated proteins were obtained by scanning gels treated with either Coomassie Blue, Procion Blue or TCA, at 590 nm, 602 nm or 280 nm respectively, using a chromoscan in transmission.

The distances migrated by separated proteins in the separation gel were measured either directly on stained gels, or from the densitometric scans. The relative mobility (Rm value) of each protein was determined from the ratio between the distance migrated by the protein and the distance migrated by the tracking dye.

(5) Quantitative Evaluation of the Sub-Units by Direct Densitometry of Stained Gels:

Rod gels (12 x 0.5 cm) were prepared and electrophoresed as described in section V B1 using the dissociating system (T = 17.6% $^{w}/_{v}$ C = 0.45% $^{w}/_{w}$) described in Section V B.2.

Sub-unit ratios were determined from quantitative measurements of the amount of dye bound to each sub-unit. These were obtained by integrating the densitometric scans of the gels (qv V B.4). To minimise errors due to selective dye binding by different protein species, Procion Brilliant Blue, Coomassie Blue R.250 and TCA were used (qv V B.4). Coomassie Blue and Procion Blue deviate markedly from Beers Law at high protein concentrations (Kruski and Narayan, 1968) so that for these two stains, sub-unit ratios were determined after electrophoresis of serial dilutions of a protein solution. Protein quantities ranging between 5 and 100 µg (d.b.) in a constant volume of 5 µl were applied to a series of gels which were prepared and electrophoresed simultaneously, since peak area is affected both by variation in gel concentration, and by the distance the protein migrates into a gel (Kruski and Narayan, 1968). (The gels were also stained and destained for the same periods of time before densitometric analysis).

(6) Estimation of Sub-Unit Molecular Weight:
(Weber and Osborne, 1969; Dunker and Rueckert, 1969)

Slab gels were prepared using the dissociating discontinuous systems (qv V B.2(i)) and the dissociating continuous system (qv V B.2(ii)).

Calibration curves relating the log of molecular weight with relative mobility (qv V B.4) were constructed for each gel system used, using combinations of the following standard proteins (molecular weights are given in parenthesis):
lysozyme (14300), haemoglobin (15500), myoglobin (17200), ß-lactoglobulin (18400), chymotrypsinogen (25700), carboxypeptidase A(34600), aldolase (40000), ovalbumin (43000), catalase (60000) and BSA (68000). Polymers of myoglobin and haemoglobin were prepared with gluteraldehyde according to the method of Payne, (1973). The protein samples were loaded on to gels in equal volumes of exactly 5 µl so that the tracking dye front was both sharp and straight. The molecular weights of sub-units in the storage protein preparation were obtained by reference to the calibration curves, two of which are given in fig. 6 and fig. 7.

C. Isoelectric Focussing Polyacrylamide Gel Electrophoresis:

(1) Non-dissociating Systems:

(i) Rods of Polyacrylamide Gel:
(Wrigley, 1968; Drysdale, 1975; Leaback and Wrigley, 1976)

Rods of polyacrylamide (13 x 0.5 cm; T = 6.2% $^{w}/_{v}$, C = 3.2% $^{w}/_{w}$) containing 2% $^{w}/_{v}$ carrier ampholytes (pH range 3-10) were photopolymerised with 0.005% $^{w}/_{v}$ riboflavin. Protein solutions contained 10% $^{w}/_{v}$ sucrose and 2% $^{w}/_{v}$ carrier ampholytes; 10 µl aliquots were loaded to the top of gels and overlayered each with a 40 µl aliquot of a protective solution which contained 5% $^{w}/_{v}$ sucrose, 2% $^{w}/_{v}$ carrier ampholytes. The anode solution consisted of 0.1 M sulphuric acid, the cathode, 1M ethylenediamine.

Gels were prefocussed at 4 mA/cm^2 for 30 min., then protein samples were loaded, and focussed at 0.7 watt/cm^2 (constant wattage) for 6h at

Fig. 6

Calibration curve relating the molecular weight of standard proteins with their electrophoretic mobility relative to bromophenol blue in the SDS discontinuous buffer system (acrylamide concentration 17.6 x 0.45). Protein standards, in order of decreasing size, are: bovine serum albumin, ovalbumin, carboxypeptidase A, chymotrypsinogen, β-lactoglobulin, myoglobin and lysozyme.

Fig. 7

Calibration curve relating the molecular weight of standard proteins with their electrophoretic mobility relative to chymotrypsinogen in the SDS-phosphate continuous buffer system (acrylamide concentration, 7.7 x 2.6). Protein standards in order of decreasing size are: myoglobin pentamer, hemoglobin pentamer, myoglobin tetramer, bovine serum albumin, hemoglobin tetramer, catalase, myoglobin trimer, hemoglobin trimer, ovalbumin, aldolase, myoglobin dimer, hemoglobin dimer, chymotrypsinogen, β-lactoglobulin, myoglobin and hemoglobin.

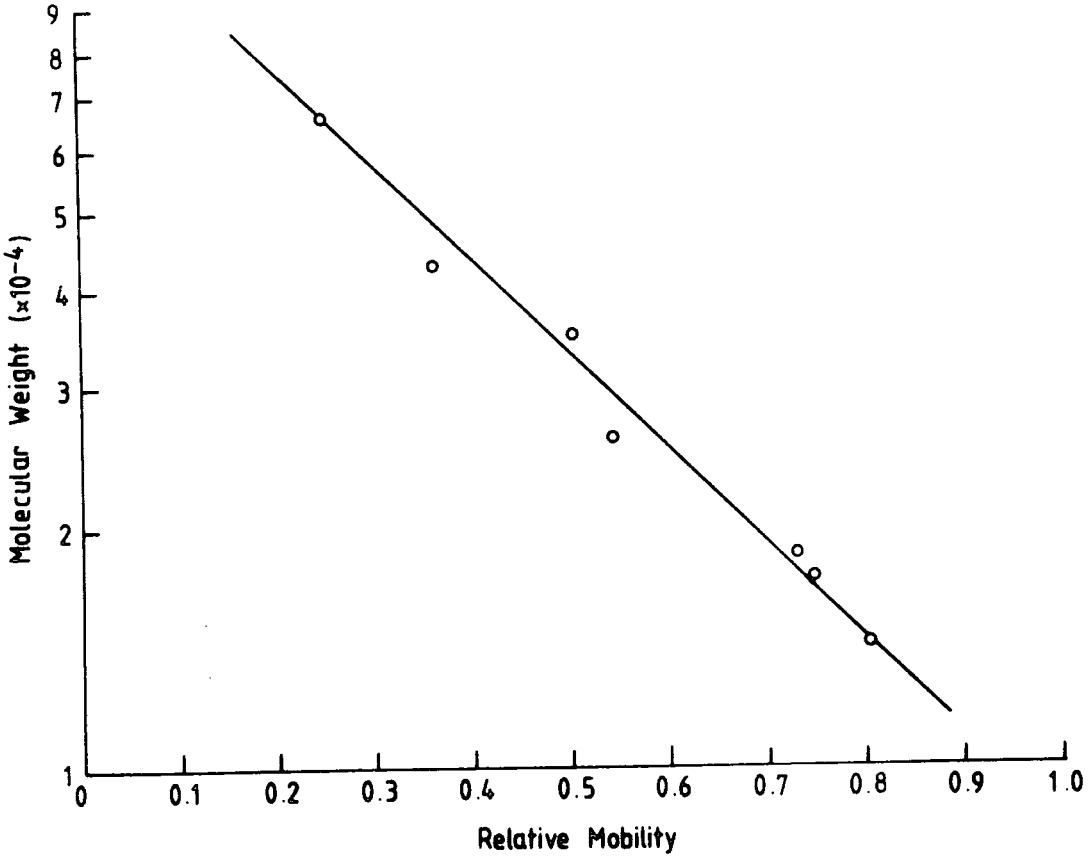

Molecular Weight (×10⁻⁴)
9
8
7
6
5
4
3
2
1
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
Relative Mobility

Fig. 6

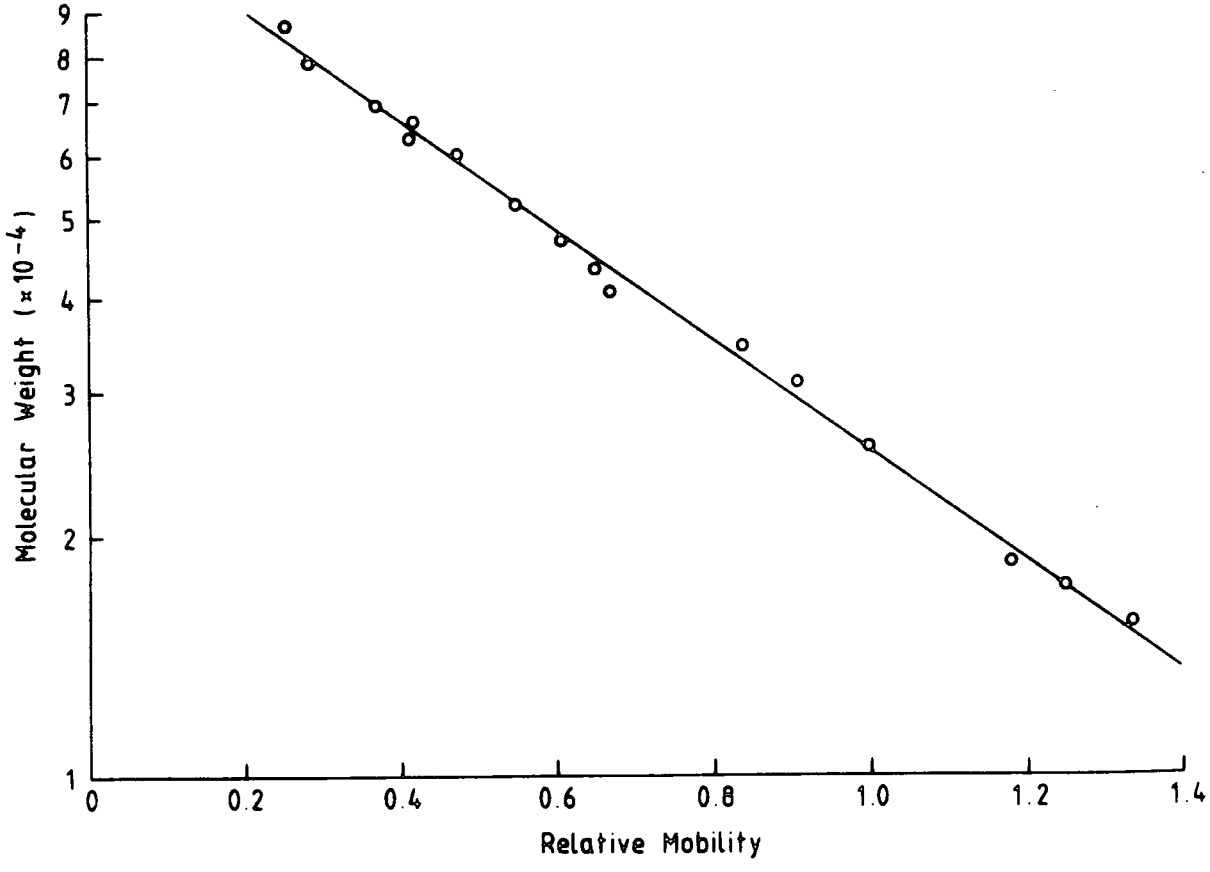

Molecular Weight (×10⁻⁴)
9
8
7
6
5
4
3
2
1
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
Relative Mobility

Fig. 7

room temperature. Some of the gels were stained after 3h, 4h and 5h of focussing to observe the progress of the focussing bands. Marker solutions of haemoglobin and cytochromec were focussed simultaneously to assess approximately when focussing was complete.

(ii) Slabs of Polyacrylamide Gel:
(Awdeh et al, 1968)

A polyacrylamide solution (T = 7.7% $^{W}/_{V}$, C = 3% $^{W}/_{W}$) containing 2% $^{W}/_{V}$ carrier ampholytes pH range 3-10, 12.5% $^{W}/_{V}$ sucrose and 0.06% $^{W}/_{V}$ ammonium persulphate was polymerised between two thin glass plates (10x15 cm) with a perspex spacer (1 cm x 0.5 cm) sealed between them.

Focussing was carried out at 4^{o}c on a flat bed apparatus. The slabs were prefocussed at 260 V for ten minutes then protein solutions (2 mg/ml in distilled water) were applied to positioned Whatmann 3 mm filter wicks (Wadström and Smyth, 1973) until they were just saturated (about 15 µl). Their positions were determined by prior experiment. The samples were electrophoresed at 10 watts for one hour, then the filter wicks were removed, and focussing continued for three hours at 15 watts. Coloured marker solutions of haemoglobin were applied at either end of the slab to observe the progress of focussing.

(2) Dissociating System:
(O'Farrell, 1975)

A polyacrylamide solution (T = 6.0% $^{W}/_{V}$, C = 6.6% $^{W}/_{W}$) containing 6M urea, 2% $^{W}/_{V}$ carrier ampholytes pH 3-10, 0.01% $^{W}/_{V}$ ammonium persulphate and 0.05% $^{V}/_{V}$ TEMED was polymerised in pyrex tubes (12 x 0.5 cm). The gels were overlayered with "lysis buffer" containing 2% $^{W}/_{V}$ carrier ampholytes, 9.5 M urea and 5% $^{V}/_{V}$ 2-ME, and prefocussed at 200 V for 15 minutes, 300 V for 30 minutes, then 400 V for 15 minutes. The lysis buffer was removed, and 5 µl aliquots of protein solutions (10 mg/ml), prepared and incubated in lysis buffer

for two hours at room temperature, were loaded on to the gels. These were overlayered with a protective solution of 9M urea, 1% $^{w}/_{v}$ carrier ampholytes. Gels were focussed for 12h at 400v, then one hour at 800v. The anode solution consisted of 0.1M phosphoric acid, the cathode, 1M ethylenediamine.

(3) Detection of Focussed Proteins and Determination of Isoelectric Point:

Focussed bands were detected using (more usually) the staining procedure of Vesterberg, (1971) or the following procedure: Gels were washed overnight in several changes of a solution consisting of 30% $^{v}/_{v}$ methanol, 20% $^{w}/_{v}$ TCA. They were then stained with Coomassie Blue R.250, 0.025% $^{w}/_{v}$, in 30% $^{v}/_{v}$ methanol, 10% $^{v}/_{v}$ glacial acetic acid, and destained with a solvent of the same composition by simple diffusion.

The approximate isoelectric points of stained bands were determined by reference to calibration curves relating distance along a focussed gel with pH value. The pH values of focussed gels were determined by the method of Awdeh et al, (1968) (slabs) and Wrigley, (1968) (rods).

D. Applications of Polyacrylamide Gel Electrophoresis:

(1) Two-dimensional Polyacrylamide Gel Electrophoresis:

(i) First dimension: dissociating PAGE
Second dimension: dissociating PAGE

Rod gels (12 x 0.5 cm; T = 17.6% $^{w}/_{v}$, C = 0.45% $^{w}/_{w}$) were prepared for the first dimension using the discontinuous buffer system described in section V B.2(i). Protein samples were incubated for two minutes at 100°c in sample buffer prepared without the addition of either urea or 2-ME. Electrophoresis was conducted at 4mA/cm^{2}.

On termination of electrophoresis, the gels were reemed from the tubes, and fine copper wire inserted into the anode ends. They

were incubated for 45 minutes in an "incubation buffer" consisting of 0.0625 M Tris-hydrochloride buffer pH 6.8 containing 2% $^{w}/_{v}$ SDS, 10% $^{v}/_{v}$ 2-ME. The duration of incubation was determined by prior experiment to be sufficient to ensure complete reduction of disulphide bonds without appreciable loss of protein from the gels by diffusion.

Slabs of polyacrylamide (T = 17.6% $^{w}/_{v}$, C = 0.45% $^{w}/_{w}$) were prepared for the second dimension, using the same discontinuous buffer system (qv V B.2(i)). The surface of each stacking gel was rinsed with the "incubation buffer", and a rod gel of the first dimension sealed on to the surface with 2% $^{w}/_{v}$ molten agarose prepared in "incubation buffer". A well for a control protein sample was formed in the agarose. Electrophoresis was conducted as described in section V B.2(i).

(ii) First dimension: isoelectric focussing PAGE
Second dimension: dissociating PAGE

Rod gels (14.5 x 0.25 cm) were prepared for the first dimension using the method described in V C1(i), except that 2% $^{v}/_{v}$ Nonidet-P40 was incorporated into both the acrylamide solution and sample buffer. The gels were overlayered with sample buffer (20 µl) and prefocussed at 10 mA/cm^2 for 30 minutes. The sample buffer was removed and protein samples (5 µl) were loaded and overlayered with the protective solution (20 µl). Focussing was conducted at 1.6 watt for 5h. Duplicate blank gels were used to measure the pH gradient.

Slab gels (T = 17.6% $^{w}/_{v}$, C = 0.45% $^{w}/_{w}$) were prepared for the second dimension using the discontinuous buffer system described in V B.2(i). The rod gels were incubated, and applied to the slab gels using the method described in V D.1(i).

(iii) First dimension: non-dissociating PAGE
Second dimension: dissociating PAGE

A slab gel (T = 8% $^{w}/_{v}$, C = 5% $^{w}/_{w}$) was prepared for the first dimension using the alkaline buffer system B (qv V B3(i)). Protein samples were electrophoresed at 10 mA (constant current) for the stacking gel, 20 mA for the separating gel. The gel was stained with Coomassie Blue and destained as described in section V B4. A strip of the gel (1 cm width) was rinsed in water, then incubated in 0.125 M Tris-hydrochloride buffer pH 6.8. After one hour, it was transferred to a second solution consisting of 0.0625 M Tris-hydrochloride buffer pH 6.8, 2% $^{w}/_{v}$ SDS, and 5% $^{v}/_{v}$ 2-ME and incubated for one hour.

A slab gel (T = 17.6% $^{w}/_{v}$, C = 0.45% $^{w}/_{w}$) was prepared for the second dimension using the discontinuous buffer system described in V B2(i). The gel strip (first dimension) was placed directly on to the surface of the stacking gel, and electrophoresis then conducted as described in section V B2(i).

(2) Electrophoresis on Isoelectrically Focussed Polyacrylamide Gels:

Electrophoresis was performed under non-dissociating conditions on isoelectrically focussed slab gels (T = 7% $^{w}/_{v}$, C = 3.8% $^{w}/_{w}$) using the method of Rosengren et al, (1977). Protein samples were applied to a central trough (1mm wide, running the width of the gel) prepared by slicing the gel after the pH gradient had been developed. Bromophenol blue and methyl green were added to the protein solution to track the progress of electrophoresis. The pH gradient was measured as described in section V C3, and the gels were stained and destained using the method of Vesterberg, (1971).

The method was the same under dissociating conditions, except that urea (6M) was incorporated into the acrylamide solution, and

samples were incubated for 75 minutes (20^{o}c) in a solution containing 9.5M urea, and 5% 2-ME prior to electrophoresis.

(3) Transverse Gradient Polyacrylamide Gel Electrophoresis:

Transverse gradient polyacrylamide gels were prepared using the alkaline buffer system B (qv V B3(i)), and the acid buffer systems B and C (qv V B3(ii)). The composition of the two acrylamide solutions used to prepare the gradient were:

(a) "light" solution: polyacrylamide, T = 4% $^{w}/_{v}$, C = 5% $^{w}/_{w}$
ammonium persulphate 0.14% $^{w}/_{v}$
TEMED 0.92% $^{v}/_{v}$
Separating Gel Buffer

(b) "heavy" solution: polyacrylamide, T = 15% $^{w}/_{v}$, C = 5% $^{w}/_{w}$
ammonium persulphate 0.14% $^{w}/_{v}$
TEMED 0.15% $^{v}/_{v}$
Sucrose 1.54% $^{w}/_{v}$
Separating Gel Buffer

The concentrations of polymerisation catalysts were selected by prior experiment so that the "light" solution polymerised within 10 minutes exactly, the "heavy", within 20 minutes exactly.

The gradient was prepared with the aid of a three channel peristaltic pump. Heavy solution was pumped into a mixing vessel containing the light solution, and magnetically stirred at a slow speed to avoid vortex formation and air bubbles. Acrylamide was simultaneously pumped from the mixing chamber into the base of a standard slab former.

After polymerisation, the slab was rotated 90^{o}, and a separating gel (1 cm depth) polymerised on to the upper surface of the transverse gradient. Approximately 750 µl (1mg/ml) of the protein solution (prepared in the appropriate stacking gel buffer and containing

20% $^{w}/_{v}$ sucrose) was loaded, and electrophoresis then conducted using a constant current of 15mA for the stacking gel, 25 mA for the separating gel gradient.

E. Construction of Ferguson Plots:

Ferguson plots were constructed for components of the storage protein by using data obtained after electrophoretic separation on gels of varying total (T) $\%^{w}/_{v}$ acrylamide, but with constant (C = 5% $^{w}/_{w}$) crosslinker concentration. Gels were prepared using both the alkaline buffer system (B) (qv V B3(i)) and acid buffer system (B) (qv V B3(ii)). The methods outlined by Rodbard and Chrambach, (1971) and Chrambach et al, (1976) were employed.

Stocks of all solutions were prepared and stored at 4^{o}c, and the pH and specific conductance monitored in order to maximise reproducibility between experiments. Prior to use the solutions were brought to 25^{o}c i.e. the temperature used for gel polymerisation and electrophoresis. Sixty rod gels (separating gel only) were prepared simultaneously for each system, 10 at each value of %T. The tubes (i.d. 0.5 cm, e.d. 0.7 cm, x 12 cm) were sealed at their base with parafilm since rubber appears to enhance polymerisation (Schenkein et al, 1968). They were filled with exactly 2.1 ml of acrylamide solution, and overlayered with 20 µl distilled water. Temperature control of polymerisation was achieved by conducting polymerisation in the electrophoresis apparatus. Optimum concentrations of polymerisation catalysts (table 5) for each acrylamide concentration were selected by prior experiment so that polymerisation was achieved at 10 minutes exactly (as judged by the appearance of a schlieren inter-face between water overlay and acrylamide (Kingsbury and Masters, 1970). Similar procedures have been shown to ensure at least 95% conversion of monomer to polymer (Rodbard and Chrambach, 1971), and were considered to be of

TABLE 5. Concentration of Catalysts Used to Polymerise gels of varying %T in exactly 10 min.

		Acid Buffer System[a]		Alkaline Buffer System[b]	
	%T	TEMED $\%^{V}/v$	Ammonium persulphate $\%^{W}/v$	TEMED $\%^{V}/v$	Ammonium persulphate $\%^{W}/v$
Separating Gels[c]	4	0.950	0.14	0.051	0.07
	6	0.795	0.14	0.048	0.07
	8	0.745	0.14	0.035	0.07
	10	0.725	0.14	0.031	0.07
	12	0.725	0.14	0.019	0.07
	14	-	-	0.021	0.07
	14.933	0.45	0.14	-	-
Stacking gel[d]	3.125	0.125	0.125	0.06	0.125

[a] buffer system B (qv VB3(ii))

[b] buffer system B (qv VB3(i))

[c] %C = 5

[d] %C = 20

paramount importance since facilities for the ready determination of the absolute T value of each gel, such as that described by Chen and Chrambach, (1979) were not available. After 30 minutes, the polymerised gels were rinsed and stored at $4^{o}c$ in buffer of the same final composition.

For each electrophoretic separation, a set of 12 gels representing each %T value in duplicate were selected and exactly 150 µl of the stacking gel acrylamide solution polymerised on to the surface. The gels were loaded with 20 µl aliquots of the protein solution and electrophoresed at $25^{o}c$ using a constant current of 2mA/tube (10 mA/cm^2). The composition of the protein solutions examined were determined by prior experiment, and stock solutions were maintained in sealed dark containers. They were as follows:

(a)	alkaline system: (pH 7)	ß -lactoglobulin	0.5 mg/ml
		ferritin	0.5 mg/ml
		myoglobin*	0.5 mg/ml
		storage protein	E^{1cm}_{280} 1.23 exactly (about 1.23 mg/ml)
		sucrose	20% $^{w}/_{v}$
		phosphoric acid	0.032 M
		Tris	0.0588 M

*myoglobin was included for one series of electrophoretic separations only

(b)	acid system: (pH 4.875)	ß -lactoglobulin	0.5 mg/ml
		γ -globulin	0.5 mg/ml
		storage protein	E^{1cm}_{280} 1.23 exactly
		sucrose	20% $^{w}/_{v}$
		potassium hydroxide	0.06 M
		acetic acid	0.0625 M

The internal standard proteins (ß-lactoglobulin, ferritin, myoglobin and γ-globulin) were selected according to criteria set out by Johnson, (1979).

On termination of electrophoresis, gels were reemed from the tubes and sliced at the position of the tracking dye. They were stained with Coomassie Blue and destained (qv V B.4). Rm values were determined from the densitometric scans of the gels.

Within each system, the data obtained from each electrophoretic separation were used collectively to construct Ferguson plots for the separated proteins. The slopes of the plots were obtained through unweighted linear regression (Gonenne and Lebowitz, 1975), and gave the values of the retardation coefficients (Kr values) for the corresponding protein components. The plots were extrapolated to 0%T to obtain relative measures of free electrophoretic mobility (Yo values).

Approximate estimates of the molecular radii of the storage protein components were obtained for identification purposes only by reference to simple calibration curves relating molecular radius with $\sqrt{Kr}$ for values obtained for the internal standards used in each system. The calibration curves are given in figs. 8 and 9, together with data obtained by Rodbard and Chrambach, (1971) (using the same gel systems) to substantiate the validity of the construction. The molecular radii thus obtained were compared with those of standard proteins of known molecular weight to assess approximately the molecular weights of the storage protein components. In all instances the molecular radii of the standard proteins are those calculated by Rodbard and Chrambach, (1971) and assume the molecules to be spherical and unhydrated, with partial specific volumes of 0.74.

Fig. 8 and Fig. 9

Standard curves for the estimation of molecular size.

Fig. 8: alkaline buffer system B, 5%C

Fig. 9: acidic buffer system B, 5%C

The abscissa is the geometric mean radius $\bar{R}$, values of which are based on the assumption that the protein molecules are spherical, unhydrated, and have a partial specific volume of 0.74. The ordinate is the square root of the retardation coefficient, Kr.

Values from Rodbard and Chrambach, 1971, are indicated by the symbol -□- : these were obtained using the same buffer systems and %C value.

Experimental values from this work are indicated by the symbol -O-.

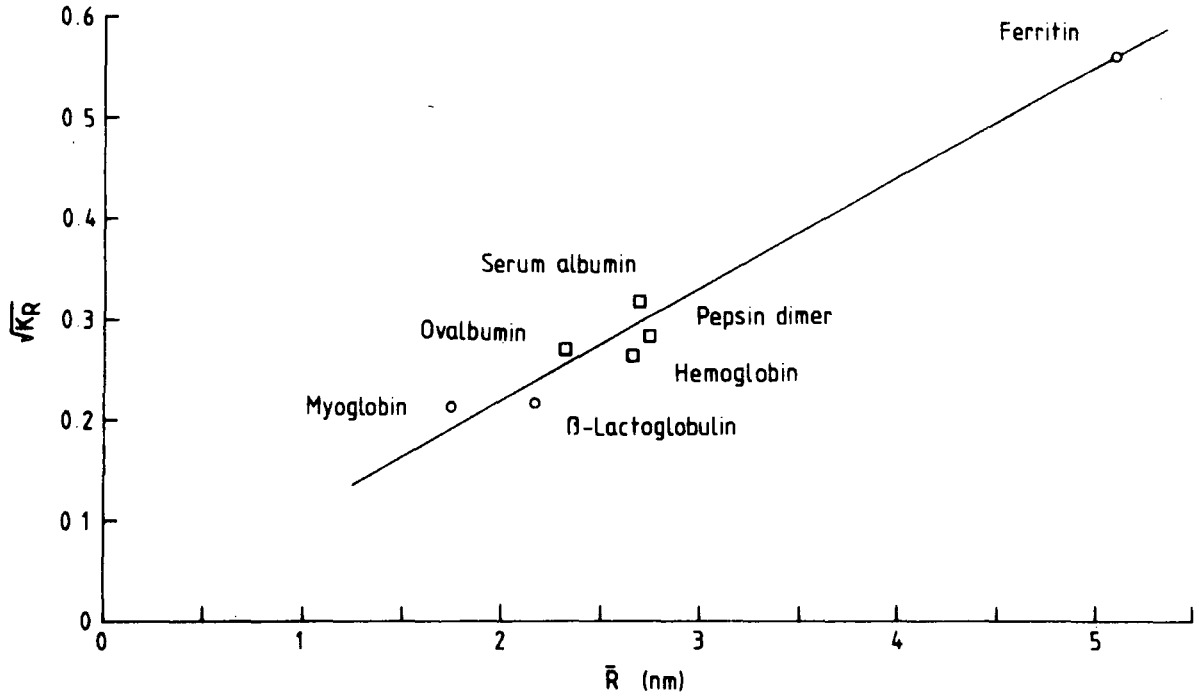

0.6
0.5
0.4
0.3
0.2
0.1
0
$\sqrt{K_R}$
Ferritin
Serum albumin
Pepsin dimer
Ovalbumin
Hemoglobin
Myoglobin
ß-Lactoglobulin
0
1
2
3
4
5
$\bar{R}$ (nm)

Fig. 8

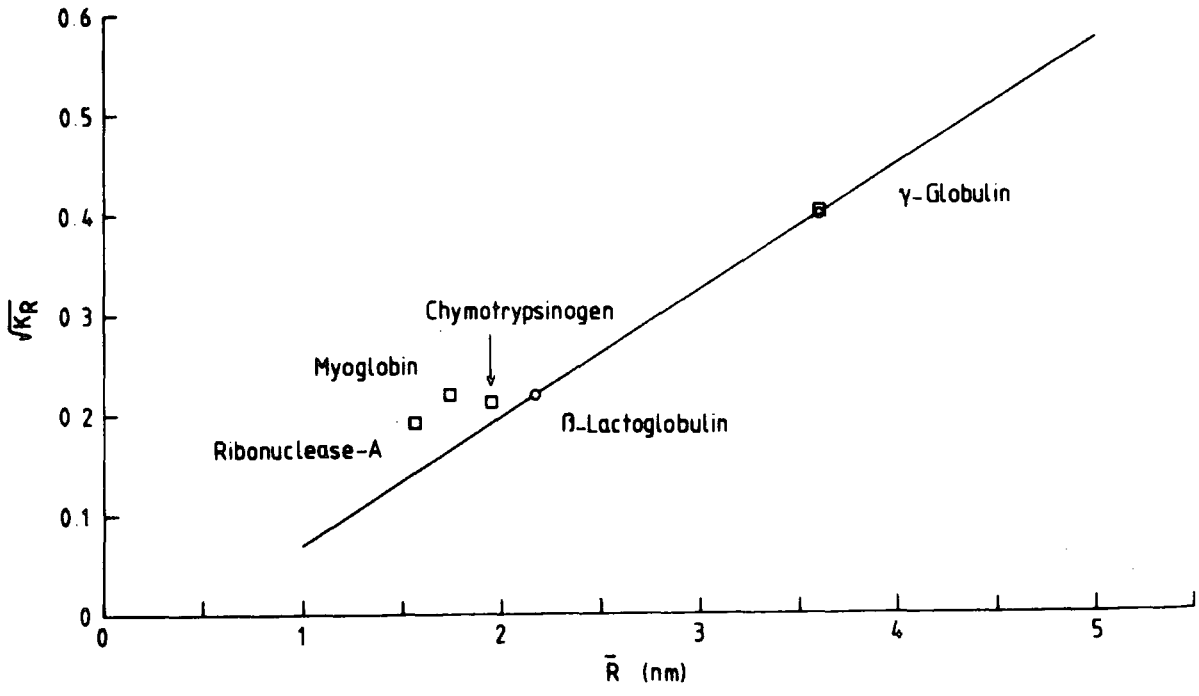

0.6
0.5
0.4
0.3
0.2
0.1
0
$\sqrt{K_R}$
γ-Globulin
Chymotrypsinogen
Myoglobin
ß-Lactoglobulin
Ribonuclease-A
0
1
2
3
4
5
$\bar{R}$ (nm)

Fig. 9

VI. ANALYTICAL ULTRACENTRIFUGATION

Analytical ultracentrifugation using the sedimentation velocity method was performed on a Christ Omega II 70000 ultracentrifuge and a Beckman Model E ultracentrifuge L.70. Constant speeds of 51,000 rpm (20°c) and 60,000 rpm (19.5°c) were maintained on the two centrifuges respectively. Sedimentation coefficients were determined as described by Schachman, 1957, from which crude estimates of molecular weight were obtained using the expression

$$\log S = 0.61 \log M - 2.27$$

which describes very approximately the relationship between sedimentation coefficient (S) and molecular weight (M) for a number of standard proteins analysed on the latter centrifuge under similar conditions (R. Pain personal communication).

VII. FURTHER CHROMATOGRAPHIC METHODS

A. Thin Layer Chromatography:

Thin layer chromatography (TLC) plates were prepared with the adsorbent kieselgur to a depth of less than 2 mm. Sample volumes of 10 µl were spotted onto the plates which were then developed in the solvent system methanol : chloroform : 17% ammonia, 40:40:20. After drying they were sprayed with 0.2% ninhydrin in acetone and the colour developed at 105°c for five minutes.

B. Adsorption Chromatography using Hydroxylapatite:

A 10 ml aliquot of the storage protein prepared by ion exchange chromatography (qv III.E) was dialysed against several changes of potassium phosphate buffer (0.06M, pH 7.9). The sample was then filtered through a millipore filter (GF/C) and introduced on to a column of hydroxylapatite (17 x 3.2 cm), packed and equilibriated with sterile-filtered phosphate buffer of the same composition. Elution commenced by pumped flow (5 ml/h/cm^2) down the column with the same buffer, for a period of five hours (200 ml); then a linear

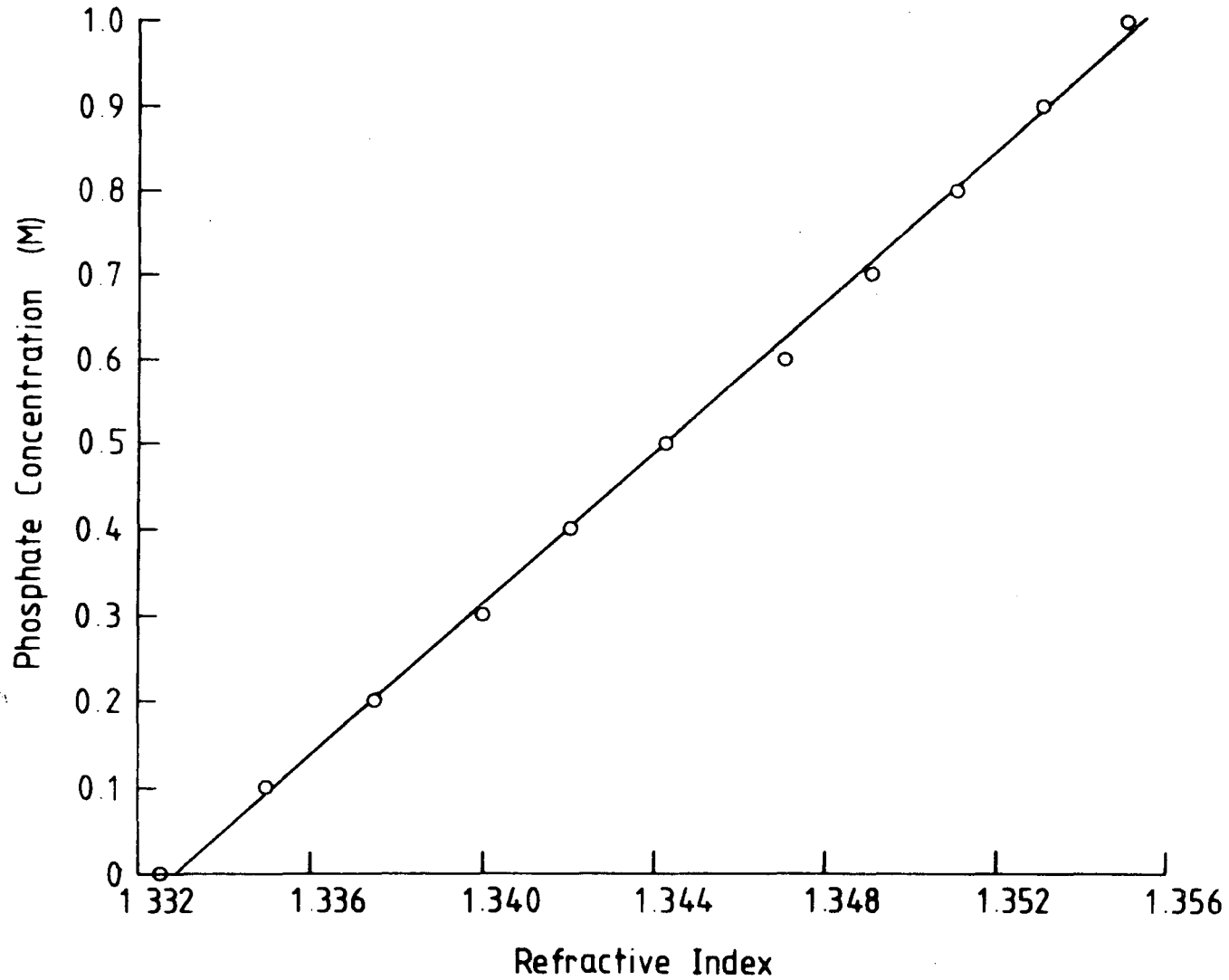


Fig. 10 Calibration curve relating values of refractive index with phosphate concentration

gradient of increasing phosphate molarity (0.06-1.0M) was developed (total volume 1000 ml). Column eluant was monitored continuously at 280 nm, and fractions collected. The gradient densities were measured by refractometry, and the corresponding concentrations of phosphate determined by reference to a calibration curve which related refractive index with phosphate concentration (fig. 10).

C. Molecular Sieve Chromatography for Distribution Analysis and Molecular Weight Estimation of the Storage Proteins in Different Buffer Systems:

(1) Bio-gel:

Columns of dimensions 60 x 2.2 cm, 45 x 2 cm and 50 x 1.6 cm were packed with Bio-gel P-150 or Bio-gel P-300 (Biorad catalogue D, 1978), and equilibriated and operated with the following buffers:

(i) 0.05M Tris-hydrochloride, 0.15M sodium chloride pH 8.3

(ii) 0.05M Tris-hydrochloride, 0.3M sodium chloride pH 8.3

(iii) 0.025M Tris-hydrochloride pH 8.3

(iv) 0.025M Tris-hydrochloride, 0.01M dithiothreitol pH 8.3

(v) 0.0125M Sodium acetate pH 4.7

(vi) 0.0125M Sodium acetate, 0.3M sodium chloride pH 4.7

(vii) 0.02M Glycine hydrochloride, 0.05M sodium chloride, pH 2.6

(viii) 0.02M Glycine hydrochloride, 0.3M sodium chloride, pH 2.6

(ix) 0.02M Glycine hydrochloride pH 2.6

Constant flow rates of 4.8 $ml/h/cm^2$ were maintained by pumped flow down the columns. Eluants were monitored continuously at 280 nm, and collected either as fractions or in a graduated measuring cylinder. The elution profiles were recorded on a chart driven at a constant speed of 10 mm/h. Each chart was calibrated with respect to volume of column eluant.

Protein solutions of constant volume (usually 1.5 ml) but varying concentration were loaded on to each column after buffer

had been drained to bed level: dilute protein solutions were concentrated using a minicon B.15 concentrator. Eluant was monitored and collected as the protein drained into the bed. In this way, the elution volume (Ve) for each protein component was determined from measurement on each chart of the distance from the origin to the mid-point of the protein peak.

An approximate estimation of the molecular weight of each protein component in the storage protein preparation was made by reference to calibration curves constructed for each buffer system which related molecular weight with the ratio of the elution volume to the void volume (Vo) for a number of standard proteins. Combinations of the following standard proteins were used for this purpose (molecular weight in parentheses): cytochrome C (12400), myoglobin (18400), soybean trypsin inhibitor (24000), chymotrypsinogen (25000), ovalbumin (43500), bovine serum albumin (67000), transferrin (76600) and γ-globulin (150000). Dextran Blue 2000 and dinitrophenyl glycine were used to determine the void volume (Vo) and buffer volume (Vi), respectively. One of the calibration curves constructed is given in fig. 11.

(2) Sephadex:

Columns of dimensions 45 x 2 cm and 56.5 x 2.2 cm were packed with Sephadex and equilibriated and operated as described in Section III.A. The following buffers were used:

(i) 0.05M Tris-hydrochloride, 0.15M sodium chloride pH 8.3

(ii) 0.1M Sodium phosphate, 0.15M sodium chloride pH 6.2

(iii) 0.02M Sodium borate, 0.15M sodium chloride, 0.1M glucose pH 7.3

Preparation of the storage protein and standard proteins, measurement of Ve, and estimation of approximate molecular weight were carried out as described in VII.C1. A typical calibration

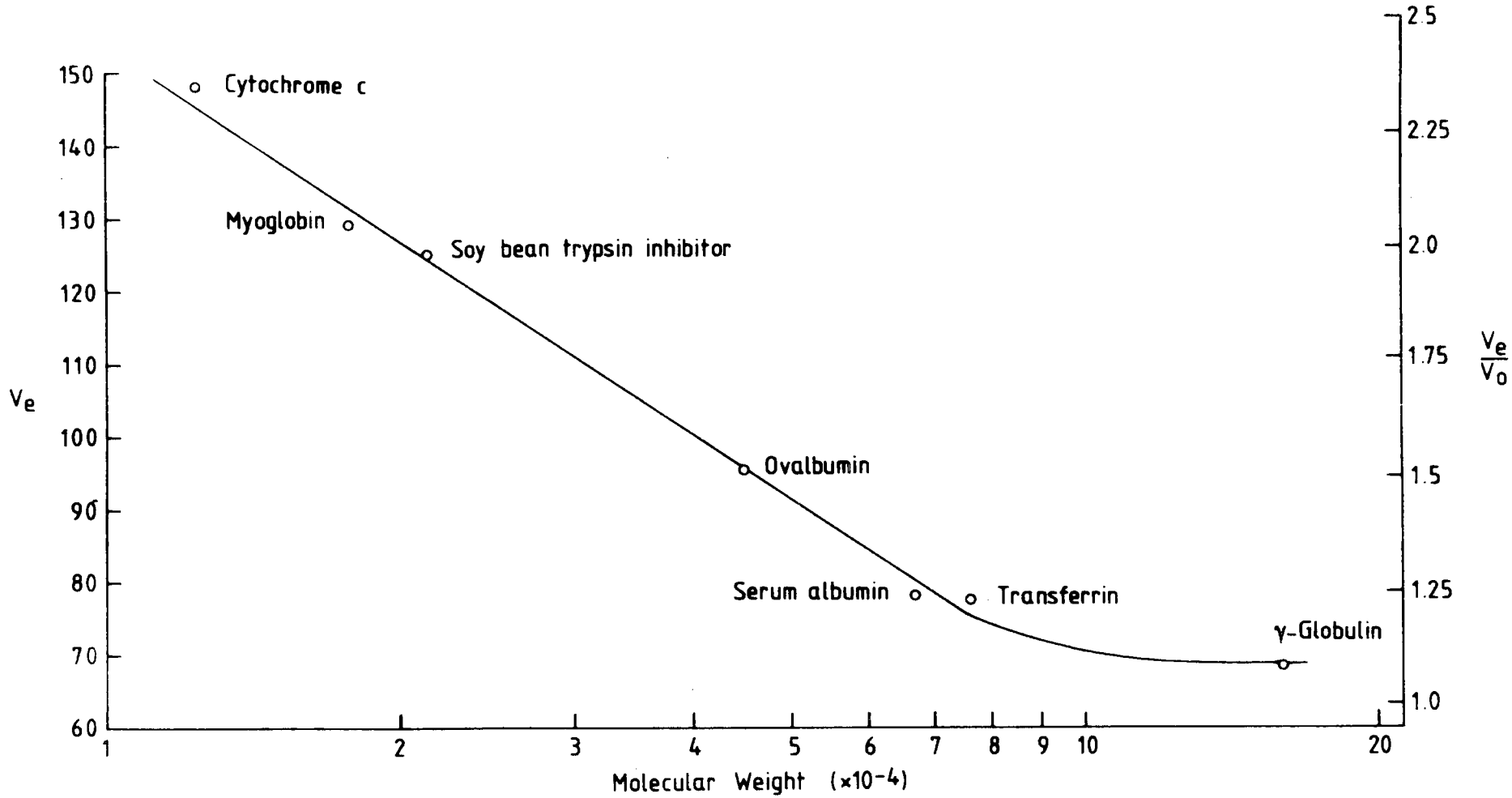


Fig. 11

Standard curve for estimation of molecular weight by molecular sieve chromatography on Biogel P-150. Column equilibriated with 0.05M Tris HCl pH 8.3 containing 0.15M NaCl. The void volume, Vo, measured with Dextran Blue 2000, was 61.7 ml. The internal volume, Vi, measured with DNP glycine, was 248 ml.

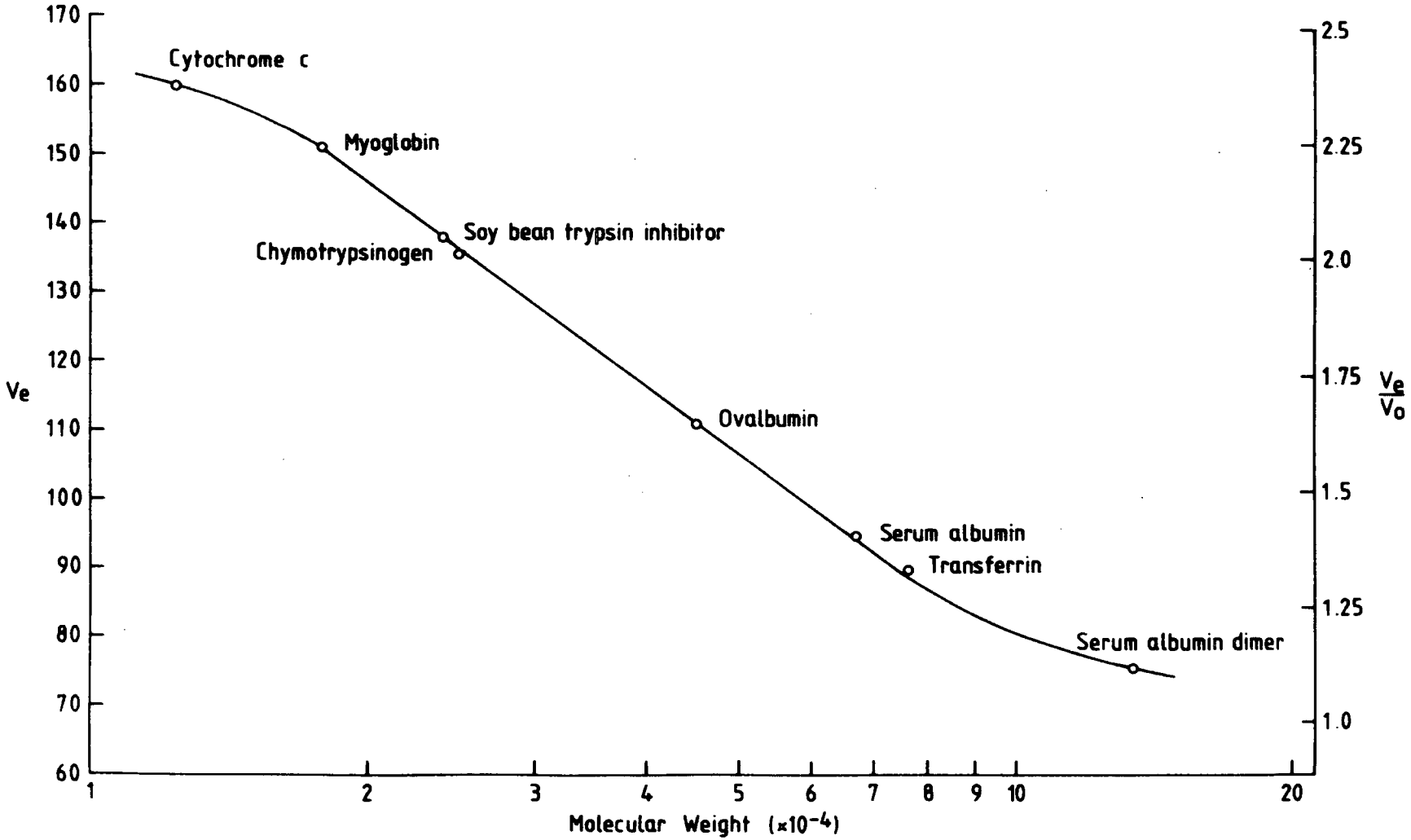


Fig. 12

Standard curve for estimation of molecular weight by molecular sieve chromatography on Sephadex G100. Column equilibriated with 0.05M Tris HCl pH 8.3 containing 0.15M NaCl. The void volume, Vo, measured with Dextran Blue 2000 was 67.5 ml. The internal volume, Vi, measured with DNP glycine, was 236.5 ml.

curve is given in fig. 12.

(3) Ultrogel:

A column of dimensions 80 x 2.2 cm was packed with ultrogel and equilibriated and operated with 0.1M Tris-hydrochloride, 0.25M sodium chloride pH 8.5. A lyophilised preparation of the storage protein was solubilised in the same buffer, and filtered through millipore filters GF/C and GF/F before being introduced on to the column. Measurement of Ve and estimation of molecular weight were carried out as described in Section VII.C1.

VIII. HISTOCHEMICAL METHODS

A. Protein:

The general distribution of protein in a mature dormant tuber was studied on fresh sections which were immersed directly in a solution of 0.025% $^{w}/_{v}$ Coomassie Blue in 50% $^{v}/_{v}$ methanol, 3.5% $^{v}/_{v}$ acetic acid and examined under a Watson dissecting microscope.

Thin sections were prepared from samples which were fixed in a mixture of 2.5% $^{v}/_{v}$ glutaraldehyde and 1.5% $^{v}/_{v}$ formaldehyde in sodium cacodylate (0.05M pH 7.0) for four hours at room temperature, washed in buffer and post fixed in 1% osmium tetroxide for four hours. After alcohol dehydration, the samples were embedded in Spurr's resin (Spurr, 1969). Sections (1 µm) for light microscopy were stained with 1% $^{w}/_{v}$ Toluidine Blue in borax and examined and photographed in a Leitz Ortholux microscope. Thin sections for electron microscopy were stained with aqueous uranyl acetate and post stained with Reynolds lead citrate: they were examined in a Philips EM 400 electron microscope at 60 KV.

B. Lignin and Starch:

Fresh sections of a mature dormant tuber were immersed

directly in concentrated HCl for five minutes, then stained with 10% $^{w}/_{v}$ phloroglucinol in 95% acidified ethanol for five minutes, and the general distribution of lignified tissue photographed.

Fresh sections were also immersed in an aqueous iodine solution for two/five minutes to visualise the starch grains.

IX. THE BREEDING PROGRAMME

A. Experimental Design and Growth Conditions:

(1) Population 1:

Tubers of the variety Nwapoko were collected from both the I.I.T.A. and local farmers. Setts of approximately uniform weight were cut and planted in four replicates within one field in a completely randomised design. Variation of environmental conditions within the field as a whole was minimised as far as possible. At the end of the season the tubers of twenty plants within each replicate were harvested (6th November, 1978), then sampled (qv IX.B).

(2) Population 2:

Tuber setts from both of the seedling derived clones W 387-3 and W 387-4 were planted together in a completely randomised design within a small uniform plot in 1978. At the end of the season tubers from four plants of each clone were harvested (20th November, 1978), weighed and sampled (qv IX.B).

(3) Population 3:

Tuber setts from 96 different seedling derived clones were planted together in a completely randomised design within a uniform plot. The tubers from a single plant of each clone which survived were harvested at the end of the season (November 1977) weighed, and one tuber from each plant then sampled (qv IX.B). In March 1978, tuber setts were prepared from the same plants analysed previously

and planted together, again in a completely randomised design within a uniform plot. At the end of the season, tubers from all the plants propagated which survived were harvested (20th November, 1978), weighed and sampled as before. Substantial loss of plant material was incurred so that at the closure of the experiment, the number of clones remaining in the population was reduced to nine.

(4) Population 4:

Tuber setts of approximately uniform weight (500g) were planted in the field in March 1978 in a completely randomised design (1 m x 1 m spacing). The field was divided into four areas since considerable variation of environmental conditions was apparent (table 6). Tubers were harvested between 20th and 30th October, 1978, weighed, cured and stored. Sampling of the tubers was carried out in three batches over a period of nine days; tubers for each batch were selected at random.

(5) Population 5:

Seeds produced by non-assortative mating between eight pairs of unselected parents in 1977 were planted in nursery beds at close spacing (27.0 x 15.5 cm) in 1978. In March 1979, both the seedling tubers from the nursery beds and tuber setts from the parents were planted together in the field (1 x 1 m spacing), using a complete randomised block design with three replications of each family. The seedling tubers were planted whole so that sett weight varied between 21 g and 591 g, whilst the parent tuber setts were cut to approximately uniform weight (200-350 g) where possible. Harvesting commenced in October, seven months after planting. The first harvest (16th October) was selectively carried out on dead plants. Each replicate (rep) was then fully harvested, rep 1 on 26th October, rep 3 on 29th October and rep 2 on 1st November. After curing the tubers were weighed then stored before

Table 6. Soil Chemical Analysis of Samples taken from the field for Population 4

Location	Area of Field	pH	% C	% Total N	Available P Bray I (µg/g)	NH_4O Ac extractable cations (µg/g)					Total Acidity (Al + H) (Me/100g)
						Ca	Mg	Mn	K	Na	
1	NE	4.5	0.73	0.316	22.0	2079	>300	130.7	705	50	0.24
2	SE	5.5	0.63	0.116	18.0	984	>300	93.6	105	263	0.26
3	NW	5.7	0.65	0.122	24.9	1672	>300	68.4	150	87	0.26
4	SW	5.6	0.75	0.120	28.2	897	>300	83.4	90	135	0.26

sampling (22nd January/5th February). A substantial loss of plant material reduced the numbers of male parents which could be analysed to five, and female parents to four.

B. Evaluation of Plant and Tuber Characters:

The methods used to record plant and tuber characters are summarised in table 7.

The sampling of all the tubers except those from population 5 (qv IX A.5) commenced on 5th December at the time of maximum consumption of yam by the Nigerian population. Between harvesting and sampling, tubers were cured at high temperature and relative humidity in plastic roofed huts for four days and then stored at 22/25°c. During this period loss of both moisture content and dry matter by respiration occurs, and in order to estimate yield in terms of total nitrogen content it has been assumed that all tubers within a population suffer the same % loss in weight.

Sampling was carried out as follows. The weight of each tuber was recorded (tuber fresh weight, table 7), then the inedible head portion was removed as for cooking and a median longitudinal slice (½"-1" thick) was peeled, diced and weighed. The sample was oven dried at 55-65°c for 24-36 hours to constant weight, and the % dry matter estimated. The entire sample was ground and a sub-sample sent to Durham for analysis of nitrogen content, carried out as described in section IV.B.

C. Statistical Methods:

(1) Analysis of Variance and Correlation of Measurements: (Bailey, 1959)

Tests of this nature were carried out using the Michigan computer programme Midas, except where indicated.

Table 7 Terminology and Methods of Evaluation of Plant and Tuber Characters

Character	Abbreviation Used in Appendices 1-10	Method of Evaluation/ Terminology
yield per plant (g)	hill wt.	fresh weight of all tubers from one plant, recorded at harvest
tuber fresh weight (g)	tuber f.w.	weight of single tuber sampled, recorded at the time of sampling
tuber dry weight (g)	tuber d.w.	calculated from values of tuber fresh weight and % dry matter
% dry matter	dm %	measured on a median longitudinal slice of tuber dried to constant weight
% nitrogen dry weight	N d.w. %	measured by the microKjeldahl technique on a dry sub-sample
% nitrogen fresh weight	N f.w. %	calculated from values of % dry matter and %N dry weight
total nitrogen content of tuber (g)	total Nt	calculated from values of %N fresh weight and tuber fresh weight
total yield of nitrogen (g) per plant	N.hill wt.	calculated from values of %N fresh weight and hill weight
tuber number	tuberno	count of number of tubers per plant
tuber shape	tubershape	subjective measurement; 1-5 ranks representing increasing length to diameter ratio
seedling tuber shape	s.lendia	length: diameter ratio of seedling tuber measured
tuber smoothness	tusmooth	subjective measurement; 1-4 ranks representing very rough (nematode susceptible) to very smooth (nematode resistant)

Table 7 (Cont.)

Terminology and Methods of Evaluation of Plant and Tuber Characters (Cont.)

Character	Abbreviation Used in Appendices 1-10	Method of Evaluation/ Terminology
plant height	height	main vine measured; 1-3 ranks; less than 1.2m(1), 1.2-2.0m(2), greater than 2.0m(3)
branch number	branchno.	subjective measurement; 1-3 ranks, few to many. Only branches with two or more expanded leaves considered
leaf number	leafno.	subjective measurement; 1-3 ranks, few to many
leaf size	leafsize	subjective measurement; 1-3 ranks representing small to large
stem colour	stemcol	subjective measurement; green(1), green-purple(2), purple-green(3), purple (4)
stem number	stemno.	count of number of stems per plant
stem diameter	stemdiam	subjective measurement made 10-15 cm above ground; 1-3 ranks representing small to large
thorniness	thorn	subjective measurement; 1-4 ranks representing very few thorns to most of the basal portion of the stem bearing thorns
virus score	virus	(subjective measurement; (1-4 ranks representing no (leaves showing symptoms (to all leaves showing (symptoms
leaf spot score	leafspot	
emergence date	em.date	1-10 ranks representing early to late emergence (Population 4)/weeks after planting (Population 5)
first branch height (cm)	ht.1.bra	height to first branch of plant measured
flowering date	fl.date	1-9 ranks representing early to late flowering. Rank 10 = nonflowering

Table 7 (Cont.)

Terminology and Methods of Evaluation of Plant and Tuber Characters (Cont.)

Character	Abbreviation Used in Appendices 1-10	Method of Evaluation/ Terminology
sex	sex	nonflowering(1), male(2), monoecious(3), female(4)
vine vigor	vigor	subjective measurement; 1-5 ranks representing increasing vigor
dry vine weight (g)	vinedw	weight of vine after harvest

(2) Estimation of Heritabilities:

Heritabilities of characters were estimated according to Falconer, (1964) using data from population 5. The design of the field into three replicates (see IX A.5) had to be discounted in order to use the maximum numbers of families in the estimations and this has required the assumption that offspring and parents are randomly distributed within the field.

Heritabilities were determined from regression coefficients, calculated from the regression of each offspring value on firstly midparent values, secondly female parent values and thirdly male parent values. The regressions were carried out using the Michigan computer programme Osiris, IV. Several of the parents were replicated and in this instance the mean value was used. Midparent values were obtained by taking the mean value of the two parents concerned. Regression coefficients calculated from offspring-midparent regressions gave direct estimates of narrow-sense heritabilities, $\frac{VA}{Vp}$, where

VA = additive genetic variance

Vp = phenotypic variance

Coefficients calculated from offspring female parent and offspring male parent regressions gave estimates of half the heritabilities, so that in these instances the values were doubled. 95% confidence limits for the estimates were calculated using results from analyses of variance of offspring values, according to the method of Colquhoun, (1971).

RESULTS

I. ANALYSIS OF YAM MEALS FROM VARIETIES NWAPOKO AND YAM 2

A. Amino Acid and Nitrogen Analyses:

The amino acid compositions and contents, together with nitrogen contents of meals prepared from harvests of both 1977 and 1979 for the variety Nwapoko, and from harvest 1975 of Yam 2 are given in table 8.

The amino acid composition of the variety Nwapoko was essentially the same after both harvests, and also very similar to that of variety Yam 2: this latter meal contained a slightly lower relative amount of arginine, and higher relative amounts of glycine and serine in particular. Aspartic and glutamic acids were the major amino acids, but the sulphur-containing amino acids were low in relative content.

However, the amino acid contents and the nitrogen contents of the individual meals differed appreciably. Thus meal prepared from Nwapoko after the harvest 1977 had amino acid and nitrogen contents which were 25% and 20% greater respectively than those of the same variety but after the harvest 1979, and 80% and 100% greater respectively than those from variety Yam 2.

The nitrogen in amino acids represented nearly the same proportion of total nitrogen in the three meals ie 71%, 68% and 66% for variety Yam 2, and variety Nwapoko, harvest 1978 and 1979 respectively; thus nearly a third of the Kjeldahl nitrogen estimates represented non-amino acid nitrogen.

B. Sulphur Content:

The total sulphur content of the meal Nwapoko (1978) was 0.059 $\pm$ 0.006% (dry weight basis), whilst the sulphur in amino

TABLE 8. Amino Acid and Nitrogen Analyses of Yam Meals [a]

Amino Acids (gaa 16 gN)[b]	Yam 2 (Harvest 1975)	Nwapoko (Harvest 1977)	Nwapoko (Harvest 1979)
aspartic acid	12.72	11.02	11.09
threonine	3.04	2.93	2.94
serine	5.92	4.10	4.08
glutamic acid	16.33	14.06	12.96
proline[d]	2.90	3.66	3.04
glycine	3.77	2.96	2.94
alanine	5.07	3.68	4.36
valine	4.36	3.75	3.72
methionine	1.14	1.27	1.14
isoleucine	3.63	3.15	3.03
leucine	6.83	5.74	5.44
tyrosine	1.75	2.19	1.71
phenylalanine	4.95	4.54	3.87
histidine	1.92	1.92	1.86
lysine	4.58	4.57	4.56
arginine	5.29	7.43	6.94
cysteic acid	0.87	0.75	1.00
Total	85.07	77.72	74.68
amino acid content (g/100g meal) (dw))	3.444	6.227	4.983
amino acid nitrogen content (g/100g meal)	0.462	0.874	0.703
total nitrogen[c] (g/100g meal)	0.648 ± 0.009	1.282 ± 0.016	1.064 ± 0.009
Crude protein content (%N x 6.25)	4.050	8.013	6.650

[a] dry weight basis

[b] mean of two 22h hydrolysates, $\pm$ 5% error: tryptophan and ammonia contents not determined.

[c] mean of several analyses $\pm$ SD.

[d] poorly resolved in analyses.

acids, determined from the amino acid composition, was 0.038 ± 0.001% (dry weight basis), a value which represented only 64% of the total sulphur content.

C. Protein Content and Composition:

(1) Time Course of Extraction of Kjeldahl and Ninhydrin N with 70% V/v Ethanol:

The colour intensity of spots on thin layer chromatography plates showed that the majority of free amino acids and peptides soluble in 70% ethanol were extracted from the meal after one hour. After a further five hours of extraction, a small amount of ethanol-soluble amino acids could still be extracted, but on further extraction no more amino acids were detected in the extracts by this method of analysis.

The nitrogen contents of ethanol extracts which were prepared after extracting the meal for six hours and two further three hour periods showed that approximately 12% of the total nitrogen in the meal was extracted after six hours. After a further three hours of extraction, a further 2% of the total nitrogen was extracted, and after another three hours of extraction only a further 0.2% of the total nitrogen could be extracted.

(2) Amino Acid and Nitrogen Analyses of Ethanol Extracts and Ethanol-extracted Meals:

Results from amino acid and nitrogen analyses of two meals (varieties Yam 2 and Nwapoko, harvest 1977) extracted with ethanol, together with the results from analysis of the ethanol extracts, both acid hydrolysed and unhydrolysed, for the meal variety Yam 2 are given in table 9.

The protein-bound amino acid contents of the two meals which were determined from the amino acid contents of the ethanol-extracted meals differed widely in absolute value but represented nearly the

TABLE 9. Amino Acid and Nitrogen Contents and Amino Acid distribution (% of total amino acids) in ethanol extracts, and ethanol-extracted meals[a]

Variety	Yam 2			Nwapoko
amino acids[b] (% of amino acids in meal)	hydrolysed ethanol extract[c]	unhydrolysed ethanol extract[c]	ethanol-extracted meal	ethanol-extracted meal
aspartic acid	18.8	11.2	80.1	85.4
threonine	5.9	6.1	92.5	73.9
serine	27.2	27.1	76.7	74.2
glutamic acid	24.2	11.1	72.8	75.4
proline	5.0	3.7	93.4	91.8
glycine	7.0	0.9	90.5	85.1
alanine	32.7	32.3	76.7	81.3
valine	5.9	5.0	92.8	91.1
methionine	1.2	1.5	100.9	93.2
isoleucine	3.5	2.9	99.3	92.4
leucine	2.4	1.6	99.0	89.2
tyrosine	2.3	1.2	88.0	42.3
phenylalanine	2.7	2.5	100.3	89.6
histidine	6.2	6.1	84.1	85.0
lysine	4.4	3.4	85.8	80.7
arginine	1.6	0.3	93.5	74.5
cysteic acid	3.2	ND	97.5	75.5
amino acid content (g/100g meal)	0.461 ± 0.026	0.313	2.949	5.058
amino acid nitrogen content (g/100g meal)	0.055	0.039	0.398	0.705
total nitrogen[e] (g/100g meal)	0.107 ± 0.004	-	0.525 ± 0.019	0.929 ± 0.023
weight (g/100g meal)	8.26 ± 0.18	8.26 ± 0.18	91.74 ± 0.18	93.95 ± 1.46

a dry weight basis prior to extraction.
b 22h hydrolysates: tryptophan and ammonia content not determined.
c In additionto the protein amino acids four other amino acids appeared in minor amounts on the trace, one of which was probably α aminobutyric acid.
d Mean of two hydrolysates $\pm$ SD
e Mean $\pm$ SD of several determinations.

same proportion of total amino acids present (qv I.A) i.e. 86% and 83% for variety Yam 2 and variety Nwapoko respectively. The relative amounts of individual amino acids in protein, expressed as a proportion of the corresponding amino acids in the meals were similar apart from the amounts of arginine, cysteic acid and threonine which were lower in the protein from variety Nwapoko. The ethanol-extracted meals of both varieties contained lesser amounts of, in particular, aspartic and glutamic acids, serine, alanine histidine and lysine compared to the unextracted meals.

The amino acids present in the ethanol extracts were considered to be primarily free amino acids, since although the total amino acid content of the hydrolysed extract was about 40% greater than that of the unhydrolysed extract, the difference was primarily due to greater relative amounts of aspartic and glutamic acids after hydrolysis. The major free amino acids in Yam 2 were aspartic acid/asparagine and glutamic acid/glutamine, serine and alanine, which together constituted 84% of the total free amino acids. The free amino acid pool also contained low levels of all the essential amino acids. (Free amino acids in Nwapoko were not determined experimentally, but can be deduced to be similar apart from larger relative amounts of arginine, threonine and cysteic acid).

The distribution of nitrogen in the two meals is given in table 10 and is compared in table 11 with values obtained by Evans (I.I.T.A. in-house report) for the distribution of nitrogen in other varieties of D.rotundata. Nitrogen in protein-bound amino acids (excluding amide N) represented 61% of the total nitrogen in Yam 2, and 55% of the total in Nwapoko, but these low values are nevertheless comparable with those determined for the different seedling-derived varieties B 72-7, M 136 and W 185 (table 11) as well as the unknown variety designated "large batch". The proportion of non-amino acid nitrogen for these different

TABLE 10. Nitrogen distribution (% of Total N) in Yam Meals

Meal	Ethanol Extract		Residue	
	Free amino acid N [a]	Non-amino acid N [b]	Protein-Bound[a] Amino Acid N	Non-amino acid (b) N
Yam 2	9	8	61	20
Nwapoko (1977) [c]	13	14	55	17

(a) Determined from 22h hydrolysates.

(b) Calculated from total nitrogen content and amino acid nitrogen content.

(c) Figures for N in ethanol extract derived by deduction.

TABLE 11. Nitrogen Content, Amino Acid Content and Nitrogen Distribution (% of Total N) in Different Varieties of D.rotundata

Variety	% N	Crude Protein Content (%Nx 6.25)	Total amino acid content (gaa/100g meal)	Protein-bound amino acid content (gaa/100g meal)	% Protein-bound amino acid N of total N	% Free amino acid N of total N	% Non-amino acid N of total N	Conversion factor to relate % N to true protein content
Yam 2	0.65	4.06	3.44	2.95	61	9	28	4.54
Nwapoko 1977	1.28	8.00	6.23	5.06	55	13	31	3.95
Nwapoko 1979	1.06	6.63	4.98	4.37[b]	57[b]	8[c]	34	4.10
B 72-7[a]	1.64	10.25	7.82	7.06	60	8	32	4.30
M 136[a]	1.83	11.44	8.50	8.08	60	4	36	4.42
W 185[a]	1.81	11.31	8.70	7.68	61	8	31	4.24
"Large Batch"[a]	0.90	5.63	4.56	3.82	56	11	33	4.24
Meal value $\pm$ SD	1.31 ± 0.46	8.19 ± 2.91	6.32 ± 2.08	5.57 ± 2.02	59 ± 3	9 ± 3	32 ± 3	4.26 ± 0.19

[a] data courtesy M. Evans, in-house report of IITA

[b] determined in cooked meal

[c] determined in cooking liquid.

varieties was also relatively constant with a mean value of 32% ± 3%. Conversion factors calculated to relate the total nitrogen content of the meals with the weight of protein-bound amino acids determined from amino acid analysis of the ethanol-extracted meals are given in table 11; the mean value of these conversion factors was 4.26 ± 0.19. Values of protein content as estimated from values of total % N x 4.26 correlated highly (r = 0.99, significant at less than 0.1%) with the values obtained by aminoacid analysis for the protein-bound amino acid content of these varieties.

D. Analysis of Cooked Yam Meal:

Results from amino acid and nitrogen analyses of the cooked yam meal (variety Nwapoko, harvest 1979), and the cooking liquid are given in table 12.

The amino acid and nitrogen contents of the cooked yam represented 88% and 89% respectively of the corresponding contents in the uncooked meal. The amounts of individual amino acids in the cooked meal expressed as a proportion of the corresponding amino acids in the meal were essentially the same as those determined to be protein-bound for this variety (qv table 9).

E. Nutritional Value:

(1) Chemical Score:

The Chemical Scores (FAO and WHO 1973) for the essential amino acids in the meals, ethanol-extracted meals and cooked meal are given in table 13.

The sulphur amino acids were the limiting amino acids with low scores of 57-60% in the meals, but higher scores (around 70%) were obtained for the ethanol-extracted meals. Tryptophan, however, was not determined.

TABLE 12. Amino Acid and Nitrogen Contents[a], and Amino Acid Distribution (% of Meal Amino Acids) of Cooked Yam[b] and Cooking Liquid

Amino Acids[c] (% of Meal Amino Acids)	Cooked Yam	Cooking Liquid
Aspartic Acid	85.0	14.2
Threonine	87.2	12.8
Serine	83.8	16.5
Glutamic Acid	85.7	15.8
Proline	92.6	7.4
Glycine	85.7	8.2
Alanine	81.1	18.9
Valine	89.5	8.5
Methionine	94.7	5.3
Isoleucine	94.6	5.0
Leucine	97.5	3.6
Tyrosine	87.7	5.3
Phenylalanine	98.1	3.5
Histidine	83.9	14.5
Lysine	90.5	8.9
Arginine	83.8	15.9
Cysteic Acid	74.6	17.9
Amino Acid Content[c] (g/100g meal)	4.371	0.591
Amino Acid Nitrogen[c] content (g/100g meal)	0.611	0.088
Total nitrogen content (g/100g meal)[d]	0.948± 0.050	0.146± 0.001
Weight (g/100g meal)[e]	87.20 ± 1.64	10.47 ± 0.89

[a] Dry weight basis.

[b] Yam variety Nwapoko, harvest 1979: the amino acid and nitrogen contents of the meal before cooking were 4.983% and 1.064% respectively (See Table 8)

[c] Mean values determined from two 22h hydrolysates.

[d] Mean of several values ± SD

[e] Mean of two values ± SD

Table 13 Chemical Scores and Levels of Essential Amino Acids in Meals, Ethanol-Extracted Meal and Cooked Meal

Essential Amino Acids	FAO pattern	Yam 2				Nwapoko, harvest 1977				Nwapoko, harvest 1979			
		Meal		Ethanol Extracted meal		Meal		Ethanol Extracted meal		Meal		Cooked Meal	
	(a)	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)
Sulphur Amino Acids	3.52	2.01	57.1	2.47	70.2	2.02	57.4	2.42	68.8	2.14	60.8	2.06	58.5
Lysine	5.44	4.58	84.2	4.85	89.2	4.57	84.0	5.10	93.8	4.56	83.8	4.66	85.7
Isoleucine	4.00	3.63	90.8	4.45	>100	3.15	78.8	4.02	>100	3.03	75.8	3.22	80.5
Leucine	7.04	6.83	97.0	8.34	>100	5.74	81.5	7.07	>100	5.44	77.3	6.00	85.2
Aromatic Amino Acids	6.08	6.70	> 100	8.04	>100	6.73	> 100	8.27	>100	5.58	91.8	5.96	98.0
Threonine	4.00	3.04	76.0	3.47	86.8	2.92	73.3	2.99	74.8	2.94	73.5	2.89	72.3
Valine	4.96	4.36	87.9	4.99	>100	3.75	75.6	4.71	95.0	3.72	75.0	3.75	75.6
Tryptophan	0.96	ND		ND		ND		ND		ND		ND	

(a) Amino acid level (g/16gN)

(b) Chemical score (%)

(2) Anti-physiological Components:

(i) Saponin Analysis:

The saponin, digitonin, caused 50% haemolysis of a 2% erythrocyte suspension at a concentration of 2.3 μg/ml. This may be compared to the yam meal Nwapoko, which, at a concentration of 46 mg/ml, caused 100% haemolysis of the erythrocytes almost immediately, indicating the presence of saponins.

(ii) Trypsin Inhibitor Analysis:

Analyses of the meals for trypsin inhibitor were negative.

(iii) Lectin Analysis:

Direct analysis of the meals was not possible due to the presence of saponins. The major protein fraction, however, was tested for lectin activity (Section V.M) and negative results were obtained.

II. EXTRACTION OF PROTEIN FROM YAM MEAL

A. Examination of the Efficiency of Extraction of Protein from Yam Meal:

Yam meals were extracted using three different extraction media; distilled water brought to pH 8.3, 0.05M sodium borate buffer pH 8.3 (low ionic strength buffer) and 1M potassium phosphate buffer pH 8.3 (high ionic strength buffer). The effect of varying the meal weight: extractant volume ratio was also tested (see Methods II B.1).

(1) Preliminary Analysis of Extracts:

Results from preliminary analyses of the extracts indicated that the low ionic strength buffer, borate (0.05M), was more efficient at extracting protein than water, at all meal weight: extractant volume ratios tested. The final pH values of the former extracts were above 7.5, whereas the extracts prepared with water had final pH values which ranged between 5.9 and 6.4. The high ionic strength buffer phosphate (1M) was considerably less efficient as an extractant, although the final pH value of these extracts remained above 8.0.

The efficiency with which borate buffer extracted protein was greatest using meal weight: buffer volume ratios of 1:10 and 1:20, compared to ratios of 1:50 and 1:100. In contrast, the efficiency of extraction of protein with water was greatest using meal weight: volume ratios of 1:50 and 1:100. These extracts had final pH values which were greater (6.2 and 6.4 respectively) than those prepared at ratios of 1:10 and 1:20 (final pH values 5.9).

All extracts were brown in colour.

(2) Protein and Nitrogen Contents of Extracts:

The protein and nitrogen contents of extracts are given in table 14. The low ionic strength buffer, borate (0.05M pH 8.3) proved to be the most efficient extractant of protein and nitrogen from yam meal, particularly when extraction was carried out at a meal weight: volume ratio of 1:20. Under these conditions approximately 89% and 82% respectively of the protein and nitrogen contents of the meal were extracted, the protein content in the meal having been estimated from direct amino acid analysis after ethanolic extraction of the meal (qv I.C2).

Approximately 78% and 73% respectively of the protein and nitrogen contents of the meal were extracted with water at a meal weight: volume ratio of 1:50. This degree of efficiency using water could not be improved by any of the other meal weight: water volume ratios tested, and was comparable to extraction using the low ionic strength borate buffer at a meal weight: volume ratio of 1:10 where approximately 82% and 71% of the protein and nitrogen contents of the meal were extracted.

The superior efficiency of extraction of protein using the low ionic strength borate buffer at the meal weight: buffer volume ratio of 1:20 was maintained when the duration of extraction was reduced from 15h to 2h.

B. Examination of the Effect of Adding Polyvinylpyrrolidone, 2-Mercaptoethanol, and Cellulose to Extraction Media:

The combinations of 5% $^{w}/v$ polyvinylpyrrolidone (PVP) and 10mM 2-mercaptoethanol (2-ME), and of cellulose and 2-ME were added to extraction media to determine whether any of these reagents were effective in reducing the brown coloration of extracts. However, no difference in colour between "treated"

and "untreated" extracts was observed, and their absorption spectra in the ultraviolet region (230-325 nm) were also similar.

The results of electropherograms showed that addition of 5% $^{w}/_{v}$ PVP and 10mM 2-ME to extraction media did not change the protein profile either quantitatively or qualitatively when extracts were electrophoresed under both dissociating and apparent non-dissociating conditions. Furthermore, the weights of lyophilised extracts prepared from treated extraction media were not significantly different to the weights of extracts from untreated extraction media ($F = 0.154$, for which $p > 20\%$).

Table 14 Protein and Nitrogen Contents in Extracts Prepared from Yam Meal

Extractant	0.05M Borate Buffer pH 8.3			Deionised Water pH 8.3		1M Phosphate Buffer pH 8.3		
duration of extraction	15h[b]		2h[c]	15h[b]		15h[b]		
weight of meal(g): extractant volume (ml)	1:10	1:20	1:20	1:50	1:100	1:20	1:50	1:100
% nitrogen in extract [a, d]	0.461 ± 0.01	0.531 ± 0.01	ND	0.472 ± 0.01	0.415 ± 0.02	0.283 ± 0.01	0.317 ± 0.01	0.287 ± 0.036
% nitrogen in residue after extraction [a]	0.255 ± 0.002	0.132 ± 0.01	ND	0.137 ± 0.002	0.224 ± 0.002	0.438 ± 0.01	0.343 ± 0.01	0.361 ± 0.05
% nitrogen extracted of total	71	82		73	64	44	49	44
% recovery of total N in meal	110	103		94	99	111	102	100
% protein in extract [a, e]	2.402 ± 0.04	2.636 ± 0.01	4.474 ± 0.15	2.287 ± 0.02	2.032 ± 0.20	ND	ND	ND
% protein extracted of total [f]	82	89	88	78	69			

a dry weight basis prior to extraction

b variety yam meal 2

c variety Nwapoko

d measurement of N by microkjeldahl technique: values include non-amino sources of nitrogen

e measurement of protein with Folin Ciocalteau reagent after TCA precipitation of protein from extract (methods IV D.1

f total protein in meal determined from amino acid analysis of ethanol-extracted meal (qv results I C.2)

Values are means of several estimates ± SD: % error in recording weight of extracts approximately 5%

III. CHARACTERISATION OF THE PROTEIN IN EXTRACTS PREPARED FROM THE MEAL, VARIETY NWAPOKO

A. Amino Acid, Nitrogen and Protein Determination:

The amino acid composition and content, nitrogen content and protein content (determined by the Lowry technique) of lyophilised extracts are given in table 15.

The results indicated that around 80% (75.4% from amino acid analysis and 79.3% using the Lowry method for protein determination) of the dry weight content of extract was attributable to protein content.

The nitrogen content determined from the amino acid composition of a 22h hydrolysate was 10.57% (dry weight basis) and represented approximately 85% of the total nitrogen content (12.51% $\pm$ 0.27% dry weight basis).

The relative amino acid composition of the extract was the same as that determined for the ethanol-extracted meal of variety Nwapoko, apart from tyrosine which was higher relatively in the extract.

B. Analytical Ultracentrifugation:

Analytical ultracentrifugation of an extract showed it to contain two components (fig. 13). The sedimentation coefficient at 19.5^{o}c of the larger component was approximately 6.63, and that of the smaller component approximately 2.93, giving calculated molecular weights of around 116000 and 30000 respectively.

C. Electrophoretic Analysis:

(1) Cellulose Acetate Membrane Electrophoresis:

The electrophoretic patterns of extracts prepared using deionised and distilled water brought to pH7, and 0.1M sodium phosphate buffer pH7, each consisted of one band only although

Table 15 Amino Acid, Nitrogen and Protein Analyses of Extracted Protein from Yam Meal Nwapoko[a]

Amino Acids	g aa/16g N[b]
aspartic acid	13.46
threonine	3.36
serine	4.66
glutamic acid	15.23
proline[c]	4.56
glycine	3.79
alanine	4.53
valine	5.11
methionine	1.74
isoleucine	4.17
leucine	7.91
tyrosine	3.76
phenylalanine	5.95
histidine	2.33
lysine	5.88
arginine	9.16
cysteic acid	0.92
amino acid content (g/100g extract)	75.44
amino acid nitrogen content (g aaN/100g extract)	10.57
total nitrogen content[d] (g/100g extract)	12.51 ± 0.27 (7)
protein content (g/100g extract)[e]	79.3 ± 5.4 (5)
weight (g/100g meal)[f]	5.524 ± 0.27 (6)

a dry weight basis

b values from a 22h hydrolysate: tryptophan and ammonia content not determined

c poorly resolved in analysis

d determined by the microkjeldahl technique: values are a mean of seven estimates for 3 extracts ± SD

e determined by the Lowry technique: values are a mean of five estimates ± SD

f values are a mean for six extract preparations

Direction of sedimentation

Fig. 13

Ultracentrifuge pattern of a lyophilised extract preparation dissolved in 0.01M phosphate buffer pH 7.2 containing 0.2M NaCl. Photograph taken 56 minutes after the centrifuge had reached a speed of 51,000 revs/min. Bar angle 77^{O}. Direction of sedimentation, left to right.

some protein remained near the origin for both extracts prepared in water, the final pH value of which was 5.8. Migration of the band was towards the anode.

(2) Polyacrylamide Gel Electrophoresis under Apparent Non-dissociating Conditions:

(i) Alkaline Buffer System:

The electrophoretic pattern (fig. 14) consisted of three approximately equally stained broad bands together with a fourth which migrated less far. The intensity of stain taken up by this latter band varied as the concentration of protein electrophoresed was altered, becoming more intensively stained with increasing protein concentration. It was also more intensively stained after lyophilization of the extract. There were also some barely detectable bands which migrated further than the above mentioned bands (illustrated more clearly in track 9 of the same figure). The electrophoretic pattern tended to be somewhat smeared.

(ii) Acidic Buffer System:

Under acidic conditions, there was one broad band together with a faintly stained band which migrated less far. The proteins in this system migrated further relative to the tracking dye than in the alkaline system (fig. 15).

(3) Polyacrylamide Gel Electrophoresis under Dissociating Conditions:

(i) Treatment of Sample with SDS and 2-ME:

The electrophoretic patterns of extracts varied depending on the conditions under which the samples were incubated in SDS and 2-ME prior to electrophoresis (fig. 16). After incubation for ten minutes at 105°C (track 5) a complex pattern was obtained which, however, consisted principally of one broad band with a sharp leading edge, Rm value 0.52, and diffuse trailing edge, Rm 0.48. The protein represented by this band is henceforth referred

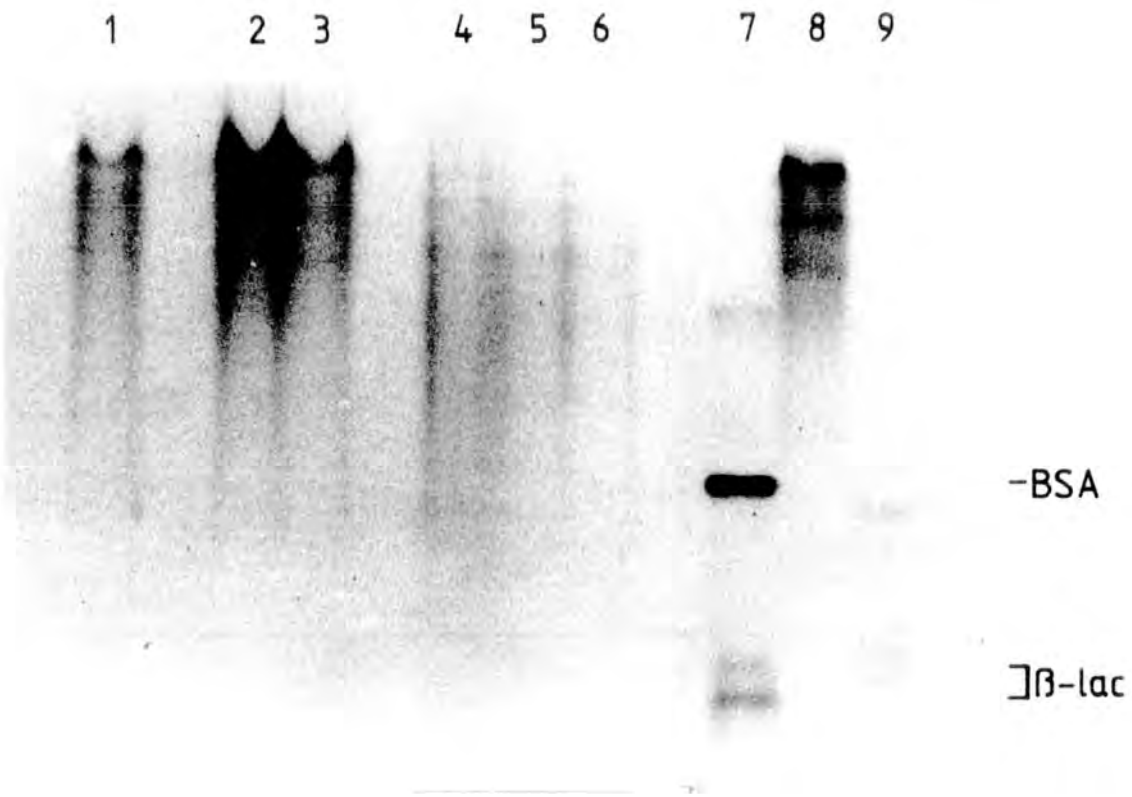


Fig. 14

Electropherogram obtained after non-dissociating PAGE (7.7 x 2.6), alkaline buffer system A.

Track 1:	lyophilised extract incubated for two minutes at 100°c in 0.06M Tris HCl pH 6.7 containing 5% v/v 2-ME.
Tracks 2 and 3:	lyophilised extract, 80 and 40 µg (d.b.) respectively.
Tracks 4, 5 and 6:	unlyophilised extract, 100, 50 and 25 µl respectively.
Track 7:	bovine serum albumin and ß-lactoglobulin.
Track 8:	lyophilised storage protein preparation.
Track 9:	lyophilised preparation of fraction D2 obtained from an extract by anion exchange chromatography (see fig. 22(b)).

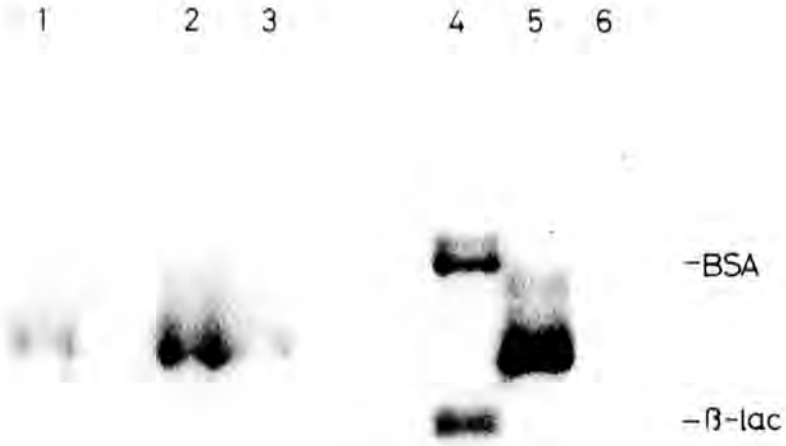


Fig. 15

Electropherogram obtained after non-dissociating PAGE (7.7 x 2.6), acidic buffer system A.

Track 1: lyophilised extract incubated for two minutes at 100°c in 0.06M acetic acid/KOH pH 6.7 containing 5% v/v 2-ME

Tracks 2 and 3: lyophilised extract, 80 and 40 µg (d.b.) respectively

Track 4: bovine serum albumin and ß-lactoglobulin.

Track 5: lyophilised storage protein preparation.

(Track 6 contained a lyophilised preparation of fraction D2 obtained from an extract by anion exchange chromatography (see fig. 22(b)) but these proteins cannot be detected in the acidic buffer system; they most probably migrated toward the anode).

Fig. 16

Electrophoretic patterns obtained for an extract preparation after PAGE (17.6 x 0.45) using the discontinuous SDS buffer system. The conditions under which the samples were incubated in sample buffer which contained either 2% $^{w}/_{v}$ SDS, 5% $^{v}/_{v}$ 2-ME, or 2% $^{w}/_{v}$ SDS alone were varied.

Tracks 1 and 2: incubation (SDS + 2-ME) of a lyophilised extract for two minutes at 100°c, 50 and 20 µg (d.b.) respectively. (See also fig. 21 track 1).

Track 3: incubation (SDS + 2-ME) of a lyophilised extract for two hours at room temperature, 50 µg (d.b.). (See also fig. 60 track 1).

Track 4: unlyophilised extract of yam meal prepared using 0.0625M Tris HCl pH 6.7, 2% $^{w}/_{v}$ SDS, 5% $^{v}/_{v}$ 2-ME. Meal extracted for two hours at room temperature, meal weight: volume ratio, 1:8 (see also fig. 60 track 2).

Track 5: incubation (SDS + 2-ME) of a lyophilised extract for ten minutes at 105°c, 50 µg (d.b.).

Track 6: in order of increasing mobility, bovine serum albumin (BSA), ovalbumin (ov), chymotrypsinogen (chy) and myoglobin (myo).

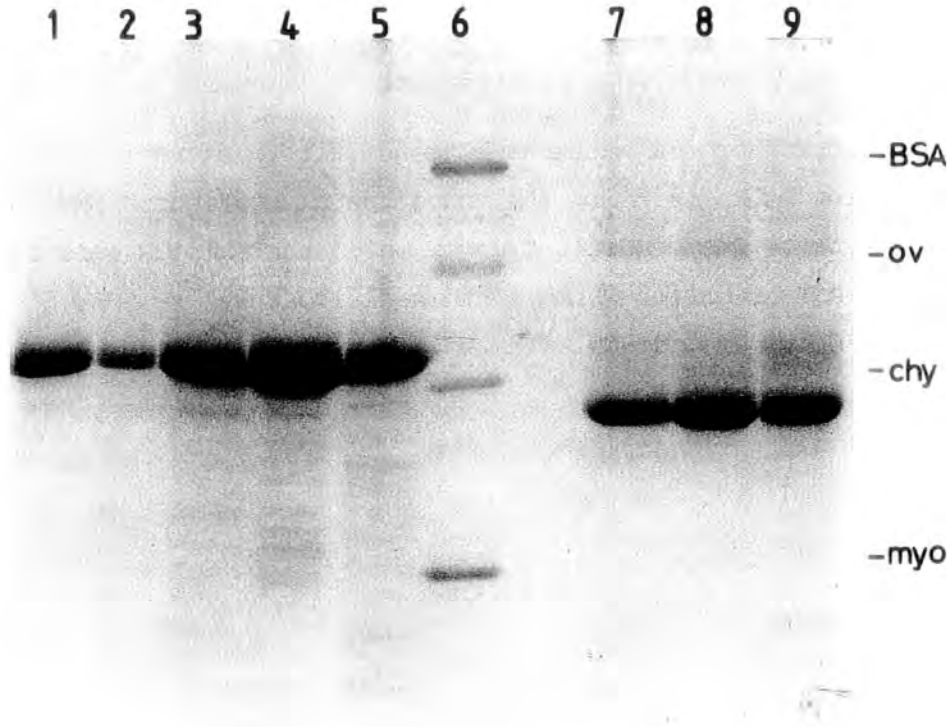
Track 7: incubation (SDS alone) of a lyophilised extract for two minutes at 100°c, 50 µg (d.b.).

Track 8: incubation (SDS alone), of a lyophilised extract for two hours at room temperature, 50 µg (d.b.).

Track 9: incubation (SDS alone), of a lyophilised extract for ten minutes at 105°c, 50 µg (d.b.).

The line diagram illustrates all the bands detected in the corresponding profiles, since many of the bands are only faintly visualised in the photograph. The molecular weight values indicated in this and subsequent electropherograms are those determined from a standard curve such as that illustrated in fig. 6.

The relative mobilities of the standard proteins illustrated in track 6 i.e. bovine serum albumin, ovalbumin, chymotrypsinogen and myoglobin are indicated. C denotes those bands which stained positively with the periodic acid-Schiff staining technique.


1
2
3
4
5
6
7
8
9
-BSA
-ov
-chy
-myo

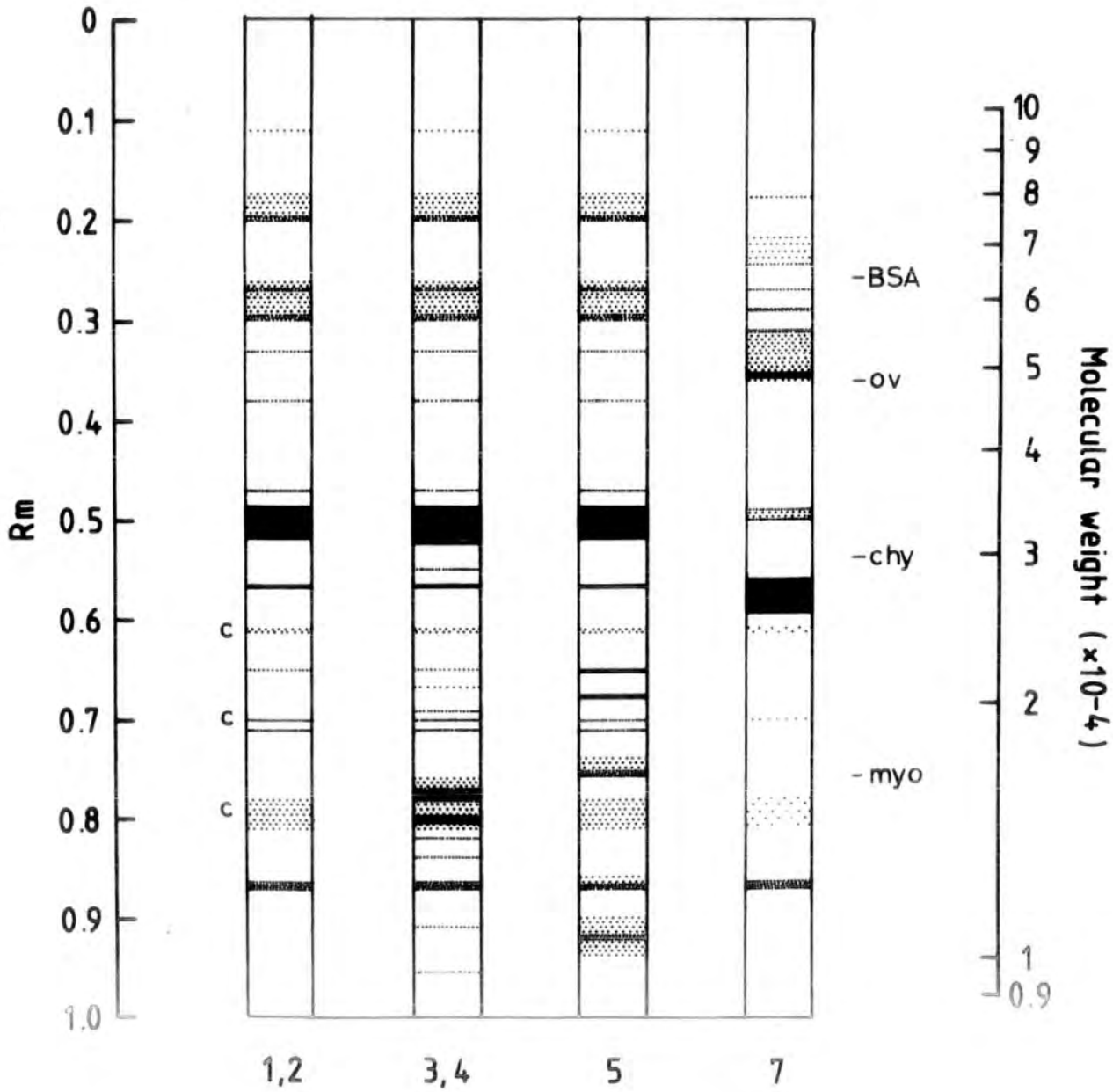

Rm
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
c
c
c
-BSA
-ov
-chy
-myo
Molecular weight (×10-4)
10
9
8
7
6
5
4
3
2
1
0.9
1,2
3,4
5
7

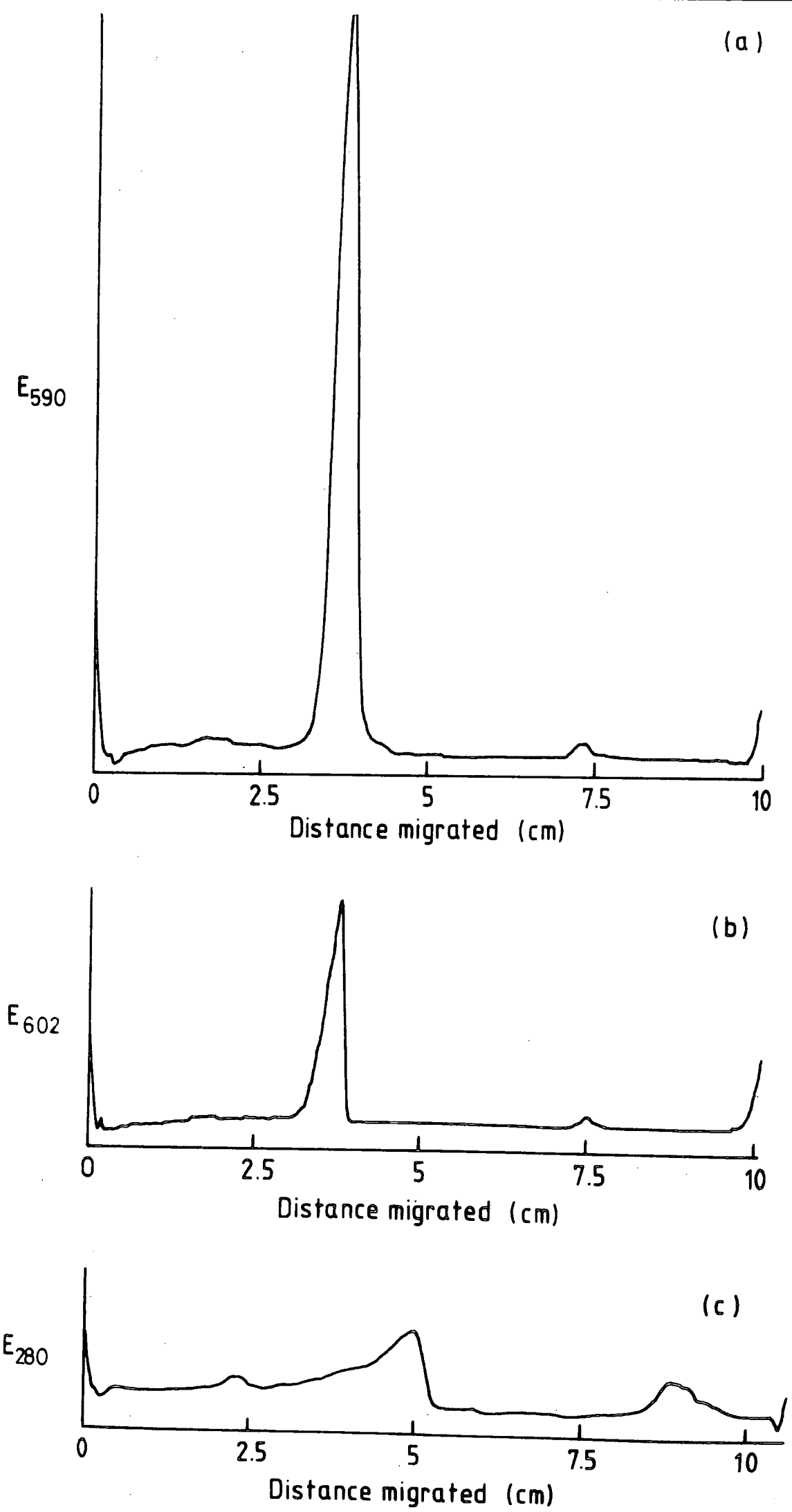
Fig. 17

Densitometric profiles of a lyophilised extract preparation electrophoresed in the discontinuous SDS buffer system on polyacrylamide gels (17.6 x 0.45), after incubation for two minutes at 100°c in 2% $^{w}/_{v}$ SDS, 5% $^{v}/_{v}$ 2-ME.

(a) 75 µg (d.b.) stained with Coomassie Blue R250

(b) 75 µg (d.b.) stained with Procion Brilliant Blue

(c) 250 µg (d.b.) precipitated with TCA


(a)
E_{590}
0
2.5
5
7.5
10
Distance migrated (cm)
(b)
E_{602}
0
2.5
5
7.5
10
Distance migrated (cm)
(c)
E_{280}
0
2.5
5
7.5
10
Distance migrated (cm)

to as the major sub-unit. There were also a large number of faintly stained bands representing smaller sub-units, together with equally faintly stained bands representing heavier sub-units.

A different, but equally complex electrophoretic pattern was obtained when the extracts were incubated for two hours at room temperature (track 3). This pattern was the same as that obtained after extraction of protein from the meal using buffer which contained 2% $^{w}/_{v}$ SDS and 5% $^{v}/_{v}$ 2-ME (track 4; see also fig. 60 tracks 1 and 2).

An even simpler electrophoretic pattern was obtained when the samples were incubated for two minutes in a boiling bath (tracks 1 and 2). This consisted principally of the one broad band representing the major sub-unit, but the number of bands which represented smaller sub-units was markedly reduced. These smaller sub-units gave bands with Rm values 0.56, 0.61, 0.65, 0.70, 0.71, 0.78-0.81 and 0.87, whilst the larger sub-units were represented by bands with Rm values 0.27-0.30, 0.18-0.20 and barely detectable bands with Rm values 0.44, 0.38 and 0.33.

A quantitative evaluation of the sub-units was carried out by direct densitometry of electrophoresis patterns obtained from an extract preparation which was incubated for two minutes in a boiling bath in the presence of SDS (2% $^{w}/_{v}$) and 2-ME (5% $^{v}/_{v}$) and then serially diluted. Using the stains Coomassie Blue and Procion Brilliant Blue, the major sub-unit represented 85 $\pm$ 1% and 81.5 $\pm$ 2% respectively of the extracted protein. The smaller sub-units represented approximately 5% (5.6 $\pm$ 0.6% and 5.0 $\pm$ 0% for the two stains respectively)of the protein, whilst the larger sub-units were determined to represent 9.3 $\pm$ 0.6% of the protein with Coomassie Blue, but 13.5 $\pm$ 2% of the protein with Procion

Brilliant Blue. Densitometric scans of the protein profiles stained with these stains as well as with TCA are presented in fig. 17.

(ii) Treatment of Sample with SDS alone:

When 2-ME was omitted from the sample incubation medium, the electrophoretic pattern obtained (fig. 16, tracks 7, 8 and 9) consisted principally of one major band which, however, migrated further than that of the major sub-unit previously identified; the Rm value of the leading edge was 0.59, that of the trailing edge, 0.56. There were also two closely migrating, lesser stained bands which migrated to approximately the same position as the major sub-unit, and had Rm values of 0.49 and 0.50, as well as a series of bands above these whose intensity of stain decreased from the smallest to the heaviest. Smaller sub-units were not readily visible apart from one with an Rm value of 0.87.

Densitometric evaluation of 37.5 µg of the extracted protein, electrophoresed and then stained with Coomassie Blue, indicated that the major band represented at least 60% of the protein, the smaller (faster migrating) about 10%, and the larger about 30%. The two bands with Rm values 0.49 and 0.50 together represented about 14% of the protein. However, densitometric analysis of Coomassie Blue uptake by protein in a concentration of more than 15 µg shows a marked deviation from Beers Law, so that the value given for the % protein represented by the major band is probably underestimated, whilst those values for the other bands, overestimated.

(4) Isoelectric Focussing:

The isoelectric focussing profile consisted of a number of

Fig. 18

Isoelectric focussing profiles of an extract (a), and the purified storage protein preparation (b).

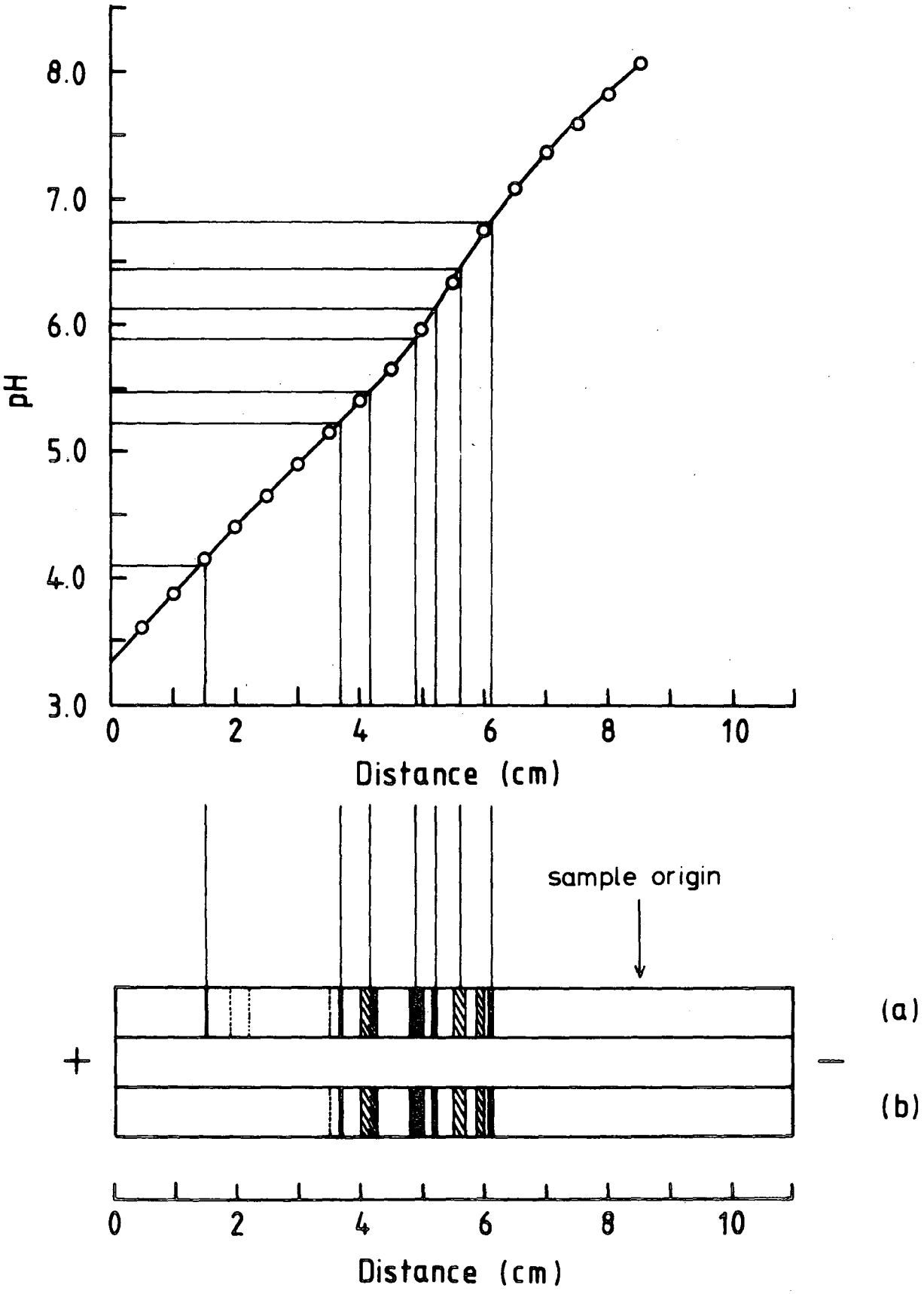
Samples focussed for one hour at 10 watts, three hours for 15 watts on slabs of PAG (7.7 x 3) prepared with LKB ampholines pH 3.5-10 (with 12.5% sucrose incorporated into the matrix).

Temperature of focussing : $4^{o}c$.

Cathode buffer : 1M ethylene diamine.

Anode buffer : 0.1M sulphuric acid.

Values for isoelectric point are indicated in the calibration curve which relates distance along the gel with pH value.


8.0
7.0
6.0
5.0
4.0
3.0
pH
0
2
4
6
8
10
Distance (cm)
sample origin
+
−
(a)
(b)
0
2
4
6
8
10
Distance (cm)

bands (fig. 18). Seven of these, and four in particular were more intensively stained. The pI values of the bands ranged approximately between 5.1 and 6.8, apart from one of the more intensively stained bands which had a pI value of about 4.1.

D. Carbohydrate Analysis:

The protein sub-units with Rm values of 0.61, 0.70 and 0.78 (see fig. 16) stained positively after Periodic acid-Schiff's staining treatment, and were thus considered to contain bound carbohydrate moieties.

E. N-Terminal Amino Acid Analysis:

(1) Apparent Non-Dissociating Conditions:

The N-Terminal amino acids were identified as glutamic acid/glutamine, and aspartic acid/asparagine, the latter as a spot of very much lower intensity.

(2) Dissociating Conditions:

The N-Terminal amino acids of the protein sub-units were identified principally as glutamic acid/glutamine, together with aspartic acid/asparagine, alanine and glycine; these latter three appeared as spots of lower intensity than the former.

F. Saponin Analysis:

The extract, after extensive dialysis against water, gave negative results when assayed for haemolytic action on red blood cells and was, therefore, considered to contain no saponins.

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IV. FRACTIONATION OF EXTRACTED PROTEINS AND CHARACTERISATION OF THE FRACTIONS

A. Fractionation by Molecular Sieve Chromatography:

(1) Fractionation:

The elution profile following molecular sieve chromatography is given in fig. 19.

Increasing the column dimensions did not alter the profile significantly, but an improved resolution could be attained with the use of the superfine grade of Sephadex: however, this applied to the first peak and shoulder only. The broad peak following appeared as a long plateau with shoulders.

Fractions were collected as indicated on the profile.

(2) Polyacrylamide Gel Electrophoresis:

PAGE was carried out on a quantitative weight basis and showed that fractions 3, 4, 5 and 6 contained relatively little protein.

Under both dissociating (fig. 20) and apparent non-dissociating conditions (alkaline and acid systems) the protein profiles were essentially the same as that of the extract (qv III C.2 and III C.3).

However, the incubation treatment imposed (10 minutes at 105°c in the presence of SDS and 2-ME) appeared to lead to the formation of sub-units smaller than the major sub-unit; these bands were not as readily visible in the extract (tracks 2-10 cf track 1). The smaller sub-units appeared in increasing amounts through the fractions.

Subsequent electrophoresis of fraction 2a incubated for only two minutes (track 11) gave essentially the same profile as the extract. Carbohydrate analysis showed that the bands with Rm values 0.61, 0.70 and 0.78-0.80 stained positively with the PAS

Fig. 19

Elution profile obtained after molecular sieve chromatography of a lyophilised extract preparation (19 mg. d.b.) on a column of Sephadex G100 (84 x 1.5 cm), equilibriated with 0.05M sodium borate buffer pH 8.3.

Flow rate 10.5 ml/h.

2 ml fractions collected.

Fig. 20

Electropherogram of fractions indicated in fig. 19 which were electrophoresed in the discontinuous SDS buffer system on PAG (17.6 x 0.45).

Samples in tracks 1-10 were incubated for 10 minutes at 105°c in the presence of 2% $^{w}/_{v}$ SDS, 5% $^{v}/_{v}$ 2-ME, prior to electrophoresis.

The sample in track 11 indicates the pattern obtained for fraction 2a after incubation for two minutes at 100°c in the presence of 2% $^{w}/_{v}$ SDS, 5% $^{v}/_{v}$ 2-ME.

ex = extract

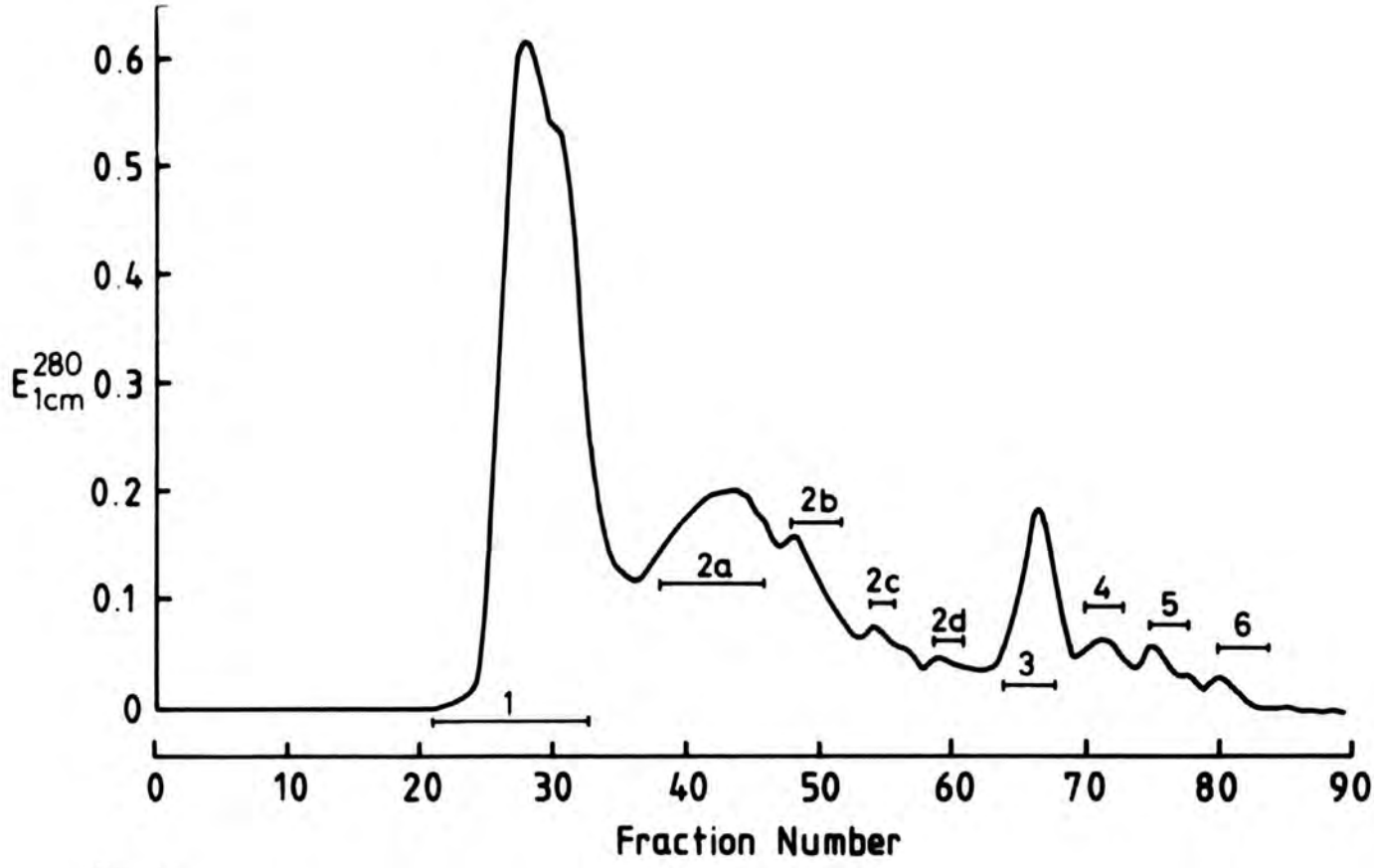

0.6
0.5
0.4
E^{280}_{1cm} 0.3
0.2
0.1
0
1
2a
2b
2c
2d
3
4
5
6
0
10
20
30
40
50
60
70
80
90
Fraction Number

Fig.19

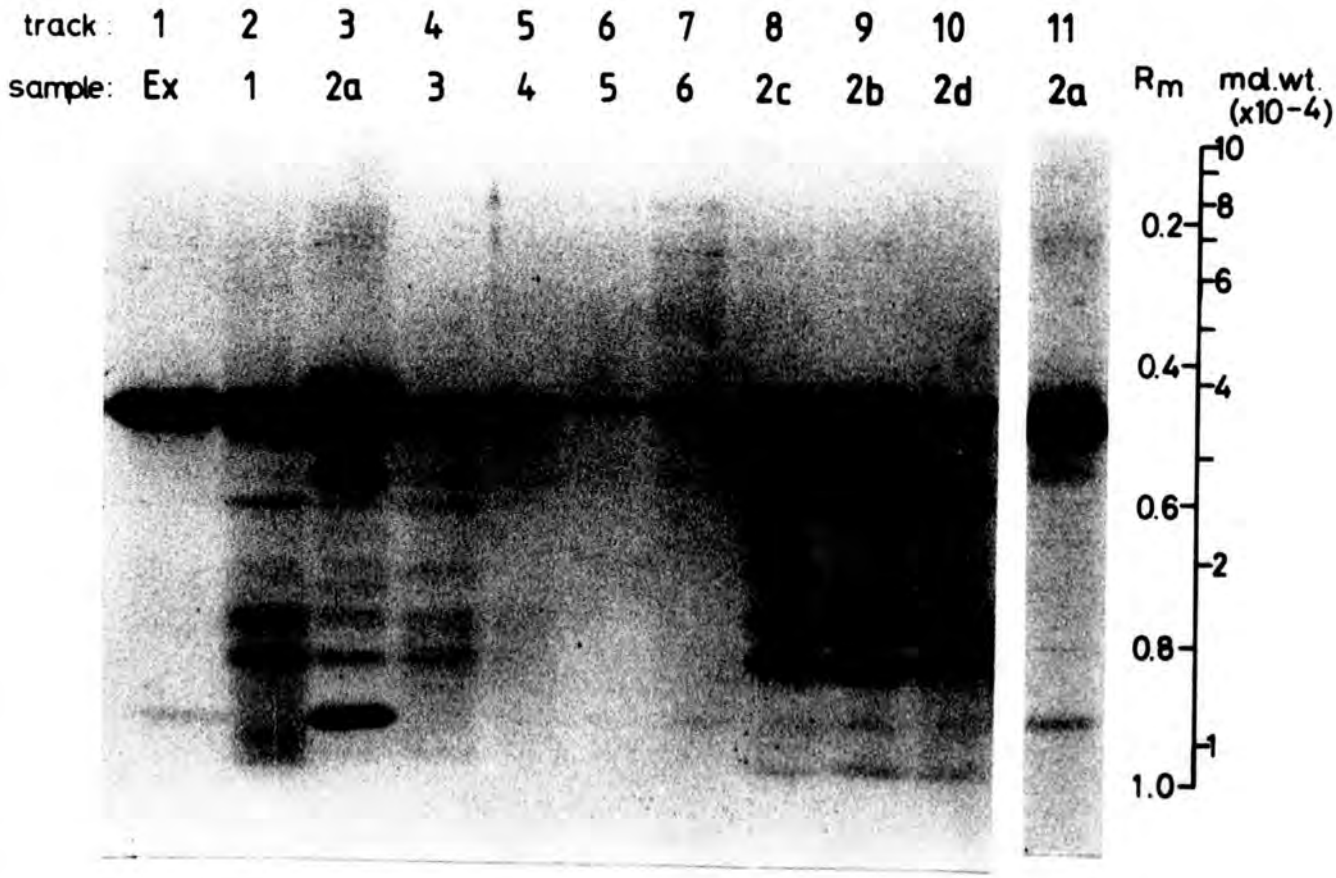

track: 1 2 3 4 5 6 7 8 9 10 11
sample: Ex 1 2a 3 4 5 6 2c 2b 2d 2a
Rm
mol.wt. (x10-4)
10
8
0.2
6
0.4
4
0.6
2
0.8
1
1.0

Fig.20

technique, as was also found for the extract.

B. Fractionation by Ammonium Sulphate Precipitation:

An electropherogram of the precipitates which formed between the limits 10-20%, 20-30%, 50-60%, 60-70%, 70-80% and 90-100% of ammonium sulphate saturation showed that each protein fraction gave the same sub-unit profile as that of the extract.

C. Fractionation by Ammonium Sulphate Precipitation followed by Dialysis against Acetate Buffer pH 5:

The % protein and the protein content of the fractions obtained (Methods III.D) are given in table 16, and an electropherogram of the sub-units in the fractions, prepared on a semiquantitative protein basis, is given in fig. 21.

The protein contents of the fractions obtained by precipitation between 0-50% and 50-100% limits of ammonium sulphate saturation represented approximately 32% and 67% respectively of the protein in the extract.

Further fractionation by precipitation in acetate buffer indicated that 78% of the total protein was insoluble in this buffer. The greater part of this fraction precipitated between the 50-100% limits of ammonium sulphate saturation.

Electrophoretic analysis of the fractions indicated that the major sub-unit was the most predominant sub-unit in each fraction. The acetate-soluble and -insoluble sub-unit profiles, however, were different to one another. Smaller sub-units with Rm values 0.61, 0.70, 0.78 and 0.87, together with larger sub-units with Rm values 0.38 and 0.33 were present only in the former (tracks 2 and 4 cf tracks 3, 5 and 7). The acetate-soluble fractions were also largely devoid of brown pigments, in contrast

Table 16 Protein Content of Fractions Obtained from an Extract by Ammonium Sulphate Precipitation followed by Dialysis against Acetate Buffer pH 5.0

	Total [a] Extract	Fractions Obtained from Extract				
level of ammonium sulphate saturation		0%-50%		50%-100%		100% saturated solution
nature of protein in acetate buffer		solution	precipitate	solution	precipitate	precipitate [b]
dry weight content (g)	0.2760	0.0318	0.0357	0.0131	0.1150	0.0174
% protein (dry weight basis)[c]	86.6 ± 5.9	96.7 ± 7.4	77.5 ± 6.4	65.3 ± 7.3	98.3 ± 6.0	12.6 ± 1.1
total protein content (mg)	239.0	30.7	27.7	8.6	113.1	2.2
protein content in fractions as % of total		17	15	5	62	1
colour of fractions	brown	white	brown	white	brown	

a volume of total extract = 124.05 ml. Data for protein content derived from 10 ml aliquot (control). The recovery of the protein and dry weight content of the extract was 93% and 94% respectively.

b precipitate and solution in diffusate combined

c determined with Folin Ciocalteau reagent using bovine serum albumin as standard

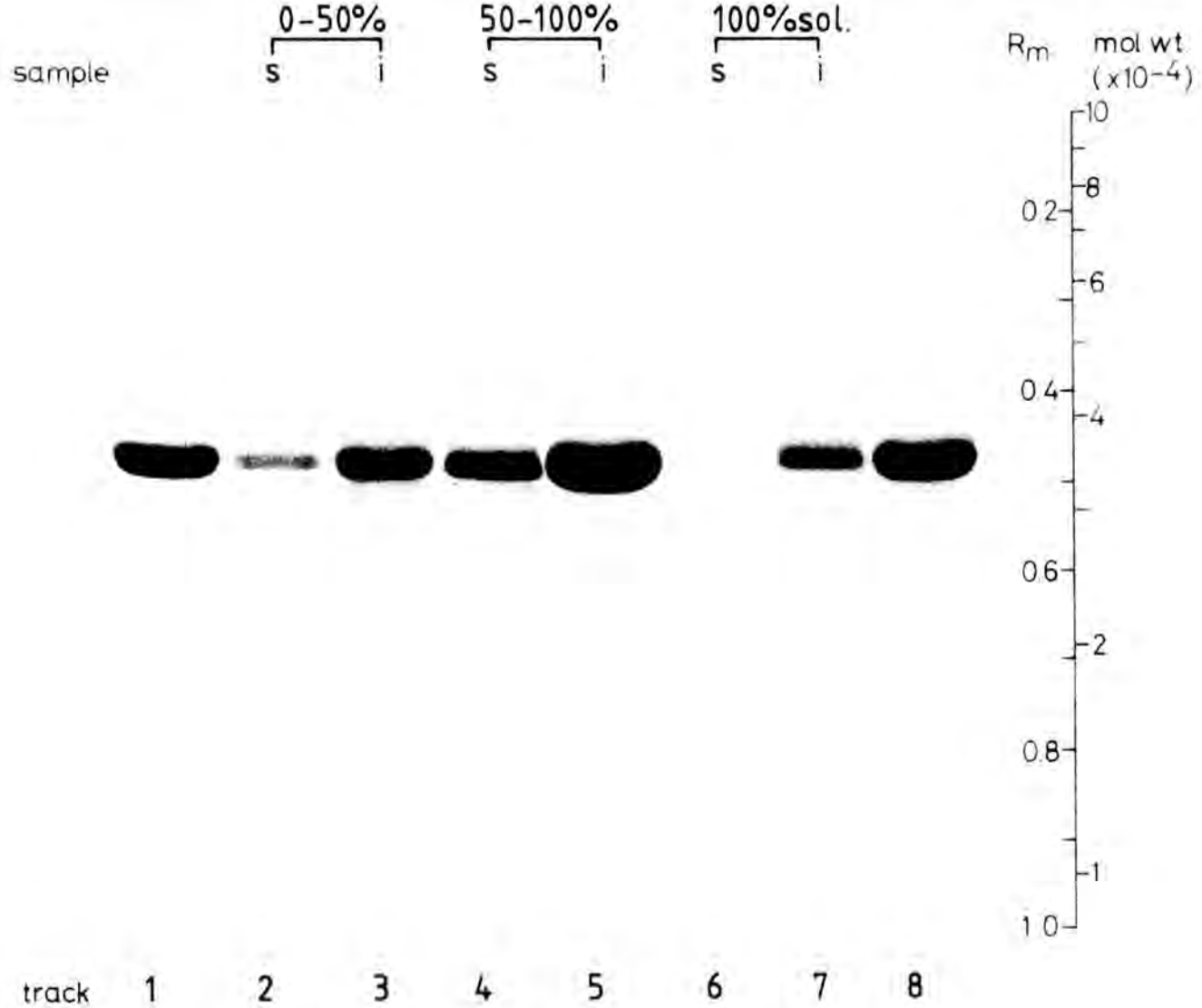


Fig. 21

Electropherogram (17.6 x 0.45) of different fractions obtained after amonium sulphate precipitation followed by dialysis against acetate buffer, pH 5.0. The fractions, after dialysis against water, were lyophilised then incubated for two minutes at 100°c in the presence of 2% $^{w}/_{v}$ SDS, 5% $^{v}/_{v}$ 2-ME before electrophoresis in the discontinuous SDS buffer system.

Track 1: total extract.

Track 2: acetate-soluble fraction)
) 0-50% cut of ammonium sulphate saturation
Track 3: acetate-insoluble fraction)

Track 4: acetate-soluble fraction)
) 50-100% cut of ammonium sulphate saturation
Track 5: acetate-insoluble fraction)

Track 6: acetate-soluble fraction)
) 100% ammonium sulphate saturated solution
Track 7: acetate-insoluble fraction)

Track 8: Borate-soluble derivative from acetate-insoluble fraction of 50-100% cut.

s = acetate soluble i = acetate insoluble

to the acetate-insoluble fractions.

The acetate-insoluble fraction from the 50-100% cut (62% of the total protein), after dialysis against water and subsequent lyophilisation, could not be fully solubilised in borate buffers which contained varying amounts (0.05M to 1.0M) of sodium chloride. The brown insoluble derivative which represented only a small fraction of the total protein, however, gave the same sub-unit profile as the soluble derivative except that the minor band Rm 0.44 was absent in the former profile.

The borate-soluble derivative gave essentially the same protein profile as the extract, both after non-dissociating PAGE, and isoelectric focussing except that in the former electropherogram, the fastest migrating faintly stained bands were absent, whilst in the latter the component responsible for the band with pI value 4.1 could not be detected.

N-Terminal analysis of the soluble derivative under apparent non-dissociating conditions revealed glutamine/glutamic acid, a lesser amount of asparagine/aspartic acid and strong traces of glycine and threonine. Under dissociating conditions, the same N-terminal amino acids were identified: glycine, however, stained more intensely than asparagine/aspartic acid, and both alanine and serine were also identified.

D. Fractionation by Ion Exchange Chromatography:

(1) Fractionation:

Elution profiles following anion exchange chromatography are given in fig. 22, and the protein content of the fractions indicated in fig. 22b, in table 17.

Fig. 22

Elution profiles obtained after anion exchange chromatography of extract preparations on a column of DE 52 (12.5 x 2.5 cm), equilibriated with 0.05M Tris HCl pH 8.3.

Flow rate 8 ml/h/cm^2.

10 ml fractions collected.

(a) Extract prepared in 140 ml 0.05M Tris HCl buffer, meal weight: volume ratio 1:20. Elution of adsorbed proteins effected with a linear salt gradient.

The gradient indicated is that measured by refractometry and reference to a calibration curve (see methods III.E).

(b) Extract prepared in 450 ml 0.05M Tris HCl buffer, meal weight: volume ratio 1:18. Elution of adsorbed proteins effected with firstly a salt step of 0.15M NaCl, then a linear salt gradient, and finally a step of 1.0M NaCl.

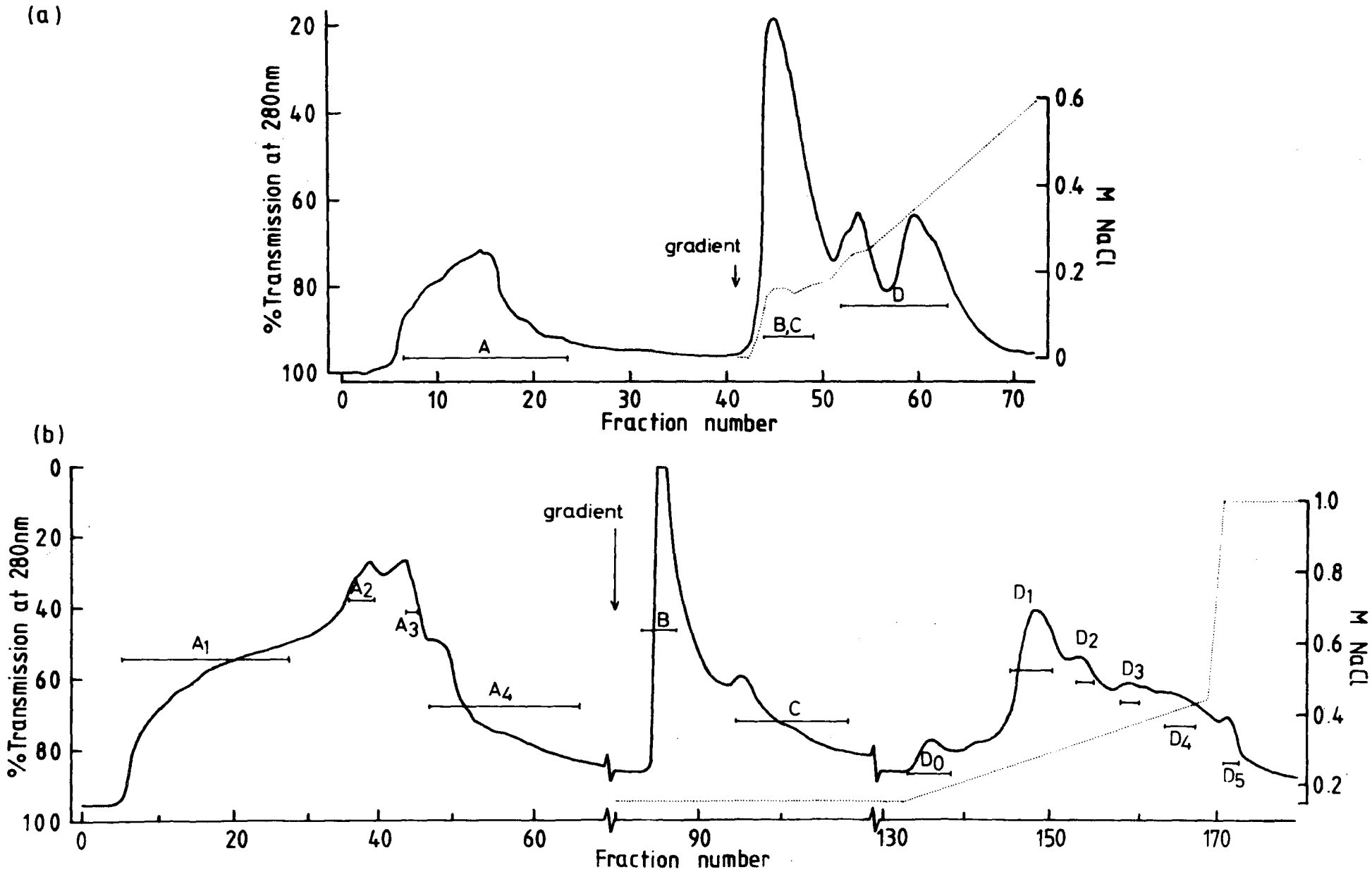

(a)
% Transmission at 280nm
20
40
60
80
100
0
10
20
30
40
50
60
70
Fraction number
gradient
A
B,C
D
0.6
0.4
0.2
0
M NaCl
(b)
% Transmission at 280nm
0
20
40
60
80
100
0
20
40
60
90
130
150
170
Fraction number
gradient
A1
A2
A3
A4
B
C
D0
D1
D2
D3
D4
D5
1.0
0.8
0.6
0.4
0.2
M NaCl

Table 17 Protein Content and Principal N-Terminal Amino Acids of Fractions obtained following Ion Exchange Chromatography of an Extract

Fractions[a]	Label	Total[b] Protein Content (mg)	Colour[c] of Lyophilisate	Principal N-Terminal Amino Acid	Number of N-Terminal Amino Acids Identified
6-28	A_1	16.4 ± 0.6	White	gly	4
37-40	A_2	33.9 ± 0.3	White +	gly	6
45	A_3	6.7 ± 0.1	White ++	gly	9
48-66	A_4	23.4 ± 0.5	White +++	glx	2
fractions 1-80 remaining	A_5	48.7 ± 8.2			
84-87	B	294.0 ± 8.8	White +	glx	1
95-106	C	12.8 ± 0.1	Brown	gly	7
134-138	D_o	0.9 ± 0.02	Grey	gly	7
146-150	D_1	1.5 ± 0.05	White ++	asx	6
154-155	D_2	5.2 ± 0.3	White +	gly	8
159-160	D_3	0.4 ± 0.02	White	asx	6
164-167	D_4	0.4 ± 0.01	White +	gly/asx	8
171-172	D_5	0.1 ± 0.00	White ++	gly	7
total extract		496.0 ± 14.1		glx	2

a fraction volume 9ml

b determined with Folin Ciocalteau reagent using bovine serum albumin as standard. Values are the mean ± SD (4)

c + indicates degree of brown colour

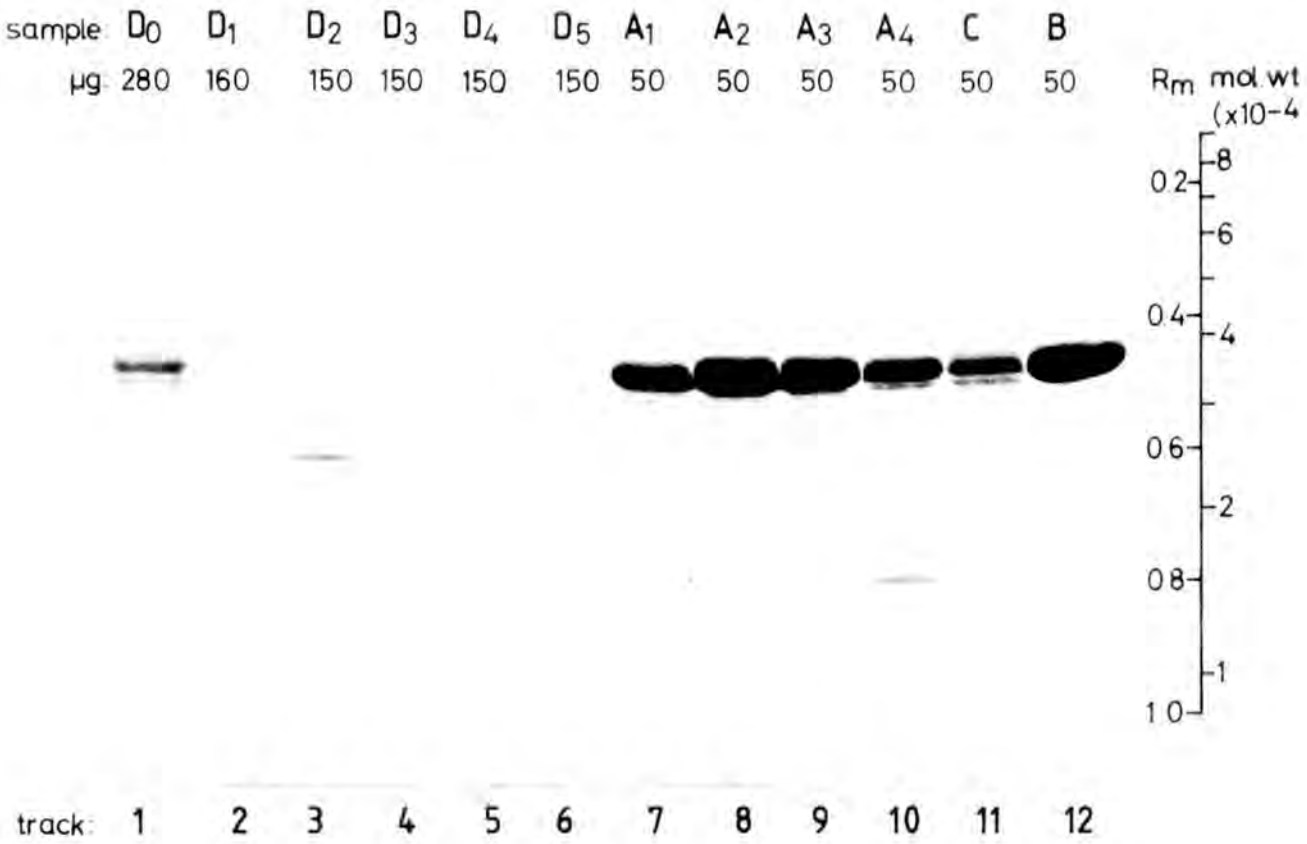


Fig. 23

Electropherogram (17.6 x 0.45) of fractions obtained by anion exchange chromatography (see fig. 22(b)).

The fractions were dialysed against water, lyophilised, then incubated for two minutes at 100°c in the presence of 2% w/v SDS, 5% v/v 2-ME before electrophoresis in the discontinuous SDS buffer system.

The numbers indicate the weight (µg) of lyophilisate electrophoresed.

Approximately 26% of the protein loaded (fractions A_1-A_5) was not absorbed to the resin. Fractions B and C, which represented 60% and 3% respectively of the total, were eluted with a salt step of molarity 0.15, whilst the remainder, 2%, (fractions D_0-D_5) were eluted with increasing salt concentration up to a molarity of 0.575. No further protein could be eluted after complete equilibriation of the column with buffer containing 1.0M salt.

The protein fractions were variously pigmented (table 17) although a significant proportion of the brown pigments remained bound to the resin.

(2) Polyacrylamide Gel Electrophoresis:

An electropherogram of the sub-unit profiles of the fractions is given in fig. 23.

Fractions A, B and C showed strong resemblance to one another, but the minor bands Rm values 0.44 and 0.38, and value 0.33 were observed only in fractions A and C respectively. In addition, fractions A_1-A_4 and C, on incubation for two minutes in SDS and 2-ME, showed a progressive tendency to form smaller sub-units the same as those observed after prolonged incubation of an extract in SDS and 2-ME at room temperature (cf fig. 16, track 3). The sub-unit profiles of fractions D strongly resembled those of the acetate soluble fractions obtained after dialysis of an extract against acetate buffer (cf fig. 21, tracks 2 and 4), but the relative stain intensity of the major sub-unit was reduced.

(3) Dialysis against Acetate Buffer pH 5.0:

The sub-unit profiles of the acetate-soluble and -insoluble fractions obtained from fractions A to D are given in fig. 24.

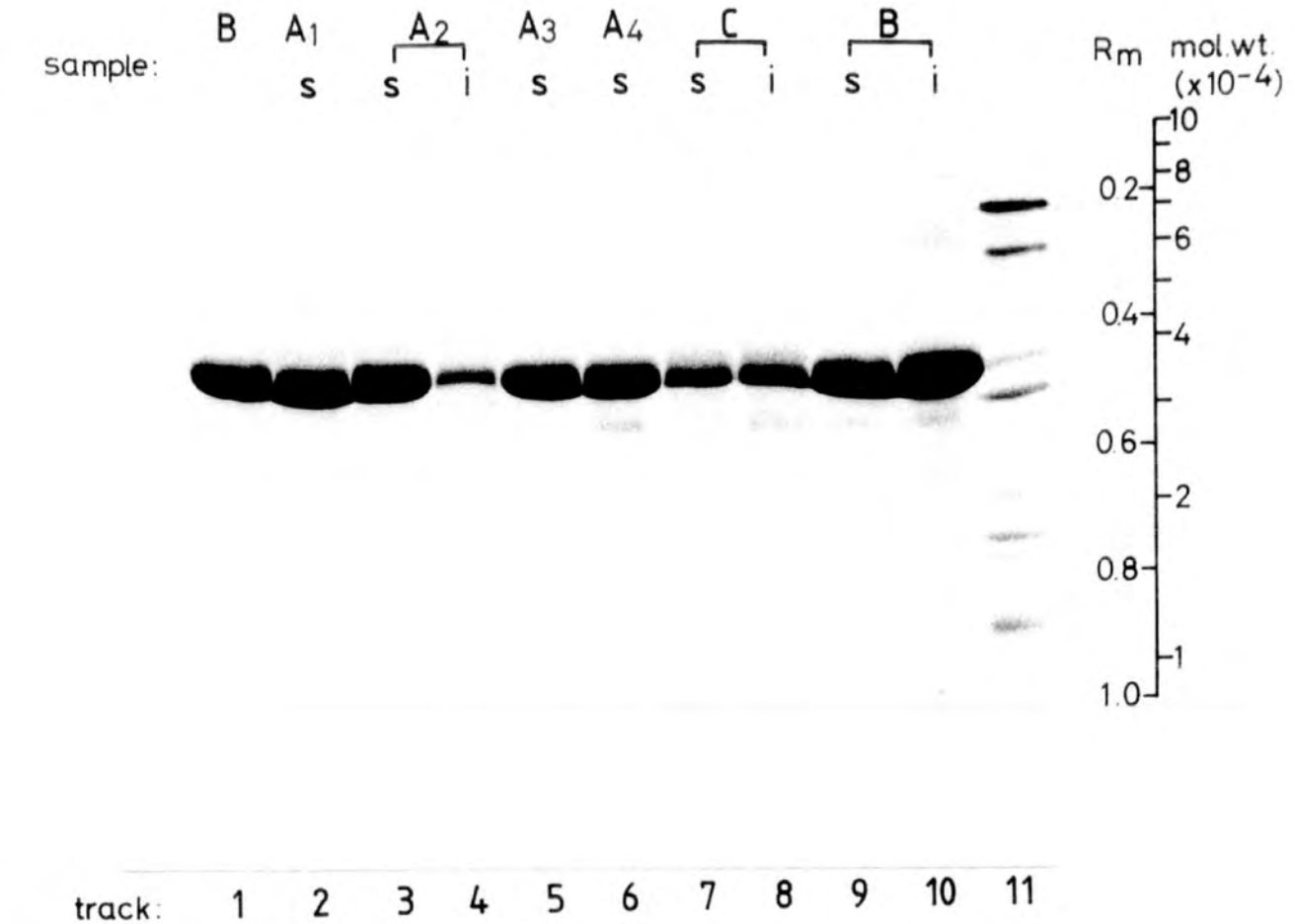
Fig. 24

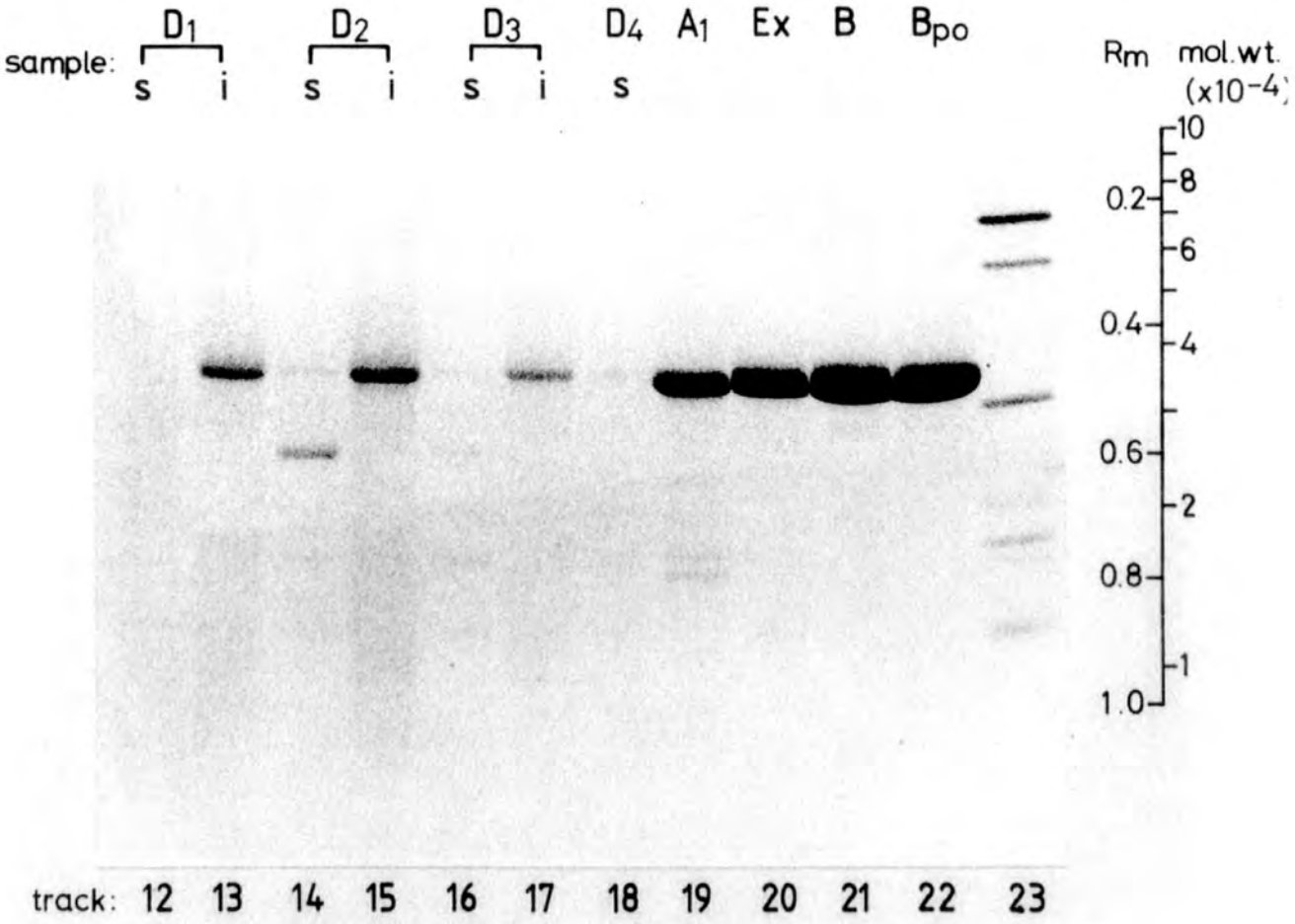
Electropherograms (17.6 x 0.45) of fractions obtained by anion exchange chromatography (see fig. 22B) which were further fractionated by dialysis against acetate buffer pH 5.0.

Electrophoretic conditions and method of sample preparation as for fig. 23.

s = acetate-soluble
i = acetate-insoluble
ex = extract
Bpo (track 22) = fraction B dialysed against 0.06M phosphate buffer pH 7.9.

Tracks 11 and 23 contain reference proteins: bovine serum albumin, ovalbumin, chymotrypsinogen and myoglobin.


sample:
B
A1
A2
A3
A4
C
B
s
s
i
s
s
s
i
s
i
track:
1
2
3
4
5
6
7
8
9
10
11
Rm
mol.wt.
(x10-4)
10
8
6
4
2
1
0.2
0.4
0.6
0.8
1.0


sample:
D1
D2
D3
D4
A1
Ex
B
Bpo
s
i
s
i
s
i
s
track:
12
13
14
15
16
17
18
19
20
21
22
23
Rm
mol.wt.
(x10-4)
10
8
6
4
2
1
0.2
0.4
0.6
0.8
1.0

Fractions A_{1-4} were primarily soluble on dialysis against acetate buffer. Fractions B and C were partially acetate-soluble, but both soluble and insoluble fractions particularly in the case of fraction B, gave the same sub-unit profiles. In fractions D_o-D_5, the major sub-unit was found in the acetate-insoluble, and the contaminating bands in the acetate-soluble fractions.

Dialysis against acetate buffer appeared to alter the sub-unit profile of fractions A and C. As demonstrated previously, the sub-unit profiles of these fractions, after incubation for two minutes in a boiling bath in SDS and 2-ME, contained a number of minor bands, but after dialysis against acetate buffer, and similar incubation treatment, these bands were not observed (see tracks 2-6 and 19).

(4) N-Terminal Amino Acid Analysis:

Fractions A and C showed considerable heterogeneity with respect to N-terminal amino acids (table 17). Glycine appeared as a spot of strongest staining intensity followed by valine, isoleucine and leucine, then serine, aspartic acid/asparagine and phenylalanine. Alanine was identified in sub-fractions A_2-A_4 but not in fraction C, whereas threonine was identified only in the latter.

By contrast only a single N-terminal amino acid was identified in fraction B, that of glutamic acid/glutamine.

The strongest staining N-terminal amino acids amongst the sub-fractions D_o to D_5 were either of glycine or aspartic acid/ asparagine. Valine, isoleucine, leucine, phenylalanine, alanine and glutamic acid/glutamine were, however, also identified.

(5) Carbohydrate Analysis:

None of the sub-units in either of fractions A or B stained

positively with the PAS technique. However, the bands with Rm values 0.61, 0.70 and 0.78 in sub-fraction D_2 did.

(6) Chromatography of Fraction B on Hydroxylapatite:

The elution profile of fraction B following chromatography on hydroxylapatite (fig. 25) consisted of a single peak, the protein composition of which was the same as that of the original as shown by PAGE under both non-dissociating and dissociating conditions, isoelectric focussing and N-terminal analysis of both the protein and sub-units. The sub-unit composition of protein represented by the plateau region leading up to the peak was also identical to that of the original, and that of the peak.

(7) Analysis of Individual Fractions Collected from Peak B:

The sub-unit profiles of 7 individual 4 ml fractions collected through peak B, which together comprised fraction B, were the same. Fraction B was considered to contain a purified preparation of the major storage proteins.

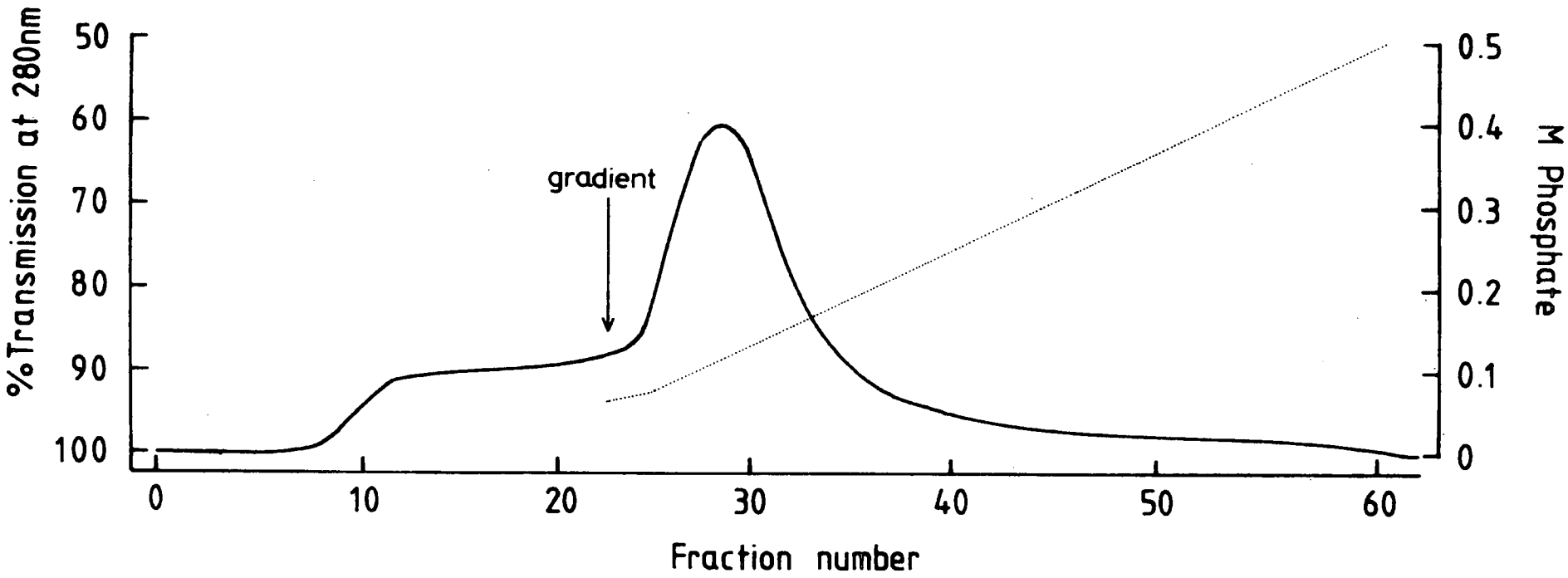


Fig. 25

Elution profile obtained after hydroxylapatite chromatography of 10ml fraction B prepared by anion exchange chromatography (see fig. 22(b)). Column equilibriated with 0.06m potassium phosphate buffer. Flow rate 40ml/h. 10ml fractions collected.

V. CHARACTERISATION OF THE STORAGE PROTEIN PREPARATION

A. Protein Determination:

The protein content, as estimated using the Lowry technique with bovine serum albumin as reference, was 101.3 ± 2.5% (14) of the dry weight content.

B. Nitrogen Determination:

The total nitrogen content was 16.03 ± 0.16% (12) of the dry weight content.

C. Amino Acid Composition:

The amino acid composition is given in table 18 (tryptophan not determined). The minimal molecular weight was determined as 10,390 ± 280. Non-polar amino acids represented 36% of the total, basic amino acids 13%, whilst the remainder were either acidic or polar.

D. Carbohydrate Analysis:

(1) Polyacrylamide Gel Electrophoresis:

Using both the periodic acid-Schiff and dansyl hydrazine staining techniques, negative results were obtained for carbohydrate associated with the protein sub-units.

(2) Gas Liquid Chromatography:

Gas liquid chromatography (GLC) analysis of the trichloroacetic acid-washed protein (see methods IV G.3) showed that only insignificant amounts of carbohydrate i.e. similar to the non-glycoprotein control, bovine serum albumin, were present.

GLC analysis of the unwashed protein indicated a total of about 2.45% carbohydrate (dry basis) in the preparation: the sugars identified were principally glucose (0.76%), galactose (0.61%), mannose (0.23%) and N-acetyl glucosamine (0.22%).

Table 18 Composition and Minimal Molecular Weight of the Storage Protein Preparation

Amino Acid[a]	Grams of[b] Amino Acid Residue per 100g Protein (d.b.)	Minimal Molecular Weight	Calculated[c] Molecular Weight	Assumed Number of Residues	Calculated number of residues for av. minimal m.w. of 10386
aspartic acid	m 13.47 ± 0.36	855	10260	12	12.1
threonine	e 3.80	2661	10644	4	3.9
serine	e 5.76	1512	10584	7	6.9
glutamic acid	m 18.36 ± 0.42	703	10545	15	14.8
proline	m 3.85 ± 0.16	2523	10092	4	4.1
glycine	m 2.81 ± 0.08	2031	10155	5	5.1
alanine	m 3.84 ± 0.08	1852	11112	6	5.6
valine	h 4.93 ± 0.35	2011	10005	5	5.2
methionine	m 2.05 ± 0.08	6400	12800	2	1.6
isoleucine	i 4.35 ± 0.10	2602	10408	4	4.0
leucine	m 7.91 ± 0.25	1431	10017	7	7.3
tyrosine	e 4.69	3480	10440	3	3.0
phenylalanine	m 7.06 ± 0.54	2085	10425	5	5.0
histidine	m 2.17 ± 0.11	6320	12468	2	1.6
lysine	m 5.03 ± 0.45	2584	10192	4	4.1
arginine	e 8.98	1740	10440	6	6.0
cysteic acid	m 0.99 ± 0.10	10419	10419	1	1.0
total	100.07[d]			92	91.21

Table 18
(Cont.)

Composition and Minimal Molecular Weight of the Storage Protein Preparation

a tryptophan and ammonia contents not determined

b values obtained from six hydrolysates, two at each of 22h, 48h and 72h duration of hydrolysis

m mean of 22h, 48h and 72h hydrolysates ± standard deviation

e extrapolation to zero time

h mean of 72h hydrolysates ± standard deviation

i mean of 48h and 72h hydrolysates ± standard deviation

c the average molecular weight for all the residues was 10650, whilst that for all the residues apart from methionine and histidine was 10386

d on the basis of nitrogen content, a 98.0% recovery may be calculated for a value of 16.03% as the total percentage of nitrogen

E. SDS Gel Electrophoresis:

(1) Treatment with SDS and 2-ME:

The sub-unit profile of the protein incubated for two minutes at 100°c in the presence of both SDS and 2-ME prior to electrophoresis consisted of the major sub-unit, three smaller minor sub-units, and some larger minor sub-units (qv Section IV.D, fraction B, figs. 23 and 24 and fig. 30a, track 4).

Denaturation by guanidine hydrochloride followed by alkylation gave the same sub-unit profile.

A quantitative evaluation of the sub-units by direct densitometry revealed that 95.2 ± 0.6%(3) and 95.5 ± 0.7%(3) of the protein stains Coomassie Blue and Procion Blue respectively were taken up by the major sub-unit, and the remainder by the larger (2.3 ± 0.3%) and smaller (2.4 ± 0.2%) sub-units.

(2) In vitro Polymerisation with Glutaraldehyde:

The sub-unit profile of the storage protein preparation treated with glutaraldehyde showed that polymers of the major sub-unit thus obtained were each aligned with all of the heavier sub-units observed in an untreated preparation (fig. 26 and fig. 27, tracks 5 cf tracks 6; fig. 28, track 2 cf track 8).

(3) Omission of 2-ME:

The sub-unit profile obtained following incubation (two minutes, 100°c) of the storage protein preparation in SDS alone was the same as that of the extract similarly treated (qv III.C3(ii)), in so far as there was one major sub-unit, the relative mobility of which was greater than that of the reduced major sub-unit but which corresponded with that of one of the minor sub-units, Rm 0.56; there were also a series of larger sub-units; but no smaller sub-

units. Two minor closely spaced larger sub-units (Rm values 0.49 and 0.50) had relative mobilities which corresponded with that of the reduced major sub-unit (see fig. 29).

Two-dimensional PAGE revealed that all the sub-units separated in SDS alone (first dimension) had the same mobility as the reduced major sub-unit in the second dimension (treatment with both SDS and 2-ME) (fig. 33). The minor sub-unit Rm 0.56 was a component of the unreduced major sub-unit (Rm 0.56-0.59) of the first dimension whilst the remaining minor sub-units Rm values 0.65 and 0.71 were derived from the two minor sub-units Rm values 0.49 and 0.50 (first dimension) respectively.

(4) Heterogeneity of the Major Sub-Unit:

After electrophoresis of less than 10 µg of the dissociated reduced protein preparation, the major sub-unit appeared as two overlapping bands, the Rm values of which, 0.49 and 0.50, corresponded with the two minor bands in the dissociated but unreduced preparation (see fig. 30a, track 2).

The minor sub-unit, Rm value 0.56 could also be detected as two closely spaced bands (fig. 30a, track 4). Similarly, the unreduced major sub-unit appeared as two bands on dilution.

However, whilst the heterogeneous nature of both the unreduced major sub-unit, and the band Rm value 0.56 became more apparent after electrophoresis on gels of varying porosity (compare tracks 9 in each of figs. 26, 27 and 28; and compare tracks 6, 6 and 2 in the three figs. respectively), this was not true of either the reduced major sub-unit or of the minor bands Rm 0.49 and 0.50 in the unreduced preparation (compare the same tracks in the same three figs.).

Fig. 26, Fig. 27 and Fig. 28

Electropherograms of approximately constant total acrylamide concentration but varying bis acrylamide concentration, prepared using the discontinuous SDS buffer system.

Fig. 26: 10.0 x 0.88

Fig. 27: 10.2 x 1.78

Fig. 28: 10.4 x 3.33

Fig. 26 and Fig. 27:

Track 1: hemoglobin

Track 2: bovine serum albumin and chymotrypsinogen

Track 3: ovalbumin and myoglobin

Track 4: β-lactoglobin and carboxypeptidase-A

Track 5: storage protein polymerised with gluteraldehyde (see methods IV.K)

Track 6: storage protein incubated for two minutes at 100°c in the presence of 2% w/v SDS, 5% v/v 2-ME

Track 7: storage protein incubated for thirty minutes at 100°c in the presence of 2% w/v SDS, 5% v/v 2-ME

Track 8: storage protein incubated overnight at room temperature in the presence of 2% w/v SDS, 5% v/v 2-ME

Tracks 9 and 10: as for tracks 6 and 8 respectively, but incubation in the absence of 2-ME.

Fig. 28:

Track 1: bovine serum albumin and chymotrypsinogen

Track 2: as for track 6 in fig. 26 and fig. 27

Track 3: ovalbumin and myoglobin

Track 4: as for track 7 in fig. 26 and fig. 27

Track 5: aldolase and carboxypeptidase-A

Track 6: as for track 8 in fig. 26 and fig. 27

Track 7: catalase and β-lactoglobulin

Track 8: as for track 5 in fig. 26 and fig. 27

Tracks 9 and 10: as for tracks 9 and 10 respectively in fig. 26 and fig. 27

Fig.26

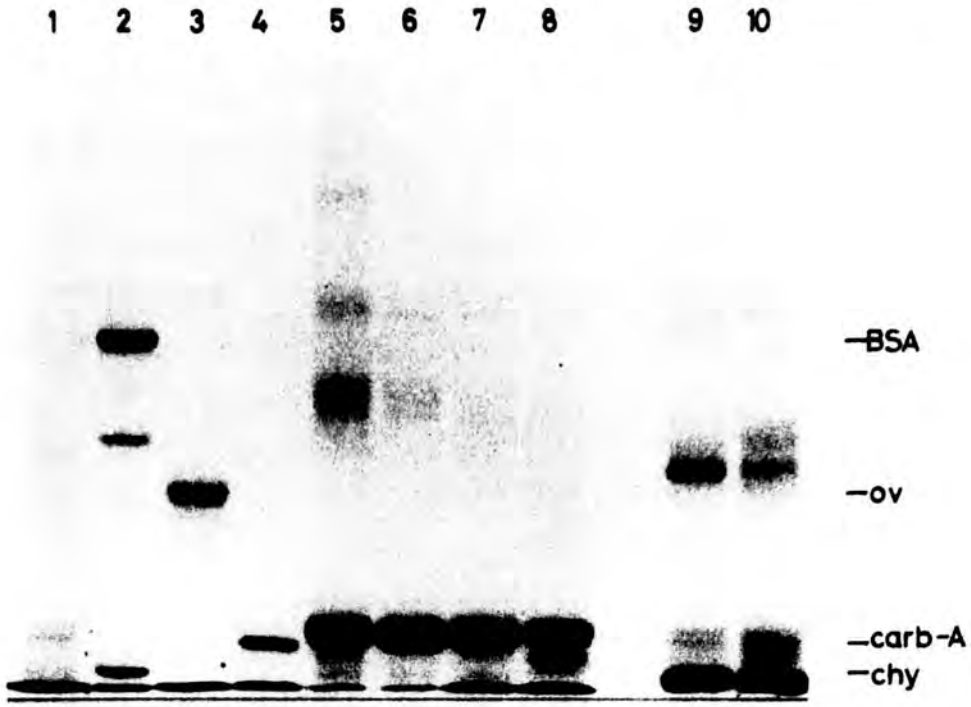


Fig.27

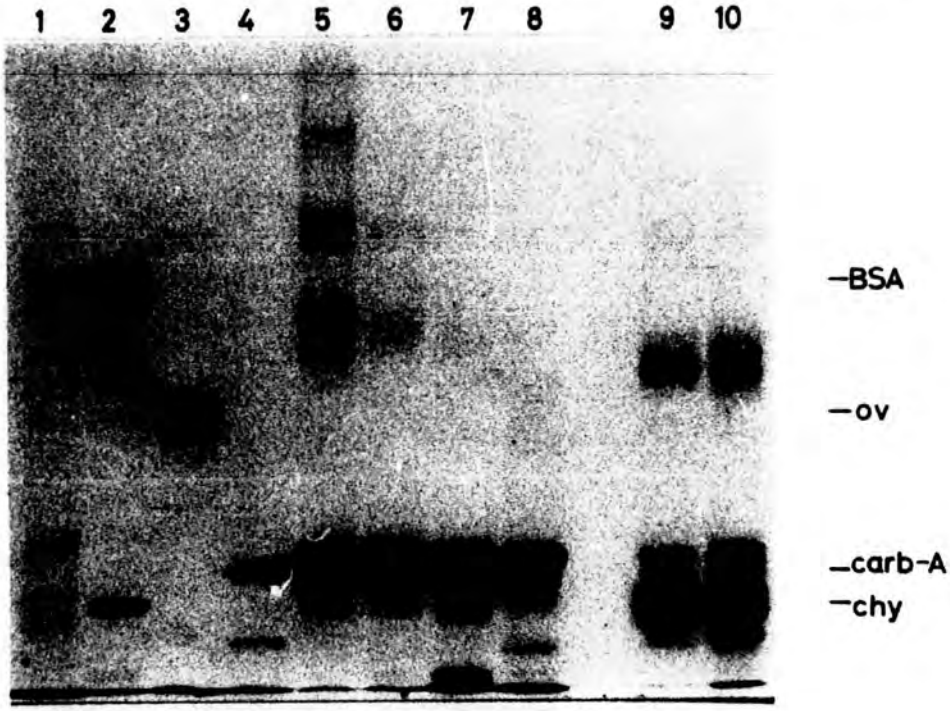


Fig.28

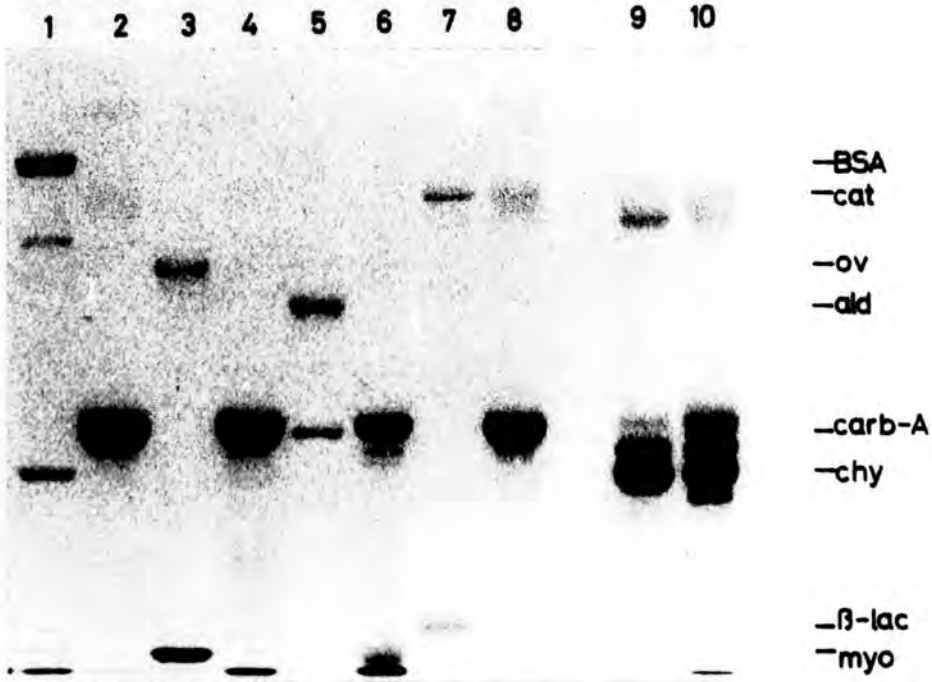


Fig. 29 and Fig. 30

Electropherograms illustrating the effect of varying the conditions of incubation of the storage protein in 2% $^{w}/_{v}$ SDS $\pm$ 5% $^{v}/_{v}$ 2-ME. (Discontinuous SDS buffer system, 17.6 x 0.45).

Fig. 29:

Tracks 1-4: incubation at 100°c in the absence of 2-ME, for 30 min., 10 min., 2 min., and 1 min. respectively.

Track 5: ultrasonication for 30 sec. followed by incubation for 2 min. at 100°c (- 2-ME).

Track 6: ultrasonication for 30 sec. (- 2-ME).

Track 7: incubation at 105°c for 10 min. (- 2-ME).

Tracks 8-9: incubation at room temperature (- 2-ME) for 16h and 6h respectively.

Track 10: as for track 6 in fig. 30(a).

Fig. 30(a):

Tracks 1, 3, 5 and 9: bovine serum albumin, chymotrypsinogen lysozyme and carboxypeptidase - A respectively.

Track 7: ovalbumin and myoglobin.

Tracks 2 and 4: 10 µg and 50 µg (d.w.) storage protein respectively, incubated for 2 min. at 100°c in SDS and 2-ME.

Track 6: incubation at 100°c for 30 min. in SDS and 2-ME.

Track 8: incubation at room temperature for 16h in SDS and 2-ME.

Fig. 30(b):

Track 1: ultrasonication for 30 sec. in SDS and 2-ME followed by incubation for 2 min. at 100°c.

Track 2: ultrasonication for 30 sec. in SDS and 2-ME.

Tracks 3 and 4: as for tracks 6 and 8 in fig. 30(a).

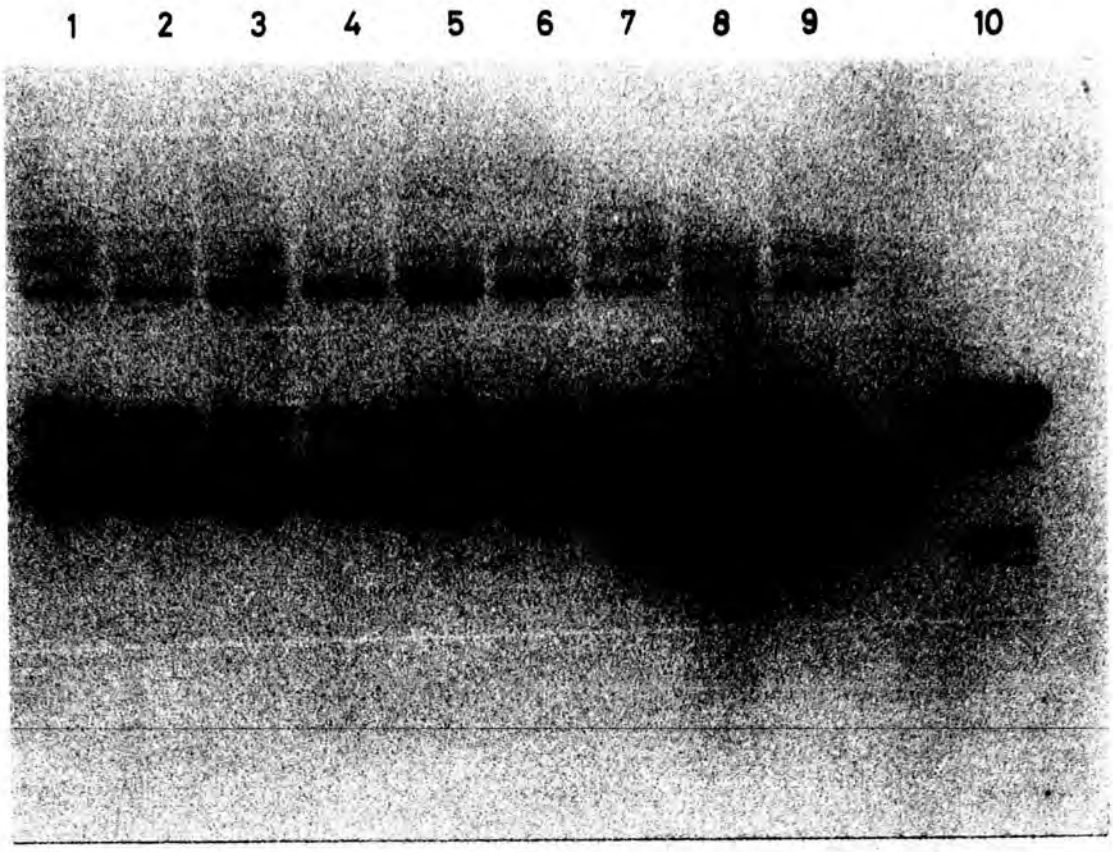


Fig.29

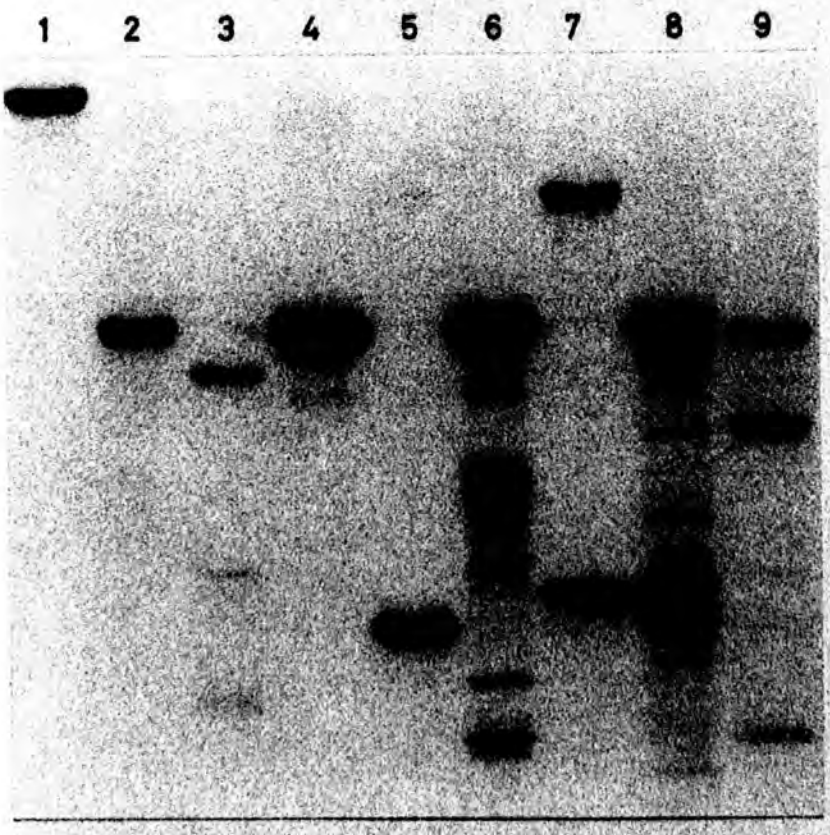


Fig.30(a)

Fig.30(b)

Fig. 31

Electropherogram illustrating the effect of varying the duration of incubation of the storage protein at 100°c in SDS and 2-ME prior to electrophoresis (discontinuous SDS buffer system, 17.6 x 0.45).

Tracks 1-6: 2 min., 4 min., 6 min., 8 min., 10 min., and 30 min. duration of incubation at 100°c respectively.

Track 7: 30 min. at 100°c in SDS, followed by the addition of 2-ME and heat at 100°c for 2 min.

Track 8: 30 min. at 100°c in 2-ME, followed by the addition of SDS and incubation at 100°c for 2 min.

Track 9: 30 min. at 100°c in the absence of either 2-ME or SDS, followed by the addition of both reagents, and incubation at 100°c for 2 min.

Track 10: 30 min. at 100°c in the presence of SDS, 2-ME and urea.

Track 11: 30 min. at 100°c in the presence of SDS alone.

Fig. 32

Electropherogram illustrating the effect of varying the duration of incubation of the storage protein at room temperature (r.t.) in SDS and 2-ME prior to electrophoresis.

Tracks 1-7: 1h, 2h, 4h, 6h, 10h, 16h and 24h duration of incubation respectively.

Track 8: 16h at r.t., followed by incubation at 100°c for 2 min.

Track 9: 16h at r.t. in SDS, followed by the addition of 2-ME and incubation at 100°c for 2 min.

Track 10: 16h at r.t. in 2-ME, followed by the addition of SDS and incubation at 100°c for 2 min.

Track 11: 16h at r.t. in the presence of SDS, 2-ME and urea.

Track 12: 16h at r.t. in the presence of SDS alone.

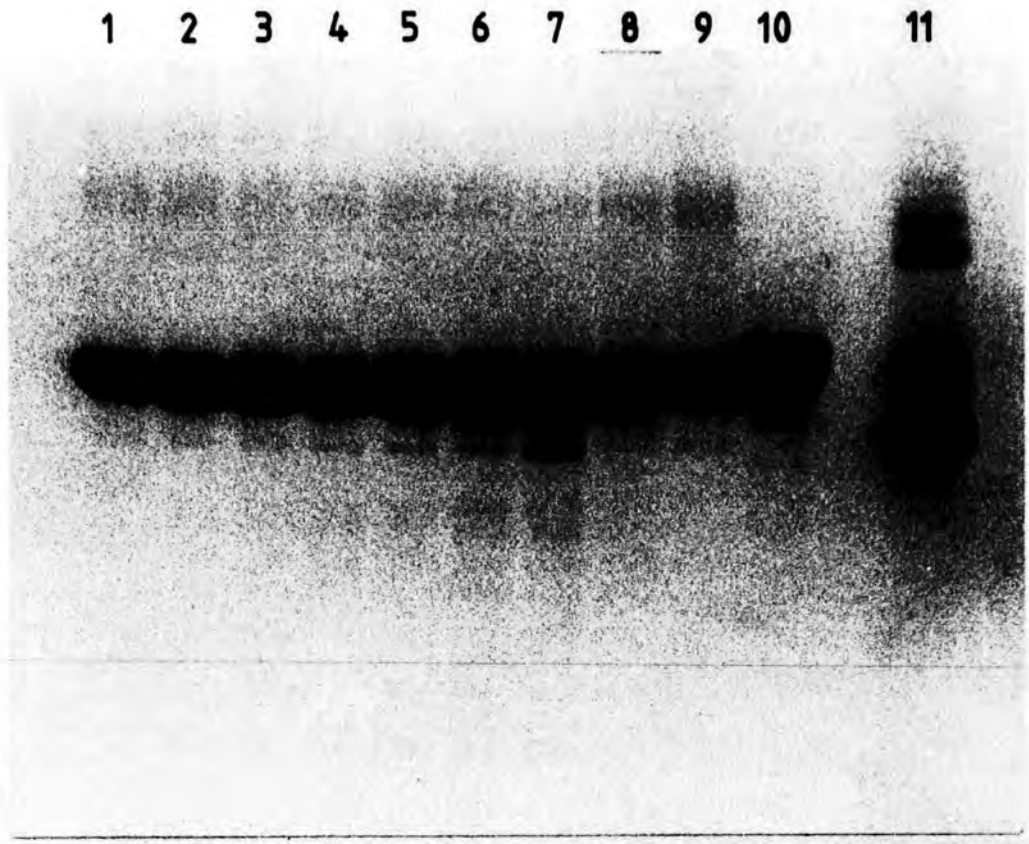


Fig. 31

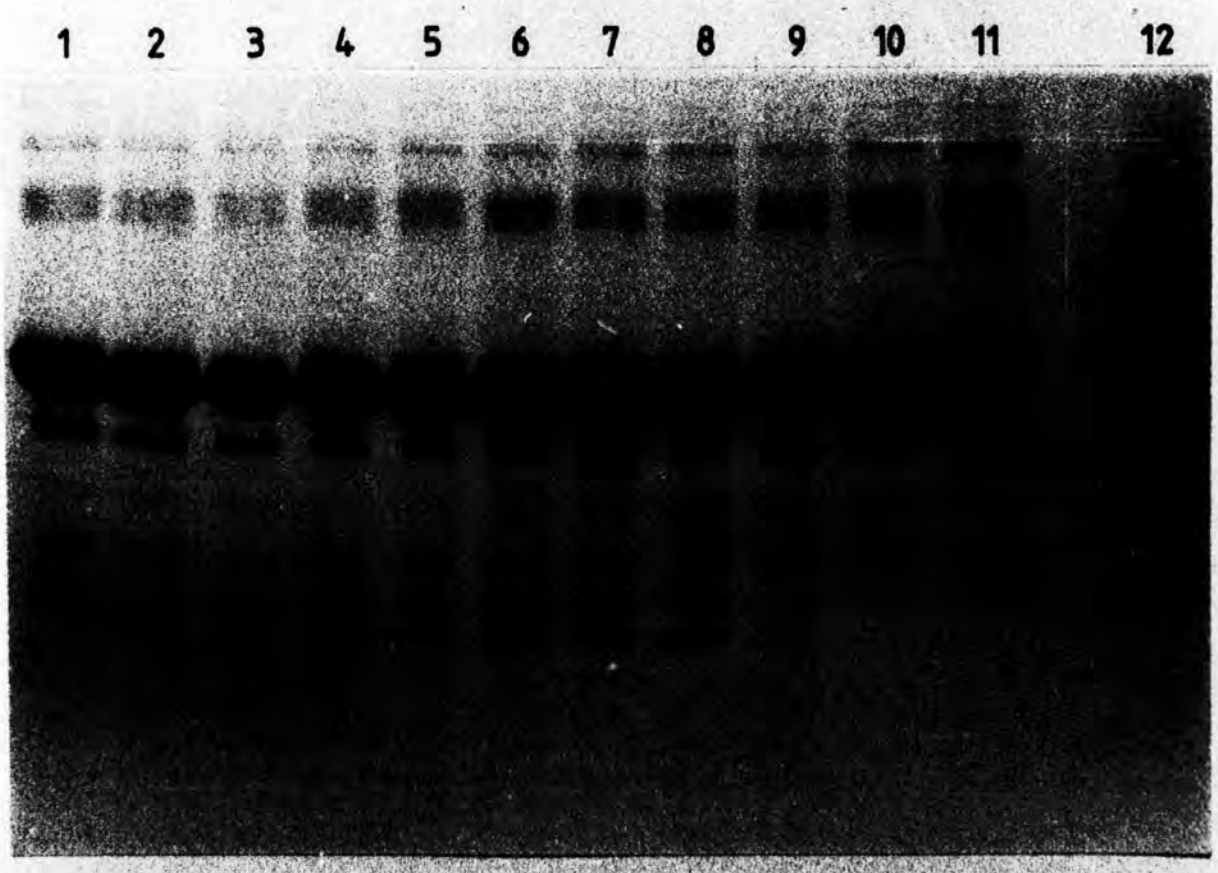


Fig. 32

Fig. 33

Separation of the storage protein preparation by two-dimensional PAGE (discontinuous SDS buffer system, 17.6 x 0.45).

First dimension: separation after pretreatment of the sample with SDS alone.

Second dimension: separation after pretreatment of the first dimensional rod gel with both SDS and 2-ME.

(See methods V D(1)).

(The lesser migrating band of the second dimension, Rm value approximately 0.33, is an artifact of the method).

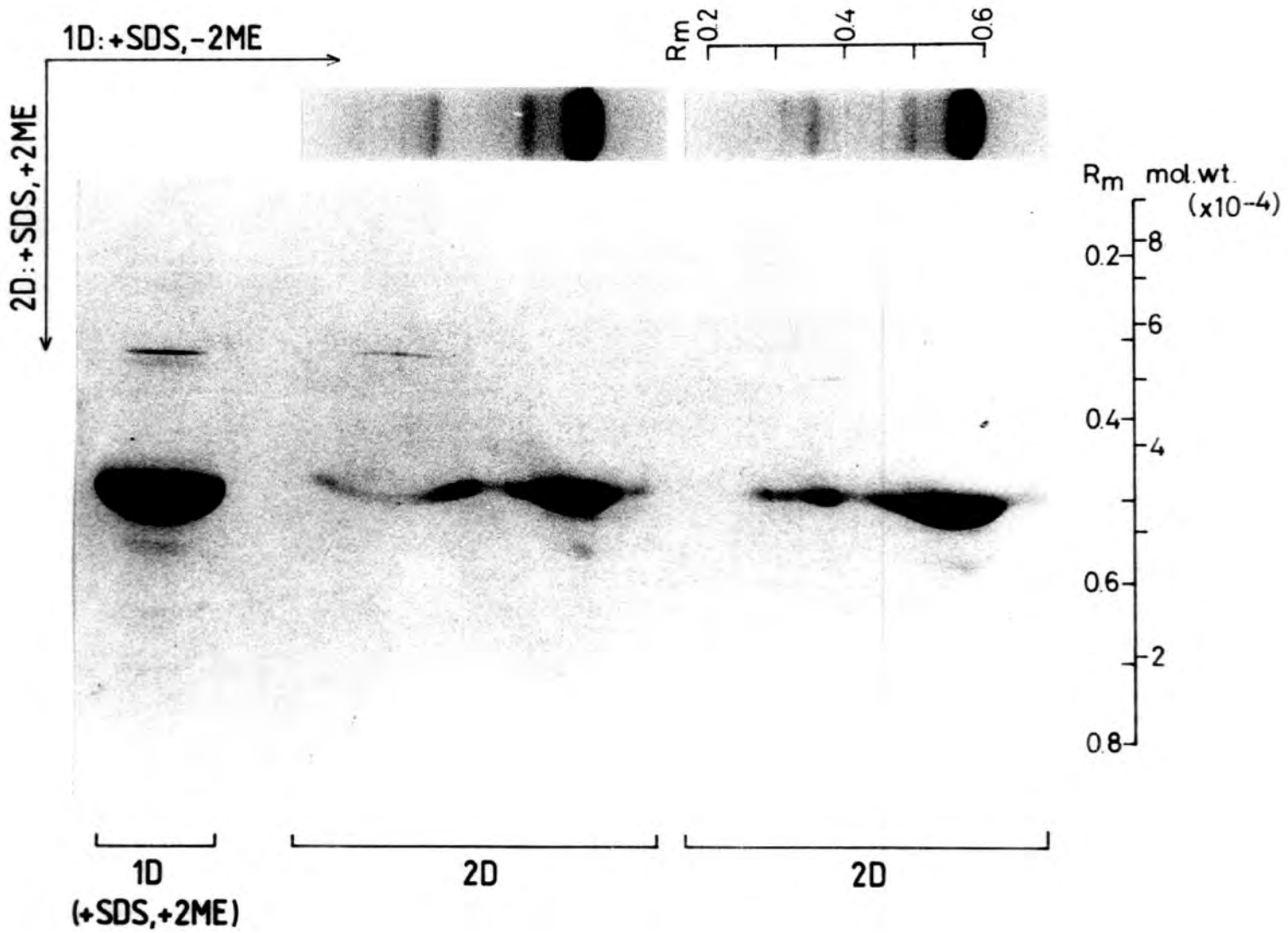

1D:+SDS,-2ME
2D:+SDS,+2ME
Rm
0.2
0.4
0.6
Rm mol.wt.
(x10-4)
0.2
0.4
0.6
0.8
8
6
4
2
1D
(+SDS,+2ME)
2D
2D

Fig. 33

Table 19 Values of Molecular Weight[a] of the Protein Sub-Units in the Purified Storage Protein Preparation

Buffer System	Discontinuous-SDS				Continuous-SDS
Acrylamide concentration	17.6 x 0.45	10.4 x 3.33	10.2 x 1.77	10.0 x 0.88	7.7 x 2.6
Regression coefficient for standard curve	0.987	0.986	0.983	0.979	0.997
Reduced major sub-unit, and unreduced minor sub-units, Rm 0.49 and Rm 0.50	33890, 32970	31730-30800	31310, 30660	31150, 30430	29230-27860
Unreduced major sub-unit, and reduced minor sub-unit, Rm 0.56	28020, 27500	27960	27300, 26400	27860, 27570	27460-26100
Reduced minor sub-unit, Rm 0.65	22620	ND	ND	ND	ND
Reduced minor sub-unit, Rm 0.71	18710	ND	ND	ND	ND

[a] Values are a mean of several estimates ± c. 1% error in measurement
ND, not determined, either because the bands were too poorly resolved (continuous-SDS system) or because they migrated with, or very closely to, the front

(5) Molecular Weights of the Sub-Units:

Values for molecular weight of the sub-units are given in table 19. These varied slightly with each system but the overall variation on the gels investigated was only around 5%. The molecular weight of the reduced major sub-unit was around 31000; when these could be measured, the two overlapping bands had molecular weights of around 31500 and 30500. These values also corresponded to the values for the minor bands observed in the unreduced preparation, Rm values 0.49 and 0.50. The unreduced major sub-unit and minor band Rm value 0.56 had a molecular weight of around 27350, and again, when these could be measured, the two bands of these two sub-units had molecular weights of around 27720 and 27100.

(6) The Effect of Different Methods of Sample Preparation on the Sub-unit Profile of the Storage Protein Preparation:

Low molecular weight bands were generated when the storage protein was boiled for six minutes or more in the presence of both SDS and 2-ME (fig. 31, tracks 3, 4, 5 and 6, cf tracks 1 and 2; see also fig. 30a, track 6). The addition of urea to the incubation medium precluded their appearance (fig. 31, track 10), as did the omission of 2-ME (fig. 31, track 11; fig. 29, tracks 1, 2 and 7) but subsequent addition of 2-ME to a sample treated in the latter manner led to their production (fig. 31, track 7). The effect, however, was reduced following similar treatment of an unlyophilised preparation.

Different low molecular weight bands were generated after incubation of the protein, again in the presence of both SDS and 2-ME, but at room temperature for extended periods of time (one hour or more); this effect increased with increasing duration of incubation (fig. 32, tracks 1-7, fig. 30a, track 8). Omission

of 2-ME from the incubation medium did not inhibit their generation (fig. 32, track 12 and fig. 29, track 8), neither did a subsequent addition of this reagent to the incubated sample (fig. 32, track 9), although in both cases the rate of their production was retarded. The presence of SDS for around one hour was necessary for their generation (fig. 32, track 10). The addition of urea (fig. 32, track 11) antagonised this effect.

The same low molecular weight bands were also generated following ultrasonication of the sample in SDS and 2-ME for 30 seconds (fig. 30b, track 2) but the effect was inhibited by subsequently boiling the sample for two minutes (fig. 30b, track 1).

F. N-Terminal Amino Acid Analysis:

The N-terminal amino acid under apparent non-dissociating conditions was identified as glutamine/glutamic acid. Under dissociating conditions traces of asparagine/aspartic acid, glycine and lesser still amounts of alanine, threonine and isoleucine were also identified.

Following separation of the storage protein preparation into acetate-soluble and -insoluble fractions (qv IV D.3) the N-terminal amino acids of the former (non-dissociating conditions) were glutamine/glutamic acid and lesser amounts of asparagine/ aspartic acid and glycine, whilst those of the latter were predominantly glycine, and lesser amounts of glutamine/glutamic acid, asparagine/aspartic acid, phenylalanine and isoleucine.

After dialysis against phosphate buffer pH7.9 however, the N-terminal amino acid was glutamine/glutamic acid (qv IV D.6).

G. Isoelectric Focussing on Polyacrylamide Gels:

The profile of the protein isoelectrically focussed in the pH range 3-10 in the presence of urea and 2-ME (fig. 35) consisted of several bands, the more heavily stained of which had isoelectric points between c. pH 5.6 and 6.8.

Isoelectric focussing of the protein under apparent non-dissociating conditions gave a similar profile but fewer minor bands were observed (fig. 34). Two of the protein species were more intensely stained than the remainder; however, these had precipitated during focussing at pH values c. 4.9 and 5.2.
In the absence of urea the isoelectric points of the several bands were estimated to lie between pH values of 4.9 and 6.8.

Two-dimensional PAGE (first dimension, isoelectric focussing under non-dissociating conditions; second dimension, SDS PAGE) showed that the proteins separable by isoelectric point all had the same mobility in the second dimension as the major sub-unit (fig. 36). Polymeric sub-units and the minor sub-unit Rm 0.56 were detected as components of all the more intensely stained charge isomers, although the latter sub-unit was more heavily stained from the isomer pI c. 4.9; the remaining two minor sub-units Rm values 0.65 and 0.71 were visible only for those isomers in greatest amount.

H. Estimation of Isoelectric Point by Electrophoresis on Cellulose Acetate Membranes:

An insensitive technique, electrophoresis of the storage protein on cellulose acetate membranes in buffers of various pH values (methods V A.2) indicated, after approximate correction for electro-osmotic effects using the observed mobility of β-lactoglobulin, that the isoelectric point of the storage protein preparation lay between pH values 5.2 and 6 (position of zero mobility).

Fig. 34

Isoelectric focussing profile of the non-dissociated storage protein preparation (see methods V C.1(i)).

Sample focussed for 6h at 0.7 watts/cm^2 in a rod of PAG (6.2 x 3.2) prepared with Pharmalyte carrier ampholines pH 3-10.

Temperature of focussing: approximately 25°c
Cathode buffer: 1M ethylenediamine
Anode buffer: 0.1M sulphuric acid

Values for isoelectric point were obtained by reference to the calibration curve indicated.

(See also fig. 18(b)).

Fig. 35

Isoelectric focussing profile of the dissociated reduced storage protein preparation (see methods V C.2).

Sample focussed for 12h at 400v, then 1h at 800v in a rod of PAG (6.0 x 6.6) prepared with LKB carrier ampholines pH 3.5-10 (1.76% $^{v}/_{v}$) and pH 4-6 (0.24% $^{v}/_{v}$) with 6M urea incorporated into the matrix.

Temperature of focussing: approximately 25°c

Cathode buffer: 0.1M ethylenediamine
Anode buffer: 0.1M phosphoric acid

Values for isoelectric point were obtained by reference to the calibration curve indicated.

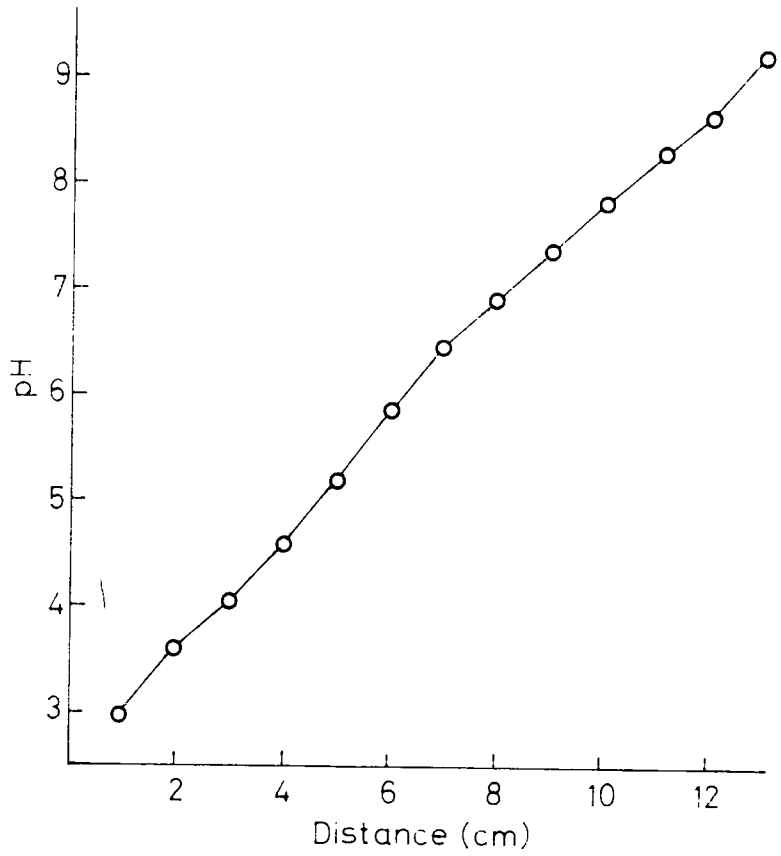

pH
9
8
7
6
5
4
3
2
4
6
8
10
12
Distance (cm)
4.9
5.2
5.5
5.8
6.1
6.8

Fig. 34

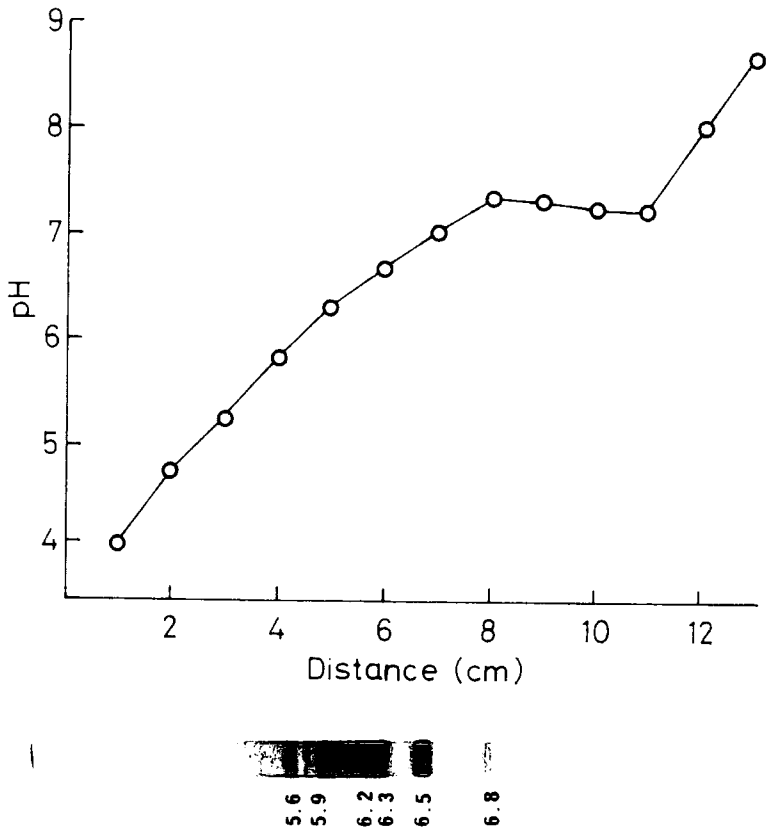

pH
9
8
7
6
5
4
2
4
6
8
10
12
Distance (cm)
5.6
5.9
6.2
6.3
6.5
6.8

Fig. 35

Fig. 36

Separation of the storage protein preparation by two-dimensional PAG techniques.

First dimension: Separation by isoelectric focussing on PAG (6.2 x 3.2) prepared with Pharmalyte carrier ampholines pH 3-10.

Second dimension: Separation, after pre-treatment of the first dimensional rod gel with SDS and 2-ME, on PAG (17.6 x 0.45), discontinuous SDS buffer system.

(See methods V D.1(ii)).

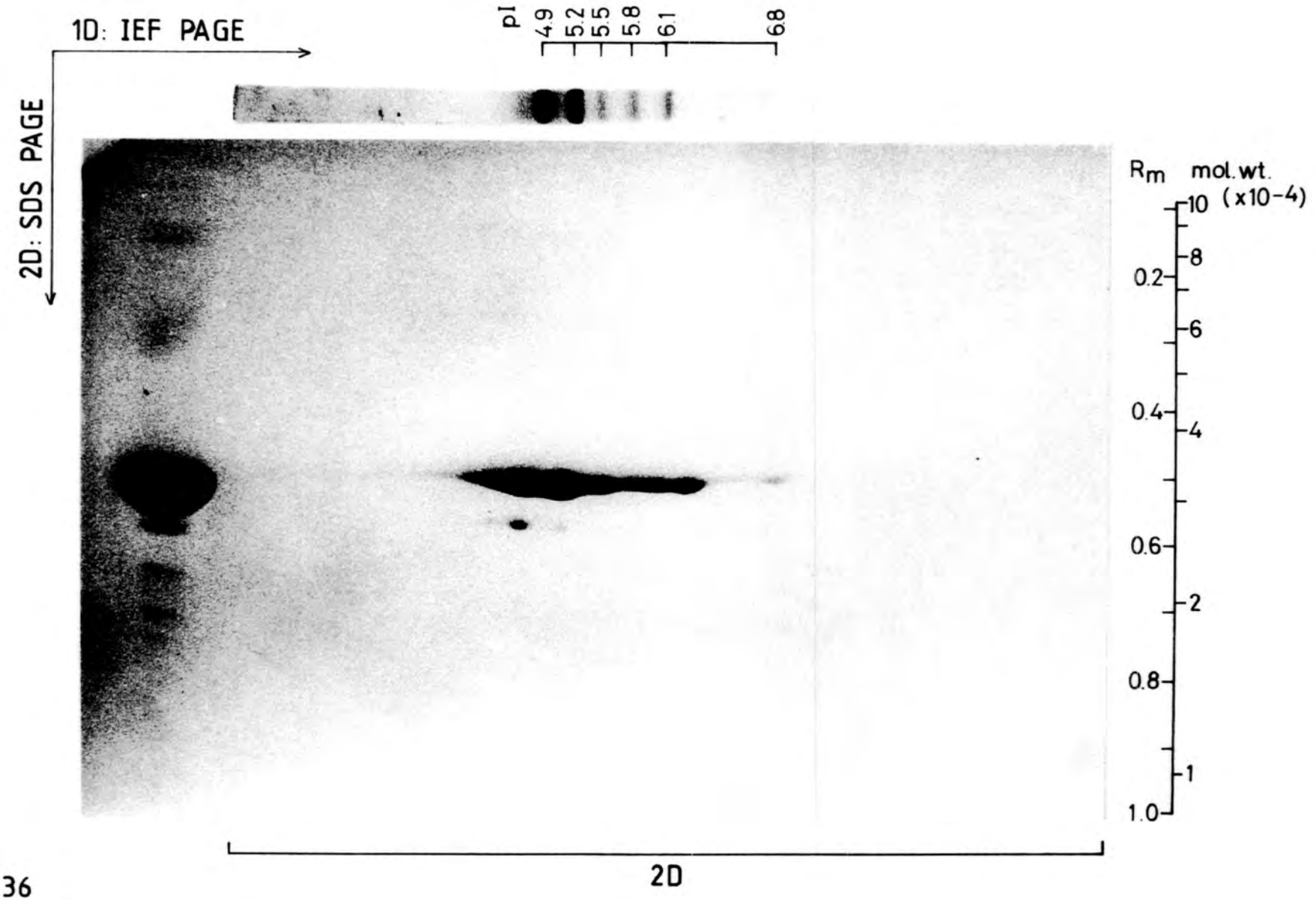

1D: IEF PAGE
pI
4.9
5.2
5.5
5.8
6.1
6.8
2D: SDS PAGE
Rm
mol.wt.
(x10-4)
10
8
0.2
6
0.4
4
0.6
2
0.8
1
1.0
2D

Fig. 36

Fig. 37

Electrophoresis of an unlyophilised preparation of the storage protein perpendicular to the pH gradient in isoelectrically focussed polyacrylamide gels.

The gels (7.0 x 3.8) were prepared with 2% $^{v}/_{v}$ Pharmalytes pH 3-10, 5mM of each of aspartic acid, glutamic acid, lysine and arginine, $\pm$ urea (6M) incorporated into the matrix (see methods V D.2).

(a) Dissociated protein profile:

Pre-treatment of the sample with 9.5M urea, 5% $^{v}/_{v}$ 2-ME for 75 minutes at r.t., followed by electrophoresis on isoelectrically focussed PAG prepared with urea incorporated into the matrix.

(b) Non-dissociated protein profile:

Electrophoresis of an untreated sample on isoelectrically focussed PAG prepared without urea.

The arrow indicates the position of sample application.

The pH gradients indicated (upper scales) were determined by reference to calibration curves relating pH value with distance along the gels.

The lower scales indicate values for isoelectric point of the protein species.

(a)

(b)

I. Electrophoresis on Isoelectrically-focussed Gels:

Protein titration curves for the storage protein, obtained under both dissociating and apparent non-dissociating conditions, are illustrated in fig. 37. The pattern was the same under both sets of conditions, and consisted of several curves most of which were united at around pH 4 and less. Above this pH the curves ran essentially parallel with one another through to the alkaline pH spectrum, some of the curves converging or diverging at several points between pH values 5.5 and 7. The mobility of the protein species in the acid range was considerably greater relative to that in the alkaline range.

Values for isoelectric point, estimated from the point of sample application, are indicated in the figure, and ranged between 5.2 and 6.9.

J. Solubility Properties:

(1) Alkaline Buffers:

The protein preparation was soluble in all buffers in the pH range 7-9, at both room temperature and $4^{o}c$ irrespective of the ionic strength of the buffer or concentration of protein.

(2) Acidic buffers:

In the presence of 0.0125 M acetate buffer pH value 4.7 approximately 14% of the protein (concentration c. 4 mg/ml) precipitated, but the precipitate could be resolubilised in acetate buffer containing 0.3M sodium chloride. The remainder of the protein formed a stable solution at room temperature, but cryoprecipitated at $4^{o}c$, becoming resoluble on warming (qv IV D.3 as well).

By contrast, the protein (concentration c. 4 mg/ml) formed a stable solution, both at room temperature and $4^{o}c$, in glycine hydrochloride butter pH 2.625 at low ionic strength (molarity 0.02)

but at high ionic strength (addition of 0.3M sodium chloride) protein was precipitated: 52% of the total after 12h dialysis against the buffer, a further 33% after 24h standing from the solution remaining, and a further 5% after an additional 24h period. The precipitated protein, however, could be resolubilised in the original low ionic strength glycine hydrochloride buffer.

K. Analytical Ultracentrifugation:

The Schlieren optics patterns obtained after ultracentrifugation of the storage protein preparation consisted of three poorly separated peaks (fig. 38). The sedimentation coefficient of protein in the largest peak was determined as 7.09 $\pm$ 0.008S at the two protein concentrations analysed, giving a very crude molecular weight estimate of 130,000; the shapes of the slower and faster moving peaks precluded a determination of the sedimentation coefficients.

A comparison between the patterns obtained at the two concentrations of protein analysed showed that the relative sizes of the peaks differed; at the lesser concentration (pattern b) the largest peak and the faster moving peak were relatively smaller compared to the slower moving peak.

L. Molecular Sieve Chromatography:

(1) Chromatography on the Polyacrylamide Matrix Bio-Gel:

(i) Molecular Size:

The elution profiles obtained after molecular sieve chromatography of the storage protein preparation under various conditions (figs. 39-43) indicated that there were several forms of the protein separable by size. By reference to calibration curves describing the elution behaviour of standard proteins (methods VII.C), one of the species was identified as the major

Fig. 38

Ultracentrifuge patterns of a lyophilised preparation of the storage protein in 0.01M Na phosphate buffer pH 7 containing 0.15M NaCl.

The sample was solubilised in the buffer (10 mg/ml, d.w.) then dialysed, using sterile technique, for 60h at 4^{o}c against the same buffer.

The solution was centrifuged for 5 minutes at 10,000g, then analysed in the ultracentrifuge.

(a) concentration x

(b) concentration 0.5x

Upper photograph taken 32 minutes after the ultracentrifuge had reached a speed of 60,000 revs./minute.
Bar angle 70^{o}.

Lower photograph taken 48 minutes after the ultracentrifuge had reached a speed of 60,000 revs./minute.
Bar angle 70^{o}.

Direction of sedimentation, right to left.

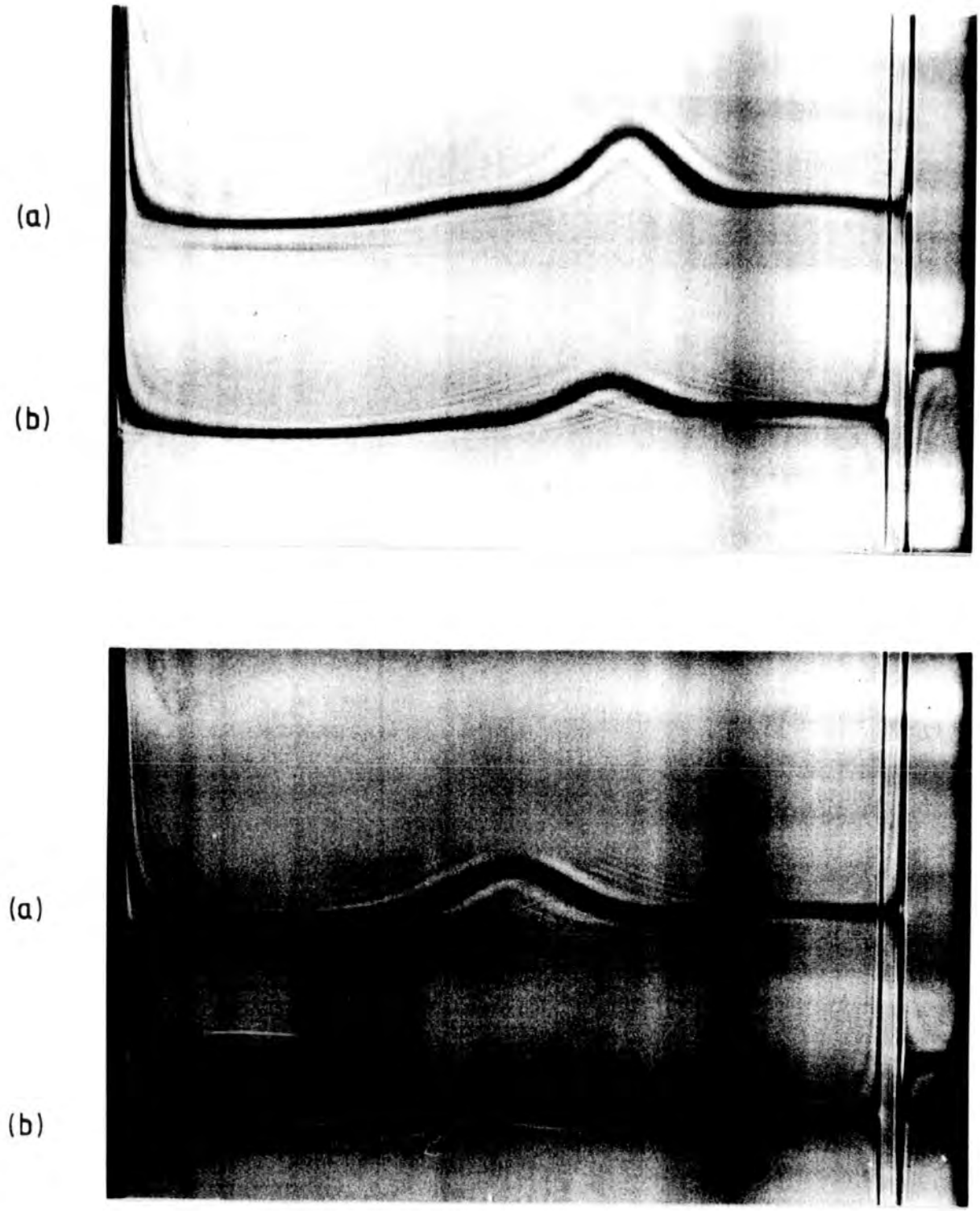

(a)
(b)
(a)
(b)
Direction of sedimentation

Fig. 39

Elution profiles obtained after chromatography of the storage protein on a column of Bio-gel P-150.

Column dimensions: 60 x 2.2 cm.

Flow rate: 5.3 ml/h/cm^2.

Eluant: 0.05M Tris-HCl, 0.15M NaCl, pH 8.3.

Sample volume: 1.5 ml.

Values for protein concentration determined from measurement of the optical density at 280 nm for which the $E^{280}_{1\ cm}$ value of 1 mg(d.w.) of the protein per ml was 0.996 $\pm$ 0.025.

(a) 7.77 mg/ml.

(b) 9.56 mg/ml.

(c) 8.92 mg/ml: Protein sample lyophilised prior to chromatography.

(d) 16.53 mg/ml.

(e) Rechromatography of 1.5 ml of the fraction indicated in fig. 39(d).

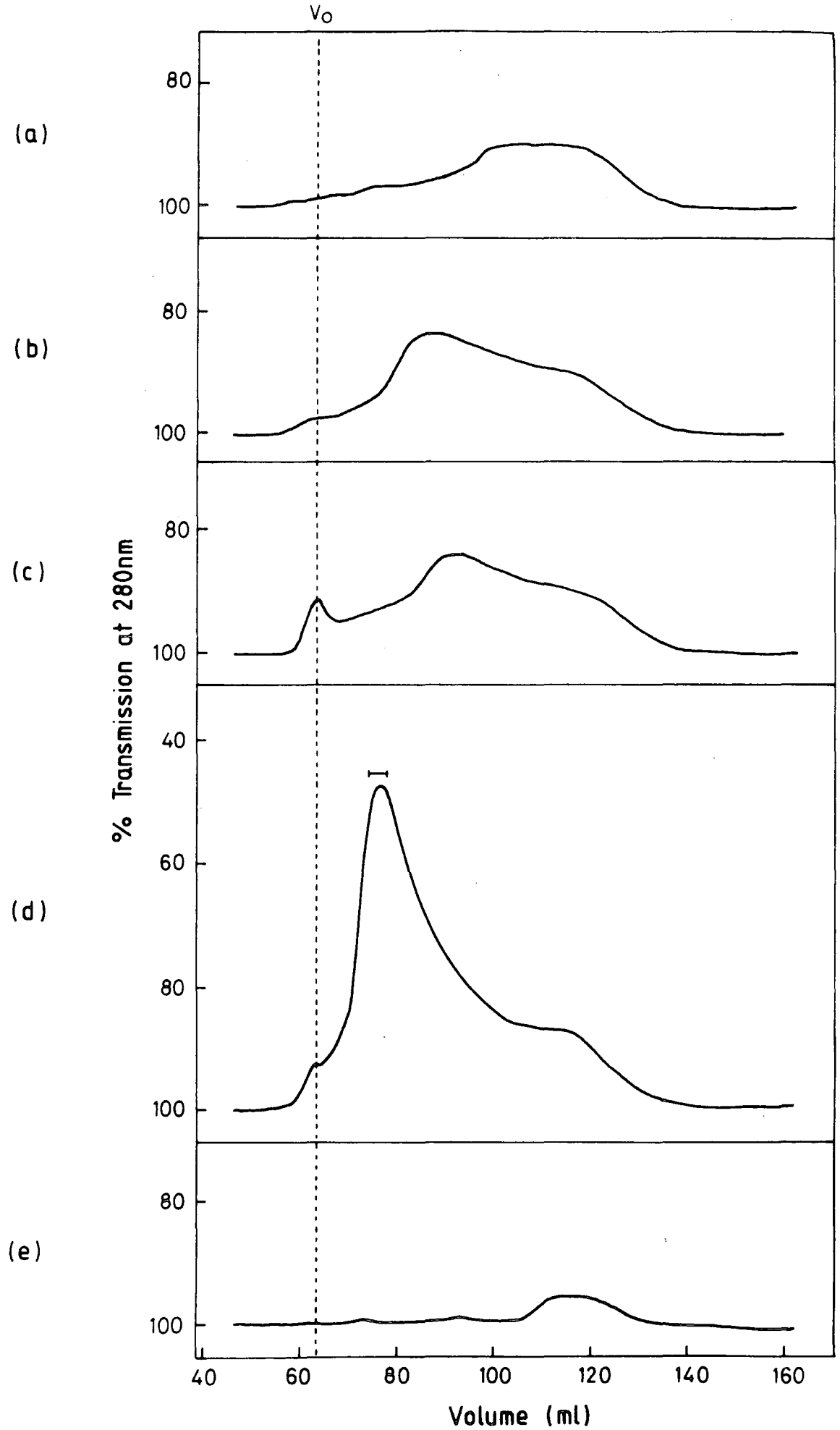

Vo
(a)
(b)
(c)
(d)
(e)
80
100
40
60
% Transmission at 280nm
40
60
80
100
120
140
160
Volume (ml)

Fig. 40

Elution profiles obtained after chromatography of the storage protein on a column of Bio-gel P-150.

Column dimensions: 45 x 2 cm.
Flow rate: 4.8 ml/h/cm^2.
Eluant: 0.05M Tris-HCl, 0.15M NaCl, pH 8.3.
Sample volume: 1.5 ml.

Values for protein concentration determined as for fig. 39.

(a) 7.74 mg/ml.

(b) 13.75 mg/ml.

(c) 8.96 mg/ml: Fractions of the monomer, collected from several chromatographic analyses, were pooled, concentrated to 6.52 mg/ml, stored for two days at $4^{o}c$, then concentrated further to 8.96 mg/ml and chromatographed the same day.

(d) 7.22 mg/ml: Fractions of the tetramer, collected from the same chromatographic analyses as for (c) were pooled, concentrated to 7.22 mg/ml, and chromatographed the same day.

(e) 0.99 mg/ml: Pooled fractions of the tetramer as for (d), but without concentration.

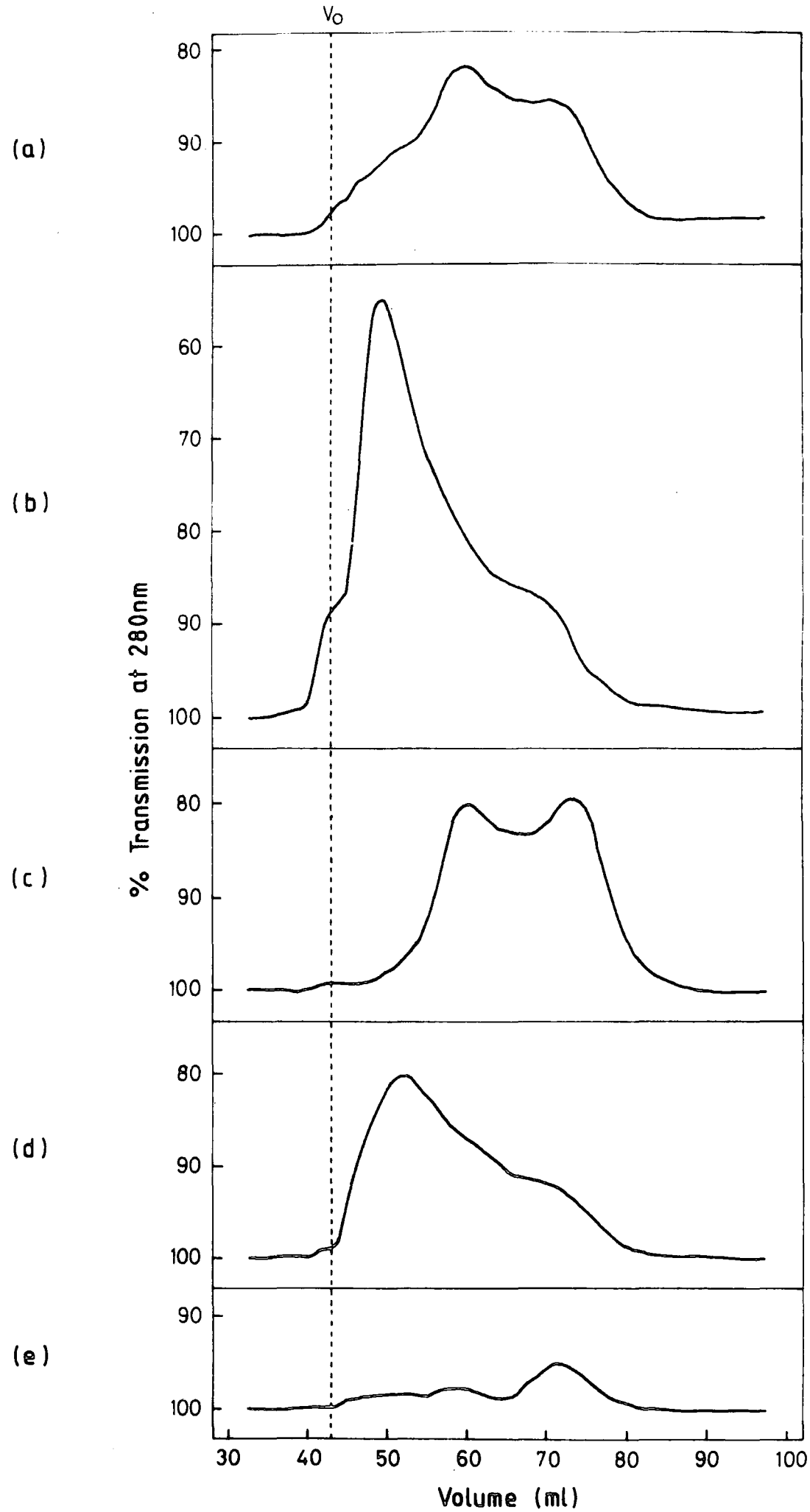

V_O
(a)
(b)
(c)
(d)
(e)
80
90
100
60
70
80
90
100
80
90
100
80
90
100
90
100
% Transmission at 280nm
30
40
50
60
70
80
90
100
Volume (ml)

Fig. 41

Elution profiles obtained after chromatography of the storage protein on columns of Bio-gel P-150, equilibriated with eluants of varying composition.

Column dimensions: 45 x 2 cm for (a)-(d); 50 x 1.6 cm for (e).

Flow rate: 4.8 ml/h/cm^2.

Sample volume: 1.5 ml

Samples were dialysed against buffer of the same composition as the corresponding eluants before determination of the protein concentration (see legend for fig. 39), and chromatographic analysis.

(a) Eluant: 0.025 M Tris-HCl, pH 8.3.
Protein concentration: 4.36 mg/ml.

(b) Eluant: 0.05M Tris-HCl, 0.3M NaCl, pH 8.3.
Protein concentration: 4.36 mg/ml.

(c) Eluant: 0.02M Glycine-HCl, pH 2.6.
Protein concentration: 4.11 mg/ml.

(d) Eluant: 0.0125M sodium acetate, pH 4.7.
Protein concentration: 3.76 mg/ml.

(e) Eluant: 0.0125M sodium acetate, 0.3M NaCl, pH 4.7.
Protein concentration: 4.43 mg/ml.

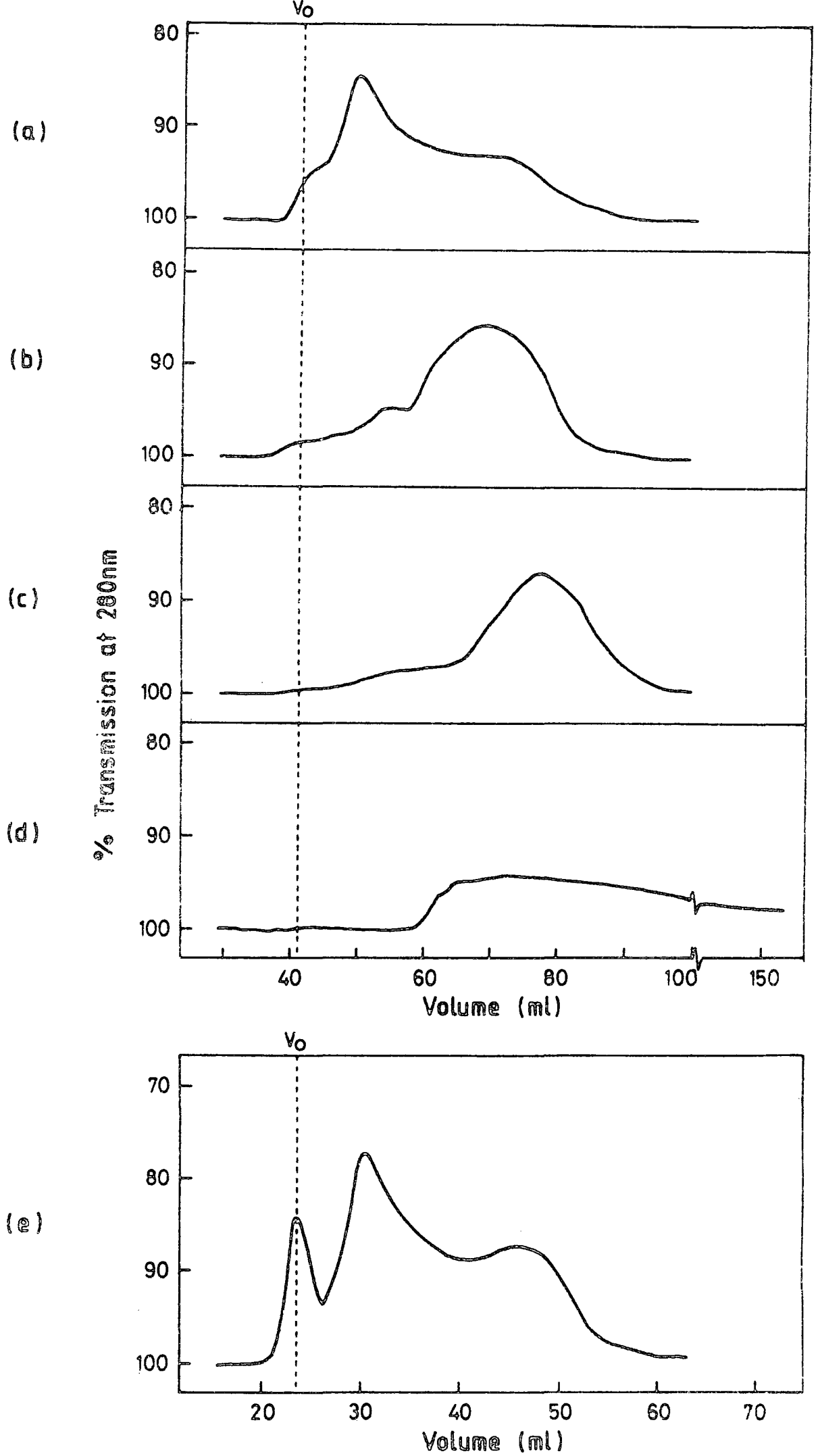

Vo
(a)
(b)
(c)
(d)
(e)
80
90
100
% Transmission at 280nm
40
60
80
100
150
Volume (ml)
70
20
30
40
50
60

Fig. 42

Elution profiles obtained after chromatography of the storage protein on a column of Bio-gel P-150.

Column dimensions: 45 x 2 cm.

Flow rate: 4.8 ml/h/cm^2.

Eluant: 0.05M Tris-HCl, 0.15 M NaCl, pH 8.3.

Sample volume: 1.5 ml.

Values for protein concentration determined as for fig. 39.

(a) Chromatography of a protein sample immediately after preparation by anion exchange chromatography (see fig. 22(b); fraction B). (The elution profile of a sample of the same protein concentration but chromatographed after 24h storage, was identical (see fig. 40(b)).

Protein concentration: 13.75 mg/ml.

(b) Chromatography of a protein sample seven days after preparation by anion exchange chromatography.
Protein concentration: 21.23 mg/ml.

Fig. 43

Elution profile obtained after chromatography of the storage protein on a column of Bio-gel P-300.
Column dimensions: 48 x 1.5 cm.
Flow rate: 4.075 ml/h/cm^2.
Eluant: 0.05M Tris-HCl, 0.15M NaCl, pH 8.3.
Sample volume: 1.5 ml.
Protein concentration: 17.43 mg/ml (see legend, fig. 39).
The elution volume, Ve, of ovalbumin (ov) is indicated.

Fig.42

(a)

(b)

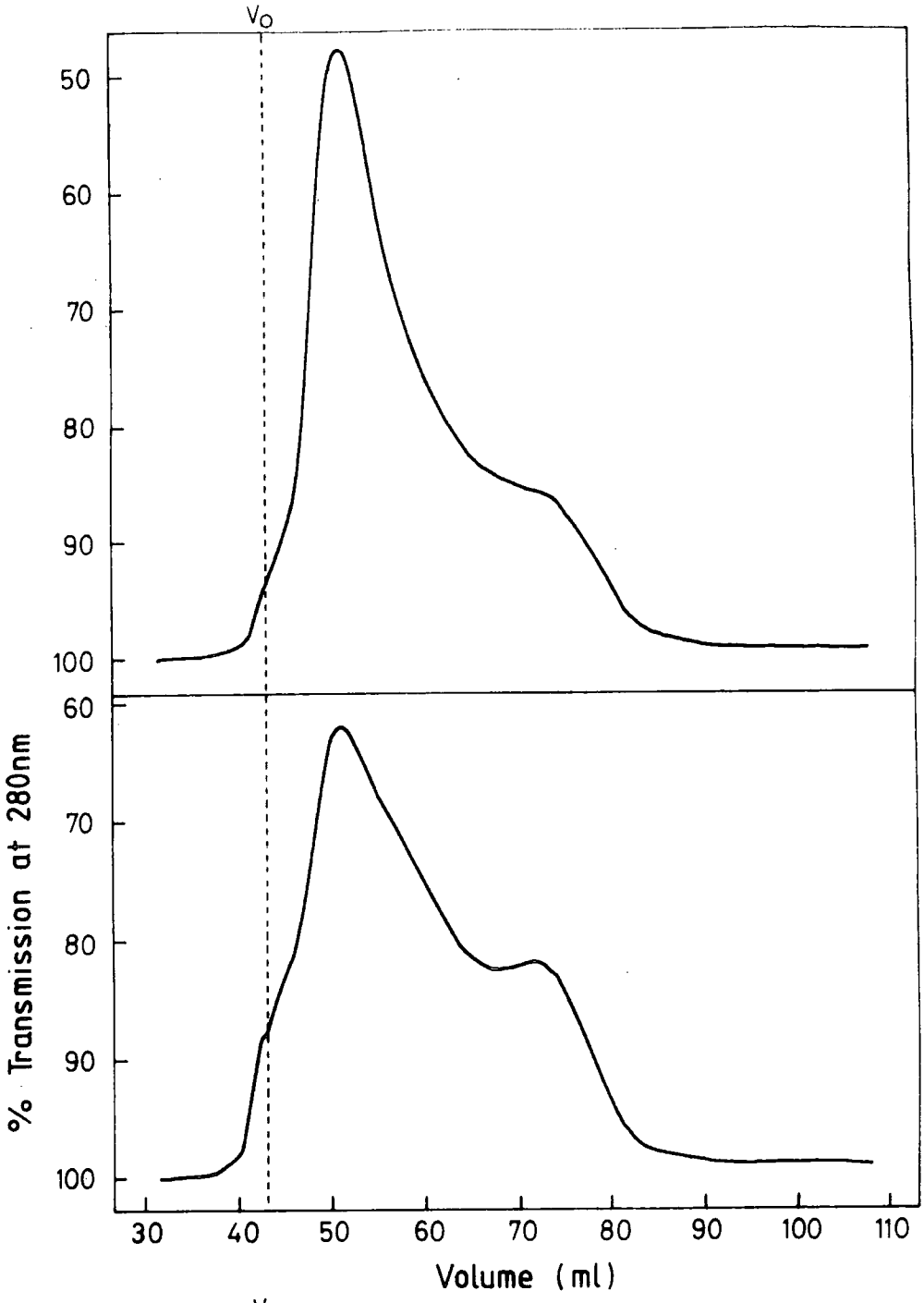
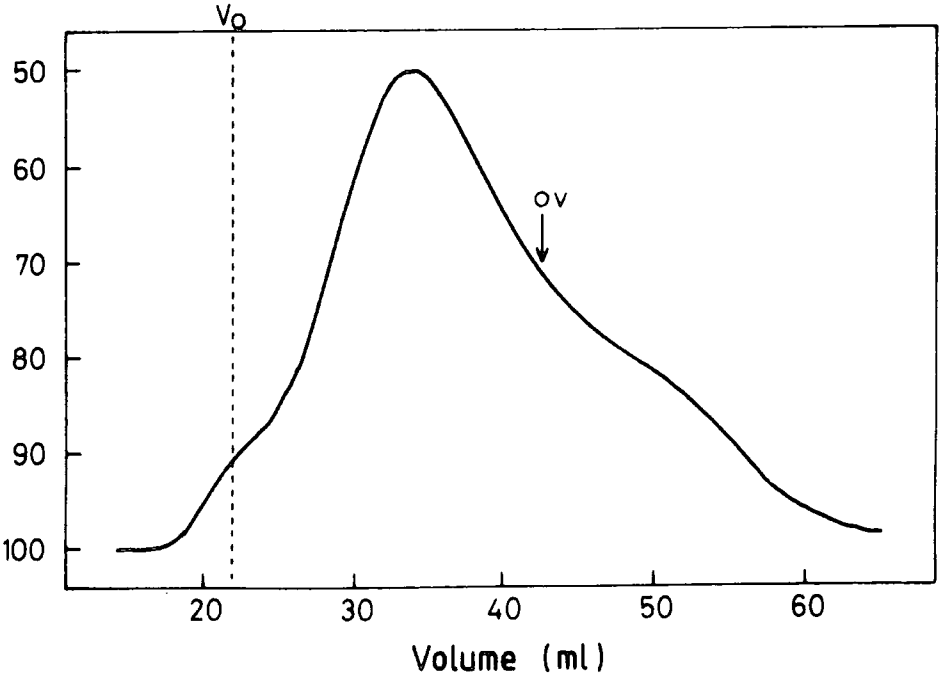


Fig.43



sub-unit (monomer); the remainder were identified as polymers formed by association of these sub-units, namely dimer, possibly trimer, tetramer, and higher polymers, some of which eluted in the void volume of a column of Bio-gel P-300 (exclusion limit c. 400,000).

The existence of a trimer was difficult to ascertain, as was the size of the larger polymers since the elution peaks of the several protein species tended to be both broad and asymmetrical. These dispersion effects became more pronounced on larger (60 x 2.2 cm) compared to smaller (45 x 2 cm) columns (fig. 39(a) cf fig. 40(a) respectively) and on columns of Bio-gel P-300 compared to Bio-gel P-150 (fig. 43 cf fig. 42 respectively).

(ii) Chromatography of a Lyophilised and Unlyophilised Preparation:

The elution profile following chromatography of a lyophilised preparation of the storage protein (fig. 39(c)) compared to that of an unlyophilised preparation (fig. 39(b)) showed that larger relative amounts of protein eluted at the exclusion limit of the column (Bio-gel P-150) in the former. Distribution analysis of the protein species under different conditions was, therefore, considered to be more profitably followed using preparations which had not been lyophilised.

(iii) Effect of Protein Concentration (Chromatography in Buffers of the same Alkaline pH Value):

The tendency for sub-units to associate was shown to increase as the concentration of protein was increased (fig. 39(a), (b), (d); fig. 40(a), (b)). This effect was largely independent of time as shown by identical elution profiles obtained following chromatography of two samples of the same concentration, the one 24h after the other, although the elution profiles of two samples chromatographed seven days after one another (fig. 42(a) and (b)) were slightly

different, the latter showing slightly lesser amounts of tetramer, and greater amounts of monomer, dimer and polymer of a higher order than tetramer.

Also the elution profiles following rechromatography of protein which had been eluted as tetramer showed the equilibrium to have shifted toward dissociation, (fig. 39(e); fig. 40(e)) whilst rechromatography of several fractions consisting of dissociated sub-units which were pooled and concentrated showed some association to have occurred (fig. 40(c)).

However, a comparison between fig. 40(a) (storage protein at an initial concentration of 7.7 mg/ml), fig. 40(d) (tetramer concentrated to 7.2 mg/ml), and fig. 40(c), (monomer concentrated to 9.0 mg/ml) showed that the association-dissociation equilibria of the three protein samples differed; that of the tetramer sample lay toward association, and that of the sample containing monomer lay toward dissociation, relative to the equilibrium existing in the storage protein sample.

(iv) Effect of Ionic Strength (Chromatography in Buffers of the same Alkaline pH Value):

The association-dissociation equilibrium was found to alter with changes in ionic strength, association being favoured at low ionic strength (fig. 41(a)), dissociation at high (fig. 41(b)).

(v) Effect of pH:

The elution profiles following chromatography in buffers of similar high ionic strength but different pH values are given in fig. 41. At pH 8.3, the association-dissociation equilibrium lay toward dissociation (fig. 41(b)), and at pH 4.7, toward association (fig. 41(e)); at pH 2.6, protein was precipitated, and could not be chromatographed.

However, chromatography in buffers of similar low ionic strength but different pH values showed that at pH 8.3 association was favoured (fig. 41(a)),at pH 4.7 protein was precipitated (fig. 41(d)), whilst at pH 2.6 dissociation was favoured (fig. 41(c)).

(vi) Chromatography in the Presence of Dithiothreitol:

A prior investigation of the concentration of dithiothreitol (DTT) required to reduce disulphide bonds was made by SDS gel electrophoresis; the concentration selected, 10mM DTT, was found to be as effective in this capacity as 5% $^{v}/_{v}$ 2-ME.

Chromatography of the storage protein in the presence of DTT gave an identical elution profile to that obtained after chromatography of the storage protein under the same conditions but in the absence of DTT.

(2) Chromatography on Polysaccharide Matrices:

The elution profiles obtained following chromatography on columns of Sephadex equilibriated with various buffers (fig. 44) showed that the greater part of the protein only started to elute at positions which, by reference to calibration curves describing the elution behaviour of standard proteins in the various buffers, implied molecular weights of around 10,000 and less, and furthermore, in all but the borate buffer (containing both glucose and sodium chloride) protein continued to be eluted well beyond the estimated internal volumes (Vi) of the columns used.

Chromatography of samples of increasing protein concentration resulted only in increased amounts of protein eluting beyond the position of the internal volume of the column (fig. 44(a) cf (b)). Subsequent chromatography of standard proteins showed that they eluted in a normal manner. SDS gel electrophoresis of fractions

Fig. 44

Elution profiles obtained after chromatography of the storage protein on a column of Sephadex G100.

Column dimensions: 45 x 2 cm
Flow rate : 6.3 ml/h/cm^2
Sample volume : 1.5 ml

Values for protein concentration determined as for fig. 39.

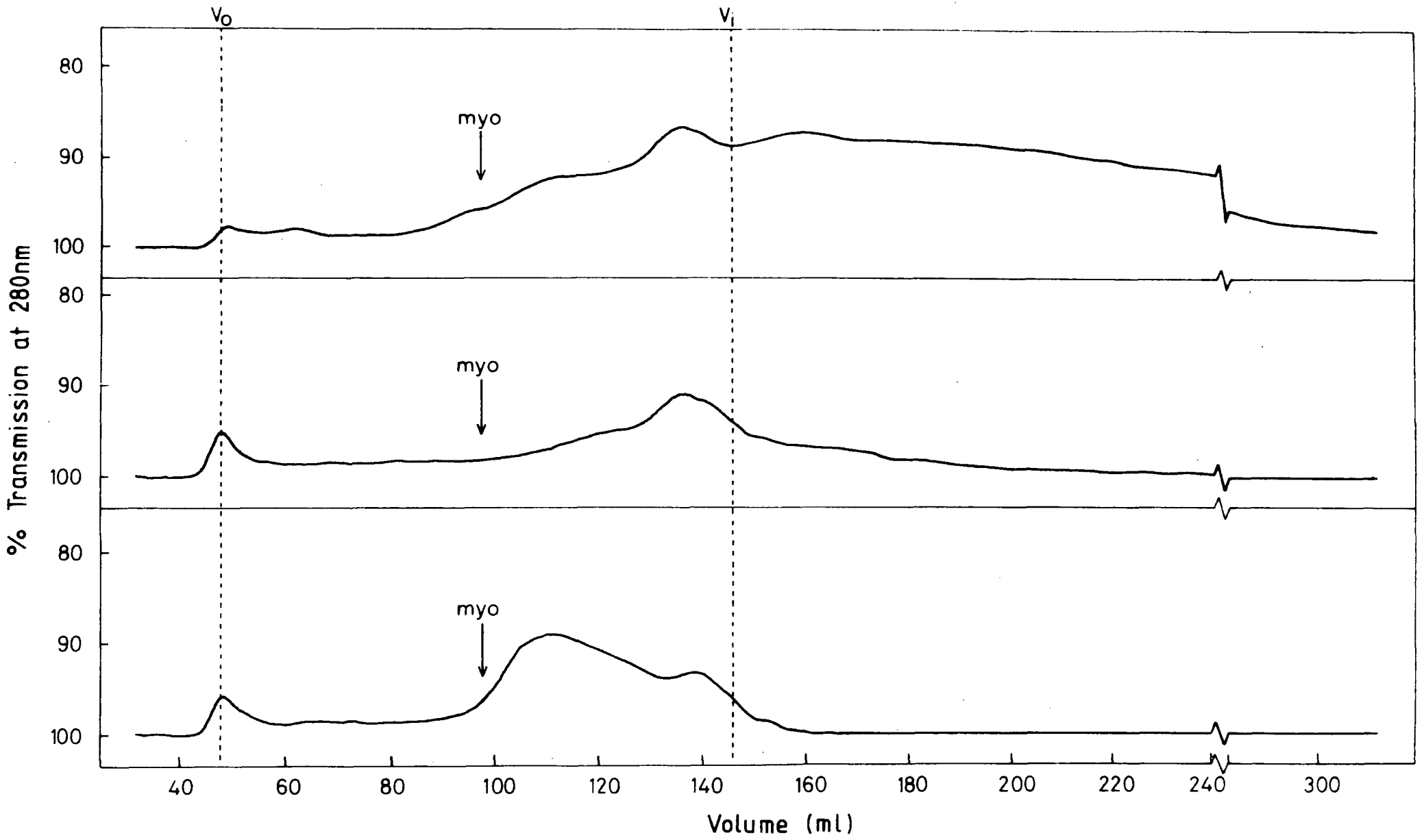
(a) Eluant: 0.05M Tris-HCl, 0.15M NaCl, pH 8.3.
Protein concentration: 24.8 mg/ml.

(b) Eluant: 0.05M Tris-HCl, 0.15M NaCl, pH 8.3.
Protein concentration: 6.6 mg/ml.

(c) Eluant: 0.02M sodium borate, 0.15M NaCl, 0.1M glucose, pH 7.3.
Protein concentration: 6.6 mg/ml.

The elution volume, Ve, of a reference protein, myoglobin (myo), is indicated.

Elution profiles obtained after chromatography of the storage protein in 0.1M sodium phosphate, 0.15M NaCl, pH 6.2 were similar to those of (a) and (b).


V_o
V_i
myo
myo
myo
80
90
100
80
90
100
80
90
100
% Transmission at 280nm
40
60
80
100
120
140
160
180
200
220
240
300
Volume (ml)

collected after chromatography of the storage protein gave the same sub-unit profiles as the original although the heterogeneous nature of the unreduced sub-unit was more marked.

The elution profile obtained after chromatography on a column of Ultrogel consisted of two poorly separated, broad and asymmetrical peaks. By reference to a calibration curve describing the elution behaviour of standard proteins, the first peak represented protein with a molecular weight of around 31,600, but the other, a molecular weight of less than 10,000.

M. Some Investigations to Determine the Cause of Interaction with Column Matrices containing Polysaccharide:

(1) Diffusion against Glycoproteins in Plates of Agarose:

Diffusion of the storage protein in a supporting medium of agarose against the glycoproteins thyroglobulin, ovalbumin and fetuin, and the lectin concanavalin A (Con A) showed that none of the proteins interacted with one another except for Con A and thyroglobulin, between which a sharp precipitation line formed.

(2) Haemagglutination Assays:

Assays of the storage protein for haemagglutination activity using rabbit blood, both trypsin treated and untreated, and solutions of Con A and saline phosphate buffer as both positive and negative agglutination controls respectively gave negative results for all of several preparations which were variously treated prior to the assays. These treatments included extensive dialysis against phosphate buffer 0.05M, pH 8.2, or borate buffer 0.05M, pH 8.4 and then dialysis against saline phosphate buffer; as well as extensive dialysis against 1M glucose in 0.05 Tris-HCl, 0.15 M NaCl, pH 8.3, then against 0.05 M Tris-HCl and finally the saline phosphate buffer.

N. Polyacrylamide Gel Electrophoresis under apparent Non-dissociating Conditions:

(1) Separation at Alkaline pH Values:

The profile obtained after electrophoresis on gels (T = 8%, C = 5%) under alkaline conditions (separation at pH 9.5) consisted of several bands (fig. 45), all of which were shown by two-dimensional PAGE (first dimension, PAGE under non-dissociating conditions, second dimension, SDS PAGE) to have the same mobility as the major sub-unit (fig. 46).

Increasing the concentration of protein electrophoresed caused a greater relative increase in the stain intensity of the slowest migrating band (fig. 45, tracks 4-7), which also increased in zone width in a manner which indicated the appearance of protein species of lesser relative mobility. In addition, above each of the succeeding bands, slightly slower migrating protein bands became progressively more intensely stained. Electrophoresis of a lyophilised preparation also showed this effect, but compared to an unlyophilised preparation of similar concentration, the slowest migrating band was always more intensely stained.

(2) Separation at Acidic pH Values:

The profile obtained after separation at acidic pH value (ionic strength 0.02) consisted of only two visible (broad) bands (fig. 47, tracks 3-5; fig. 15, track 5), both of which migrated further relative to the bands in the alkaline system. The band with greater mobility stained with considerably greater relative intensity; however, in contrast to the effect observed in the alkaline system, variation in the concentration of protein electrophoresed caused a corresponding variation in the intensity of stain taken up by both bands (fig. 47, tracks 3-5).

Fig. 45

Electropherogram (8 x 5) of the storage protein separated by non-dissociating PAGE, alkaline buffer system B (see methods V B.3(i)).

Sample volume: 20 µl.

Tracks 1-3: reference proteins: aldolase (track 1), β-lactoglobulin and γ-globulin (track 2), and ferritin and myoglobin (track 3).

Tracks 4-7: unlyophilised storage protein, 0.61 mg/ml, 1.23 mg/ml, 2.45 mg/ml and 4.9 mg/ml respectively. (Protein concentration determined from E^{1cm}_{280} values, see legend fig. 39).

Tracks 8-10: unlyophilised storage protein (1.23 mg/ml) electrophoresed together with β-lactoglobulin and γ-globulin (track 8), myoglobin and ferritin (track 9), and aldolase (track 10).

The positions of ferritin (fer), myoglobin (myo) and β-lactoglobulin (β-lac) are indicated.

See also fig. 14, track 8, for separation of a lyophilised preparation of the storage protein.

Fig. 46

Separation of the storage protein preparation by two-dimensional PAGE.

First dimension: Separation as for fig. 45. Protein concentration 2.45 mg/ml.

Second dimension: Separation, after pre-treatment of the first dimensional rod gel with SDS and 2-ME, on PAG (17.6 x 0.45), discontinuous SDS buffer system.

(See methods V D.1(iii)).

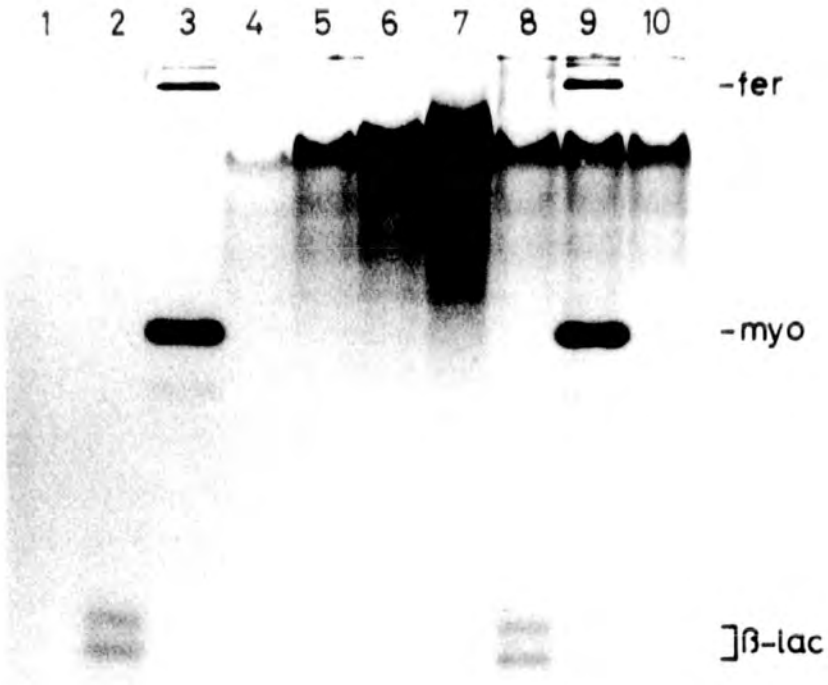


Fig.45

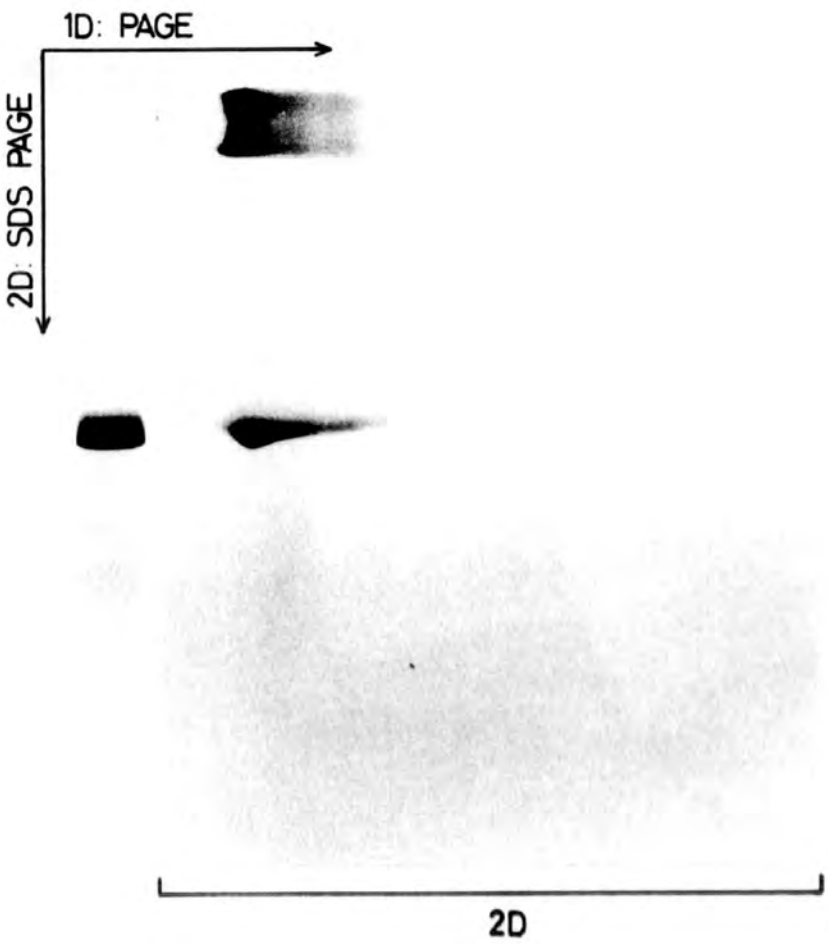


Fig.46

Fig. 47

Electropherogram (8 x 5) of the storage protein separated by non-dissociating PAGE, acidic buffer system B (ionic strength 0.0206; see methods V B.3(ii)).

Sample volume: 20 µl.

Tracks 1 and 2: reference proteins: γ-globulin (γ-gl) and β-lactoglobulin (β-lac) (track 1); ferritin (fer) and cytochrome-c (cyt-c) (track 2).

Tracks 3-5: unlyophilised storage protein, 0.31 mg/ml, 0.61 mg/ml and 1.23 mg/ml respectively. (Protein concentrations determined from E_{280}^{1cm} values, see legend fig. 39).

Tracks 6-9: unlyophilised storage protein (0.31 mg/ml) electrophoresed together with γ-globulin and β-lactoglobulin (tracks 6 and 8, 0.5 mg/ml and 1 mg/ml each protein respectively), and ferritin and cytochrome-c (tracks 7 and 9, 0.5 mg/ml and 1 mg/ml each protein respectively).

Tracks 10 and 11: as for tracks 6 and 7, but with increased concentration of the storage protein (1.23 mg/ml).

(See also fig 15, track 5 for separation of a lyophilised preparation of the storage protein).

Fig. 48

Electropherogram of the storage protein separated by non-dissociating PAGE, acidic buffer system C (ionic strength 0.0021; see methods V B.3(ii)).

Sample volume: 20 µl.

Track 1: reference proteins: γ-globulin (γ-gl) and β-lactoglobulin (β-lac).

Tracks 2-6: unlyophilised storage protein, 0.31 mg/ml, 0.61 mg/ml, 1.23 mg/ml, 2.45 mg/ml and 4.9 mg/ml respectively. (Protein concentration determined as for fig. 47).

Tracks 7 and 8: unlyophilised storage protein (1.23 mg/ml) electrophoresed together with γ-globulin and β-lactoglobulin, each at 0.5 mg/ml (track 7), then 1 mg/ml (track 8).

Track 9: as for track 7 but with increased concentration of the storage protein (2.45 mg/ml).

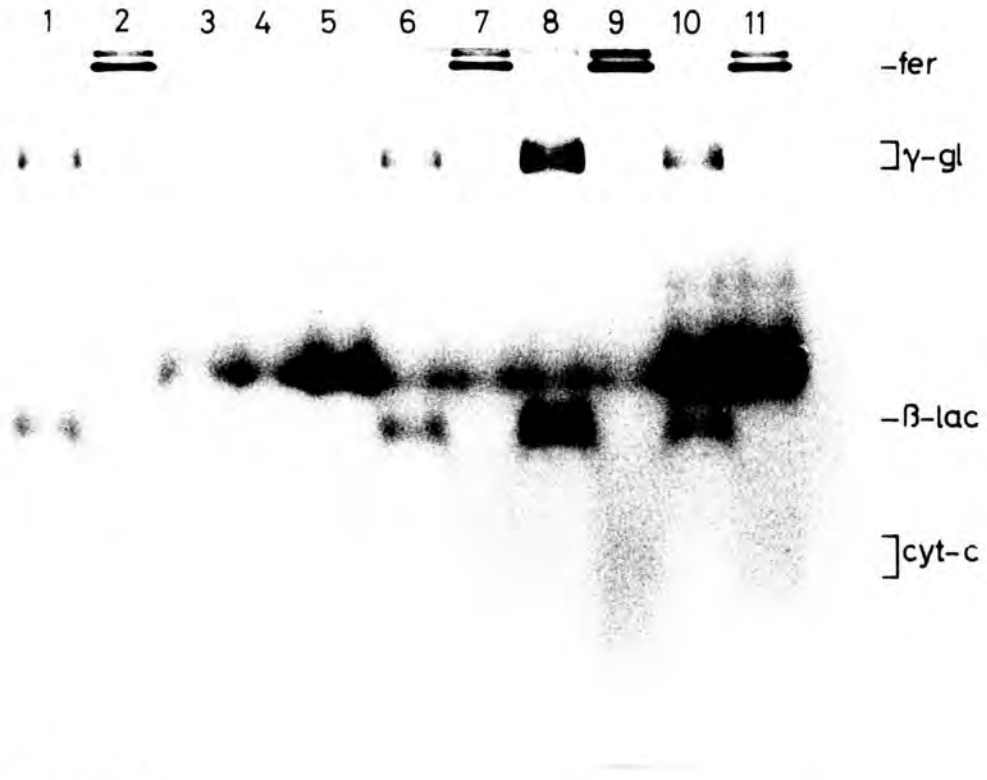


Fig. 47

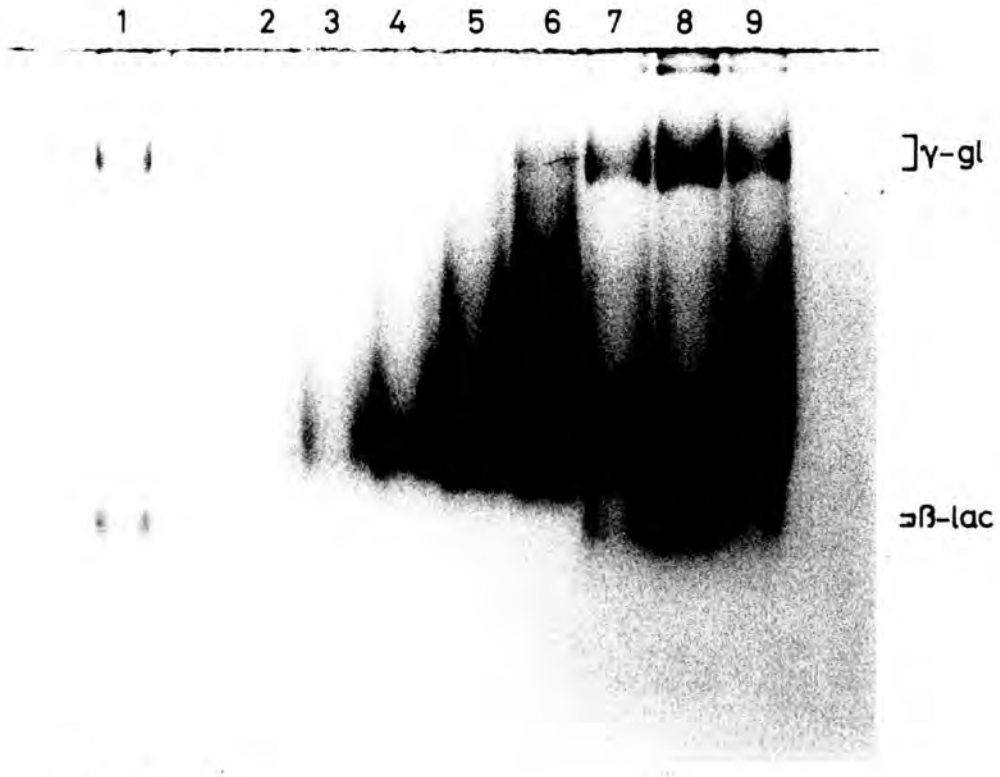


Fig. 48

Separation at low ionic strength but at the same acidic pH value and gel concentration resulted in a blurred broad zone for both bands, (fig. 48), the densitometric scan of which suggested several bands in each zone.

(3) Physicochemical use of PAGE to Elucidate the Nature of the Protein Species observed in both the Acid and Alkaline Buffer Systems:

Densitometric scans depicting the electrophoretic separation of the storage protein (constant concentration), and internal standard proteins on gels of varying total acrylamide concentration (%T) but constant cross-linking (%C) are given in fig. 49 (alkaline separation) and fig. 50 (acid separation), and results from electrophoresis on slab gels prepared with a horizontal gradient of acrylamide in fig. 51.

The two internal standards used in each system were selected after verifying, by prior experiment on gels of 8% acrylamide concentration, that they did not affect the mobility of the storage protein, and did not migrate to the same position (see fig. 45, tracks 8 and 9, and fig.47 tracks 6, 8 and 10). They were also selected on the basis of having different retardation coefficients (Kr values) and different isoelectric points, the isoelectric point of one of them being near the pH value of separation in each system. The value of the ratio of their relative mobilities (Rm) then provided sensitive standardisation for each electrophoretic run performed.

Analysis of the variation of these ratios determined for each gel concentration, duplicated within each run, and for all the electrophoretic runs, indicated (within each system, and for each gel concentration) that the variation arising from differences between electrophoretic runs was no greater than that arising from within each run. The latter source of variation was primarily due

to error in measurement of the distance migrated by the tracking dye (methods, V.E) and hence the Rm value. Rm values of protein bands from the storage protein preparation were accordingly determined after electrophoretic separation on several replicate gel concentrations, although ultimately the precision of estimates was limited because of the large number of different component protein species which migrated with similar relative mobility.

Ferguson plots constructed from the data are given in figs. 52 and 53. Good fits to the data were obtained for the standard proteins, substantiating the validity of the experimental technique.

The electrophoretic patterns obtained for the storage protein preparation in the alkaline system were complex (fig. 49). Ferguson plots (fig. 52(a)) constructed for the main components indicated firstly that there were a number of different protein species, designated in the figs. by the symbol m, which had very similar molecular surface area (plots of the same slope), but different apparent free electrophoretic mobility (different y-intercepts), hence net charge. The retardation coefficient, Kr, of these proteins (slope of the lines) was 0.057, which,by reference to a simple calibration curve relating the $\sqrt{Kr}$ and molecular radius of the standard proteins (fig. 8), indicated an approximate molecular radius of 2.17, and therefore, by comparison with the value for the molecular radius of β-lactoglobulin, a molecular weight of around 35,000, corresponding to that of the major sub-unit (monomer).

There were also two protein species (designated by the symbol d in figs. 49 and 52(a)) which again had very similar molecular surface areas, but different net charge. The retardation

coefficient of these, 0.088, corresponded approximately to that anticipated for the dimer.

The most intensely stained protein band (t) had a retardation coefficient of 0.131, identifying it as the tetramer.

In the acid system only three somewhat broad protein bands were in evidence (fig. 50), the Ferguson plots of which (fig. 53) indicated three protein species of different size. The measured retardation coefficients indicated that the most intensely stained band (m) corresponded to that of the monomer (Kr value 0.045), the second (d) to that of dimer (Kr value 0.072), and the third (t) to that of tetramer (Kr value 0.102). The width of the bands tended to point to some degree of heterogeneity of either net charge or shape amongst the three protein species.

Changes in conformation of monomer, dimer and tetramer in particular, in the different ionic environments were indicated by the differences in Kr values determined for each protein species in the acid and alkaline systems, those in the acid system being lower.

Fig. 49(a)-(f)

Densitometric profiles depicting the electrophoretic separation of protein components of the storage protein preparation, and two standard proteins, ferritin (mol. wt. 450,000, pI value c. 4.4), and β-lactoglobulin (mol. wt. 35,000, pI c. 5-5.3) on polyacrylamide gels of varying total (% T) acrylamide concentration, but constant cross-linker (% C) concentration.

(See methods V.E for details).

Buffer system: alkaline, system B.

%C: 5.

Sample volume: 20 µl.

Storage protein concentration: 1.23 mg/ml (determined from E_{280}^{1cm} value, see legend fig. 39).

Ferritin concentration)
β-lactoglobulin concentration) 0.5 mg/ml (d.b.)

m_1-m_6 : monomer components of the storage protein preparation.

d_1, d_2 : dimer components of the storage protein preparation

t : tetramer

fer : ferritin

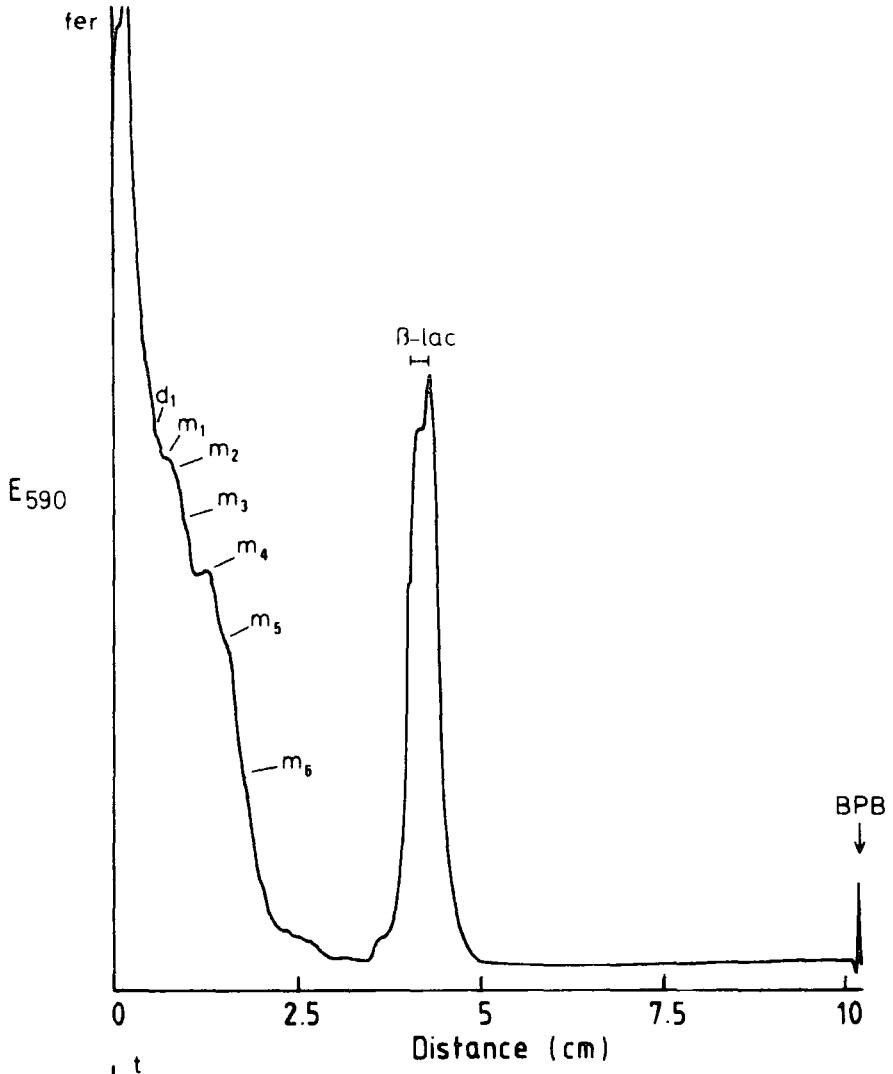
β-lac : β-lactoglobulin

BPB : bromophenol blue

The identification of monomer, dimer and tetramer components in the storage protein preparation has been based upon results obtained after construction of Ferguson plots for the individual protein components separated. (See fig. 52(a) and text).

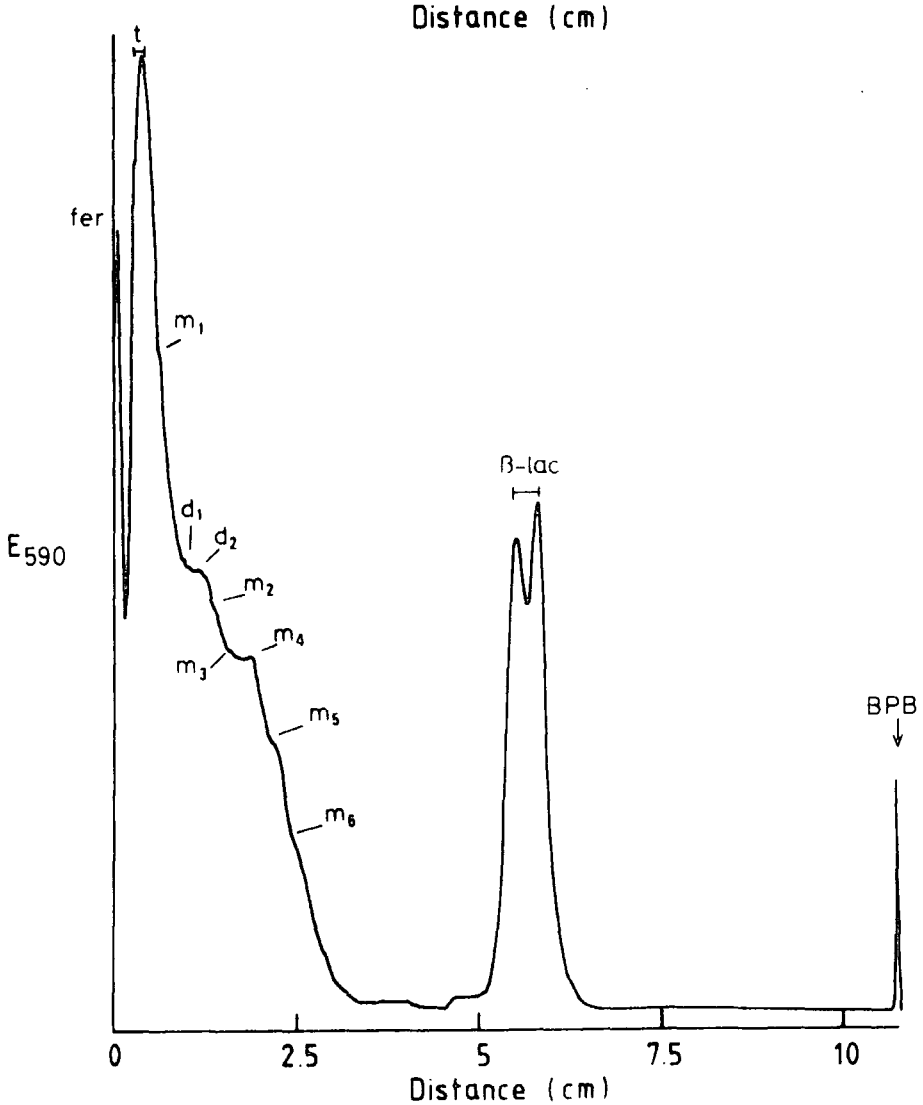
(a)

T=14%
C= 5%



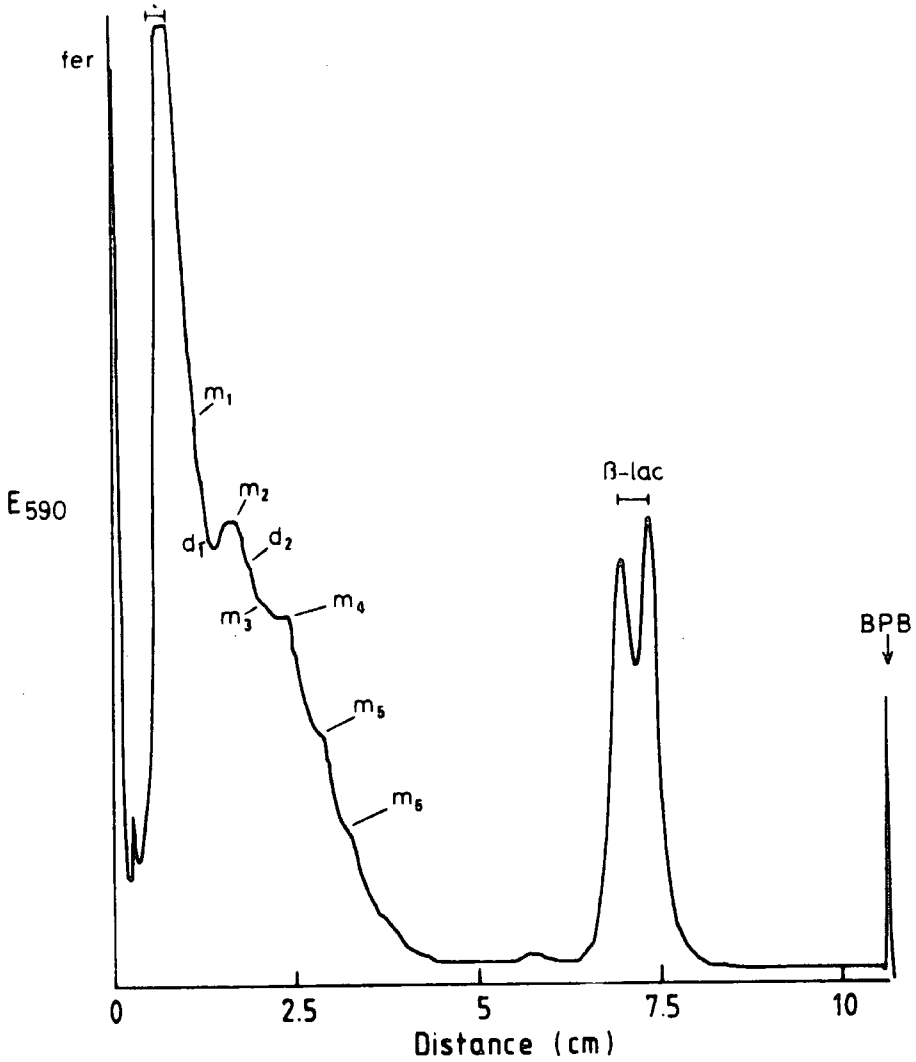
(b)

T=12%
C= 5%



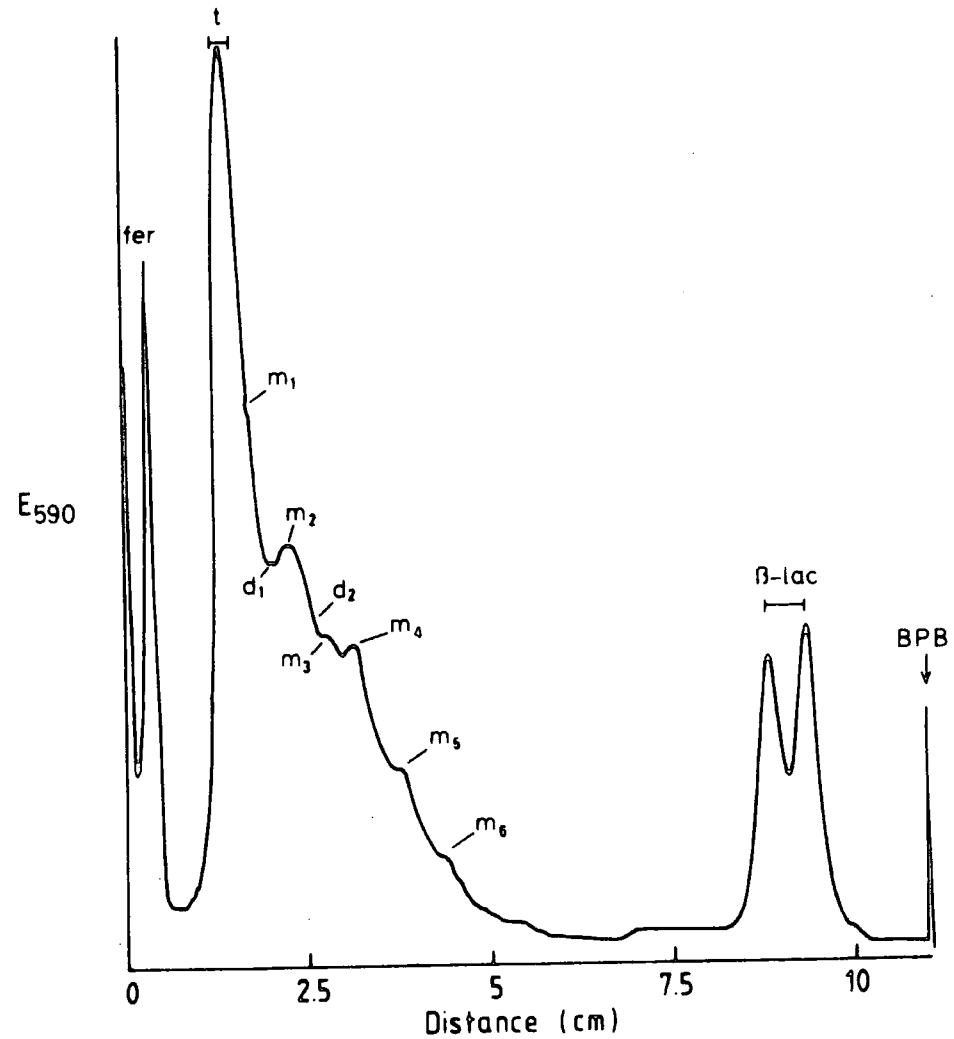
(c)

T = 10%
C = 5%



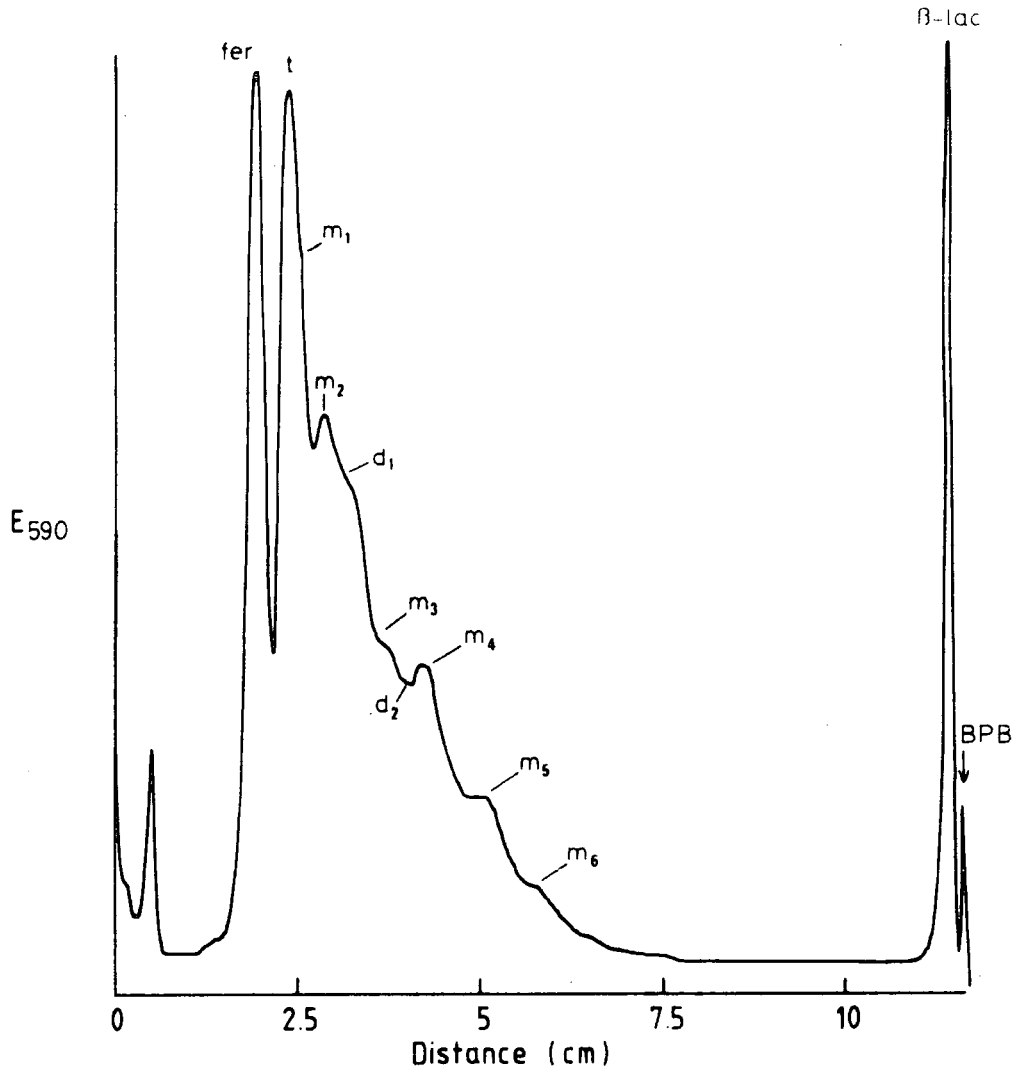
(d)

T = 8%
C = 5%



(e)

T = 6%
C = 5%


ß-lac
fer
t
m_1
m_2
d_1
m_3
m_4
d_2
m_5
m_6
BPB
E_{590}
0
2.5
5
7.5
10
Distance (cm)

(f)

T = 4%
C = 5%

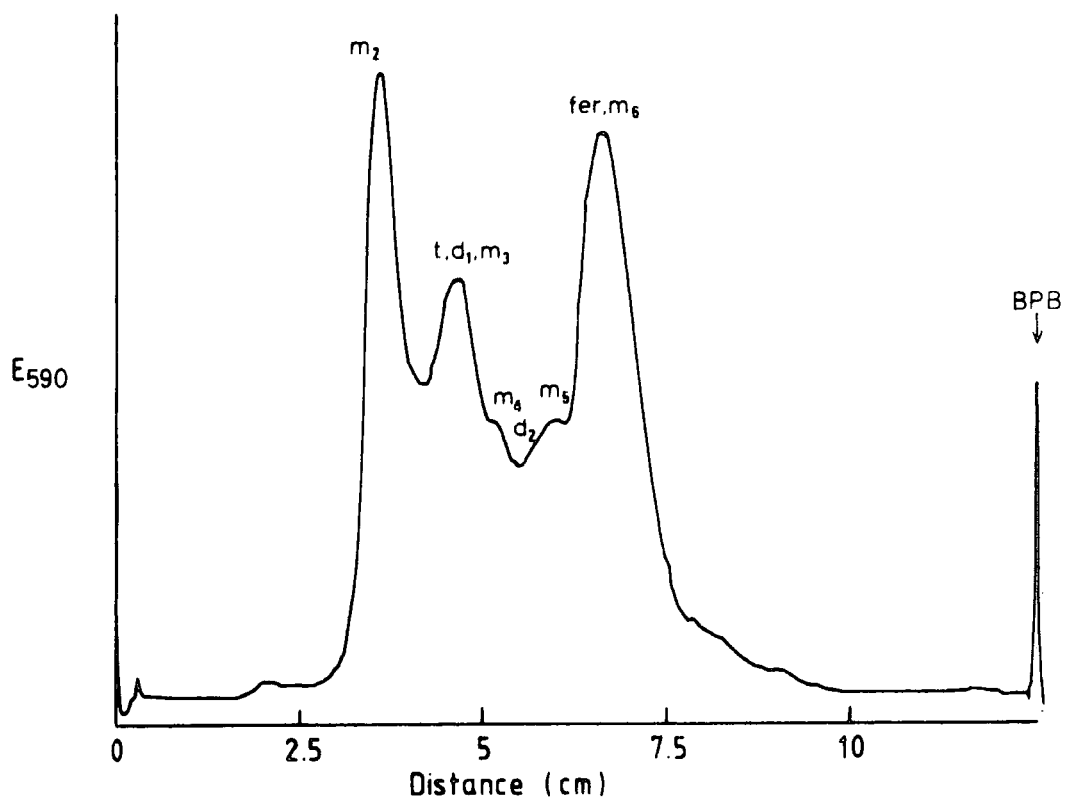

m_2
fer, m_6
t, d_1, m_3
m_4
m_5
d_2
BPB
E_{590}
0
2.5
5
7.5
10
Distance (cm)

Fig. 50(a)-(f)

Densitometric profiles depicting the electrophoretic separation of protein components of the storage protein preparation, and two standard proteins, γ-globulin (mol. wt. 160,000, pI value c. 8.6) and ß-lactoglobulin (mol. wt. 35,000, pI value c. 5-5.3) on polyacrylamide gels of varying total (% T) acrylamide concentration, but constant cross-linker (% C) concentration.

(See methods V.E for details).

Buffer system: acidic, system B.

% C: 5

Sample volume: 20 µl

Storage protein concentration: 1.23 mg/ml (determined from E_{280}^{1cm} value, see legend fig. 39).

γ-globulin concentration)
ß-lactoglobulin concentration) 0.5 mg/ml (d.b.)

m: monomer

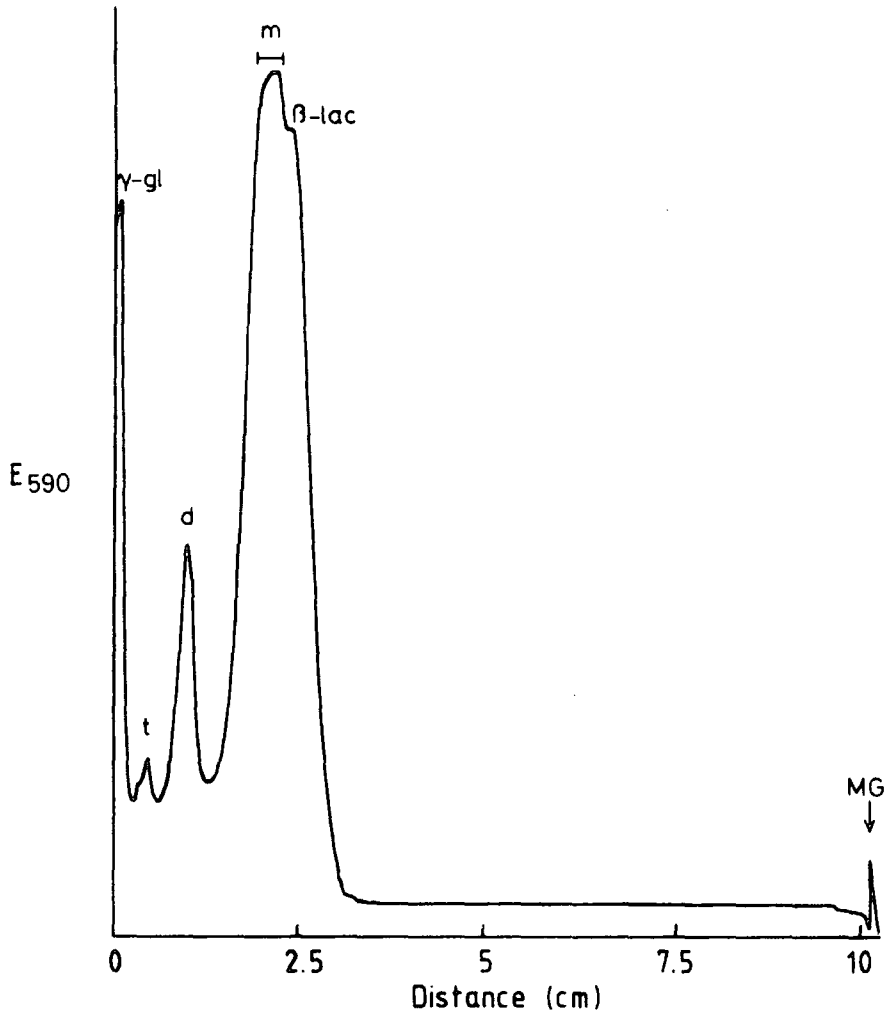
d: dimer

t: tetramer

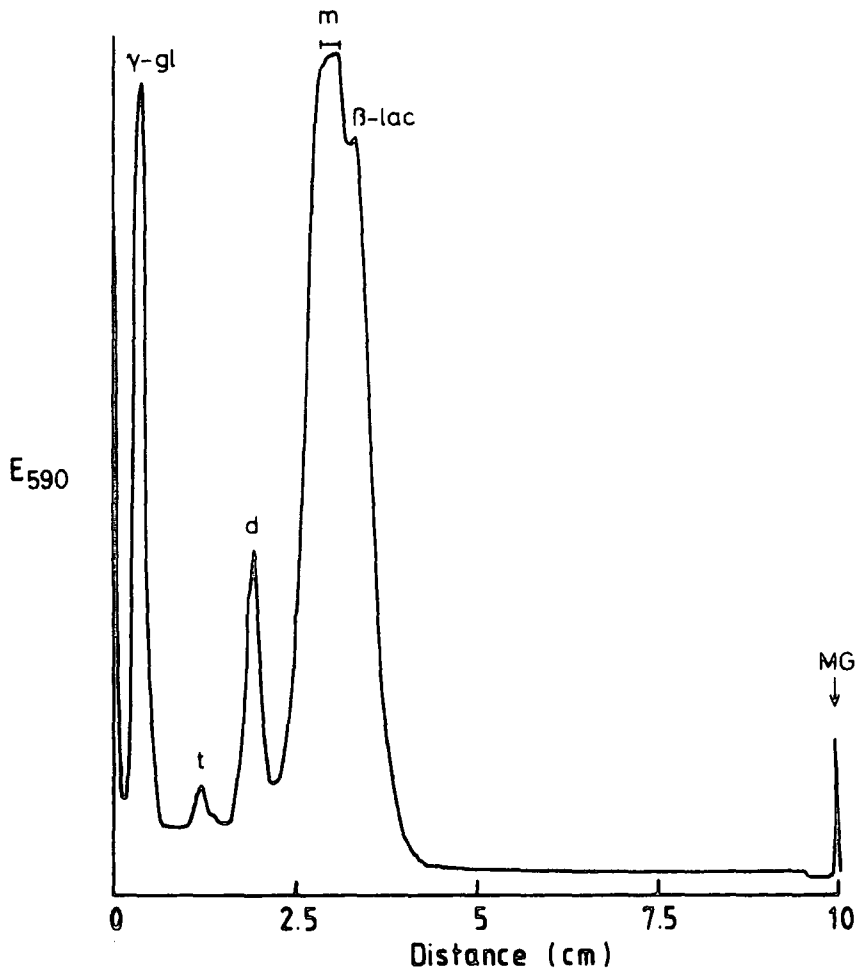
MG: methyl green

The identification of monomer, dimer and tetramer components in the storage protein preparation has been based upon results obtained after construction of Ferguson plots for the individual protein components separated (see fig. 53, and text).

(a)
T=15%
C= 5%



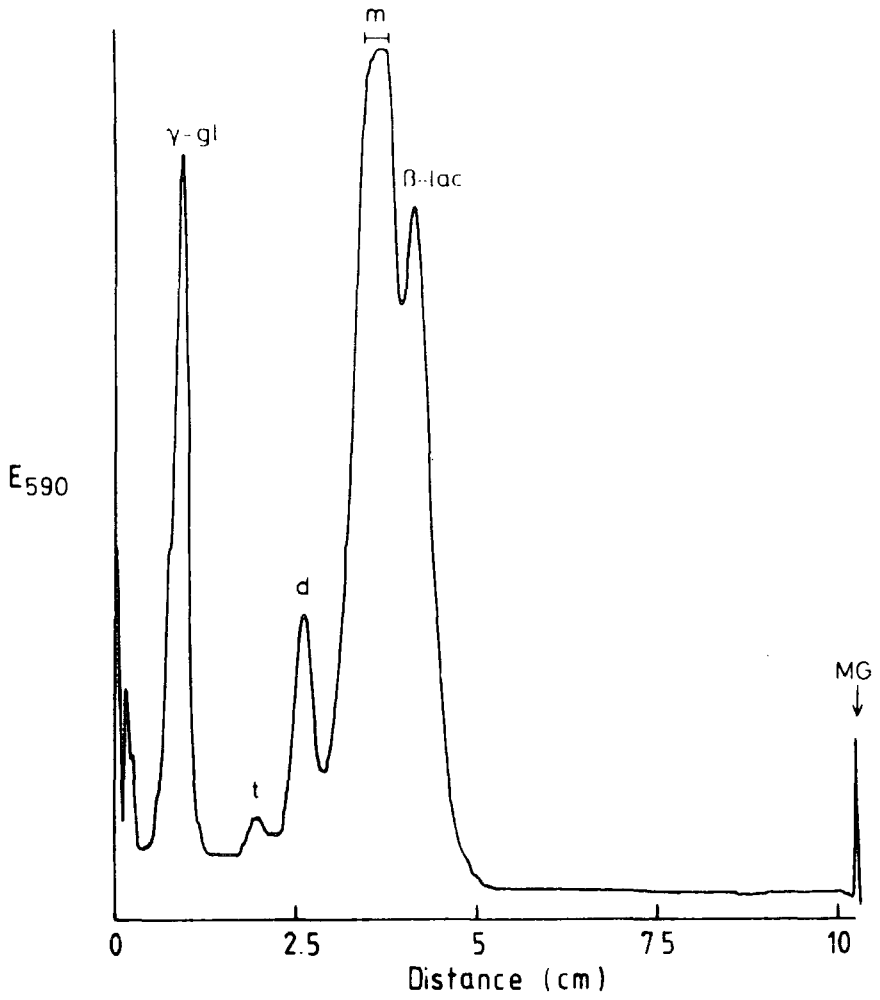
(b)
T=12%
C= 5%



(c)

T=10%

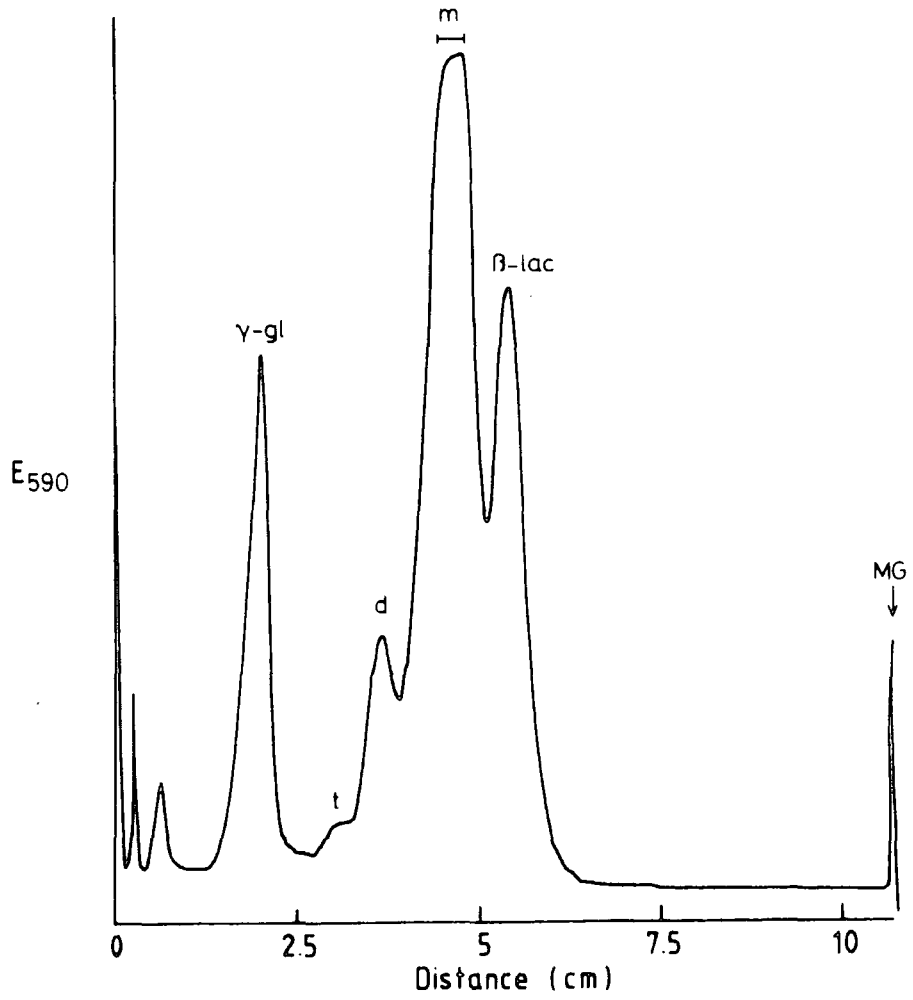
C= 5%



(d)

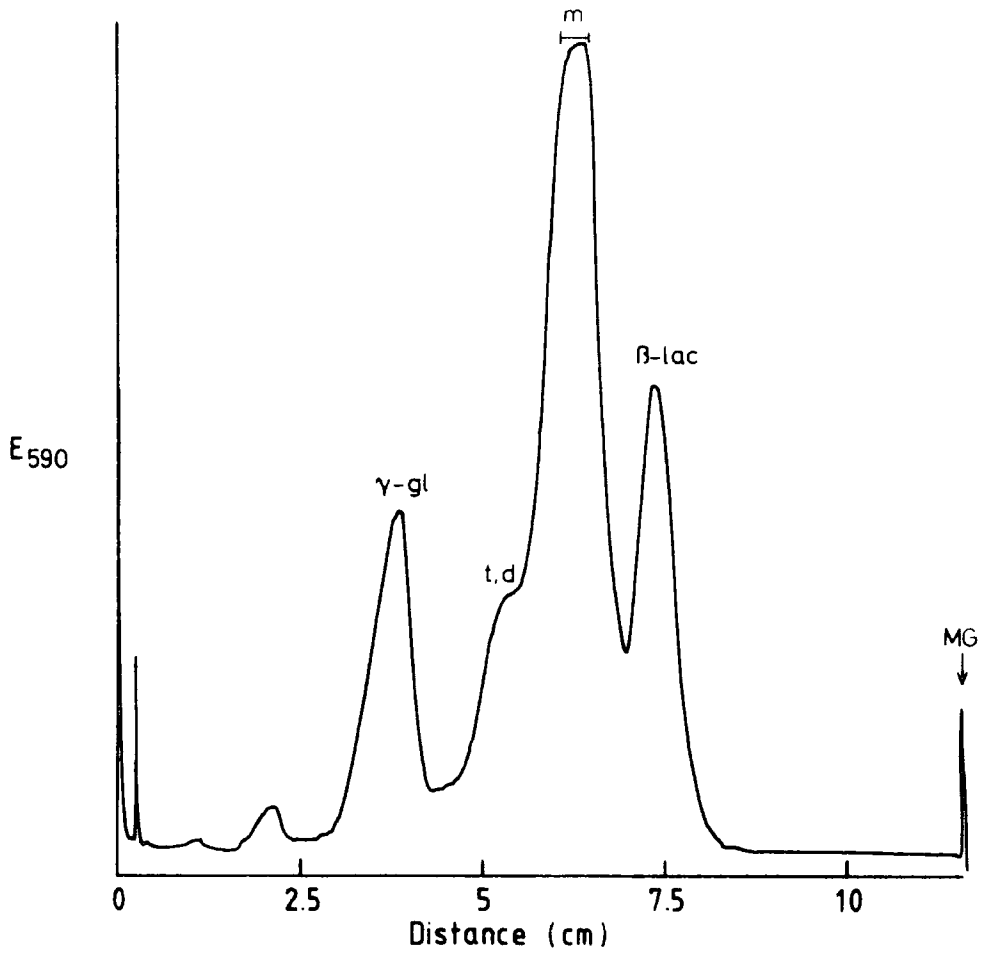
T=8%

C=5%



(e)

T=6%
C=5%


m
ß-lac
γ-gl
t,d
MG
E_{590}
0
2.5
5
7.5
10
Distance (cm)

(f)

T=4%
C=5%

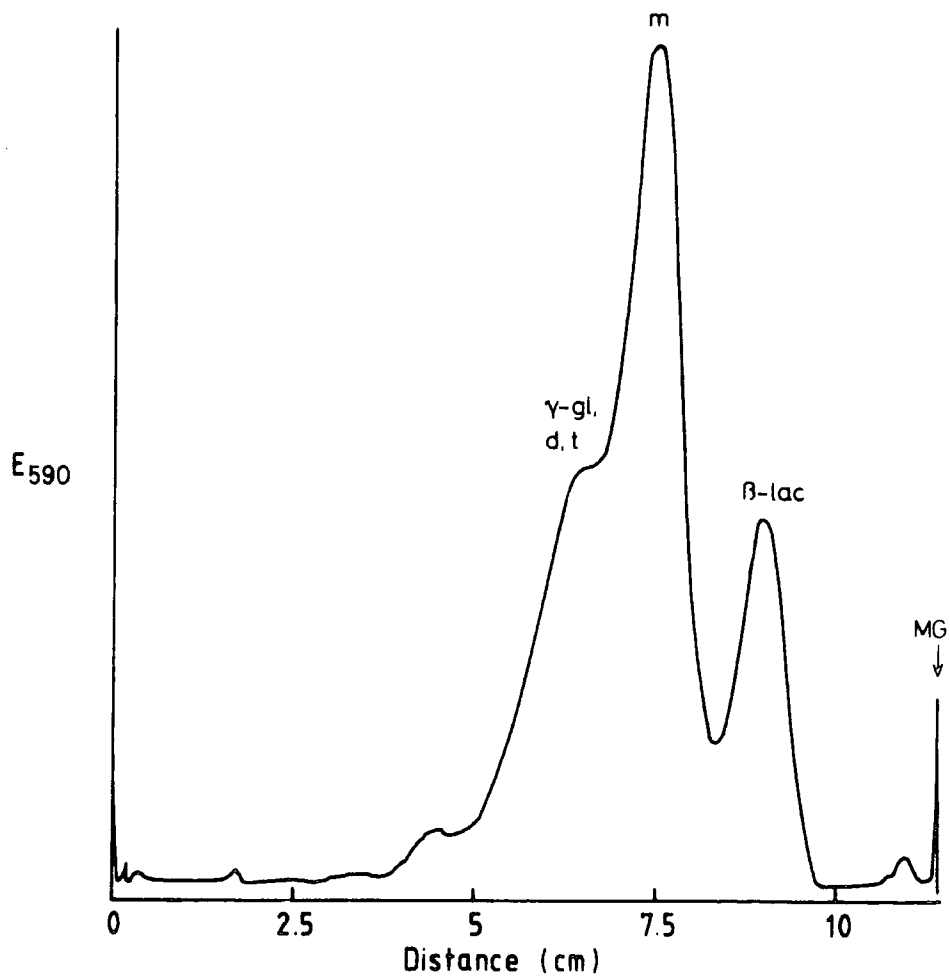

m
γ-gl,
d, t
ß-lac
MG
E_{590}
0
2.5
5
7.5
10
Distance (cm)

Fig. 51

Electrophoretic separation of the storage protein preparation (fig. 51(a)), and of the storage protein preparation together with two standard proteins, γ-globulin (γ-gl) and β-lactoglobulin (β-lac) (fig. 51(b)) on a transverse gradient of PAG concentration, from T = 15% to T = 4%. (See methods V D.3).

% C: 5%

Sample volume: 750 µl

Storage protein concentration: 1.23 mg/ml (determined from E_{280}^{1cm} value, see legend fig. 39)

(a) Alkaline buffer system B.

(b) Acidic buffer system B. The bands corresponding to the two standard proteins, γ-gl and β-lac are indicated.

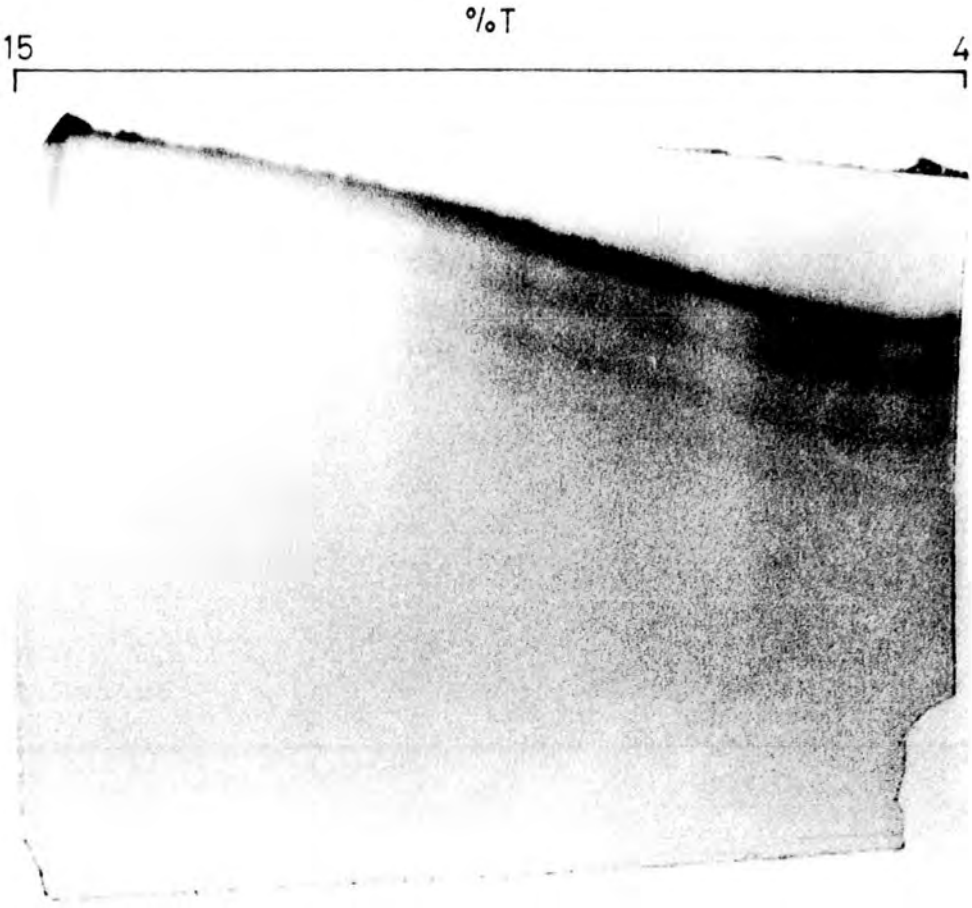


Fig. 51(a)

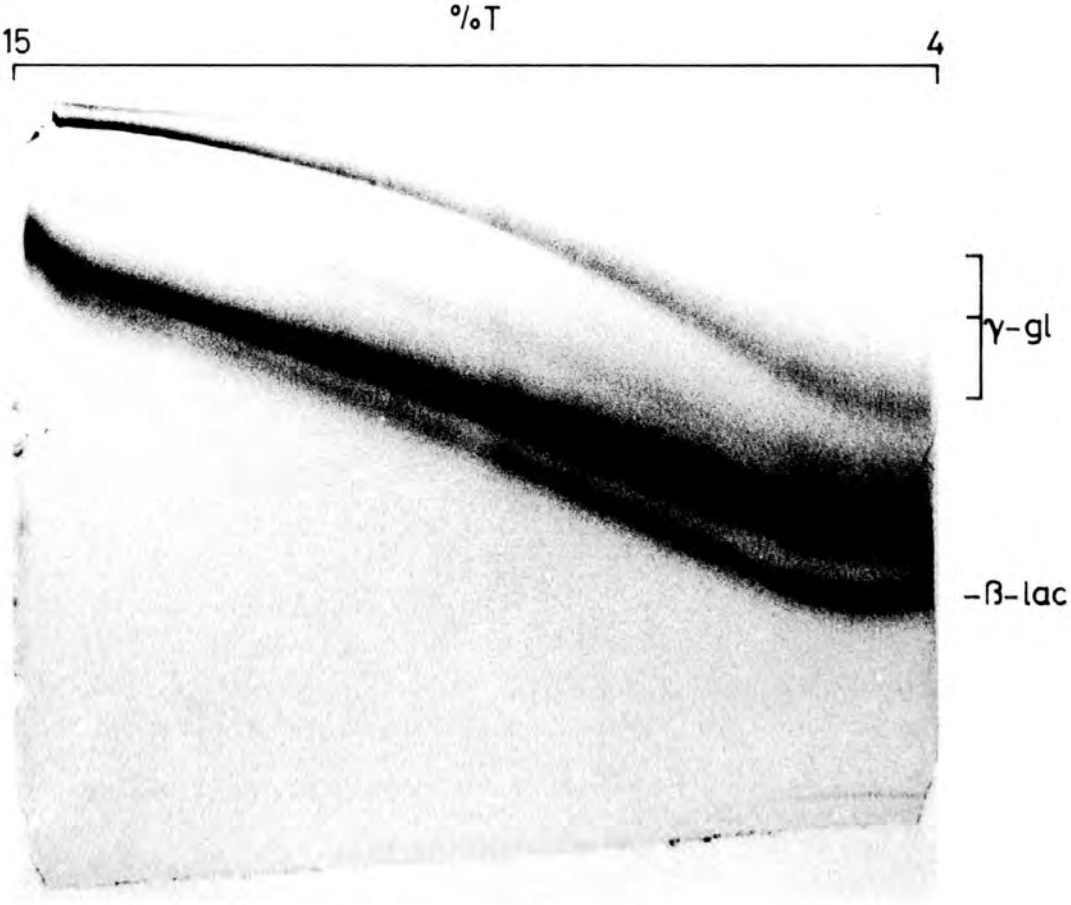


Fig. 51(b)

Fig. 52(a)

Ferguson plots constructed by regression analysis for the main protein components of the storage protein preparation separated by non-dissociating PAGE, alkaline buffer system B.

Rm values were obtained from analysis of densitometric profiles such as those depicted in fig. 49(a)-(e). (Data from separation on T = 4% gels (fig. 49(f)) has been excluded from the plots because of the greater deviation of the assigned value of T, 4%, from the true value).

Experimental details and symbols as for fig. 49.

Fig. 52(b)

Ferguson plots constructed by regression analysis for protein standards which were electrophoresed simultaneously with the storage protein components illustrated in fig. 52(a).

Data for the protein standard myoglobin was obtained from one set of gels only, since the migration position of this protein corresponded with those of some of the storage protein components on some of the gels analysed.

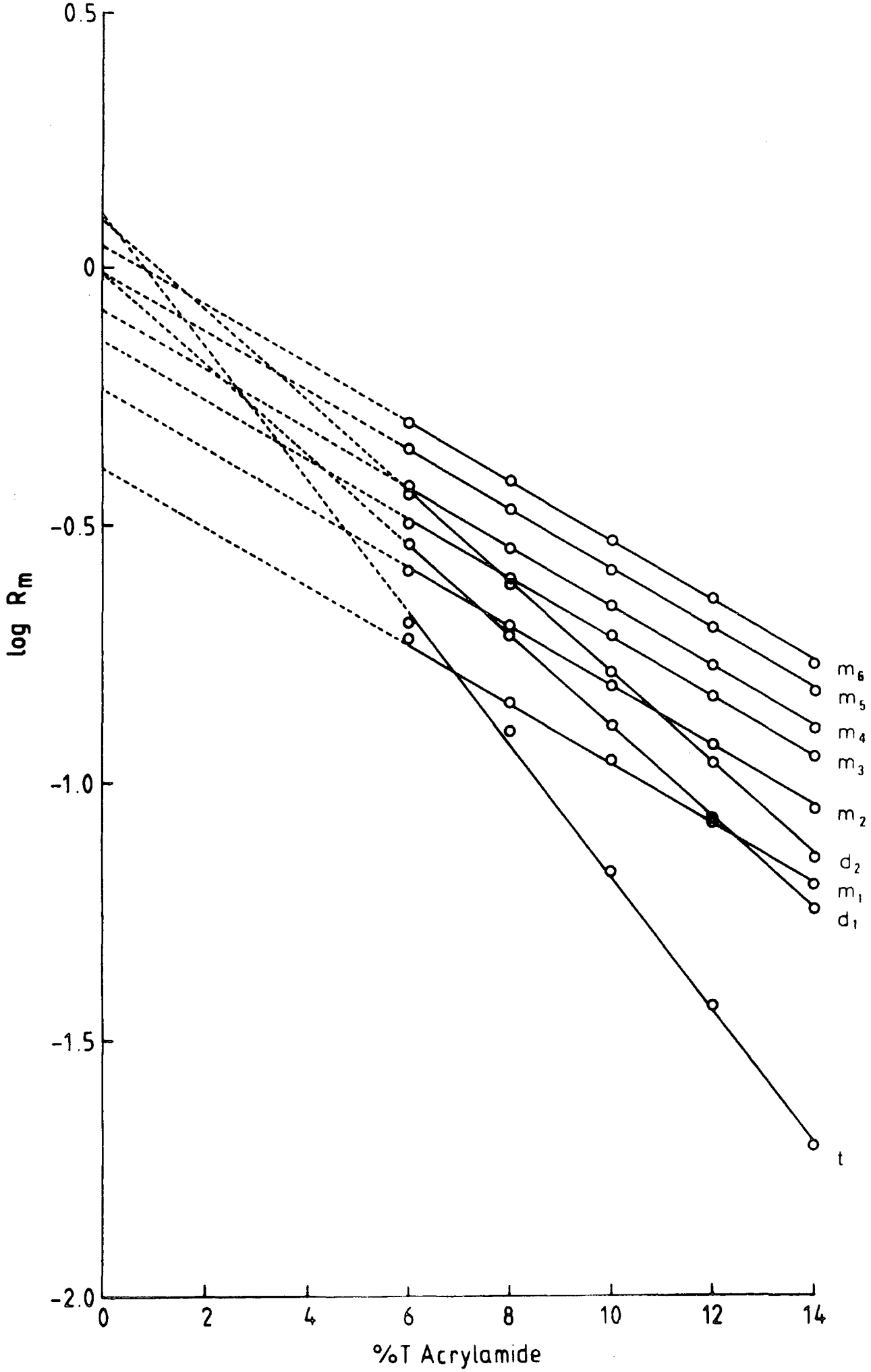

0.5
0
-0.5
-1.0
-1.5
-2.0
log R_m
0
2
4
6
8
10
12
14
%T Acrylamide
m_6
m_5
m_4
m_3
m_2
d_2
m_1
d_1
t

Fig. 52(a)

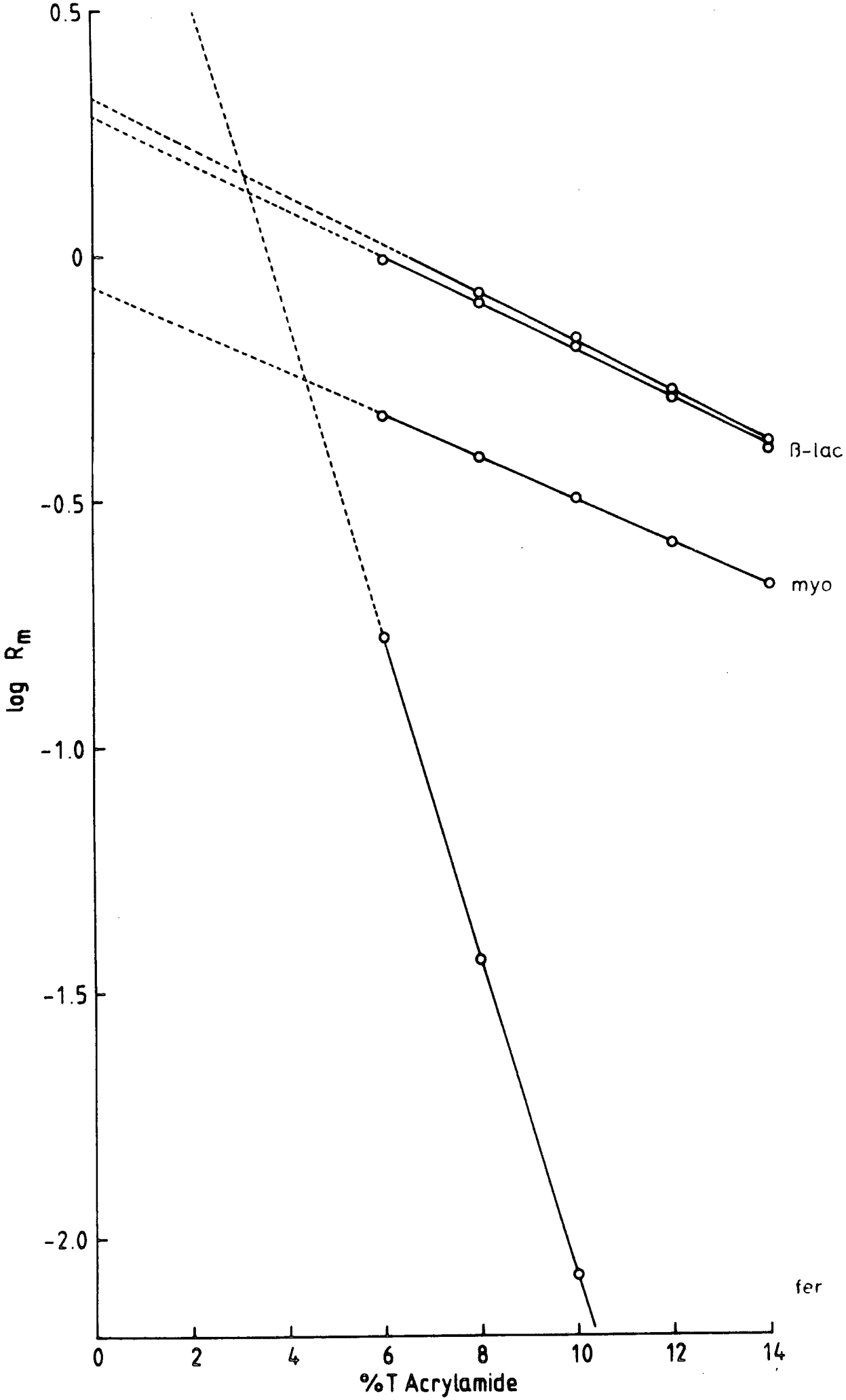


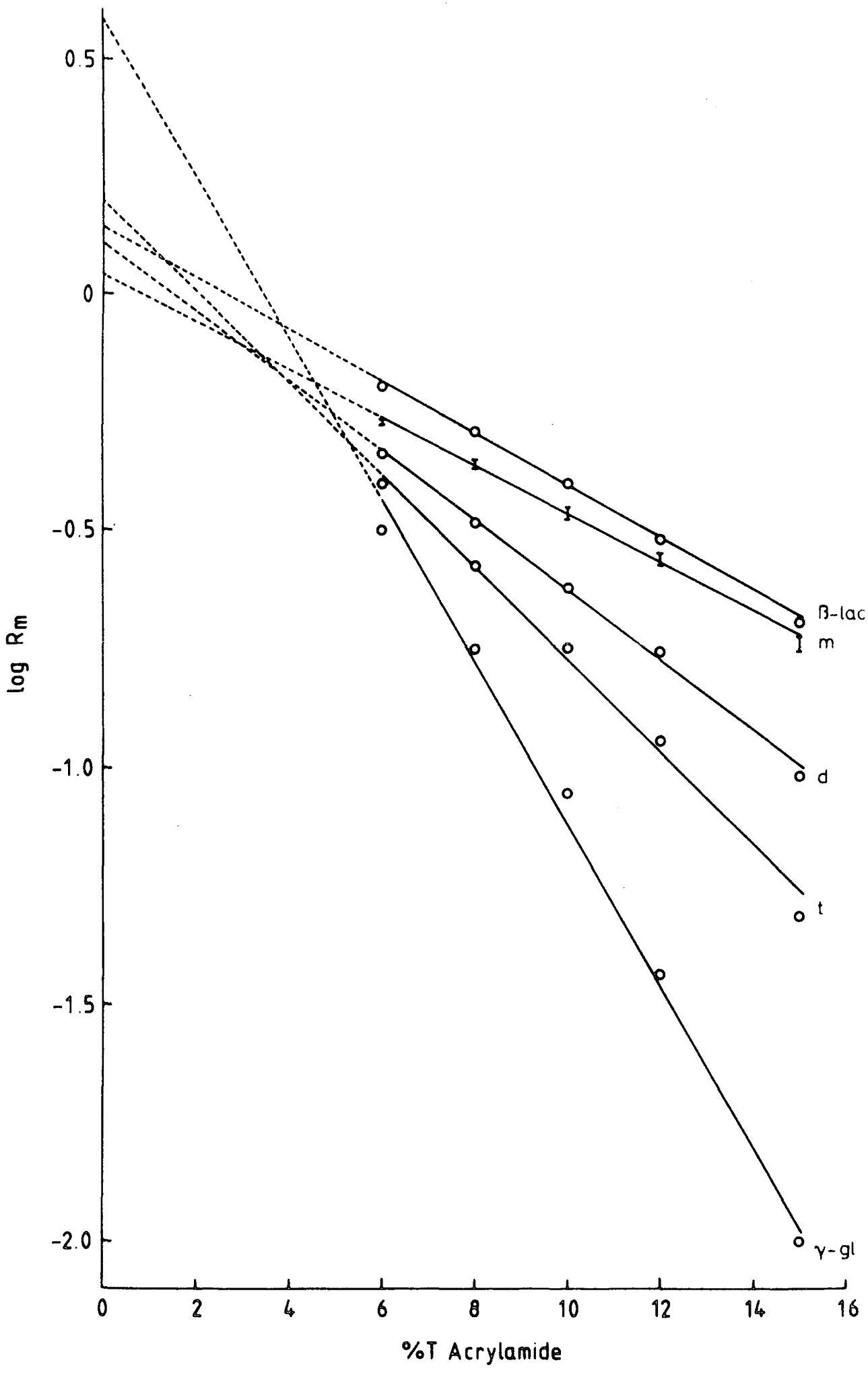
Fig. 52(b)

Fig. 53

Ferguson plots constructed by regression analysis for the main protein components of the storage protein preparation and for the protein standards, γ-globulin and β-lactoglobulin, which were electrophoresed simultaneously with the storage protein preparation under non-dissociating conditions, acidic buffer system B.

Rm values obtained, as for fig. 52, from analysis of densitometric profiles such as those depicted in fig. 50(a)-(e) only.

Experimental details and symbols as for fig. 50.


0.5
0
-0.5
-1.0
-1.5
-2.0
log Rm
0
2
4
6
8
10
12
14
16
%T Acrylamide
ß-lac
m
d
t
γ-gl

VI. HISTOCHEMICAL STUDIES

A. Protein:

(1) General Distribution within the Tuber:

The distribution of uptake of the protein stain Coomassie Blue in a horizontal section of a mature dormant tuber showed that the subepidermal layer stained more intensely for protein than the storage tissue, which was uniformly but faintly stained. Vascular bundles also stained more intensely, but this may have been due to imbibition of stain.

(2) Light Microscopy:

Sections from the storage tissue which were stained with the protein stain toluidine blue (fig. 54) showed a uniform intracellular distribution of, for the most part, spherical, protein "aggregates" of various sizes within the storage cells. Fig. 54(a) shows that neither xylem vessels nor adjacent parenchymal cells appeared to contain these aggregates.

(3) Electron Microscopy:

Electron micrographs showed that the protein aggregates appeared to be contained within both cellular protein vacuoles (fig. 55(a)) and cytoplasmic vesicles (fig. 55(b) and (c)). Fig. 55(d) shows several of the vacuolar protein aggregates contained within a single vacuole, none of which appeared to be surrounded by a limiting membrane. Protein aggregates contained within the cytoplasm may have contracted during fixation in a manner similar to that of the starch grain shown in the same electron micrograph (fig. 55(b), so that the apparent absence of a limiting membrane may be artifactual. The electron micrographs show few mitochondria and no apparent endoplasmic reticulum, both typical of non-metabolically active tissues.

Fig. 54

Sections examined by light microscopy of a mature dormant tuber illustrating the intracellular distribution of protein bodies (aggregates).

(a) magnification x 200

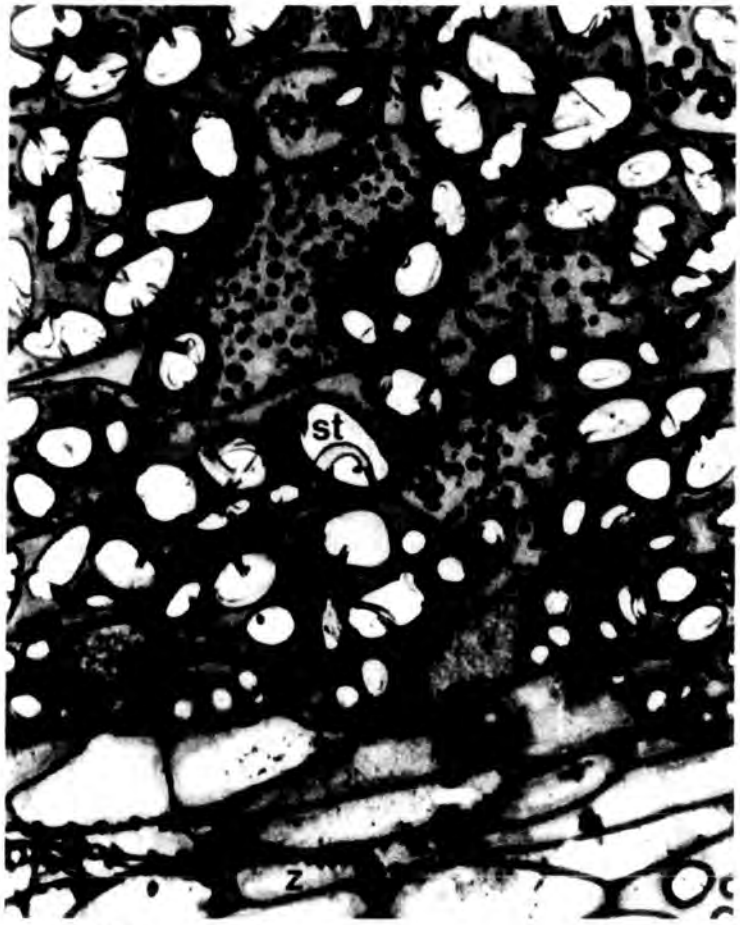
(b) magnification x 300

pb = protein body

st = starch grain

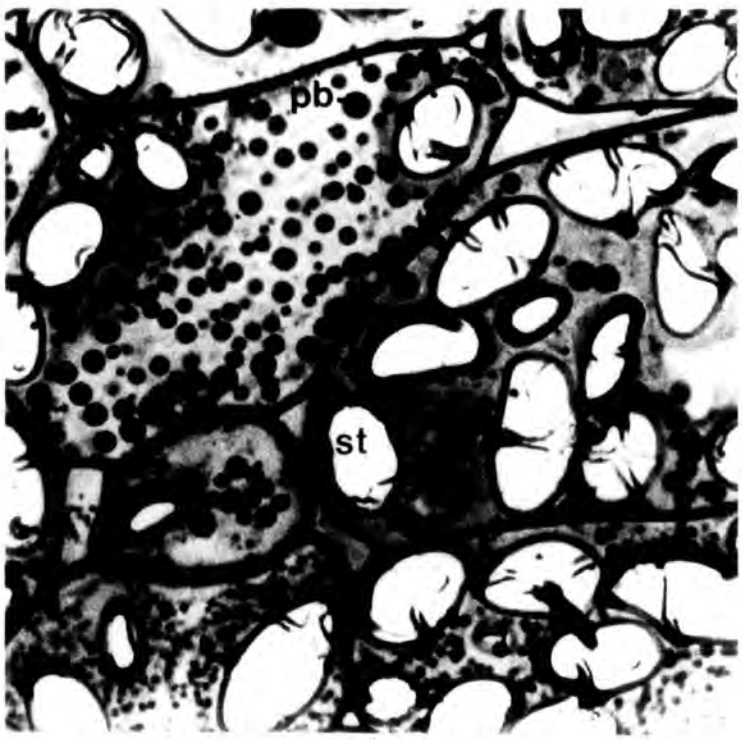
z = xylem

(a)



75µm

(b)



50µm

Fig. 55

Electron micrographs illustrating protein bodies (aggregates) within both cellular protein vacuoles ((a) and (d)), and cytoplasmic vesicles ((b) and (c)).

(a) magnification x 7500

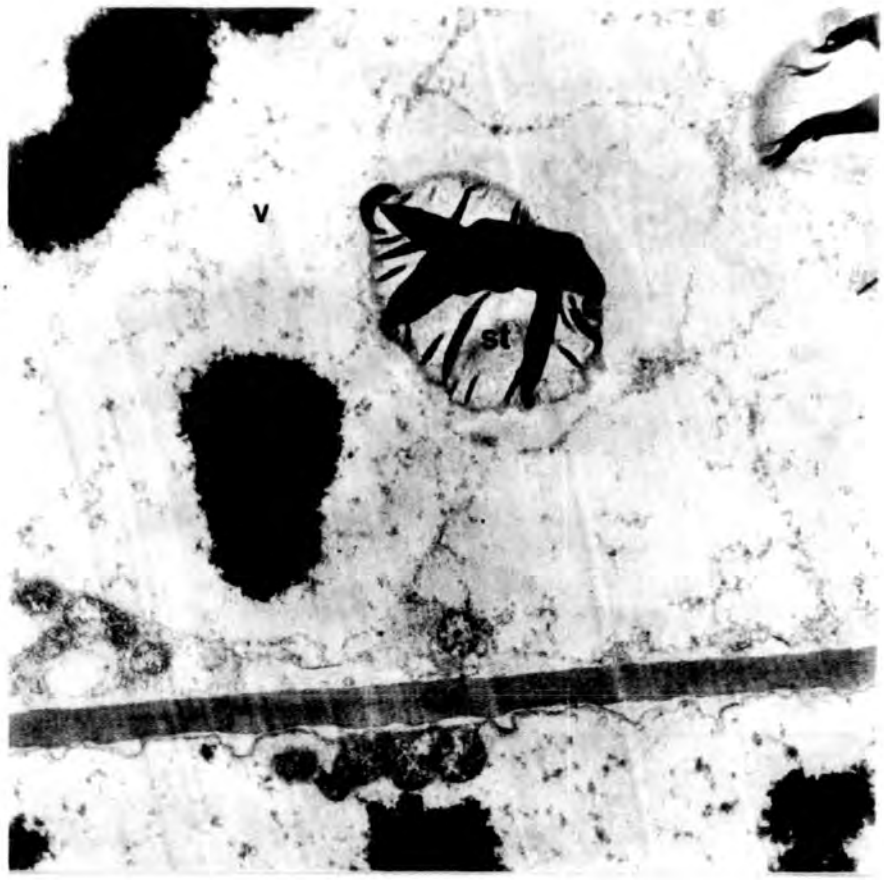
(b) magnification x 11250

(c) magnification x 15000

(d) magnification x 7500

pb = protein "body"
st = starch grain
cw = cell wall
v = protein vacuole

(a)

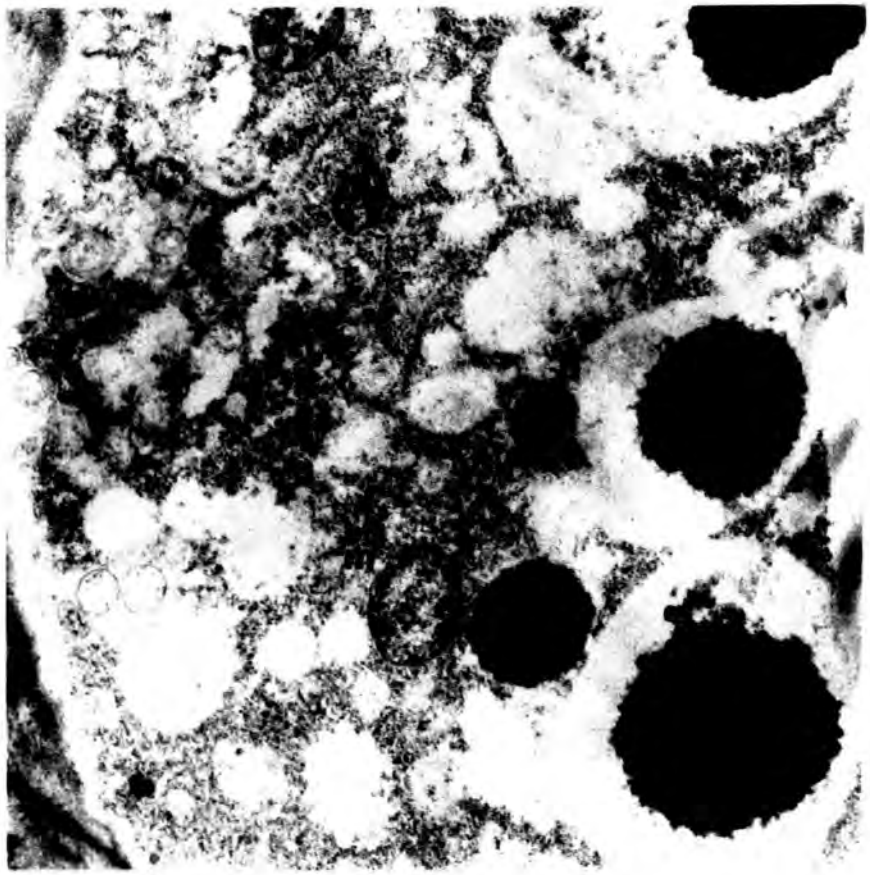


2µm

(b)

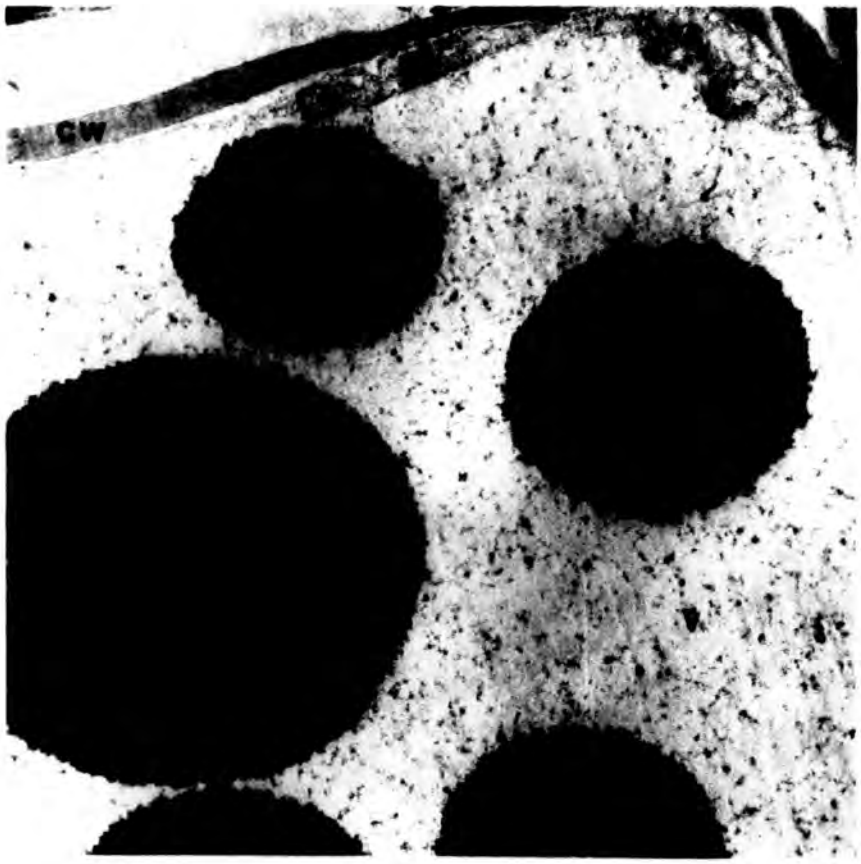
2µm

(c)



1μm

(d)



2μm

(4) Extraction of Protein Aggregates:

An attempt to isolate the protein aggregates was made using methodology commonly used to isolate protein bodies (methods III.F).

After the preliminary centrifugation steps, a small cream compact pellet was obtained (12,000g pellet) whose protein content, however, (determined by the Lowry method, table 20) was only c. 55% (dry basis), that of the supernatant remaining being c. 70% (d.b.).

The 12,000g pellet resuspended in buffer was centrifuged on a stepped gradient of sucrose, and the ensuing fractions (depicted on the gradient in fig. 56) were analysed for protein content using the Lowry method (bovine serum albumin as reference) and then by SDS PAGE on a quantitative protein basis.

The sub-unit profile of the fraction which sedimented at the 70-90% sucrose interface (E) was found to be identical to that of the purified storage protein (fig. 57, tracks 6 and 12, cf tracks 1 and 11). However, the protein content of this fraction was only c. 64% (d.b.). This was nevertheless greater than that of the other fractions analysed (table 20) which also contained the major sub-unit but additional minor sub-units as well (fig. 57, tracks 2, 3, 4, 5 and 7).

The 70-90% sucrose interface fraction, believed to contain a partially purified preparation of the protein aggregates, was examined by electron microscopy after appropriate prior treatment (methods III.F). Fig. 58 indicates that the protein aggregates had dispersed considerably, in keeping with the observation that they may not have been surrounded by a limiting membrane. A somewhat distorted mitochondrion, and some unidentified cellular bodies are also shown, but starch grains and cellular debris were also noted to be present in the preparation.

Fig. 56

Fractionation by sucrose density gradient centrifugation of the 12000g pellet which was prepared from a fresh extract of a mature dormant tuber (variety Nwapoko) by the method described in methods III.F.

The steps of sucrose concentration used to prepare the gradient, i.e. 30% $^{w}/_{v}$, 70% $^{w}/_{v}$ and 90% $^{w}/_{v}$ in 0.1M phosphate buffer pH 7.5, are indicated.

(a), (b) and (c) illustrate fractionation of increasing concentrations (x, 2x and 8x) of the 12000g pellet which was resuspended in 0.05M phosphate buffer, 0.5M sucrose pH 7.5 prior to centrifugation and overlayered with 15% $^{w}/_{v}$ sucrose in 0.1M phosphate buffer.

Fig. 57

Electropherogram (17.6 x 0.45, discontinuous SDS buffer system) of fractions A-F indicated in fig. 56.

Tracks 1 and 11:	lyophilised preparation of the purified storage protein preparation.
Tracks 2-7:	fractions A-F respectively.
Track 8:	12000g pellet(p).
Track 9:	supernatant(s) remaining after separation of the 12000g pellet from the extract.
Track 10:	extract.
Track 12:	fraction E as in track 6.

All fractions were dialysed against water and lyophilised prior to electrophoretic analysis.

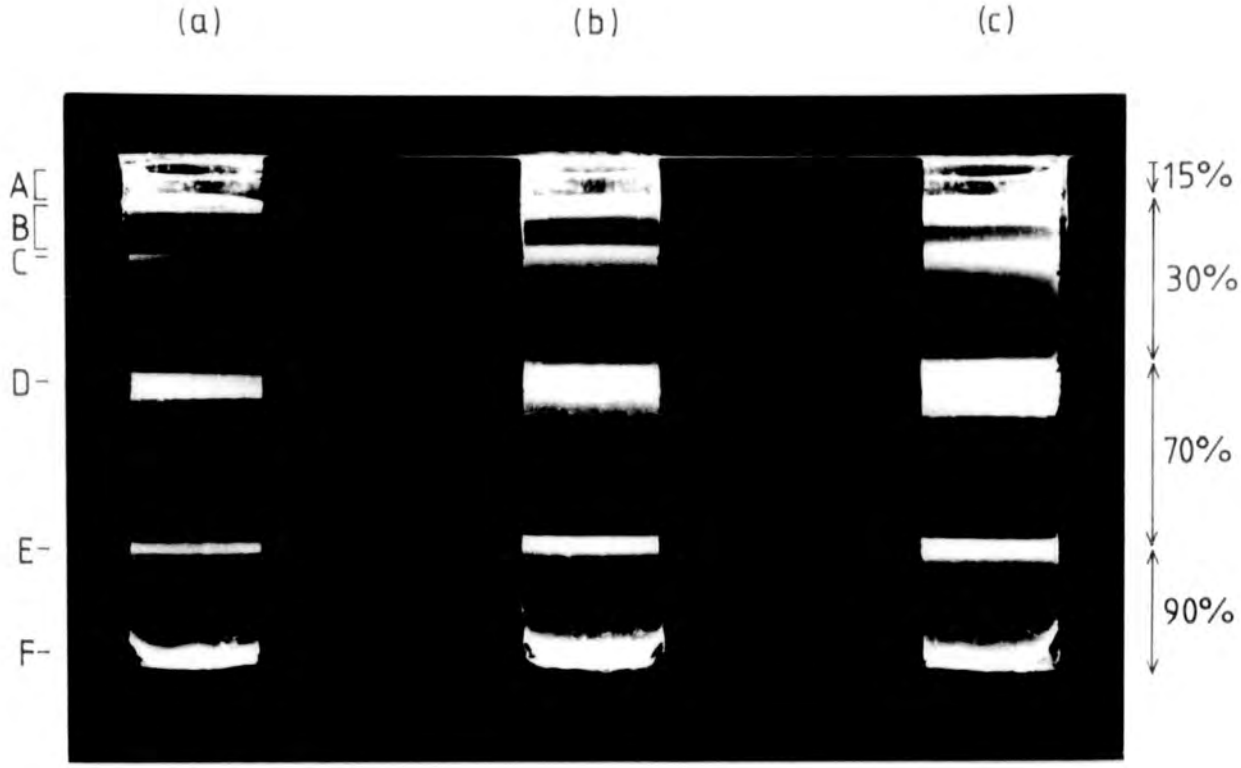

(a)
(b)
(c)
A
B
C
D
E
F
15%
30%
70%
90%

Fig. 56

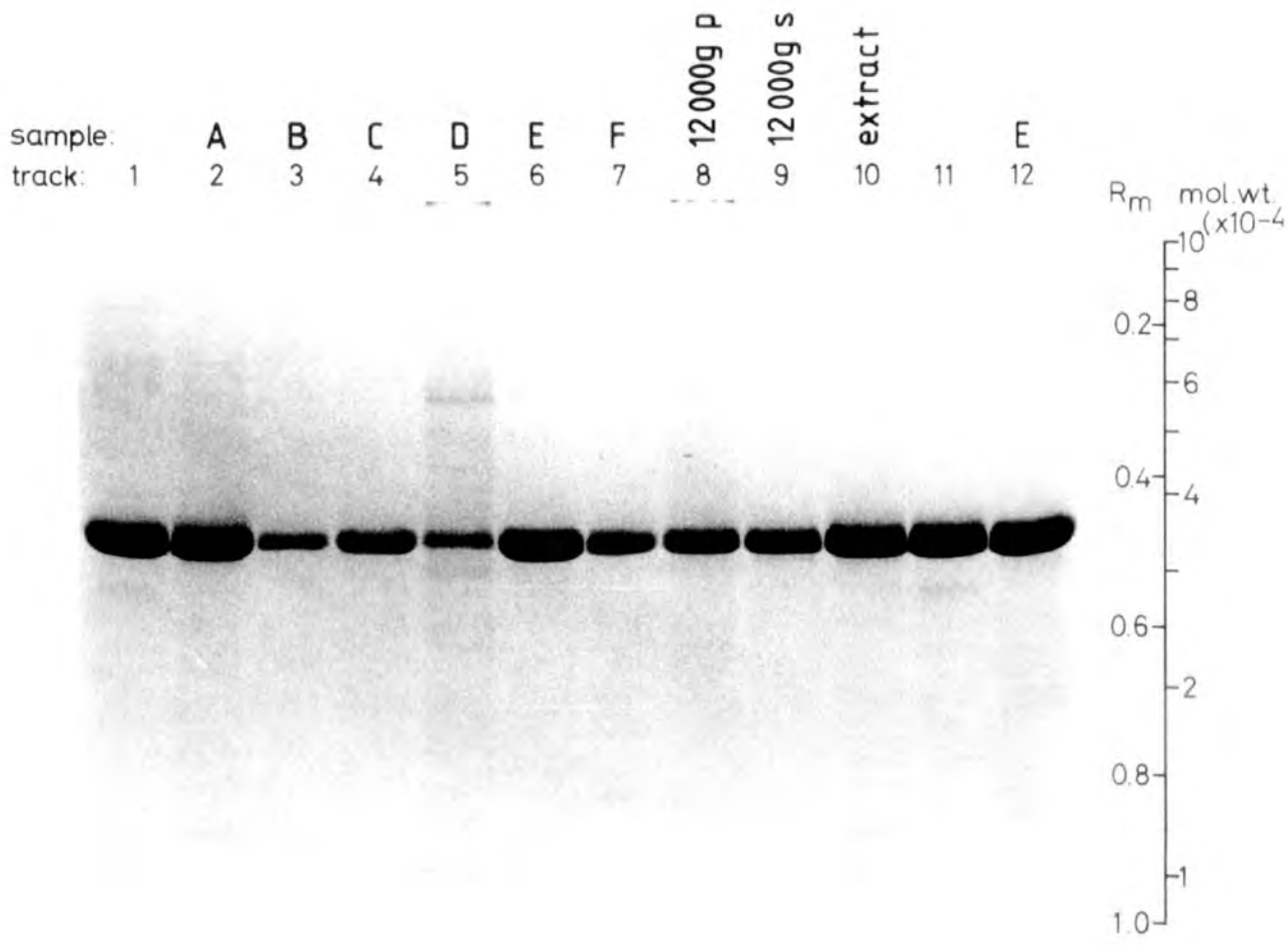

sample: A B C D E F 12000g p 12000g s extract E
track: 1 2 3 4 5 6 7 8 9 10 11 12
Rm
mol.wt. (x10-4
10
8
6
4
2
1
0.2
0.4
0.6
0.8
1.0

Fig. 57

Table 20 Protein Content of Fractions Isolated from a Stepped Gradient of Sucrose

Fractions	% protein (dry basis)[a]
lipid containing [b,c]	58.3 ± 0.2 [e]
band within 70% [c] zone of sucrose	40.1
band on 70-90% [c] sucrose interface	63.6 ± 2.4 [d]
band at bottom of [c] gradient	14.1
12,000g pellet	54.5 ± 0.4 [e]
supernatant remaining after separation of 12,000g pellet	70.0
extract	79.0
purified storage protein (control)	101.6 ± 2.7 [d]

[a] determined using the Folin Ciocalteau reagent with bovine serum albumin as standard

[b] topmost band on gradient determined to contain lipid after paper translucence test

[c] refer to fig. 56

[d] mean of two determinations ± standard deviation

[e] mean of two readings for one sample ± standard deviation

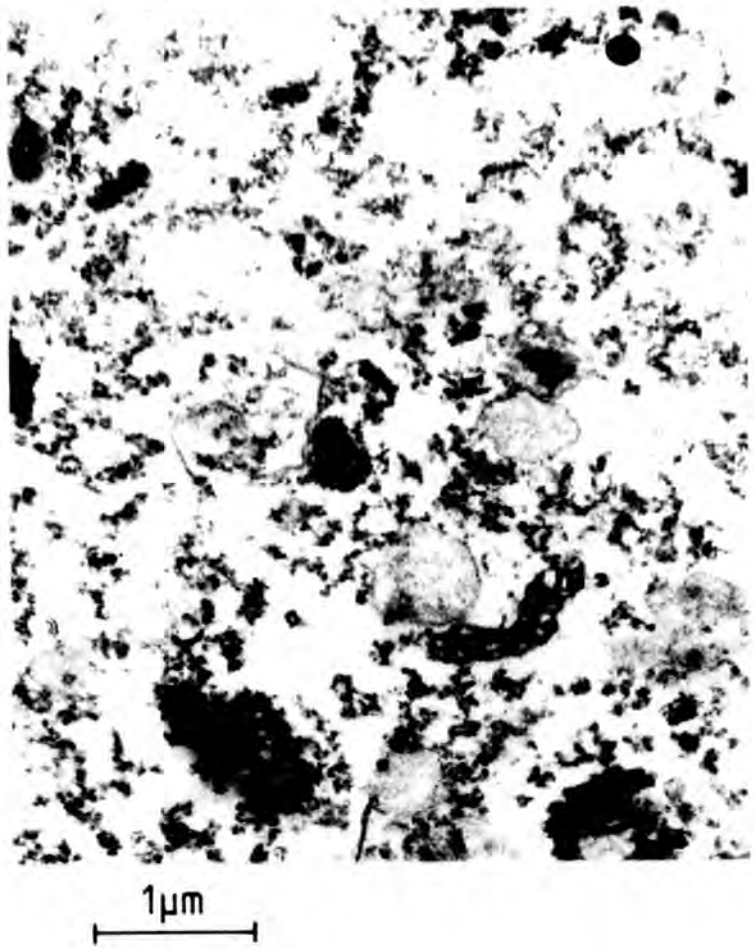


Fig. 58

Electron micrograph of the 70-90% sucrose interface fraction (see fig. 56), prepared for microscopy by the method described in methods III.F.

Magnification: x 20,000

m = mitochondrion

B. Lignin and Starch:

Vertical and horizontal sections of the tuber stained with phloroglucinol (fig. 59) showed the presence of long strands of lignified tissue associated with the vascular bundles, distributed throughout the storage tissue. The bundles were of greater number and lesser diameter (12 μm) in both the outer, and lower, (younger) regions of the tuber compared to the inner and upper (older) regions (c. 16 μm diameter).

Starch grains were typically egg-shaped, measuring an average 5 μm in length, although just below the subepidermal layer, the average length was less than 0.1 μm.

C. Browning at Cut Surfaces:

The extent to which tuber flesh browned at cut surfaces varied markedly between varieties; the variety Nwapoko showed very little browning, but those seedling varieties illustrated in fig. 1 showed quite extensive browning, varying in colour from brown and purple to orange and yellow. Fig. 59(a) illustrates the extent of browning in a tuber from clone R16-214-127 after ½h exposure of the cut surface to the atmosphere.

Fig. 59

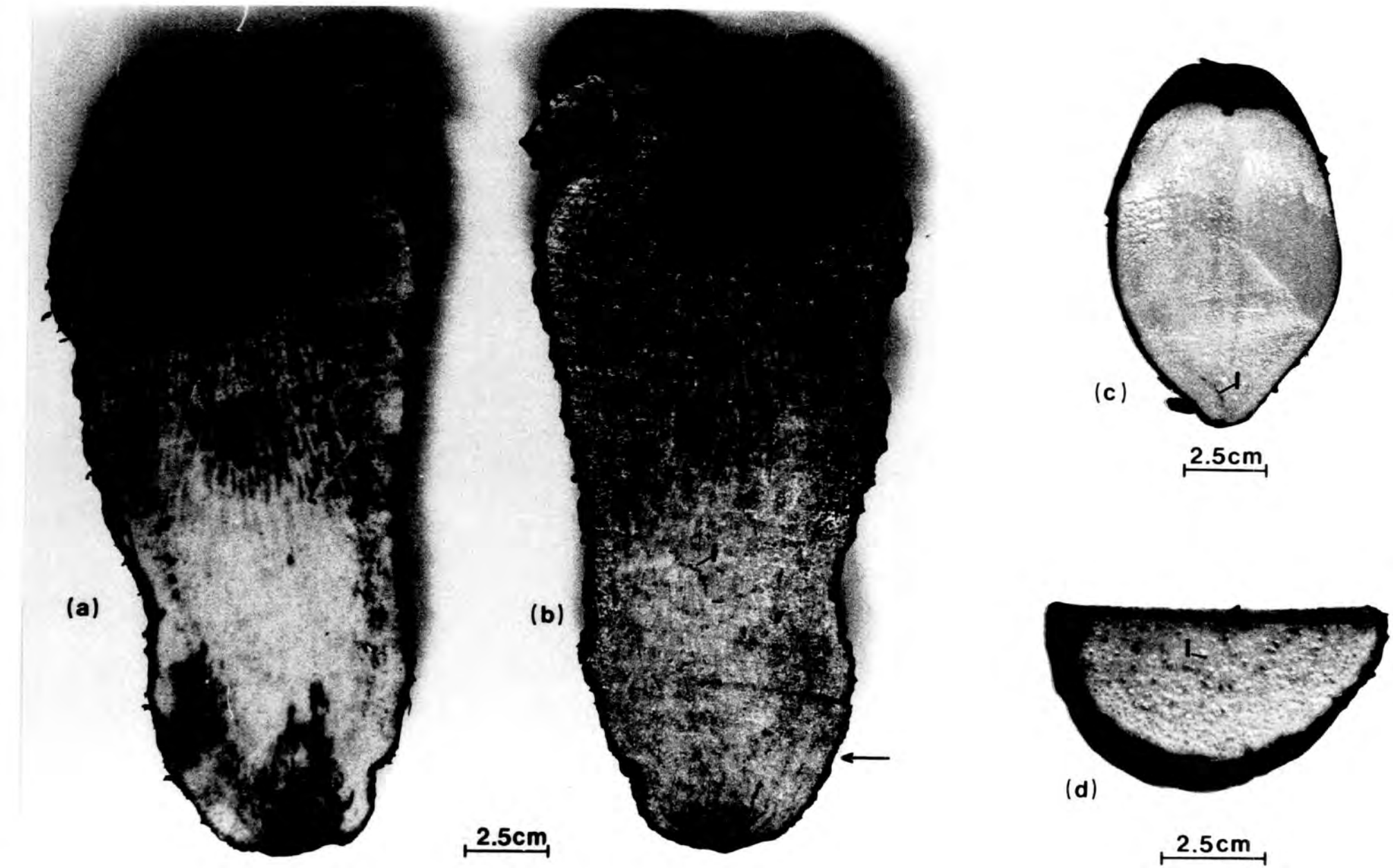
(a) L.S. of a tuber from clone R16-214-127 illustrating the extent and distribution of browning after ½h exposure of the cut surface to the atmosphere.

(b) L.S. of the same tuber as in (a), stained with phloroglucinol.

(c) L.S. of a potato tuber stained with phloroglucinol.

(d) T.S. of the yam tuber illustrated in (b), taken from the position indicated by the arrow, and stained with phloroglucinol.

l = lignified tissue


(a)
(b)
2.5cm
(c)
2.5cm
(d)
2.5cm

VII. THE BREEDING PROGRAMME

The range of values of crude protein content obtained for the different populations studied, together with the mean value and standard deviation are given in table 21.

A. Examination of Environmental Variation:

(1) The Variety Nwapoko (population 1):

Statistical analysis of the variance between and within replicates of each of the tuber characters evaluated (table 22) indicated that there was insignificant variation of % dry matter content between replicates. However, those characters pertaining to nitrogen content and tuber weight varied significantly. These latter characters appear to be strongly influenced by a source of environmental variation which was not identified in the design of the experiment.

In order to assess the extent of "developmental" variation and to ascertain the reliability of using a single tuber from plants which produce two or more tubers, variance arising from differences between replicates was separated from the total, and the variance between plants and between tubers of a plant analysed in a hierarchical analysis of variance of each character (table 23). These analyses indicated that the variation between plants was the same as that between the tubers of any one plant for each character evaluated. Since the population is assumed to be genetically uniform, it can be predicted that in genotypically mixed populations the variation between tubers is likely to be insignificant compared to the variation between genotypes, and multiple measurements will, therefore, give little gain in precision.

(2) Two Seedling-derived Clones (population 2):

Statistical analysis of data obtained for these two clones

Table 21 Crude Protein Content (%N (d.w.) x 6.25) in Populations of Dioscorea rotundata

Population	Number of Cultivars	Mean value ± standard deviation (No. of samples)	Minimum Value	Maximum Value
1.[a] (Nwapoko, harvest 1978)	1	4.26 ± 0.54 (103)	3.25	6.31
2.[b]	2	4.96 ± 0.73 (16)	3.81	6.25
3.[c] (harvest 1977)	9	5.74 ± 1.27 (9)	3.94	8.28
3.[c] (harvest 1978)	9	5.18 ± 0.76 (17)	3.94	7.06
4.[d]	81	4.83 ± 0.88 (81)	3.03	6.56
5.[e] (offspring)	194	5.98 ± 1.00 (194)	3.36	9.01
5.[e] (parents)	9	6.06 ± 1.21 (29)	4.33	9.43

a data in appendices 1 and 2

b data in appendices 3 and 4

c data in appendix 5

d data in appendix 9

e data in appendix 6

Table 22 Results from Analyses of Variance of Tuber Values (variety Nwapoko), between Replicates compared to Within

Character	F ratio[b]	Significance
% nitrogen (d.w.)	8.194	0.0001 *
% nitrogen (f.w.)	6.139	0.0007 *
total nitrogen content of tuber	2.652	0.0528 *
tuber fresh weight	2.876	0.0400 *
% dry matter	1.120	0.3449

a data in appendix 1

b degrees of freedom (D.F.) between replicates = 3
D.F. within replicates = 99

* significant at 5% level or less

Table 23 Results from Analyses of Variance of Tuber Values (variety Nwapoko), between plants compared to Between Tubers of a Plant (Within Plants), With Effects of Replicates Eliminated[a]

Character	F ratio[b]	Significance
% nitrogen (d.w.)	0.719	> 0.4
% nitrogen (f.w.)	1.005	> 0.2
total nitrogen content of tuber	0.699	> 0.4
tuber fresh weight	0.797	> 0.4
% dry matter	1.429	> 0.2

a Data in appendix 1. Data from only those plants which produced more than one tuber were used in the analyses.

b D.F. between plants within locations = 14

D.F. within plants (between tubers of a plant) = 23

Table 24 Results from Analyses of Variance of Tuber Values between clones W387-3 and W387-4 (population 2), compared to within[a]

Tuber Character	F ratio[b]	Significance
% nitrogen (d.w.)	0.500	0.4910
% nitrogen (f.w.)	2.735	0.05-0.20
total nitrogen content of tuber	4.913	0.025-0.05 *
tuber fresh weight	3.211	0.0948
% dry matter	0.7114	0.4132

a Data in appendix 3

b D.F. between clones = 1
D.F. within clones = 14

* Significant at less than 5%

Table 25 Results from Analyses of Variance Between Plants within clones (W387-3 and W387-4, population 2) compared to Between Tubers of a Plant (within Plants) with Effects of Differences between Clones Eliminated[a]

Tuber Character	F ratio[b]	Significance
% nitrogen (d.w.)	3.430	0.05 *
% nitrogen (f.w.)	2.957	0.05-0.20
total nitrogen content of tuber	2.179	0.05-0.20
tuber fresh weight	1.640	>0.20
% dry matter	12.323	0.001-0.01 **

a Data in appendix 3

b D.F. between plants within clones = 6
D.F. within plants (between tubers of a plant) = 8

* Significant at 5% level and less

** Significant at 1% level and less

indicated firstly that the variation of % nitrogen (d.w. and f.w.), weight, and % dry matter content, was as great within the clones as between (table 24). This was not true, however, for the character nitrogen content; the analysis based on this character indicated a significant difference between clones.

Results from a second set of analyses in which the variation between plants and between tubers of a plant was examined (table 25) indicated, as did the analyses performed on the variety Nwapoko (qv), that multiple measurements of nitrogen, weight and % dry matter on a plant producing more than one tuber will give little gain in precision. However, it was anticipated that since each clone is genetically uniform, the variation between plants within clones should be insignificant when compared to that between the tubers of a plant. For the characters % dry matter, and % nitrogen (dry weight) the converse was obtained; there was a significant difference between plants. Results for the characters % nitrogen (fresh weight) and nitrogen content also indicated a difference, although for each analysis this was significant only between 5% and 20%. These characters appear to be strongly influenced by an as yet "intangible" source of environmental variation which may considerably reduce the precision of any genetical studies.

B. Analysis of Measurements of % Nitrogen (d.w.) and Yield between Years and between Clones of Population 3:

Measurements of % nitrogen (dry weight) and yield made on nine plants representing nine different clones in 1977, and on their vegetative progeny in 1978 are given in appendix 5.

The correlation between the two years for each character was found to be statistically insignificant; the correlation coefficient for % nitrogen was -0.100 (14 degrees of freedom),

whilst that of yield was -0.276 (13 degrees of freedom).

Secondly, analysis of the data for 1978 indicated that for both % nitrogen and yield, variation between the clones was not significant compared to that between the plants in each clone. With eight degrees of freedom between the clones, and seven within the clones, the F ratios for % nitrogen and yield were 1.28 and 3.44 respectively.

C. Estimation of the Heritabilities of % Nitrogen, % Dry Matter and Yield in Population 5:

Measurements of yield, and of the tuber characters % nitrogen and % dry matter which were evaluated for five full sib families of offspring, their male parents and four of the female parents in population 5 are given in appendix 6. The midparent and parent values used in the regression analyses to determine heritabilities are given in appendix 7.

A preliminary analysis of the offspring data was carried out to examine the variability of these characters. The analyses indicated that the differences between families of both % nitrogen and % dry matter were highly significant (less than 0.1% probability for each character, table 26). By contrast differences between the offspring families in terms of the character yield were significant only between 5% and 20%.

The variability of parent values for these three characters was also analysed since estimates of heritabilities partly depend on the magnitude of variability between parents. These analyses (table 26) indicated a highly significant difference of % dry matter between parent clones compared to within, but the differences of % nitrogen were significant only between 5% and 20%. The difference of yielding ability between female parent

Table 26 Results from analyses of variance of tuber values between and within Offspring Families and between and within Parents. (Population 5). [a]

Code	Five offspring families (50, 51, 52, 53, 54)		Four offspring families (51, 52, 53, 54)		All parents (2, 4, 5, 6, 9, 10, 11, 12, 13)		Female parents (4, 5, 10, 11)		Male parents (2, 6, 9, 12, 13)	
Character	F ratio	Significance	F ratio	Significance	F ratio	Significance	F ratio	Significance	F ratio	Significance
% nitrogen (d.w.)	5.45	0.0004 ***	7.05	0.0002 ***	2.20	0.0703 *	2.48	0.1582	2.30	0.1098
yield	1.72	0.1471	2.25	0.0839 *	2.28	0.0643 *	1.04	0.4390	3.98	0.0232 **
% dry matter	32.11	0.0000 ***	45.12	0.0000 ***	4.42	0.0033 ***	9.64	0.0104 **	2.86	0.0636 *
sett weight	3.22	0.0139 **	4.58	0.0041 ***	4.76	0.0022 ***	2.54	0.1523	2.89	0.0614 *
D.F. between	4		3		8		3		4	
D.F. within	189		166		20		6		14	

[a] Data in appendix 6

* significant between 5% and 10%

** significant between 1% and 5%

*** significant at less than 1%

Table 27 Correlation of Characters with Sett Weight (Population 5)

Character	Correlation coefficient (r)	T-statistic	Significance[b]
% nitrogen (d.w.)	0.1522	2.2898	0.0230 **
% dry matter	-0.0196	-0.2907	0.7715
yield	0.4112	6.7052	0.0000 **

** significant at less than 5%

a correlation matrix in appendix 8

b degrees of freedom = 221

Table 28 Values of Heritability determined from the Regression of Offspring on Parent(s) Values (Population 5)

Method of Estimation / Character	Offspring on Midparent regression	Offspring on female parent regression	Offspring on male parent regression
% nitrogen (d.w.)	0.58 ± 0.39	0.33 ± 0.44	0.17 ± 0.25
yield	0.02 ± 0.31	- 0.05 ± 0.37	0.41 ± 0.84
% dry matter	0.98 ± 0.17	1.35 ± 0.23	1.53 ± 0.48

Values ± 95% confidence limits of the estimate, calculated according to Colquhoun, (1971).

The analyses of variance are set out in table 29.

Table 29(a) Results from Analyses of Variance of Offspring Values of % Nitrogen (Population 5)

Regression Analysis		DF	MS	F	Significance
Offspring on Midparent	regression	1	0.20084	8.43	0.001-0.01 **
	deviations from linearity	2	0.15161	6.36	0.001-0.01 **
	between offspring	3	0.16802	7.05	0.0002 **
	within offspring	166	0.02383		
Offspring on Female Parent	regression	1	0.05205	2.18	>0.05
	deviations from linearity	2	0.22600	9.48	<0.001 **
	between offspring	3	0.16802	7.05	0.0002 **
	within offspring	166	0.02383		
Offspring on Male Parent	regression	1	0.04203	1.78	>0.05
	deviations from linearity	3	0.15741	6.68	<0.001 **
	between offspring	4	0.12856	5.45	0.0004 **
	within offspring	189	0.02358		

* significant between 1% and 5%

** significant at less than 1%

Table 29(b) Results from Analyses of Variance of Offspring Values of % dry matter (Population 5)

Regression Analysis		DF	MS	F	Significance
Offspring on Midparent	regression	1	1839.035	122.86	<0.001 **
	deviations from linearity	2	93.483	6.25	0.001-0.01 **
	between offspring	3	675.340	45.12	<0.001 **
	within offspring	166	14.968		
Offspring on Female parent	regression	1	1908.089	127.48	<0.001 **
	deviations from linearity	2	58.956	3.94	0.01-0.025 *
	between offspring	3	675.340	45.12	<0.001 **
	within offspring	166	14.968		
Offspring on Male parent	regression	1	617.071	38.5	<0.001 **
	deviations from linearity	3	479.976	29.97	<0.001 **
	between offspring	4	514.260	32.11	<0.001 **
	within offspring	189	16.016		

* significant between 1% and 5%

** significant at less than 1%

Table 29(c) Results from Analyses of Variance of Offspring Values of Yield (Population 5)

Regression Analysis		DF	MS	F	Significance
Offspring on Midparent	regression	1	1.6444×10^4	0.01	> 0.05
	deviations from linearity	2	5.6303×10^6	3.38	0.025–0.05 *
	between offspring	3	3.759×10^6	2.25	0.0839
	within offspring	166	1.6672×10^6		
Offspring on Female parent	regression	1	1.1797×10^5	0.07	> 0.05
	deviations from linearity	2	5.5795×10^6	3.35	0.025–0.05 *
	between offspring	3	3.759×10^6	2.25	0.0839
	within offspring	166	1.6672×10^6		
Offspring on Male parent	regression	1	1.5221×10^6	0.91	> 0.05
	deviations from linearity	3	3.3197×10^6	1.99	> 0.05
	between offspring	4	2.8795×10^6	1.72	0.1471
	within offspring	189	1.6734×10^6		

* significant between 1% and 5%

clones was insignificant, but between male parent clones, significant (2% probability).

One further set of preliminary analyses was carried out on the character sett weight. Sett weight was calculated to be significantly positively correlated with both the characters % nitrogen and yield (table 27). It was not significantly correlated with % dry matter, however. Since the progeny setts were planted whole, a considerable range of sett weight (21-591 g) existed, and analysis of this variation between and within offspring families (table 26) indicated a significant difference in sett weight between families. Variation between parent sett weights was also analysed; these indicated a significant difference between parent clones compared to within the clones. A consideration of the variability amongst male parents alone indicated differences between the clones which were significant with a probability of 6%; there were however no significant differences between female parent clones.

"Narrow sense" heritabilities of the characters % nitrogen, % dry matter and yield are given in table 28, together with their 95% confidence limits. The significance of each regression coefficient used to determine heritability was tested, in an analysis of the variance, against the "error" described by the within offspring component of variance. These analyses are given in table 29.

The heritability of % dry matter was determined to be in the order of 100% from each regression analysis, and each regression coefficient calculated was found to be highly significant.

By contrast, the character yield had a heritability approaching zero in each estimation except that based on the

regression of offspring values on male parent values. However, this latter estimate had broad confidence limits, whilst the regression coefficient used to determine the heritability was not significantly different from zero.

The heritability of % nitrogen was calculated as 0.58 $\pm$ 0.39 from the offspring midparent regression. The analysis of variance test (table 29) indicated that this estimate, equal in this instance to the regression coefficient, was highly significant (probability between 0.1% and 1%). There was, however, no significant difference between it and the components of variance described by the "deviations from linearity" in the regression analysis. By contrast, the heritabilities calculated from the offspring-female parent, and offspring-male parent regression coefficients were 0.33 $\pm$ 0.44 and 0.17 $\pm$ 0.25 respectively, and in each case the regression coefficient was not significant.

D. Correlations between the Character % Nitrogen and Other Characters of the Tuber and Vine:

(1) Population 4:

Correlations between characters were evaluated using data recorded for 81 plants in population 4 (appendix 9). Since the field had been divided into four distinct areas (methods IX A.4) correlations were also evaluated on plants within each area, although as indicated in table 30 the numbers of individuals in each of the five families comprising this population were not replicated between areas.

Firstly, the variability of % nitrogen was examined between and within families (table 31). The results obtained from an analysis of the variance of % nitrogen values recorded for plants in location 3, which had a more even representation of individuals

within each family, indicated significant differences between families and suggested genotypic variation of this character within the population. The differences between families in each of the remaining areas and within the population as a whole, however, were not significant.

Significant positive correlations were found between the character %N (both d.w. and f.w. basis) and each of the characters leaf spot score and total nitrogen content of the tuber when data for all the plants was evaluated (table 30). The character % dry matter was positively correlated with % N measured on a fresh weight basis, but negatively correlated with %N measured on a dry weight basis, although only the former correlation was significant. Each of the characters stem diameter and seedling tuber shape were significantly positively correlated with %N measured on a dry weight basis, but not with %N measured on a fresh weight basis. There were no further significant correlations between % nitrogen and other plant and tuber characters within the population as a whole.

However, when correlations were evaluated within each location, none of the above correlations were consistently significant (table 30). For example, none of these correlations were significant when evaluated for plants in location 2, but the majority were significant in location 3. Furthermore three correlations which were non-significant for the population as a whole were evaluated as being significant in one each of the locations. These were between % nitrogen (d.w. basis) and tuber shape (location 1), % nitrogen (d.w. basis) and tuber number (location 2) and % nitrogen (d.w. basis) and tuber smoothness (location 3).

Table 30 Correlations between % nitrogen and vine and tuber characters evaluated from plants in Population 4

Analysis / Character[a]	All plants irrespective of location		Location 1		Location 2		Location 3		Location 4	
	%N d.w.	%N f.w.	%N d.w.	%N f.w.	%N d.w.	%N f.w.	%N d.w.	%N f.w.	%N d.w.	%N f.w.
total N in tuber	0.33 **	0.35 **	0.50 *	0.58 **	- 0.04	- 0.02	0.51 *	0.46 *	0.12	0.18
leaf spot score	0.28 **	0.34 **	0.16	0.21	0.12	0.18	0.38 *	0.53 *	0.53 *	0.46
% dry matter	- 0.19	0.36 **	- 0.35	0.26	- 0.31	0.24	- 0.25	0.42 *	0.28	0.59 *
stem diameter	0.23 *	0.15	- 0.09	0.04	0.24	0.13	0.64 **	0.43 *	0.28	0.15
seedling l:d.	0.25 *	0.19	0.23	- 0.11	0.14	0.16	0.19	0.13	0.48 *	0.58 *
tuber shape	0.20	0.13	0.40*	0.36	0.19	0.08	0.02	- 0.28	0.03	0.19
tuber smooth-ness	0.09	0.08	- 0.12	0.06	0.11	0.19	0.62 **	0.40	- 0.21	- 0.23
tuber number	0.07	- 0.01	- 0.17	- 0.16	0.54 *	0.21	- 0.02	0.01	0.13	- 0.04

Table 30 (Cont.)

Correlations between % nitrogen and vine and tuber characters evaluated from plants in Population 4[a] (Cont.)

Character \ Analysis [a]	All plants irrespective of location		Location 1		Location 2		Location 3		Location 4	
	%N d.w.	%N f.w.	%N d.w.	%N f.w.	%N d.w.	%N f.w.	%N d.w.	%N f.w.	%N d.w.	% N f.w.
Number of Plants in Families (family 201	5		1		1		3		0	
(family 208	7		2		2		2		1	
(family 213	10		3		1		4		2	
(family 214	7		2		0		3		2	
(family 215	52		16		14		10		12	

* significant between 1% and 5%

** significant at less than 1%

[a] correlation matrices in Appendix 10

Table 31 Summary of Analyses of Variance of the Tuber Character % Nitrogen, between and within families (Population 4)

Analysis	% Nitrogen (dry basis)		% Nitrogen (fresh basis)	
	F ratio	Significance	F ratio	Significance
All plants[a]	1.11	0.3567	0.17	0.9548
Location 1[b]	0.88	0.4944	0.68	0.6159
Location 2[c]	0.63	0.6092	0.53	0.6721
Location 3[d]	3.80	0.0219 **	3.78	0.0224 **
Location 4[e]	1.82	0.1935	1.74	0.2074

** significant at less than 5%

Degrees of freedom (a) between = 4, within = 76
(b) between = 4, within = 19
(c) between = 3, within = 14
(d) between = 4, within = 17
(e) between = 3, within = 13

Data in Appendix 9

(2) Population 5:

Correlations between characters were evaluated using data recorded for 223 plants in population 5 (table 32). Each of the characters yield, % dry matter and stem number were significantly negatively correlated with % nitrogen (d.w. basis). Sett weight was significantly positively correlated (qv VII.C) but there was no correlation with the character "weeks to emerge", dry vine weight or tuber number.

E. SDS-Extraction of the Protein from Five Varieties of Yam Tubers:

An electropherogram of the SDS extracts obtained from five different varieties of yam tubers (fig. 60) indicated that the sub-unit composition of the protein from each was essentially the same, and the same as that from variety Nwapoko, harvest 1977.

Table 32 Correlations between % Nitrogen (d.w.) and Characters Evaluated in Population 5[a]

Character	Correlation Coefficient
yield	-0.1706 *
% dry matter	-0.2232 **
sett weight	+0.1447 *
weeks to emerge	-0.0502
stem number	-0.1587 *
tuber number	-0.0443
dry vine weight	-0.0661

* significant between 1% and 5% (220 degrees of freedom)

** significant at less than 1%

[a] correlation matrix in Appendix 8: Data in Appendix 6

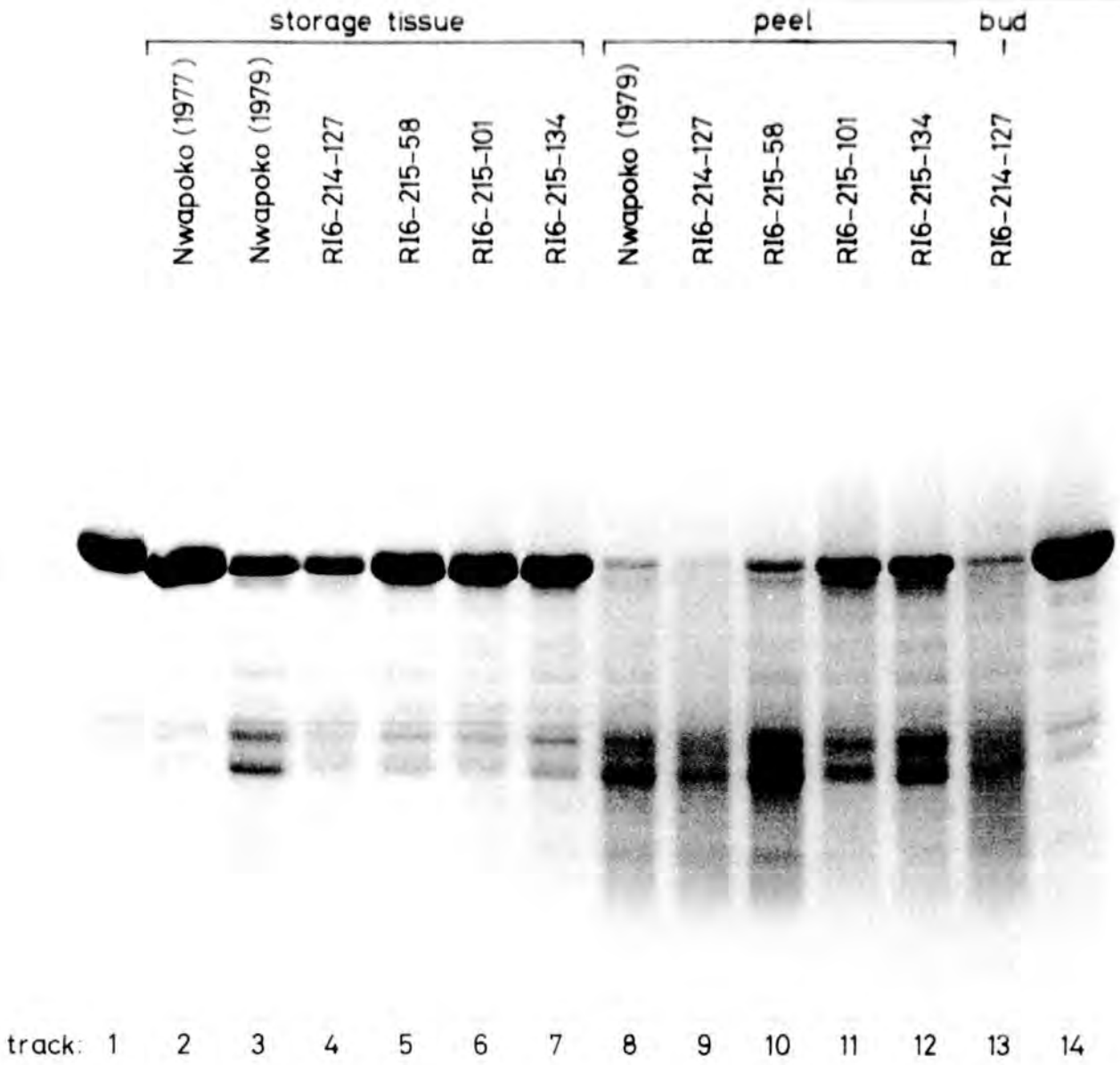


Fig. 60

Electropherogram (discontinuous SDS buffer system, 17.6 x 0.45) of protein samples prepared from different cultivars of yam (D.rotundata).

The meals were extracted with 2% $^{w}/v$ SDS, 5% $^{v}/v$ 2-ME in 0.0625M Tris-HCl pH 6.7 for two hours at room temperature at a ratio of 1:8 $^{w}/v$; the extracts were clarified by centrifugation at 10,000g, and 10 µl aliquots electrophoresed. A lyophilised extract preparation (see methods section II B.3) from the variety Nwapoko was simultaneously incubated for two hours in buffer of the same composition before electrophoretic analysis.

Tracks 1 and 14: lyophilised extract preparation from Nwapoko (harvest 1977).

Tracks 2-13: SDS extracts prepared from meal samples as indicated.

DISCUSSION

In order to evaluate the efficiency of extraction of protein from the yam tuber, it was necessary firstly to ascertain the amount of protein present *in vivo*. In this respect, estimation of Kjeldahl nitrogen, and amino acid analysis are probably the most direct techniques available. However, Desborough and Weiser, (1974) considered that neither of these techniques gave reliable estimates of protein content in potato tubers, and a similar situation may exist in yam tubers. Indeed in potatoes, the non-protein nitrogen content may vary from 40-60% of the total (Neuberger and Sanger, 1942; Mulder and Bakema, 1956) depending not only on the variety Talley *et al*, 1970;Li and Sayre, 1975; Kiryukhin *et al*, 1975) but also on the level of mineral nutrition (Hoff *et al*, 1971; Courtrez-Geerinck, 1975; Klein *et al*, 1980) soil type (Augustin, 1975; Kiryukhin *et al*, 1975) growing temperature (Vigue and Li, 1976) developmental stage (Snyder and Desborough, 1978a) and other factors. The approach adopted, therefore, was to extract any non-protein nitrogen compounds from the meals and then determine protein content either by amino acid or nitrogen analysis. Aqueous ethanol (70-80%) has been recommended for this purpose by various workers (Thompson and Steward, 1951; Bhatty and Finlayson, 1973; Kapoor *et al*, 1975a). However, since free amino acids have been shown to be extracted at different rates with this solvent (Woodward and Rabideau, 1953; Oland and Yemm, 1956; Talley *et al*, 1958) a preliminary investigation was undertaken to determine approximately the end-point in extraction of non-protein nitrogen in yam: these results (section I.C) indicated that the final procedure adopted (19h) was acceptable.

Amino acid analysis of both the hydrolysed and unhydrolysed ethanolic extracts indicated that proteins in the meal were not extracted by this method, although a few small peptides, including

probably glutathione, a tripeptide which has been identified in sprouting potato tubers (Guthrie, 1932), may have been present in the extracts. Similar results were obtained by Kapoor et al, (1975a) who found that only 2-4% of the amino acid content of ethanolic extracts prepared from potatoes was precipitable with 15% TCA and Li and Sayre, (1975) who reported that the nitrogen content of their ethanolic extracts from potato, after dialysis against water, was zero.

Various workers (Vickery et al, 1935; Gilbert et al, 1949; Talley et al, 1956; Oreskes and Kupfer, 1967; McKerrow and Robinson, 1971) have reported the conversion of glutamine and asparagine to pyrrolidone carboxylic acid and 4 carboxy-azetidinone respectively at elevated temperatures. However, the recovery of amino acid content in the ethanolic extract and extracted meal together represented 99% of the total, so that the extent of conversion of these amides during extraction may have been minimal.

Figures for protein content were considered to be more precisely estimated from the weight of amino acids recovered in the extracted meals, than from estimation of Kjeldahl nitrogen content, for although the former figure may be underestimated from an analysis of a 22h acid hydrolysate alone (Moore and Stein, 1963), the large difference between the value for nitrogen in amino acids, which however excludes values for amide N, and the Kjeldahl N value suggested even so that there may be non-amino sources of nitrogen in extracted meals.

Having thus established a satisfactory analytical procedure, factors influencing the solubility of the proteins in extractants were investigated (see section II).

Potato proteins have commonly been extracted with dilute salt solutions, the quantity of nitrogen in such extracts representing 90-96% of the total (Neuberger and Sanger, 1942; Levitt, 1951; Nakasone et al, 1972; Miedema et al, 1976). Neuberger and Sanger determined that 77% of the total heat-coagulable protein could be extracted with water alone, but recommended the use of 2% salt solutions for improved efficiency of extraction. The extraction methods of Kapoor et al, (1975b) included the use of 55% isopropanol and 0.2% sodium hydroxide to extract the prolamine and glutelin fractions respectively of the potato, and in this way, they were able to extract 87-90% of the total "true" protein content of the potato; the prolamine and glutelin fractions together, however, represented only 9-13% of the total protein content, similar to the figures obtained for these two fractions by Lindner et al, (1960).

In contrast to recommendations put forward for efficient extraction of potato proteins, Purcell et al, (1972) reported that the efficiency of extraction of proteins from sweet potato, using water alone, could not be improved by adjusting either the pH or salt concentration of this extraction medium: they were able to extract 79.9% of the total (crude) protein content by their methods.

Yam proteins have been extracted with moderate success using either water alone (Hisatake, 1964), or water followed by 4% NaCl solution (Miège, 1978). In the former instance, approximately 75% of the total nitrogen content was extracted, whilst in the latter, 70-83%.

In this work, the yam proteins were less soluble in water than in dilute buffer, particularly under those conditions where the pH value of the extraction medium fell to below 6, or where the ionic strength, as contributed by the meal, was lowest (see section II.A).

Similar solubility characteristics have been reported for the plant storage proteins in general (Smith and Circle, 1938; Pusztai, 1965; Stegemann, 1975) and have largely reflected both the "globulin" nature of the proteins (Osborne, 1924), as well as the approach to isoelectric point and consequent precipitation. The formation of both protein-phytate (Smith and Rackis, 1957) and protein-tannin or -quinone (Patil and Zucker, 1965; Anderson, 1968; Van Sumere et al, 1975) complexes are also increased with decreasing pH values below 6-7, and the possibility of their formation in the water extracts may also have contributed to the decreased solubility of the yam proteins in this medium.

The final method used to extract the proteins from yam proved to be as efficient, with regard to the extraction of nitrogen content, as that described by Miège, (1978), and whilst the figures obtained for protein content (89% of the total protein content) may be overestimated, particularly since the presence of phenolic complexes associated with protein may interfere with the Lowry method for protein determination (Potty, 1969), they nevertheless indicated that the greater part of the proteins present were extracted. Furthermore, no additional protein sub-units were detected when SDS and 2-ME were incorporated into the extraction medium (fig. 60, tracks 1 and 2). The use of these latter two reagents has enabled the extraction of all or nearly all of the proteins from various seeds (Bietz and Wall, 1975; Hussein et al, 1977). A comparison of the amino acid composition of an extract with that of the ethanol-extracted meal provided further confirmation that a representative sample of the yam tuber proteins were extracted.

The formation of phenolic complexes during the extraction of plant proteins is a common phenomenon, and although various methods have been described to inhibit their formation (Pierpoint, 1966;

Loomis and Battaile, 1966; Anderson, 1968), none of these have been universally successful in application. Potato tubers, for example, contain several different phenolic compounds (Hanson and Zucker, 1963), as well as possibly different forms of diphenol oxidases (Patil and Zucker, 1965). Attempts to minimise the extent of browning of potato extracts have generally centered around the use of various reducing agents, in particular, sodium hydrosulphite. Metabisulphite has been recommended for effective inhibition of potato phenoloxidase activity (Stokes et al, 1968), although more recently, Seibles, (1979) found DIECA to be considerably more efficient in this respect. Use of either of these two reagents may prove to be more successful in minimising the browning of the yam extracts than that of the reducing agent, 2-ME. Polyvinylpyrrolidone has been shown to be an effective adsorbent of some phenolic compounds (Loomis and Battaile, 1966) but was ineffective as applied here. The pH optimum for the adsorption of the principal phenolic compounds in tobacco was found to be 3.5 (Anderson and Sowers, 1968), a pH value which would clearly have been unsuitable for the efficient extraction of the yam proteins.

Electrophoretic analysis of the extracted yam proteins indicated that only one protein sub-unit (hereafter referred to as the major sub-unit) was a principle component of the greater proportion of the proteins observed after both non-dissociating electrophoresis and isoelectric focussing. Selective dye-binding by different protein sub-units in the extract did not occur significantly, as was proved by the use of two of the more sensitive general protein stains, Coomassie Blue and Procion Blue, which exhibit different binding capacities for proteins, as well as by the use of TCA (see fig. 17).

The quantitative evaluation of proteins after electrophoretic separation is beset with various problems (Dravid et al, 1969; Kruski and Narayan, 1968; Gorovsky et al, 1970), and in order to quantify

the proportion of protein in an extract represented by the major sub-unit, densitometric analysis from a dilution series was undertaken in order to overcome that problem relating to the large difference in stain intensity between the major sub-unit and other sub-units. Even so, the heavier sub-units which migrated less far than the major sub-unit may have appeared in smaller proportion than they actually were, and vice versa for the smaller sub-units, (Kruski and Narayan, 1968). Association-dissociation effects dependent on, for example, protein concentration, were assumed to have been eliminated by preparing the dilution series in the presence of SDS so that evaluation of peak areas from such a series was considered to be valid. One further problem encountered related to the relative ease with which artifactual sub-unit profiles could be generated for the extracted proteins. Weber et al, (1972) have reported that the production of small sub-units may appear as a result of non-enzymatic degradation incurred with prolonged heat treatment during the incubation of some proteins with SDS. Artifactual sub-unit profiles may also be generated if the duration of incubation with SDS is extended without heat treatment: this was considered to be indicative of enzymatic degradation. Both these effects were identified for the yam proteins (fig. 16).

With appropriate precautions, i.e. standardised protein-SDS incubation conditions, it was estimated that 82-85% of the extracted protein was represented by the major sub-unit. It seemed possible, therefore, that some of the proteins separated by isoelectric focussing could be charge isomers of one another. The reduction in the number of proteins observed after acid gel electrophoresis compared to that after alkaline gel electrophoresis for gels of the same sieving capacity also suggested some degree of

charge isomerism amongst the proteins. Desborough and Peloquin, (1968), and Semikhova et al, (1969) have similarly observed a larger number of proteins (25-30) in potato extracts after separation in an alkaline gel system, but far fewer (4-14) in an acid gel system. The former workers, however, proposed that the acid gel system excluded many proteins, thus allowing fewer to migrate and so resulting in the simpler patterns (Desborough and Peloquin, 1969a). Such an explanation could, to some extent, be feasible, since the majority of potato proteins are acidic with isoelectric points which lie between pH4 and pH5.2 (Seibles, 1979). However, these values are similar to those estimated for the yam proteins (qv III C.4), but in this instance, none of the main yam protein fractions were retrograde in the acid gel system (see fig. 15). Accordingly, the simpler patterns obtained in the acid system relative to the alkaline were considered, as mentioned above, to indicate some degree of charge isomerism between the main yam proteins, the charge effects of which were masked after electrophoresis in the former system. Recently, Stegemann et al, (1973) have demonstrated that the majority of the main potato proteins differ by virtue of small charge differences which are largely due to differences in degree of amidation. It is possible, therefore, that the explanation provided to account for the yam electrophoretic patterns obtained in the two systems may also apply to some extent to the differences of the patterns observed for the potato proteins.

In addition to possible charge isomerism, the results obtained after ultracentrifugation suggested that some of the yam proteins could also represent oligomeric forms.

In summary, the implication from the above results was that there were several proteins each consisting principally of the major sub-unit, and in order to test this theory, a number of

separation methods were examined and compared for their effectiveness, measured both in terms of quantity of the isolate, and minimal effect on structural integrity, in isolating those proteins comprised of the major sub-unit.

Fractionation methods used to separate the potato protein fractions, up until very recently have concentrated on the exploitation of differences in solubility characteristics of a mixture of proteins, using, in particular, the Osborne, (1924) classification method or modifications of it. Although Osborne himself considered that there was only one protein in the potato tuber, a globulin termed "tuberin" (Osborne and Campbell, 1896), subsequent workers have recognised further fractions, namely, a water soluble "albumin" fraction, a water insoluble "globulin" fraction, and, less frequently, "tuberin", a globulin which is more easily soluble in water than the true globulin (Groot et al, 1947; Levitt, 1951; Lindner et al, 1960; Nakasone et al, 1972; Kapoor et al, 1975b; Seibles, 1979). However, different relative amounts of these fractions have been obtained by different workers, and furthermore the amino acid compositions of these fractions have been reported to be similar (Lindner et al, 1960; Kapoor et al, 1975b). Thus Seibles, (1979) considered that although a fractionation scheme based on the Osborne solubility criteria may have use in the preliminary fractionation of potato proteins, sharp distinctions between solubility classes were unreliable because of the complexity and "extreme instability" of these proteins.

Using the Osborne solubility criteria, the yam proteins comprised of the major sub-unit were largely determined to be "globulin" type proteins, although they also made up the greater proportion of the "albumin" fraction (fig. 21). Miège, (1978) similarly found that the yam proteins, after dialysis against water,

were mainly globulins, although in their work these represented on average 95% of the total protein. This contrasts to the situation in potatoes, where, for example, Kapoor et al, (1975b) and Seibles, (1979) determined that the greater proportion (75%) of potato proteins were albumins, although both Levitt (1951) and Nakasone et al, (1972) obtained nearly equal amounts of albumin and globulin. The "tuberin" fraction of potato proteins has been prepared by ammonium sulphate precipitation of an extract between the limits 40-60% (Lindner et al, 1960; Kapoor et al, 1975b). However, the yam fractions obtained from different ammonium sulphate "cuts" were all of similar sub-unit composition. Furthermore, the greater proportion of "globulins" were precipitated between higher limits (50-100%) of ammonium sulphate saturation: in general, the albumins are usually the last proteins to be salted out (Keller and Block, 1960). A possible explanation for this behaviour may lie with the fact that ammonium sulphate precipitation was carried out in buffer at pH 8.3. Both associated and dissociated forms of the protein were later shown to be present at this pH value, but with increasing salt concentration, or on dilution, the equilibrium was shifted toward dissociation: the associated proteins may be more susceptible to salting out phenomena than the dissociated proteins.

Whilst this fractionation scheme proved to be effective in isolating a large proportion of those proteins comprised of the major sub-unit, N-terminal analysis of the fraction revealed some degree of heterogeneity amongst the proteins. It is possible that these N-termini were undetected in the extract. Equally, however, the observed heterogeneity may have been introduced during the procedure as a result of proteolyticactivity (Lazarus et al, 1966; Clark and Jakoby, 1970; Lederer and Jacq, 1971; Diezel et al, 1973). Pusztai and Duncan, (1971), for example, have shown that proteolytic

activity, measured in terms of both autodigestion and digestion of denatured protein, reached a maximum in germinating seeds of Phaseolus vulgaris at pH values of 5.0-5.5. A similar situation may exist in the yam extracts, so that without resort to protease inhibitors, the structural integrity of the proteins may be adversely affected by this method of purification. Accordingly, further purification methods were investigated.

Gel filtration techniques using columns of Sephadex have been successfully applied by Nakasone et al, (1972) to isolate different protein fractions from potatoes, as judged by non-dissociating gel electrophoresis. However, these methods proved to be ineffectual in isolating any of the yam "storage" proteins free from other proteins, (see section IV.A), and furthermore appeared to increase the sensitivity of the eluted proteins to some form of (non) enzymatic degradation in SDS (fig. 20).

The isoelectric focussing profile of the extracted proteins under non-dissociating conditions had indicated that the majority of proteins were acidic in nature, but more importantly that the more intensely stained proteins were largely adjacent to one another and in a relatively restricted pH range (pH values 5-6.8), so that methods employing anion exchange chromatography were considered to hold potential for isolating these proteins. In fact, most of the proteins which contained principally the major sub-unit were effectively isolated from others by use of this method (fraction B, fig. 22(b)). No further purification of these proteins, measured in terms of the sub-unit profile, could be achieved by any of the other protein fractionation methods used in this work. Furthermore, the ionexchange method appeared to have minimal effect on the structural integrity of these proteins as judged by N-terminal amino acid analysis; in addition it did not,

for example, require the use of buffers of extreme acid or alkaline pH values which may have increased protein-phytin or protein-tannin complex formation, or non-enzymatic deamidation (Gilbert and Greenstein, 1949; McKerrow and Robinson, 1971; Robinson et al, 1973) and it also effectively separated phenolic compounds in the extract from the proteins. Since the proteins in fraction B represented by far the largest proportion of the protein in the extract, they are referred to as the "storage proteins" (see later). Those proteins comprised of the major sub-unit which were not adsorbed to the column (in fraction A) are not considered to necessarily represent different proteins of a less acidic nature, since the extracted proteins were applied directly to the column without prior dialysis against the starting buffer, and only low concentrations of salt (0.15M) were required to desorb the proteins in fraction B, i.e. fraction A may simply represent "overload". However, some differences were observed between the unadsorbed proteins and those in fraction B. Smaller "sub-units", whose mobilities corresponded with those produced artifactually for the extracted proteins after extended incubation in SDS at 25^{o}c, were present, and N-terminal amino acid analysis showed several additional amino acids, of which glutamine/glutamic acid, the only N-terminal amino acid in fraction B, was not especially prominent. Proteolytic degradation is the most likely explanation to account for these results. The proteins in fraction C were also largely comprised of the major sub-unit, but these, in addition to showing evidence of similar "proteolytic degradation", were brown coloured, indicative of the presence of quinones or tannins which may have been complexed with the protein.

It is surprising that the protein in both fractions A and C should show evidence of "proteolytic degradation" but not that in

B, the more so since under different circumstances i.e. after incubation in SDS and 2-ME at 25°c the B fraction protein showed evidence of similar "proteolytic degradation" (fig. 30) i.e. similar polypeptide products. These fragments are considered to have been produced enymatically since various methods which have been shown to denature proteases i.e. heat treatment at 100°c (Pringle, 1970; Van Heyningen, 1972), treatment with guanidine hydrochloride (Pringle, 1970; Weber et al, 1972) or treatment with urea (Matsubara and Feder, 1971), were effective in preventing their appearance in the fraction B protein after SDS incubation. Initially it might be thought that treatment with SDS, which is also a protein dissociating agent, should have given the same results as the other treatments. However, many proteases are only slowly inactivated with this reagent (Nelson, 1971; Van Heyningen, 1972; Diezel et al, 1973), and this may also be true of the situation encountered here. Indeed, the rate of proteolytic degradation observed in the storage protein fraction (B) was considerably retarded after 6h incubation with SDS and 2-ME. It is surprising that proteolytic activity was only observed in the presence of SDS, whilst at the same time SDS slowly inhibited proteolytic activity; the former result would imply, however, that some degree of denaturation of the substrate caused by SDS and enhanced in the presence of 2-ME is a pre-requisite for proteolytic degradation to ensue. It is not clear whether the source of proteolytic activity is a protease contaminant which shows strong interaction with the proteins comprised of the major sub-unit, or indeed, whether these proteins themselves are capable of auto-digestion under certain conditions.

Characterisation of the Storage Proteins:

Although fractionation of the storage protein preparation (fraction B) could be achieved by isoelectric focussing and by

non-dissociating electrophoresis, the use of two-dimensional techniques demonstrated unequivocably that each component gave the same sub-unit profile in which the major sub-unit represented by far the greatest amount (96%) of the protein. The larger minor sub-units were demonstrated to represent polymers of the major sub-unit by virtue of the correspondence in mobility with gluteraldehyde-treated protein. They are, however, not linked by disulphide bonds, unlike the dimer sub-units observed for the potato storage proteins (Stegemann et al, 1973) since they were still present in the profile after reduction with 2-ME followed by alkylation. They represented around 2% of the total protein and may be in vitro products of purification which, for some reason, could not be dissociated with SDS. Two of the smaller minor sub-units (Rm values, 0.65 and 0.71) are considered to represent chain scission fragments produced in vitro after treatment of the major sub-unit with reducing agents; they were not evident in the sub-unit profile for the storage proteins treated with SDS alone, but after treatment of the SDS-protein complexes with 2-ME, and by the use of two-dimensional electrophoretic techniques they were shown to be derived from a small fraction of the major sub-unit (qv V E.3) which appears to have intrachain disulphide bonds reduced prior to 2-ME treatment, as judged by relative mobility values. The argument for chain scissions is supported by the relative ease with which fragments could be generated from the (reduced) major sub-unit in the presence of SDS at elevated temperatures for six minutes or more (fig. 30). Chain scissions may also be responsible for the additional N-terminal amino acids observed under dissociating conditions, since this treatment includes performic acid oxidation of the protein, a treatment which has been shown to cause chain scissions of some proteins (Arndt and Berg, 1970) as well as treatment with SDS.

Without such treatment, however, only a single N-terminal amino acid could be identified, that of glutamine/glutamic acid.

The third smaller sub-unit (Rm value 0.56) corresponded in relative mobility to that of the unreduced major sub-unit, and is considered to represent a fraction of the major sub-unit for which intra-chain disulphide bridges (if present) could not be reduced, for this smaller sub-unit was still present in the sub-unit profile after alkylation of the 2-ME-treated protein. It could not be separated from the storage protein fraction by a number of fractionation methods (qv) and furthermore, two-dimensional isoelectric focussing-SDS electrophoresis showed that it was a component of at least two of the storage proteins, and possibly more (fig. 36). It may represent a genetic variant of the major sub-unit.

The molecular weight of the major sub-unit was determined by the method based on SDS gel electrophoresis, which has been shown to give reliable estimates of molecular weight for a number of different proteins electrophoresed in both the continuous (Shapiro et al, 1967; Weber and Osborn, 1969; Dunker and Rueckert, 1969; Shapiro and Maizel, 1969) and discontinuous (Laemmli, 1970; Neville, 1971; Ames, 1974) SDS buffer systems. The method is, however, dependent on a universal mechanism for SDS binding to protein, such that the binding ratio, on a gram to gram basis, is identical for all proteins. Whilst this has been shown to be true for a large variety of proteins (Reynolds and Tanford, 1970; Nelson, 1971; Pitt-Rivers and Impiombato, 1968) there are some situations where abnormal SDS binding can occur, for example, when there are restrictions on the conformational freedom of the protein. These include protein modification, such as succinylation (Pitt-Rivers and Impiombato, 1968) or maleylation (Tung and Knight, 1971),

extensive chemical crosslinking (Griffith, 1972), failure to completely reduce disulphide bonds (Pitt-Rivers and Impiombato, 1968; Reynolds and Tanford, 1970; Griffith, 1972) or an inherent rigidity of the polypeptide chain, as is found, for example, with collagen which has a high imino acid residue content (Furthmayr and Timpl, 1971). Highly charged proteins, for example pepsin, may also show abnormal SDS binding (Nelson, 1971), as well as glycoproteins (Schubert, 1970; Voyles and Moskowitz, 1974). In addition, there are some proteins, e.g. histones, which show normal SDS binding but the intrinsic charge of the protein is such as to influence electrophoretic mobility (Hayashi et al, 1974).

The storage protein preparation did not contain covalently attached carbohydrate moieties (qv V.D), and exhibited no unusual features with regard to either charge (qv V.G) or amino acid content (qv V.C). After complete denaturation with both SDS and 2-ME, with or without subsequent alkylation, the molecular weight of the sub-unit was estimated as 31,000. However, without prior treatment with 2-ME, the sub-unit showed an increased electrophoretic mobility i.e. a molecular weight of 27,350. A similar result has been obtained for a number of proteins containing intrachain disulphide bridges (Griffith, 1972), although for some proteins the presence of intact intrachain disulphide bridges has either no effect on their electrophoretic mobility in SDS, or causes decreased mobility (Dunker and Rueckert, 1969; Griffith, 1972). It is considered here, that an intact intrachain disulphide bond imposes conformational restrictions on the sub-unit, and under these situations, the protein molecule is more compact, offering less frictional resistance to passage through the gel than the reduced sub-unit. To support the notion that there is an intrachain disulphide bridge in the polypeptide chain is the fact that the

minimal molecular weight of the protein, based on only one cysteine (cysteic acid) residue per molecule is 10,400, which, when equated with a sub-unit molecular weight of 31,000, argues for the presence of three cysteine residues per molecule, two of which may be oxidised together as cystine.

It is interesting to note that, in the absence of disulphide bond reduction, the amount of SDS-treated protein representing dimer in particular is increased. Also under other conditions which commonly favour dissociation of proteins into sub-units i.e. extreme acid pH values (Klotz et al, 1970), both the 31,000 sub-unit and a small fraction of dimer were identified (fig. 41(c)). It is possible that a limited degree of disulphide bond formation can occur between sub-units, which could probably be effected through the third cysteine residue on each sub-unit. However, as will be noted, this is not the chief mechanism by which association of the sub-units occurs. An alternative explanation is that a small fraction of the dimer of the 31,000 sub-unit exists with strong non-covalent bonding between sub-units and that this bonding is not disrupted until the native conformation of the component sub-units is destroyed by intrachain disulphide bond reduction. Under these circumstances, SDS alone, for example, may be ineffective in causing total dissociation of the protein. SDS has been shown to be ineffective in causing total dissociation of some oligomeric proteins (Katzman, 1972).

One further aspect of the SDS treated protein concerns the appearance of two overlapping bands after electrophoresis of a dilute protein preparation in the more sensitive discontinuous buffer system. This is considered to reflect the differences of intrinsic charge of the major sub-unit, a property which is discussed elsewhere, rather than heterogeneity of size, since although SDS

binding is considered, in general, to swamp out the intrinsic charge of proteins (Dunker and Rueckert, 1969; Reynolds and Tanford, 1970; Shapiro and Maizel, 1969) there are instances where intrinsic charge effects contribute markedly to electrophoretic mobility, e.g. the histones (qv), and in fact, for polypeptides of less than 10,000, intrinsic charge effects can become more important determinants of electrophoretic mobility in SDS than size alone (Swank and Munkres, 1971). Furthermore, if there was size heterogeneity in the major sub-unit, it would be anticipated that, after SDS electrophoresis in gels of varying bis concentration, the effect of which has been shown to cause a dramatic alteration in gel sieving capacity (Hedrick and Smith, 1968; Johnson, 1979), some degree of separation of the sub-units would be achieved. There was, however, no alteration in the appearance of the major sub-unit, and this in itself supports the argument for charge isomerism as the main cause for the heterogeneity of the sub-unit indicated after SDS electrophoresis.

Investigations of the quaternary structure of the native proteins by molecular sieve chromatography and by analytical ultracentrifugation revealed a heterogeneous mixture of proteins with regard to size. In view of the fact that the protein preparation was comprised of only one sub-unit size of 31,000 (henceforth referred to as the monomer), the observed heterogeneity in this instance must reflect interaction of sub-units to form polymers. In the analytical ultracentrifuge, the approximate sedimentation coefficient of the schlieren boundary corresponding to the largest peak yielded a molecular weight in the order of that anticipated for a tetramer. However, both the diffuse and asymmetrical nature of the peaks, together with the fact that with a two-fold increase in protein concentration, the area of the slowest peak representing most probably both monomer and dimer (Cann, 1970) remained relatively

constant whilst that of the faster sedimenting peaks increased, suggested, in accordance with the predicted behaviour of interacting molecules discussed by Cann, (1970), that a reversible association-dissociation reaction existed between sub-units, the rate of which, nevertheless, was relatively slow compared to the duration of ultracentrifugation. Confirmation of these observations, i.e. that monomeric sub-units could indeed associate to form dimers, possibly trimers, tetramers and, to a lesser extent, higher polymers, was provided by results obtained from molecular sieve chromatography, a technique which has been demonstrated to be comparable to ultracentrifugation in terms of both the nature and accuracy of the information which may be derived for association-dissociation reactions (Ackers and Thompson, 1965; Tan et al, 1975). However, it is apparent from these studies that the association-dissociation behaviour of the sub-units is complex. It appears to depend not only on protein concentration, but also on pH value and ionic strength of the environment and probably also partly on the starting sub-units, which, as will be noted, exist as charge isomers of one another. Thus, for example, individual samples of monomer, tetramer and the initial storage protein preparation, when chromatographed under the same conditions with regard to protein concentration, pH value and ionic strength of buffer, gave different association-dissociation equilibrium positions to one another (qv V L.1(iii)).

Covalent intermolecular disulphide bond formation between sub-units does not appear to be involved in the association mechanism between sub-units; this is evident both from the fact that interconversion of species could be achieved relatively rapidly by simple alteration in protein concentration, as is shown, for example, by the increased degree of interaction observed

between species when factors such as column dimensions or flow rate were altered in favour of increased zone dispersion effects (Morris and Morris, 1976), and also from the fact that the same elution profiles were obtained whether chromatography was carried out in the presence or absence of DTT, a reagent which is effective in reducing disulphide bonds (Konisberg, 1972). However, as noted earlier, under some circumstances there may possibly be a limited degree of intermolecular disulphide bond formation between dimers. Electrostatic forces may be involved to some extent in the mechanism of interaction, as suggested, for example, by the almost total dissociation of sub-units effected at pH 2.6 (fig. 41(c)), a pH value in which the net charge of the sub-units was demonstrated to be relatively large by comparison with that at pH 8 (qv V.I), and also perhaps by some of the effects produced by change in ionic strength. However, other bonding mechanisms, e.g. hydrophobic or hydrogen, must clearly also be involved, and in view of the complexity of the interactive forces demonstrated to exist between other proteins which exhibit association-dissociation behaviour (Reithel, 1963), it would be naïve at this stage to attempt to elucidate further the nature of the bonding mechanisms involved. Also, until the microenvironment of the proteins in vivo can be defined, it is clear that no definite statement concerning the quaternary structure(s) of the native proteins should be made.

It is of interest that, whilst investigation of the proteins by molecular sieve chromatography could be effectively carried out using columns of polyacrylamide gel (Bio-gel), such investigations using columns of Sephadex in particular, but also Ultrogel, a composite of agarose and polyacrylamide were notably unsuccessful since the proteins exhibited marked adsorbence phenomena on these matrices.

Adsorbence properties during chromatography on Sephadex have been observed for a number of different proteins (Glazer and Wellner, 1962; Miranda et al, 1962) including haemagglutinins of both plant (Agrawal and Goldstein, 1965; 1967; Howard and Sage, 1969; Tichá et al, 1970; Fialová et al, 1975) and animal (Rosen et al, 1974; Simpson et al, 1974) origin, and also for certain amylases (Dellweg et al, 1975). They have also been observed for a number of lower molecular weight solutes, e.g. certain inorganic anions (Gelotte, 1960; Sinibaldi and Leder, 1975); non-polar molecules or molecules bearing non-polar groups (Marsden, 1965; Eaker and Porath, 1967; Determann and Lampert, 1972; Brook and Munday, 1970); as well as for a number of aromatic and heterocyclic compounds (Gelotte, 1960; Marsden, 1965; Eaker and Porath, 1967; Marklová and Hais, 1977) including flavonoids (Woof and Pierce, 1967) and tannins (Somers, 1966; King and Pruden, 1970). The adsorbence phenomena observed have been differently attributed to the following:

1. Electrostatic interaction between basic compounds and ionised carboxyl groups on dextran chains (Gelotte, 1960).
2. Hydrogen bonding between substituents of aromatic compounds or the hetero-atom of heterocyclic compounds (Woof and Pierce, 1967; Brook and Housley, 1969b; Brook and Munday, 1970; Williams, 1972) and the hydroxy-ether linkage between dextran chains (Determann and Walter, 1968); or interaction involving the π-electron system of such compounds (Janson, 1967; Brook and Housley, 1969a). It should be noted, however, that the exact nature of aromatic adsorption is not clear (Haglund, 1978).
3. A specific affinity for dextran residues, exhibited by some haemagglutinins and amylases as noted above.
4. Hydrophobic interactions between non-polar groups (Determann and Lampert, 1972; Marsden, 1977) or inorganic anions (Sinibaldi and Leder, 1975) and possibly the non-polar faces

of anhydroglucose residues of Sephadex (Marsden, 1977).

In this instance, the adsorbence phenomenon exhibited by the yam proteins cannot be attributed to electrostatic interaction: not only did the various eluants used contain electrolytes, but also both the protein and columns were buffered at pH 8 or thereabouts so that "ion exclusion" conditions were operating; furthermore, the present dextran gels contain much lower concentrations of ionizable carboxyl groups relative to the early gels (Janson, 1967) and have consequently a much lower capacity for electrostatic interaction than formerly.

If hydrogen bonding mechanisms or π -electron interactions between for example, aromatic amino acid residues or bound phenolic complexes on the surface of the protein molecules and the Sephadex matrix are evoked to explain the adsorption phenomena, then it would be anticipated that similar phenomena would be observed on polyacrylamide gels which also contain sites for such interactions, namely carbonyl and amido groups. In fact, a number of workers (Sun and Sehon, 1965; Schwartz et al, 1965; Schwartz and Zabin, 1966; Simkin, 1970) have shown that aromatic, heterocyclic and other compounds are indeed adsorbed on these gels, most probably by hydrogen bonding or π -electron interaction (Williams, 1972). However, in only one instance was "adsorption" of some, but not all, of the proteins observed during chromatography on polyacrylamide gels; this occurred in low ionic strength buffers at pH 4.7 (fig. 41(d)), a pH value very near the isoelectric points of the isomers. Since some precipitation of the protein in this solution occurred prior to chromatography, the effect observed may well represent further precipitation of some of the isomers during chromatography, rather than "adsorption" of the nature described above.

Proteins which show a specific affinity for the dextran residues of Sephadex are characteristically potent haemagglutinins, as demonstrated, for example, by discoidin (Simpson et al, 1974), concanavalin A (Agrawal and Goldstein, 1967) and some of the lentil phytohaemagglutinins (Vančurová et al, 1976). However, the yam proteins showed no haemagglutinating activity toward rabbit erythrocytes, and furthermore showed no interaction with any of the three glycoproteins tested (section V.M), unlike concanavalin A which showed a specific interaction toward one of these glycoproteins, thyroglobulin. Nevertheless, the elution profile obtained after chromatography in the presence of glucose (fig. 44(c)) showed that whilst the proteins still exhibited marked adsorption properties with Sephadex, the extent of interaction was not as great as formerly observed. A similar phenomenon, however, has been reported for the hydrophobic interaction exhibited by essentially non-polar solutes toward the Sephadex matrix (Marsden, 1977). Marsden has suggested that in this instance, the reduction in adsorption observed with glucose in the eluant may well be due to competition between hydrophobic interaction sites, i.e. the non-polar faces of the anhydroglucose residues of Sephadex and the non-polar faces on the glucose molecules in the eluant. It may well be that some degree of the adsorption phenomenon exhibited by the yam proteins can be attributed to hydrophobic interaction by non-polar amino acid residues or non-polar groups on amino acids. Although it is commonly believed that non-polar groups are in general, in the interior of protein molecules (Lehninger, 1975), Klotz, (1970), has reported that hydrophobic side chains occur much more frequently on the surface of the protein than had been assumed. Furthermore, Keshavarz and Nakai, (1979) have proposed that the non-polar portion of polar side chains, e.g. the four methylene groups of the lysine side chain, could also participate

in hydrophobic interactions. These workers have also reported that the measured effective hydrophobicities of a number of different proteins cannot be related to their average hydrophobicities calculated from their amino acid compositions. Thus whilst the relative amounts of e.g. non-polar amino acids in the amino acid composition of the yam proteins were not unusual by comparison with those in, for example, the FAO standard protein, this clearly gives no indication of the effective hydrophobicity of the proteins.

The 31,000 sub-unit shows charge heterogeneity and at least six different charge isomers were separated by electrophoresis on isoelectrically focussed gels under dissociating conditions (fig. 37(a)). An identical result was obtained when the protein was analysed under non-dissociating conditions (fig. 37(b)), proving that isomers only of the same charge can associate to polymers. The fact that additional bands were not seen on the latter gel, i.e. there was no discrimination between monomer, dimer, etc., of the same charge isomer, is probably due to the fact that gels of low sieving capacity were used, and indeed, with reference to the Ferguson plots, it is clear that improved resolution between monomer and polymers can only be achieved with the use of gels of at least 10% total acrylamide concentration, together with bisacrylamide concentrations (%C) of around 5% which favour decreased pore size (Morris and Morris, 1971; 1976). In addition, the equilibrium between association and dissociation of the sub-units shows a marked dependence on protein concentration, so that if both the sieving capacity of the gels were increased, and samples of increased protein concentration were used, it might then be possible to demonstrate polymers of identical charge isomers. Even so, it must be borne in mind that pH also has a marked effect on the association-

dissociation equilibrium and hence, as the gel consists of a continuous pH gradient between values of around 3 to 10, individual bands will broaden and coalesce according to the equilibrium position at that pH value.

Values of isoelectric point for the charge isomers were estimated both from the pH-mobility curves described above, and also by the more commonly used method of isoelectric focussing in polyacrylamide gels. The differences in values obtained (see section V.G, V.I and also fig. 18), although small, are considered to highlight some of the drawbacks of the latter technique. These arise in the main from the fact that proteins must be electrophoresed through polyacrylamide gels which stabilize the pH gradients. In order to achieve adequate mechanical stability, the gels are commonly prepared with acrylamide concentrations around 5-8% (Drysdale, 1975; Leaback and Wrigley, 1976), but as such they exert a significant sieving effect on all but the smallest polypeptides (Rodbard and Chrambach, 1970) and consequently extend the time required for proteins to reach equilibrium. Thus, whilst there may be no apparent alteration in band position after a few hours of focussing, equilibrium may not be reached until after 50h (Finlayson and Chrambach, 1971). However, with increased duration of focussing, a "pH plateau" commonly develops in the centre of the gel (Miles et al, 1972; Rilbe, 1977), resulting in a greater separation of proteins with pI values near the pH value of the plateau and a compression of distal bands.

Another drawback of the latter technique is that proteins which are unstable at any pH value along the gradient may precipitate before the equilibrium position is reached, so that unless a solubilising agent is incorporated in the medium, erroneous values for isoelectric point may be obtained. This is illustrated by the

values obtained for isoelectric point of the isomers focussed in the absence of urea (fig. 34). Urea is commonly used to circumvent this problem, but this reagent in turn may alter the isoelectric point of some proteins (Ui, 1971; Salaman and Williamson, 1971) as well as to carbamylate α -amino groups under certain circumstances (Cole and Mecham, 1966; Cejka et al, 1968), and has also been shown to raise the ampholine pH gradient (Josephson et al, 1971).

Problems such as these are not encountered with the former technique, and furthermore artifacts which may be introduced as a result of complex formation between protein and ampholytes (Williamson and Salaman, 1973), or reaction of protein with the gel catalyst persulphate (Maurer, 1971) are eliminated. In addition, the pH gradient has been demonstrated to be stable for at least one hour of electrophoresis (Righetti et al, 1978), with only slight variation in the region of the gel which is adjacent to the anode during focussing (Rosengren et al, 1977). Consequently, the method used in this work to determine the pH gradient will probably give a reliable estimate of the pH gradient at the point of sample application. The values obtained for isoelectric point by this method must nevertheless still be regarded as approximate since although the pH gradient was measured at 25^{o}c, electrophoresis was conducted at 4^{o}c to minimise the risk of denaturation: the pI values thus obtained may differ slightly from the true values at 25^{o}c (Fredriksson, 1977).

The presence of charge isomers explains why so many components were observed after PAGE in the alkaline buffer system (fig. 45), since this technique fractionates proteins on the basis of both molecular size and charge, and consequently separation of at least some of the differently chargedmonomer, dimer, etc., components would

be anticipated. Confirmation that the observed components do indeed represent charge isomers of monomer, dimer and tetramer was obtained when Ferguson plots were constructed for the individual components. Such plots are described by the following:

$$\log M = \log Mo - KrT$$

where M is electrophoretic mobility; Mo, the free electrophoretic mobility; Kr, the retardation coefficient; and T, the total acrylamide concentration. This relationship for electrophoretic mobility was first recognised by Ferguson, (1964) and subsequently Hedrick and Smith, (1968) and its validity has now been confirmed for a wide range of undenatured proteins (Morris, 1966; Cheever and Lewis, 1969; Rodbard and Chrambach, 1970; Morris and Morris, 1971).

In view of this relationship, it is apparent that charge isomers will yield a series of parallel lines each with the same slope (Kr value) since their mobility relative to one another will be independent of the sieving action of the gels, whilst size isomers with the same charge/mass ratio will yield a series of non-parallel lines which extrapolate to a common value on the y-intercept (0%T) corresponding to their free electrophoretic mobility. The fact that size isomers in this work did not extrapolate to a common value on the y-intercept must be attributed, in part, to the difficulty in assigning precise values for acrylamide concentrations. Although conditions of polymerisation were standardised as far as possible (Kingsbury and Masters, 1970), nevertheless even under these circumstances, 100% polymerisation may not be achieved (Rodbard and Chrambach, 1971; Chen and Chrambach, 1979). Furthermore, Morris and Morris (1971) have shown that gels of decreasing crosslinking from C = 7% to C = 1% exhibit

a correspondingly increased tendency for water regain from buffer solutions, so that the nominal values indicated for these gels of C = 5% may, in fact, be overestimated by as much as 20-30%. In addition, mobilities were measured relative to the tracking dyes which are also retarded and unstacked to some extent in discontinuous buffer systems (Rodbard and Chrambach, 1971) and thus do not correctly represent the ion boundary. Correction can be made to relate relative mobility to absolute electrophoretic mobility (Rodbard and Chrambach, 1971), but the principal aim of this investigation was not to derive absolute values for free electrophoretic mobility, but rather to distinguish charge from size isomers.

The value of Kr has been demonstrated to be a linear function of molecular weight for spherical proteins ranging from 50,000 to 500,000 (Hedrick and Smith, 1968; Cheever and Lewis, 1969); but for proteins below 50,000 the relationship is non-linear (Gonenne and Lebowitz, 1975). Consequently, the identification of the size isomers was approached on the basis that values of Kr determined in gels of low %C (1-5%) are linearly related to the molecular radii of spherical molecules by the following:

$\sqrt{Kr} = c\ (\bar{R} + r)$, where c is a constant for any specified electrophoretic system (buffer, pH value, ionic strength, temperature, %C), $\bar{R}$ is the geometric mean radius, and r, the effective radius of the gel fibre (Rodbard and Chrambach, 1970). The validity of this relationship has been demonstrated experimentally by a number of workers (Rodbard and Chrambach, 1971; Morris and Morris, 1971; Gonenne and Lebowitz, 1975). The values of $\bar{R}$ thus obtained were compared with those of standard proteins to assess approximately the order of size. More precise values of $\bar{R}$ will obviously be obtained with improved calibration curves, but the objective in

this instance was merely to distinguish between monomer, dimer and tetramer.

The results obtained confirm the effect of pH value, firstly, on the association-dissociation behaviour of sub-units i.e. the equilibrium between association and dissociation lies toward association in alkaline pH values, dissociation in acidic; and secondly, on the degree of charge difference between isomers i.e. these are greater in alkaline pH values than in acidic (cf fig. 37). In addition, the effect of protein concentration observed in fig. 45 can be appreciated since the appearance of the new bands with increasing protein concentration correspond to the appearance of dimers and tetramers as identified by the Ferguson plots: this result is again in accordance with the effect of protein concentration on the association-dissociation behaviour of the sub-units.

In common with many other storage proteins (see for example, Boulter and Derbyshire, 1971), the amino acid composition of the yam "storage proteins" indicates high relative amounts of aspartic and glutamic acids (and/or their amides), as well as comparatively large relative amounts of basic amino acids, but low levels of the sulphur amino acids (table 18). Further information concerning the amino acid composition of the protein can be obtained from an examination of the pH-mobility curves (fig. 37). In relation to the amino acid composition, the overall shape of these curves, both in the presence and absence of urea, does not, in fact, reflect the high content of acidic amino acids, but rather, larger relative amounts of basic amino acids: this result can be accommodated with the amino acid composition if, in fact, the greater proportion of the acidic amino acids are indeed amidated. This feature, i.e. a high degree of amidation, together with the fact that the isolated

proteins represent by far the greatest amount of protein in the tuber (qv), are amongst the main reasons for classifying them as "storage proteins" (see for example Stegemann, 1975).

The pH-mobility curves also indicate that the differences between the individual charge isomers identified are probably due, to a large extent, to small differences in relative content of carboxyl and neutral groups, since the individual curves were essentially united around pH values of 4 and less, corresponding to the pH range in which β and γ (and α) carboxyl groups are protonated, but above pH 5, the curves ran essentially parallel to the limit of the gradient, pH value 8 or thereabouts. The "neutral" groups involved may well be amidated forms of these residues. In addition, however, the curves indicate that some of the isomers may also differ in relative content of other charged amino acids. Based on predictions made by Righetti et al, (1978) for the shapes of curves which ensue when charged amino acids in proteins are substituted for differently charged or neutral amino acids, the increase in zone width between isomers of pI values 6.1 and 6.5 at pH 4.8 would tend to suggest that the former isomer has a greater relative amount of basic e.g. lysine or arginine, and lesser relative content of acidic amino acids than the latter; whilst the unification of some of the curves in the region of pH values 6-7 would imply that there may be small differences in content of imidazolyl groups amongst some of the isomers. Although these interpretations are necessarily tentative at this stage, the overall implication is that the charge isomers of the 31,000 sub-unit differ by virtue of small differences, possibly in degree of amidation, but also in relative content of other amino acids.

Since the storage proteins thus classified represent by far the largest amount of extractable protein in the tuber, it would be anticipated that the "protein aggregates" identified in the storage tissue (figs. 54 and 55) represent the sites of deposition of these proteins. In the attempt to prove this by isolating and analysing the aggregates, however, disappointing results were obtained, but such results would nevertheless be in keeping with the observation that the protein aggregates may not, in fact, be bound by a limiting membrane, unlike the protein bodies seen in a wide range of seeds (see for example Harris and Boulter, 1976; Harris and Juliano, 1977). Little work has been undertaken as regards the localisation of the potato storage proteins, although Marinos, (1965) identified membrane-bound cubical crystals in the outer cortex of the tuber: these contained a heterogeneous mixture of protein components considered to represent a form of storage protein (Hoff et al, 1972), but differing, however, to the storage proteins extracted from the storage tissue of the tuber (Stegemann, 1975).

Some of the similarities between these latter potato storage proteins and the yam storage proteins described in this work are noteworthy. Thus, for example, Stegemann et al, (1973) have shown that the extractable potato proteins were separable on the basis of charge by isoelectric focussing, but that the main proteins had the same sub-unit composition. Moreover, in contrast to the wide range of sub-unit size found in the proteins of, for example, maize seeds (Stegemann, 1975; 1977; 1978), there were only three principle sub-units of molecular weight 16,800, 18,000and 19,500, a fraction of the 16,800 sub-unit of which was split after reduction with 2-ME to give two sub-units, 13,800 and 10,000. The larger sub-units in the electropherogram, molecular weights 34,500, 36,500 and 39,500,

which were about one-tenth the concentration of the major protein sub-units, were considered to be dimers linked by disulphide bridges (see also Seibles, 1979). In the yam, however, although disulphide bridges were present, these were, in the main, intramolecular.

The charge differences between the main potato proteins were considered to be due, to some extent, to differences in degree of amidation (Macko and Stegemann, 1969; Stegemann et al, 1973), but these proteins also differed, but within very narrow limits, by virtue of differences in relative amino acid content (Stegemann, 1978), similar to the conclusion drawn for the differences between the yam charge isomers. Association-dissociation behaviour amongst the potato proteins, however, has not as yet been identified, and indeed, the three main proteins separated by PAGE in alkaline pH values were identified as charge, not size, isomers (Stegemann, 1978; 1979).

One further observation is that whilst different varieties of potato may be specifically identified by characteristic, genetically determined electropherograms obtained after non-dissociating PAGE of the extractable tuber protein (Desborough and Peloquin, 1966; 1968; 1969a,b; Loeschke and Stegemann, 1966; Semikhova et al, 1969; Zaccharius et al, 1971; Czupryn and Tockzo, 1974; Stegemann and Loeschke, 1977), the different varieties all yield, in fact, the same SDS-PAG profile, i.e. the protein sub-units in each variety have the same size, and fall into two size categories, one around 16,800 and one around 30,000 (Stegemann et al, 1973; Seibles, 1979). Stegemann, (1978) furthermore has reported that the main proteins of different varieties separated by non-dissociating PAGE differ only by virtue of very small differences in their amino acid

composition, and has suggested that in view of the limited variability in protein composition demonstrated between varieties, breeding for higher protein quality in potatoes will not be very successful. Although electropherograms for different yam varieties prepared by non-dissociating PAGE were not investigated, it is noteworthy that each variety analysed by SDS-PAGE gave the same sub-unit profile (fig. 60). This result suggests that there may be limited variability in protein composition of different yam varieties, and although only a few varieties were analysed, it may well be that the potential for breeding yams with improved protein quality is limited. Indeed, the amino acid profiles of the two varieties analysed here, compared with those reported by Evans (I.I.T.A. in-house report, 1976) for varieties produced from seed, and those reported by Francis et al, (1975) (table 33) also suggest limited variability of amino acid composition in yams, and in accord with the conclusion of Francis et al, (1975), on the basis of amino acid composition alone, there seems to be little evidence to substantiate the idea (Splittstoesser, 1977) that yam breeding experiments could be successful in producing a better balanced protein.

Perhaps more attention should be focussed instead on the reports that (cooked) yam meals may have adverse effects on the growth of experimental animals (Womack et al, 1977; Maranon et al, 1977). The results reported by Maranon et al, however, were obtained from experiments using chicks, and, whilst the diets per se may have contained anti-physiological components, diets containing potato have also been reported to have a detrimental effect on the growth of chicks (D'Mello et al, 1973). In the latter instance, however, the authors attributed their results to the adverse physiological properties of ground potato, i.e. the

Table 33 Comparison of the amino acid composition of yams

Amino Acids	Amino Acid Composition (g/16gN)							
	Busson[a] (1965)	Francis et al (1975)	The present study			Evans (1976)		
	Average of 11 Samples	Average of 7 Samples	Nwapoko (1977)	Nwapoko (1979)	Yam 2	B72-7	M136	M185
aspartic acid	10.5	10.0	11.0	11.1	12.7	9.6	10.8	11.7
threonine	3.7	3.4	2.9	2.9	3.0	2.9	2.9	3.5
serine	5.1	5.0	4.1	4.1	5.9	4.2	4.0	4.9
glutamic acid	12.7	12.4	14.1	13.0	16.3	15.0	14.8	14.6
proline	4.0	3.3	3.7	3.0	2.9	2.6	3.1	2.4
glycine	3.9	3.6	3.0	2.9	3.8	3.1	3.1	2.8
alanine	4.2	4.4	3.7	4.4	5.1	3.4	3.5	4.1
valine	4.7	4.1	3.8	3.7	4.4	3.6	3.9	3.6
methionine	1.7	1.2	1.3	1.1	1.1	1.4	1.4	1.3
isoleucine	3.8	3.3	3.2	3.0	3.6	3.0	3.3	2.5
leucine	6.7	5.8	5.7	5.4	6.8	5.4	5.6	4.6
tyrosine	3.2	3.3	2.2	1.7	1.8	2.4	2.1	2.6
phenylalanine	4.8	4.6	4.5	3.9	5.0	4.1	4.7	3.6
histidine	1.9	1.9	1.9	1.9	1.9	1.3	1.6	1.7
lysine	4.2	4.3	4.6	4.6	4.6	4.3	4.4	4.0
arginine	7.7	7.7	7.4	6.9	5.3	8.9	6.3	9.0
cystine	1.2	1.3	0.8	1.0	0.9	1.1	1.2	1.1
tryptophan	0.9	1.2	ND	ND	ND	ND	ND	ND

[a] cit. Francis et al, 1975

chicks had difficulty in consuming the diet. In consideration of the overall similarity in composition between yam and potato, this may also be true in the experiments conducted by Maranon et al, (1977).

The results of Womack et al, (1977) indicated that cooked, but not uncooked, meal affected adversely the growth of rats. The cooked meals were prepared from tubers which were peeled, cubed, cooked until soft, drained, mashed and then dried at 60°c. In contrast, these authors observed that meals which were prepared from tubers which were similarly peeled and cubed, but then autoclaved at 121°c for ten minutes, then lyophilised and ground, did not have a detrimental affect on the growth of rats. These authors suggested that "toxicity was added during the cooking and drying". However, it should be noted that in these experiments, only the protein content, measured as %N x 6.25, of the prepared meals was recorded, and not the amino acid composition.

Whilst it is generally recognised that the sulphur amino acids and possibly tryptophan are the limiting amino acids in yams when the total amino acid content is analysed (see introduction), the results obtained in this work after cooking (see section I.D) indicated a substantial loss (around 18%) of cysteine (cysteic acid) to the cooking liquid, as well as losses of other essential amino acids including threonine (13%), the second or third limiting amino acid in this cultivar. These results suggest that in nutritional experiments account must be made for the fact that a proportion of the essential amino acid content of yams may be lost after cooking. The total loss of amino acids recorded (12%) was higher than that reported by Splittstoesser, (1976) (3-8%), and probably reflects, in part, the use of dried yam in this work;

however, losses of amino acids after cooking may also be related to the dry matter content of the tubers, as has been reported for potatoes. Thus Jaswaal, (1973) reported a loss of as much as 40% of the total amino acid content in potatoes with a low specific gravity (% dry matter), but only half this amount (20.6%) for potatoes with a high specific gravity.

Studies on the presence of anti-physiological components in yams indicated that yam meals contain large quantities of saponins, but saponins in general do not have an adverse effect after ingestion (Birk, 1969). Trypsin inhibitor activity was not detected, neither was lectin activity, although Miège, (1978) reported the presence of trypsin inhibitor activity in some of the yam cultivars studied. Further investigation of possible anti-physiological components in a variety of yam cultivars is required.

Breeding for improved protein content requires a simple, rapid and inexpensive method which gives reliable estimates of this character, and probably the most widely used method is that of Kjeldahl. Indeed, this method has been used extensively to assess "protein content" in yams (Oyenuga, 1968; Martin and Thompson, 1971a, b; 1972; Splittstoesser et al, 1973a, b; Francis et al, 1975; Baquar and Oke, 1976). However, protein content of yams measured by this method is greater, i.e. over-estimated, than that determined from amino acid analysis (Martin and Thompson, 1972; Martin and Splittstoesser, 1975), and this is further exemplified by the data in table 11. Some of this discrepancy must be attributed to the fact that the amino acid measurements did not allow for any unhydrolysed peptides in the 22h hydrolysate, or for amino acids which were destroyed e.g. tryptophan, although the content of this latter has been reported

to be low (Francis et al, 1975; Splittstoesser and Martin, 1975). However, the major part of the discrepancy arises from the fact that the Kjeldahl method is based on measurements of N, and as such will include values for inorganic and non-amino acid organic N, i.e. not all of the N measured is proteinaceous.

A second consideration of the Kjeldahl method for estimating "protein content" is that it includes values for free amino acids which may contribute little to the diet if they are lost during food preparation (Jaswaal, 1973; Desborough and Weiser, 1974). Whilst Francis et al, (1975) reported little or no loss of N content in yams after cooking, this contrasts with the results of Splittstoesser, (1976), and also with the results reported here (see section I.D).

Various other methods, such as measurement of heat-coagulable protein in an extract either directly (Van Gelder and Kretchning, 1973; Miedema et al, 1976), or indirectly (Hoff, 1975), measurement using dye-binding methods (Kaldy et al, 1972; Desborough, 1975; Snyder and Desborough, 1978b) or measurement of N after ethanolic extraction (Vigue and Li, 1975) have been developed to overcome similar problems encountered in screening for "protein" content in potato. However, the data collated in table 11 for different yam varieties presenting a wide range (0.65-1.83%) of total N content tends to suggest that the nitrogen in free amino acids in yam is not as high as that in potato, which is estimated to be around 23% (Desborough and Weiser, 1974), and furthermore, that the proportion of non-protein N for different yam varieties is relatively constant, in contrast to the wide range of non-protein N found in potatoes (qv). Consequently, estimates of %N using the Kjeldahl method will probably reflect protein content in yams reasonably well, as has been observed by Martin and Thompson,

1972, and Martin and Splittstoesser, 1975, and estimates of true protein content from %N values could be obtained simply by use of a conversion factor. Indeed the conversion factor calculated from the data presented here correlated highly ($r = 0.99$, significant at less than 0.1%) with protein-bound amino acid content determined from amino acid analysis.

Breeding efficiency will also be dependent on the method of tuber sampling. In potatoes, for example, both the free amino acid content (Courtrez-Geerinck, 1975) and protein content (Stegemann et al, 1973) have been reported to be greater in the centre of the tuber than in the periphery, although differences between areas in protein composition were minor (Erjfalt and Skude, 1970) and not affected by environmental variation (Stegemann et al, 1973).

Martin and Thompson (1971a) have reported that inner and upper (head) regions i.e. morphologically older regions of yams had only insignificantly greater amounts of N content than outer and lower (tail) regions. However, Ferguson et al, (1980) have recently reported that a definite gradient of N content existed between head and tail regions, with head regions having as much as 2.4 to 2.7 times greater N content than tail regions. In this study, samples were prepared from peeled median longitudinal slices of the tuber, and consequently, any variation which may exist in protein distribution within a tuber should largely have been overcome.

A further consideration with regard to sampling methods is related to the fact that yam plants may produce more than one tuber, and if developmental variation between tubers of a plant is significant, then it may become necessary to analyse each tuber of

any one plant in order to improve precision. However, the results obtained using data from populations 1 and 2 (section VII.A) tend to indicate that developmental variation between tubers is small, and consequently multiple measurements would give little gain in precision.

Environmental variation caused by, for example, variation in nutritional and climatic conditions, is a source of error which may markedly reduce precision in genetical studies, and consequently the aim of the breeder is to reduce this as far as possible. In yams, the influence of various environmental factors on N content can be appreciated to some extent from values obtained for %N for the variety Nwapoko. This variety is considered to be a single clone, since it is non-flowering, and cannot, therefore, be sexually reproduced i.e. none of the observed variation can be attributed to genetic sources. The crude protein content (%N (d.w.) x 6.25) for the sample analysed in 1977 (section I.A) was 8.01%, a value approaching that of the highest value (9.43%) recorded for any plant in this study. However, the mean value for samples analysed in 1978 was only 4.26%, the highest value for these samples being only 6.31% (table 21), and this is in contrast again to the value obtained for the sample analysed in 1979 (6.65%, section I.A). The range of values obtained for this clone was 3.25-8.01%, which is in fact greater than the range of values observed for any of the genetically mixed populations except population 5, where the range was 3.36%-9.43%.

The N content in potatoes has been shown to be significantly influenced by various environmental factors, such as location, mineral nutrition, soil type, growing temperature, etc. (qv), and although the non-protein N content is influenced to a greater

extent than the protein content, this too varies significantly with varying environmental conditions (Mulder and Bakema, 1956; Zaccharius et al, 1971; Desborough and Weiser, 1972; 1974; Stegemann et al, 1973; Klein et al, 1980). Few such studies have been undertaken with yams to test the effect of varying environmental factors on tuber N content, although Sobulo (1972) reported significant differences (p 0.01) in N content with varying applications of N fertilization. Even so, many of the external causes of environmental variation such as those described for potato are well recognised amongst breeders, and in these studies, have been experimentally controlled as far as possible (see methods IX.A).

However, there may be other sources of environmental variation which are not so readily recognized, and depend on the organism studied. For example, in yams the weight of planting material (sett weight) has been reported to significantly influence yielding ability, the use of larger setts giving rise to higher yields (Onwueme, 1972), probably because such setts produce more numerous and more vigorous sprouts than smaller setts (Onwueme, 1973). Indeed Wilson and Victor (1980) have reported that in their yam populations, sett weight was highly significantly correlated with not only tuber weight and tuber number, but also plant height, branch number, leaf number, leaf size and stem diameter. The influence of sett weight on %N content was not investigated by these authors but, using data from population 5, it is apparent that sett weight is also significantly positively correlated with % N content. Thus, unless uniform sett weights are used, this factor may contribute significantly as a source of environmental variation. In populations 2 and 3, the weight of setts planted was not strictly controlled, and this factor may consequently

have contributed to the fact that variation between genetically different clones was not significantly different to that between plants of a clone. (Section VII A.2 and B).

Another source of environmental variation peculiar to the yam may also arise from the use of either head, tail or middle tuber portions for propagation. Ferguson et al, (1980) have shown that setts taken from the head sections rather than middle or tail, have a greater reserve of N and P which are important for root and shoot development in the early stages of growth, and furthermore, that these setts have a higher dry matter and energy content, which may indicate that they also have a greater potential for the supply of carbohydrate for the development of the young plant. Indeed, Onwueme, (1973) has reported that setts prepared from head pieces sprouted more rapidly than either middle or tail pieces. Although the influence of head, middle or tail pieces as planting setts on % N content in tubers was not investigated in this study, it has been suggested (Wilson, personal communication) that the significant differences observed between replicates of the clone Nwapoko (Section VII A.1) has probably arisen from a non-random use of head, middle and tail portions. This reason has also been suggested to explain the significant variation found between plants of a clone in population 2, and clearly may also have confounded results from other experiments. Thus, for example, the results obtained from population 3 suggest that the character % N is not in fact genetically determined, for no correlation between values for plants analysed in 1977 and again in 1978 existed ($r = -0.1$ 14 D.F.). This is highlighted by values obtained for plant YB-6 which gave the highest value of % N (1.33%; crude protein content 8.28%) for the population in 1977, but only half this value (0.73%,

crude protein content 4.56%) in 1978. Obviously variance arising from genotype-environment interaction cannot be discounted, but even so, the use of varying sett weights, and indiscriminate use of head, tail and middle pieces for propagation may also have enhanced environmental sources of variation sufficiently to mask any genetic properties of the population.

An analysis of the variation between half-sib families in population 4, for which uniform sett weights were used, indicated, however, that there was some degree of genotypic variation in this population for the character % N. In order to test this observation, i.e. that genotypic variation of the character % N may well exist among yam breeding populations, and further, to assess whether this character showed any (narrow-sense) heritability, i.e. whether any of the variance within a population could be described as additive, an experiment was carried out to measure both parent and offspring values of plants in a specified population (population 5). The results obtained indicated significant variation between offspring families for this character which,as with population 4, suggested the existence of genotypic variation. The heritability estimate obtained from the offspring midparent regression, which, according to Falconer, (1964) yields, in general, a more precise estimate of heritability than either offspring-male or -female regressions, was 0.58, but had broad ($\pm$ 0.33) confidence limits. This would suggest that at least some degree of genetic progress could be made with regard to increased N content by selection and breeding. However, this result must nevertheless be viewed with reservations, since the experimental design entailed the use of setts of varying

weights, and there were significant differences between both offspring families and between parents in sett weights planted. Consequently, in view of the strong correlation between sett weight and %N, the effect of varying sett weight, as a potential source of environmental variation, may have confounded the analysis by masking or enhancing the heritability of this character. In addition, whilst the possible effect on N content from the use of setts prepared from head, middle or tail portions of a tuber was eliminated for the offspring by the use of "wholes", i.e. intact tubers were planted, this was not true for the parent clones. This factor may have contributed to the fact that the variation of %N values between parent clones compared to within was non-significant (table 26).

The heritabilities of % dry matter and yielding ability were also estimated for this population: high significant estimates of heritability were obtained for the former character, similar to results reported by Sanford et al, (1971) for potato, and consequently it would appear that selection and improvement of this character in breeding experiments could be successful. No significant variation of yielding ability was observed between offspring families, and accordingly, estimates of heritability were very low and non-significant. This character, in general, has been shown to be poorly heritable in other crops as well (Simmonds, 1979).

Correlations between %N values and other plant characters were determined for plants in population 4 to ascertain, for example, whether the character N content could be evaluated indirectly by virtue of a strong significant correlation with another character more readily evaluated. However, it must be borne in mind that the observed "phenotypic" correlations are brought about by two causes: firstly, a genetic cause which acts chiefly through pleitropy, although a correlation can also be brought about through linkage

disequilibrium. Thus, if a gene which influences two or more characters is segregating, it will cause simultaneous variation in both characters. The second cause is environmental, and if two characters are correlated for environmental reasons, they will be influenced by the same differences of environment.

These relationships can be expressed by the following:

$$r_p = h_x h_y r_a + e_x e_y r_e$$

Where r_p is the phenotypic correlation between two characters x and y; r_a, the genetic correlation of x and y, expressed as a correlation of their breeding values; r_e, the environmental correlation of x and y; h_x and h_y, the square root of the heritabilities of characters x and y; and e_x and e_y, the square root of 1-heritability of characters x and y (Falconer, 1964).

From this relationship it is apparent that a phenotypic correlation indicates neither the sign nor magnitude of a genetic cause of correlation. Furthermore, if the genetic and environmental sources of correlation affect two characters through different physiological mechanisms such that there is a genetic correlation which is large and positive, and an environmental correlation which is large and negative, one may not observe any phenotypic correlation. In this experiment, correlations were evaluated in four distinct locations, separable on the basis of soil analyses (table 6) and by visual characteristics. Thus if, for example, a correlation between two characters remained significant after analysis in each location, it could be deduced that the correlation probably had a strong genetic basis. However, the population consisted of five half-sib families which were unevenly replicated within each location so that consequently the probable basis for a significant

phenotypic correlation cannot be discerned.

Even so, it is of interest that a significant positive correlation existed between %N evaluated on a fresh weight basis and % dry matter, both for the population as a whole, and for the "populations" in two of the four locations. This situation is similar to that reported for various potato populations (Fitzpatrick et al, 1969; Miedema et al, 1976), and would suggest that if breeding for increased solids content were possible, as was suggested from the results for population 5, and indeed further suggested for this population since the differences of % dry matter between half-sib families was significant ($p = 0.0046$, location 3), some improvement in N content could be achieved. However, in view of the comparable situation in potatoes (Fitzpatrick et al, 1969), it is probable that any increase in total solids would be primarily reflected by an increase in starch content rather than non-starch (including N) content, so that the overall gain in N content with increased solids would only be small. Indeed, as has again been found for potatoes (Fitzpatrick et al, 1969; Talley et al, 1970; Li and Sayre, 1975; Miedema et al, 1976), the correlation between %N on a dry basis and % dry matter is negative, and although not significant for this population, was significant for populations 3 and 5 ($p = 0.01$-0.001, $p = 0.0007$ respectively) i.e. %N evaluated on a dry basis tends to be higher in tubers with low solids content. Consequently, breeding for improved N content by this method would probably show little promise for success.

These correlations between %N and % dry matter raise the question as to whether %N should be evaluated on a dry or fresh basis. Obviously, if yams were selected on the basis of high %N content of the dry matter, there would be a strong tendency to

select for yams with low solids content, and consequently reduce the yields of solids content. Furthermore, there could also be a tendency to select for immature tubers if these contain, as has been proposed for immature potato tubers (Snyder and Desborough, 1978a) a higher proportion of non-storage tissue than in mature tubers, and consequently, relatively lower concentrations of dry matter (see also Ferguson et al, 1980) and therefore higher concentrations of N.

The alternative, i.e. selection on the basis of high %N of the fresh weight, however, may favour tubers with an increased solids: N content ratio, as discussed above. This would also be true if selection were undertaken on the basis of total N in a tuber, an index which incorporates the weight of a tuber, a value which is of considerable economic importance in the yam breeding programme; or, as another alternative, to selection on the basis of total N on the "hill weight" (total weight of tubers per plant). The latter index would probably be preferable to the former in so far as a strong negative phenotypic correlation ($p = 0.0001$, population 4), existed between tuber number and single tuber weight, but the latter index as such suffers further from the fact that selection on this basis may be favouring plants producing large and/or many tubers with only moderate %N evaluated on a dry or fresh basis.

These considerations must be related to the priorities of the breeding programme. If the aim is to select specifically for varieties with increased N content, but without reduced dry matter content, it would probably be best to evaluate %N both on a dry and fresh weight basis, and to select for those plants which have a high N content on both bases.

However, the results of these studies have confirmed that objectives to increase N content, which appears to be very largely environmentally determined, or to breed for a nutritionally better balanced protein, the potential for which, as discussed earlier, appears to be limited, should not be classed amongst the main priorities of the programme. At this stage, these should be to increase yield and to improve other plant characteristics.

Consequently the monitoring of N content on the basis of %N of the fresh weight, or in fact on the index N content of the hill weight would suffice to ensure that tuber yield is not increased to the detriment of tuber protein content which would otherwise aggravate further, the problem of protein deficiency in the diet.

APPENDIX

Abbreviations used in the following appendices are listed in table 7.

Data obtained for plants of Population 1 (Variety Nampoon)

PLANTNO	REP	TUBERNO	DM%	N%DW	N%FW	TOTALNT	TUBERFW	TUBERDW
1	1	1	38.900	.66000	.25674	9.9487	3875.0	1507.4
1	2	1	38.000	.64000	.24320	5.6544	2325.0	883.60
1	4	1	37.800	.68000	.25704	8.4181	3275.0	1237.9
2	3	1	40.100	.73500	.29473	9.2105	3125.0	1253.1
3	1	1	39.300	.64000	.25152	5.4077	2150.0	844.95
4	3	1	39.700	.57000	.22629	7.4110	3275.0	1300.2
4	4	1	38.700	.67000	.25929	9.3993	3625.0	1402.9
5	1	1	39.800	.54000	.21492	9.2953	4325.0	1721.3
5	3	1	39.000	.65000	.25350	11.407	4500.0	1755.0
6	2	1	38.800	.61000	.23668	12.071	5100.0	1978.8
6	3	1	37.700	.77000	.29029	7.7653	2675.0	1008.5
6	4	2	36.700	.75000	.27525	2.0644	750.00	275.25
6	4	3	39.800	.93000	.37014	2.6835	725.00	288.55
6	4	1	37.600	.95000	.35720	5.3580	1500.0	564.00
7	1	1	37.200	.68000	.25296	8.7904	3475.0	1292.7
7	2	1	38.000	.63000	.23940	3.9501	1650.0	627.00
7	4	1	33.500	1.0100	.33835	2.0301	600.00	201.00
7	4	2	35.600	.69000	.24564	6.2024	2525.0	898.90
8	2	1	36.800	.70000	.25760	9.6600	3750.0	1380.0
9	2	1	37.800	.64000	.24192	10.403	4300.0	1625.4
9	3	1	34.100	.65000	.22165	8.8106	3975.0	1355.5
9	4	1	37.500	.68000	.25500	4.7813	1875.0	703.13
9	4	2	34.900	.80000	.27920	3.2806	1175.0	410.07
10	1	1	38.900	.62500	.24312	5.7134	2350.0	914.15
11	1	1	34.100	.68000	.23188	4.0579	1750.0	596.75
11	1	2	36.800	.62000	.22816	4.7914	2100.0	772.80
13	2	3	35.200	.64500	.22704	2.4407	1075.0	378.40
13	2	2	38.800	.57000	.22116	11.456	5180.0	2009.8
13	2	1	35.800	.61000	.21838	6.8790	3150.0	1127.7
13	3	1	39.200	.68000	.26656	6.1975	2325.0	911.40
14	3	1	39.700	.59000	.23423	4.8603	2075.0	823.77
16	1	1	35.000	.77000	.26950	7.8155	2900.0	1015.0
17	4	1	38.000	.64000	.24320	7.7216	3175.0	1206.5
19	3	1	37.100	.85000	.31535	11.037	3500.0	1298.5
19	4	1	37.000	.72000	.26640	7.7256	2900.0	1073.0
20	1	2	34.900	.62000	.21638	1.8933	875.00	305.38
20	1	1	35.000	.64000	.22400	4.6480	2075.0	726.25
20	1	3	34.900	.65000	.22685	3.3460	1475.0	514.77
20	3	2	35.900	.62500	.22437	4.3192	1925.0	691.07
20	3	1	36.600	.68500	.25071	5.7037	2275.0	832.65
20	4	1	36.400	.71000	.25844	4.0704	1575.0	573.30
21	2	1	37.400	.84000	.31416	10.289	3275.0	1224.8
21	3	1	37.900	.74000	.28046	7.7126	2750.0	1042.3
22	2	1	37.600	.65000	.24440	7.9430	3250.0	1222.0
22	3	1	41.200	.52000	.21424	7.0699	3300.0	1359.6
23	2	1	38.100	.69000	.26289	3.4833	1325.0	504.82
24	1	1	37.900	.69000	.26151	3.3343	1275.0	483.22
24	2	1	38.200	.65000	.24830	6.5179	2625.0	1002.8
25	1	1	38.500	.60000	.23100	3.8692	1675.0	644.88
25	3	1	37.800	.67000	.25326	11.713	4625.0	1748.3
26	1	2	35.400	.74500	.26373	3.1648	1200.0	424.80
26	1	1	37.100	.65000	.24115	4.2201	1750.0	649.25
26	2	2	39.300	.55500	.21811	5.5074	2525.0	992.32
26	2	1	37.800	.66000	.24948	2.9690	1150.0	434.70
26	3	1	36.900	.62000	.22320	13.615	6100.0	2196.0
26	4	1	38.600	.66000	.25476	9.4898	3725.0	1437.8
27	1	1	36.700	.62000	.22754	4.0957	1800.0	660.60
27	2	1	36.300	.57000	.20691	4.2417	2050.0	744.15
28	1	1	39.000	.65000	.25350	.91260	360.00	140.40
28	4	1	33.500	.83500	.27972	3.6364	1300.0	435.50
28	4	2	35.000	.80000	.28000	4.9000	1750.0	612.50
29	4	1	36.500	.74000	.27010	3.8489	1425.0	520.13
30	1	1	33.600	.61000	.20496	3.7405	1825.0	613.20
30	2	1	36.900	.67000	.24723	3.5848	1450.0	535.05
30	4	1	36.900	.66000	.24354	3.1660	1300.0	479.70
30	4	2	35.800	.90000	.32220	2.0943	650.00	232.70
31	2	1	37.600	.80000	.30080	7.5952	2525.0	949.40
31	4	2	34.200	.74000	.25308	2.6573	1050.0	359.10
31	4	1	36.200	.71500	.25883	3.6236	1400.0	506.80
32	4	1	36.100	.59000	.21299	6.9222	3250.0	1173.3
33	1	1	36.700	.69000	.25323	2.2158	875.00	321.13
34	2	2	36.200	.60500	.21901	6.2965	2875.0	1040.8
34	2	1	36.200	.73000	.26426	3.8978	1475.0	533.95
34	3	1	38.700	.65000	.25155	8.8671	3525.0	1364.2
35	3	1	38.900	.61000	.23729	6.8221	2875.0	1118.4
36	1	1	37.100	.62500	.23187	4.6375	2000.0	742.00
37	2	1	35.700	.54000	.19278	8.8197	4575.0	1633.3
37	3	1	39.500	.71000	.28045	5.5389	1975.0	780.13
37	4	1	35.700	.77000	.27489	2.7489	1000.0	357.00
38	2	1	34.200	.75500	.25821	4.7769	1850.0	632.70
38	4	1	36.700	.69000	.25323	5.7610	2275.0	834.92
39	1	1	37.400	.65000	.24310	6.1383	2525.0	944.35
39	3	1	38.700	.70000	.27090	6.2984	2325.0	899.77
40	1	1	40.300	.67000	.27001	8.0328	2975.0	1198.9
40	3	1	38.300	.56000	.21448	7.2923	3400.0	1302.2
40	4	1	34.000	.81500	.27710	2.6324	950.00	323.00
40	4	2	36.800	.70000	.25760	8.1788	3175.0	1168.4
41	1	2	36.000	.61000	.21960	7.2468	3300.0	1188.0
41	1	3	37.200	.61000	.22692	7.1480	3150.0	1171.8
41	1	1	33.700	.66500	.22410	5.7707	2575.0	867.77
42	1	1	37.400	.65000	.24310	3.4034	1400.0	523.60
42	2	1	36.800	.64000	.23552	3.2384	1375.0	506.00
42	3	1	38.800	.63500	.24638	3.6957	1500.0	582.00
42	3	2	36.800	.68000	.25024	.67565	270.00	99.360
43	4	1	38.100	.71000	.27051	4.5987	1700.0	647.70
44	3	3	35.000	.80000	.28000	1.4700	525.00	183.75
44	3	1	35.200	.65000	.22880	1.9448	850.00	299.20
44	3	2	27.300	.63000	.17199	.36118	210.00	57.330
44	4	1	35.900	.63000	.22617	6.4458	2850.0	1023.1
45	2	1	33.200	.71000	.23572	1.8858	800.00	265.60
45	2	2	37.000	.72000	.26640	11.522	4325.0	1600.3
46	2	1	36.700	.78000	.28626	9.0888	3175.0	1165.2
46	4	1	37.500	.66000	.24750	3.8362	1550.0	581.25

Appendix 2

Mean value and standard deviation of tuber characters evaluated from plants of the variety Nwapoko (Population 1)[a]

Tuber Character	Replicate	N	Mean	Standard Deviation
tuber fresh weight (g)	1	26	2155.2	966.1
	2	25	2686.2	1301.2
	3	24	2661.7	1421.7
	4	28	1894.6	986.3
	total	103	2331.3	1207.2
% dry matter	1	26	36.877	1.895
	2	25	36.975	1.431
	3	24	37.467	2.798
	4	28	36.464	1.579
	total	103	36.926	1.979
% Nitrogen (dry weight basis)	1	26	0.64846	0.04589
	2	25	0.66440	0.07657
	3	24	0.66583	0.07647
	4	28	0.74446	0.01030
	total	103	0.68248	0.08670
% Nitrogen (fresh weight basis)	1	26	0.23890	0.01781
	2	25	0.24543	0.02782
	3	24	0.24921	0.03204
	4	28	0.27098	0.03590
	total	103	0.25161	0.03142
total Nitrogen content of tuber (g)	1	26	5.1399	2.3423
	2	25	6.5628	3.1495
	3	24	6.6583	3.4877
	4	28	4.9384	2.3191
	total	103	5.7843	2.9113

[a] data listed in Appendix 1

Appendix 3

Data obtained for plants of Population 2

	CLONENO	PLANTNO	TUBERNO	TUBERFW	DM%	N%DW	N%FW	TOTALNT
W387	3	1	2	625.00	37.400	.73000	.27302	1.7064
	3	1	3	260.00	37.000	.80000	.29600	.7696
	3	2	1	800.00	37.700	.82000	.30914	2.4731
	3	2	2	625.00	37.600	.69000	.25944	1.6215
	3	2	3	550.00	38.600	.66000	.25476	1.4012
	3	3	1	600.00	36.000	.66500	.23940	1.4364
	3	3	3	725.00	37.100	.67000	.24857	1.8021
	3	4	1	625.00	32.900	.94000	.30926	1.9329
	3	4	2	700.00	33.900	.82000	.27798	1.9459
	3	4	3	460.00	31.400	1.0000	.31400	1.4444
	3	4	4	1225.0	34.600	.78000	.26988	3.3060
	4	1	3	900.00	41.000	.61500	.25215	2.2693
	4	2	1	800.00	34.600	.86000	.29756	2.3805
	4	2	2	470.00	31.600	.98000	.30968	1.4555
	4	3	1	975.00	40.700	.86000	.35002	3.4127
	4	4	1	1475.0	37.900	.81000	.30699	4.5281

Appendix 4

Mean value and standard deviation of tuber characters evaluated from plants of the seedling-derived clones W387-3 and W387-4 (Population 2)

Tuber Character	Clone	N	Mean	Standard Deviation
tuber fresh weight	W387-3	11	654.09	237.41
	W387-4	5	924.00	363.41
	total	16	738.44	299.15
% dry matter	W387-3	11	35.836	2.3071
	W387-4	5	37.160	4.0402
	total	16	36.250	2.8814
% Nitrogen (d.w.)	W387-3	11	0.77955	0.11310
	W387-4	5	0.82500	0.13304
	total	16	0.79375	0.11714
% nitrogen (f.w.)	W387-3	11	0.27740	0.026257
	W387-4	5	0.30328	0.034951
	total	16	0.28549	0.03064
total nitrogen content of tuber	W387-3	11	1.80359	0.65422
	W387-4	5	2.80922	1.18602
	total	16	2.11785	0.94456

Appendix 5

Data obtained for plants of Population 3

Year	1977		1978		Coding for 1978	
Character / Clone	Yield	%N d.w.	Yield	%N d.w.	Plant	Tuber
Futun 2	2220	0.89	2485 190 3095	0.92 1.13 0.865	A B C	5/6 1 ½
W305-2	2120	0.79	2925 1570 1570	0.63 [a] 0.79 [a] 0.74	A B B	2/2 ¼ 2/4
W512-2	1220	0.63	5300 5050	0.68 0.97	A B	1 5/5
W512-5	2260	0.80	6330 4595 5950	0.77 0.86 0.82	A B C	3/3 ¾ 2/3
W516-1	2140	1.09	4575 2625	0.78 0.80	A B	3/3 1
W565-3	2330	0.86	1550	0.78		1/3
AB 6	1810	0.84	2075	0.995		1
AB 11	2330	1.035	3850	0.83		1
YB 6	N.D.	1.325	2750	0.73	B	1

a The mean, 0.765, of these two values used to represent this plant

Appendix 6

Data obtained for plants of Population 5

Offspring code 50 from ♀7×♂9

FAM.NO	N%DW	HILLWT	TUBERNO	DM%	SETTWT	WKEMERGE	STEMNO	VINEDW
C50	1.0880	2575.0	1.0000	34.770	236.00	1.0000	1.0000	47.260
C50	1.0260	875.00	1.0000	37.070	130.00	7.0000	1.0000	41.800
C50	.84200	1225.0	1.0000	29.440	99.000	5.0000	1.0000	27.140
C50	.90900	1325.0	1.0000	37.300	79.000	5.0000	1.0000	49.550
C50	.88900	1850.0	1.0000	39.150	88.000	5.0000	2.0000	81.350
C50	.78400	1225.0	2.0000	36.780	47.000	5.0000	1.0000	25.500
C50	.82100	5600.0	1.0000	30.580	468.00	5.0000	4.0000	124.91
C50	1.0870	3750.0	2.0000	39.750	238.00	4.0000	2.0000	116.36
C50	.87700	2525.0	1.0000	37.560	202.00	4.0000	1.0000	141.45
C50	1.1550	3800.0	1.0000	38.020	238.00	6.0000	1.0000	219.75
C50	1.2510	2450.0	1.0000	35.530	65.000	4.0000	1.0000	40.040
C50	1.0780	3150.0	1.0000	38.170	175.00	5.0000	1.0000	67.620
C50	.77500	1350.0	1.0000	43.570	224.00	5.0000	1.0000	82.990
C50	1.2460	2300.0	2.0000	25.190	408.00	3.0000	1.0000	117.13
C50	1.0470	3450.0	1.0000	34.950	147.00	4.0000	2.0000	100.43
C50	.94100	4065.0	3.0000	32.970	255.00	4.0000	4.0000	79.960
C50	1.0040	2225.0	1.0000	39.480	79.000	4.0000	1.0000	52.100
C50	.79600	550.00	2.0000	44.260	54.000	5.0000	2.0000	15.970
C50	.91700	1950.0	2.0000	36.040	373.00	4.0000	2.0000	57.380
C50	.75800	925.00	1.0000	40.910	21.000	8.0000	1.0000	23.000
C50	.86800	900.00	1.0000	24.540	60.000	6.0000	2.0000	21.100
C50	1.0380	4000.0	1.0000	36.590	478.00	2.0000	2.0000	175.14
C50	1.1020	3325.0	1.0000	36.260	247.00	4.0000	2.0000	112.59
C50	1.1130	3800.0	1.0000	39.080	591.00	4.0000	1.0000	274.60

Offspring code 51 from ♀5×♂6

FAM.NO	N%DW	HILLWT	TUBERNO	DM%	SETTWT	WKEMERGE	STEMNO	VINEDW
E51	.90600	1725.0	1.0000	42.690	115.00	3.0000	2.0000	136.22
E51	.95400	3800.0	1.0000	44.430	158.00	3.0000	2.0000	184.75
E51	.70900	2115.0	2.0000	42.520	90.000	4.0000	2.0000	58.250
E51	.96700	1475.0	1.0000	35.460	192.00	5.0000	1.0000	63.250
E51	.99800	2500.0	2.0000	40.330	220.00	5.0000	1.0000	82.260
E51	.93600	2950.0	3.0000	44.140	317.00	4.0000	2.0000	201.75
E51	.80900	2350.0	1.0000	39.160	240.00	5.0000	2.0000	69.230
E51	.81500	2900.0	1.0000	33.370	181.00	4.0000	2.0000	94.680
E51	.96900	3525.0	1.0000	38.140	166.00	6.0000	1.0000	118.25
E51	1.0560	2125.0	1.0000	44.670	276.00	5.0000	2.0000	104.75
E51	.83300	2950.0	1.0000	32.940	104.00	5.0000	2.0000	96.540
E51	1.0360	1450.0	2.0000	39.820	129.00	5.0000	3.0000	54.380
E51	1.1280	1450.0	2.0000	38.110	129.00	5.0000	3.0000	54.380
E51	.77700	4225.0	2.0000	33.140	218.00	5.0000	3.0000	126.74
E51	.89600	4580.0	3.0000	39.650	465.00	5.0000	2.0000	246.15
E51	1.1620	400.00	1.0000	38.700	215.00	5.0000	1.0000	29.250
E51	1.0490	2595.0	3.0000	32.500	311.00	5.0000	2.0000	154.91
E51	.81800	1900.0	2.0000	26.500	105.00	5.0000	1.0000	38.000
E51	.76200	2880.0	1.0000	42.710	226.00	5.0000	2.0000	70.600
E51	.76800	3300.0	1.0000	38.850	126.00	2.0000	1.0000	58.000
E51	1.0700	2925.0	2.0000	38.490	170.00	3.0000	2.0000	206.70
E51	.77000	2935.0	4.0000	39.160	183.00	4.0000	1.0000	88.650
E51	.67000	2625.0	1.0000	38.600	290.00	5.0000	1.0000	86.050
E51	.91900	2950.0	1.0000	33.410	217.00	6.0000	1.0000	59.150
E51	.88200	1875.0	1.0000	40.130	278.00	4.0000	2.0000	98.160
E51	.99200	1400.0	2.0000	40.730	140.00	3.0000	2.0000	85.330
E51	1.0770	1800.0	1.0000	41.990	244.00	3.0000	1.0000	120.62
E51	.92500	3775.0	2.0000	35.670	232.00	5.0000	1.0000	123.66
E51	.93300	2425.0	1.0000	34.380	177.00	4.0000	2.0000	37.800
E51	.90700	3660.0	2.0000	44.380	174.00	4.0000	4.0000	119.65
E51	.87500	6350.0	3.0000	36.990	333.00	5.0000	2.0000	200.05
E51	1.1330	4525.0	1.0000	31.620	351.00	1.0000	2.0000	105.63
E51	.99300	2400.0	1.0000	39.610	85.000	5.0000	2.0000	83.990
E51	.76300	1525.0	1.0000	42.820	128.00	5.0000	1.0000	50.120
E51	.92100	4100.0	5.0000	35.150	297.00	4.0000	2.0000	96.350
E51	.94200	3450.0	1.0000	38.730	151.00	5.0000	2.0000	113.12
E51	.76000	3575.0	1.0000	38.990	284.00	4.0000	1.0000	169.99
E51	.91100	2300.0	2.0000	35.590	231.00	5.0000	4.0000	92.580
E51	1.0130	3250.0	2.0000	42.550	163.00	5.0000	1.0000	159.04
E51	.81800	3775.0	1.0000	39.330	208.00	4.0000	2.0000	127.28
E51	1.2280	3275.0	1.0000	35.660	418.00	5.0000	1.0000	165.84
E51	.97400	2300.0	1.0000	37.190	77.000	5.0000	2.0000	58.250
E51	.84900	2950.0	1.0000	35.820	278.00	4.0000	3.0000	105.25
E51	.92800	5650.0	3.0000	37.370	267.00	3.0000	3.0000	199.23
E51	.75600	2325.0	2.0000	39.100	155.00	5.0000	2.0000	70.090
E51	1.0150	5100.0	1.0000	35.460	390.00	4.0000	1.0000	186.60
E51	.71600	3225.0	1.0000	43.920	161.00	7.0000	1.0000	67.070

Offspring code 52 from ♀10×♂13

FAM.NO	N%DW	HILLWT	TUBERNO	DM%	SETTWT	WKEMERGE	STEMNO	VINEDW
F52	.76000	800.00	1.0000	40.170	44.000	5.0000	1.0000	17.200
F52	.81700	2495.0	2.0000	37.440	179.00	5.0000	2.0000	155.55
F52	.93500	3825.0	3.0000	35.040	233.00	5.0000	4.0000	82.920
F52	.83900	2250.0	1.0000	38.160	126.00	5.0000	2.0000	64.000
F52	1.0130	4850.0	1.0000	37.880	235.00	4.0000	2.0000	207.60
F52	.92600	2565.0	1.0000	43.280	121.00	2.0000	1.0000	66.740
F52	1.0470	380.00	1.0000	37.370	303.00	5.0000	1.0000	25.000
F52	.77200	3820.0	3.0000	37.430	189.00	5.0000	4.0000	123.20
F52	.86200	2515.0	3.0000	36.800	185.00	3.0000	2.0000	142.83
F52	1.1020	2025.0	2.0000	43.490	114.00	5.0000	3.0000	80.680
F52	.81400	825.00	1.0000	36.960	54.000	5.0000	2.0000	18.610
F52	1.0850	4275.0	2.0000	32.290	334.00	5.0000	3.0000	297.30
F52	.94400	4150.0	2.0000	40.940	231.00	4.0000	2.0000	213.95
F52	.80400	3200.0	1.0000	35.690	187.00	2.0000	2.0000	157.25
F52	.83400	1975.0	1.0000	34.460	132.00	6.0000	1.0000	90.780
F52	1.0170	4950.0	2.0000	35.330	178.00	3.0000	2.0000	132.26
F52	.85100	5350.0	2.0000	37.830	197.00	4.0000	4.0000	103.07
F52	1.0300	1425.0	1.0000	37.540	82.000	7.0000	2.0000	63.920
F52	.86600	700.00	1.0000	37.760	46.000	5.0000	1.0000	16.550
F52	1.0310	3700.0	1.0000	36.470	104.00	3.0000	2.0000	113.15
F52	.67100	2310.0	1.0000	38.410	145.00	3.0000	2.0000	92.100
F52	.95200	1975.0	2.0000	29.930	133.00	5.0000	1.0000	62.640
F52	.91300	2625.0	2.0000	36.690	228.00	5.0000	3.0000	79.320
F52	.71100	2400.0	1.0000	30.380	115.00	4.0000	2.0000	69.610
F52	.79300	3750.0	1.0000	38.610	197.00	3.0000	1.0000	218.30
F52	.72400	1175.0	1.0000	39.990	105.00	6.0000	2.0000	58.880
F52	.75000	3500.0	1.0000	25.670	186.00	5.0000	2.0000	133.46
F52	.73700	5400.0	2.0000	34.230	102.00	5.0000	5.0000	197.32
F52	1.1250	1050.0	2.0000	32.560	110.00	4.0000	2.0000	37.640
F52	.86100	3325.0	1.0000	33.620	132.00	4.0000	3.0000	129.52
F52	.78700	1310.0	2.0000	35.980	86.000	6.0000	2.0000	26.890
F52	.79200	2125.0	1.0000	36.840	69.000	4.0000	2.0000	35.750
F52	.82300	1150.0	1.0000	45.550	79.000	2.0000	2.0000	37.220
F52	.80400	2850.0	1.0000	34.860	186.00	5.0000	2.0000	116.05
F52	.90000	2825.0	1.0000	38.630	71.000	5.0000	2.0000	139.32

Offspring code 53 from ♀11×♂12

FAM.NO	N%DW	HILLWT	TUBERNO	DM%	SETTWT	WKEMERGE	STEMNO	VINEDW
G53	1.0260	3400.0	1.0000	25.830	322.00	3.0000	2.0000	73.400
G53	.98900	2225.0	1.0000	26.480	133.00	4.0000	1.0000	42.220
G53	1.4290	2480.0	2.0000	23.080	169.00	5.0000	2.0000	47.620
G53	1.2730	1350.0	1.0000	25.930	130.00	4.0000	2.0000	48.970
G53	1.0130	5275.0	2.0000	36.010	259.00	3.0000	2.0000	180.99
G53	.94100	2325.0	1.0000	28.530	98.000	5.0000	1.0000	34.080
G53	.80300	600.00	1.0000	27.510	65.000	5.0000	2.0000	12.190
G53	.71700	335.00	2.0000	24.600	42.000	7.0000	1.0000	12.010
G53	1.1160	1100.0	1.0000	32.320	54.000	5.0000	2.0000	21.690
G53	1.0670	1225.0	1.0000	27.070	135.00	9.0000	1.0000	22.870
G53	.90800	3790.0	2.0000	26.200	270.00	2.0000	2.0000	115.04
G53	1.3690	1625.0	1.0000	22.190	150.00	6.0000	1.0000	30.380
G53	.78100	3950.0	1.0000	37.200	342.00	5.0000	1.0000	113.99
G53	.82700	4510.0	1.0000	26.990	159.00	5.0000	1.0000	105.54
G53	1.1500	1275.0	1.0000	25.430	246.00	6.0000	1.0000	61.230
G53	1.1790	3150.0	1.0000	38.760	365.00	6.0000	2.0000	89.910
G53	.86000	1550.0	1.0000	25.740	108.00	3.0000	2.0000	31.530
G53	1.3070	2500.0	1.0000	35.030	179.00	6.0000	1.0000	67.250
G53	1.1190	4250.0	2.0000	27.460	335.00	5.0000	3.0000	170.00
G53	.60700	2985.0	2.0000	31.700	278.00	4.0000	2.0000	75.330
G53	.98200	2100.0	2.0000	30.670	173.00	6.0000	1.0000	38.390
G53	.86200	2525.0	1.0000	30.210	95.000	3.0000	2.0000	85.020
G53	.90000	2830.0	1.0000	30.710	314.00	4.0000	2.0000	106.30
G53	.89800	2150.0	3.0000	33.190	251.00	4.0000	3.0000	80.280
G53	1.0400	3225.0	3.0000	26.560	393.00	4.0000	3.0000	68.880
G53	1.1270	2250.0	1.0000	36.460	230.00	3.0000	1.0000	101.56
G53	.93900	1350.0	2.0000	37.790	134.00	7.0000	3.0000	54.000
G53	1.0210	1550.0	1.0000	31.920	130.00	3.0000	1.0000	42.020
G53	.83700	3175.0	1.0000	25.360	197.00	4.0000	1.0000	42.000
G53	.94200	3775.0	1.0000	29.340	275.00	5.0000	1.0000	99.230
G53	1.0400	1225.0	1.0000	27.720	126.00	4.0000	1.0000	27.670
G53	1.1540	1340.0	1.0000	31.970	68.000	4.0000	1.0000	24.020
G53	1.3840	1350.0	1.0000	25.870	113.00	5.0000	2.0000	44.370
G53	1.1490	2425.0	2.0000	28.370	247.00	3.0000	2.0000	71.890
G53	.92100	600.00	1.0000	31.820	52.000	5.0000	1.0000	15.690
G53	1.1470	1485.0	1.0000	32.960	116.00	3.0000	2.0000	45.950
G53	1.0780	1200.0	1.0000	27.490	63.000	4.0000	1.0000	37.580
G53	.99200	925.00	1.0000	36.440	108.00	4.0000	1.0000	37.100
G53	1.0160	4975.0	1.0000	27.150	352.00	4.0000	1.0000	131.65
G53	.72200	2675.0	1.0000	27.210	106.00	3.0000	1.0000	50.130
G53	.89800	2525.0	2.0000	28.770	375.00	5.0000	5.0000	97.760
G53	.91400	1800.0	1.0000	35.270	134.00	4.0000	2.0000	51.370
G53	1.1820	2760.0	1.0000	31.350	134.00	4.0000	2.0000	42.840
G53	.73500	1450.0	1.0000	30.610	49.000	8.0000	2.0000	22.240
G53	1.4410	775.00	1.0000	25.760	102.00	4.0000	1.0000	18.340
G53	.93200	1950.0	1.0000	29.440	153.00	4.0000	1.0000	57.520
G53	1.1920	1575.0	1.0000	32.380	117.00	3.0000	1.0000	43.150
G53	.93500	2250.0	1.0000	31.530	290.00	6.0000	2.0000	84.900
G53	.86500	5925.0	1.0000	31.610	184.00	4.0000	2.0000	136.02
G53	1.0180	1150.0	1.0000	28.160	76.000	6.0000	1.0000	32.330

Offspring code 54 from ♀4×♂2

FAM.NO	N%DW	HILLWT	TUBERNO	DM%	SETTWT	WKEMERGE	STEMNO	VINEDW
H54	.53700	1600.0	1.0000	38.390	86.000	5.0000	1.0000	45.570
H54	.90400	2700.0	1.0000	31.840	120.00	7.0000	2.0000	79.470
H54	.86700	1635.0	1.0000	36.990	125.00	7.0000	1.0000	43.820
H54	.87200	6850.0	1.0000	42.880	155.00	4.0000	2.0000	329.50
H54	1.0950	2790.0	1.0000	38.060	169.00	6.0000	1.0000	112.46
H54	1.3260	2725.0	1.0000	34.000	304.00	6.0000	1.0000	125.55
H54	1.0020	1375.0	1.0000	40.660	132.00	4.0000	1.0000	55.500
H54	1.1620	2425.0	1.0000	35.160	80.000	5.0000	2.0000	53.000
H54	.81700	1475.0	1.0000	40.150	110.00	6.0000	1.0000	29.830
H54	.93800	1700.0	2.0000	36.450	122.00	5.0000	2.0000	43.500
H54	.99800	1570.0	1.0000	37.990	127.00	4.0000	2.0000	65.800
H54	1.0050	1350.0	1.0000	35.890	103.00	5.0000	2.0000	72.550
H54	.95100	675.00	1.0000	34.340	122.00	4.0000	1.0000	43.400
H54	1.0020	4250.0	3.0000	29.280	212.00	3.0000	2.0000	194.55
H54	1.0560	2350.0	1.0000	33.740	230.00	1.0000	1.0000	58.640
H54	1.2260	1635.0	1.0000	41.640	198.00	5.0000	1.0000	79.400
H54	.77500	1295.0	2.0000	37.890	151.00	6.0000	3.0000	60.870
H54	1.0720	2150.0	2.0000	35.610	73.000	4.0000	2.0000	40.010
H54	1.1640	1900.0	1.0000	36.310	120.00	5.0000	1.0000	57.970
H54	.83800	1975.0	1.0000	38.510	159.00	5.0000	1.0000	172.03
H54	1.2130	1975.0	1.0000	34.880	190.00	4.0000	1.0000	63.140
H54	1.1430	800.00	1.0000	39.340	95.000	4.0000	1.0000	24.540
H54	.94000	2900.0	1.0000	35.850	109.00	5.0000	2.0000	128.40
H54	.90700	3380.0	2.0000	29.470	104.00	5.0000	2.0000	68.400
H54	.86500	5100.0	1.0000	39.990	493.00	4.0000	1.0000	263.40
H54	.91300	4500.0	1.0000	30.270	188.00	2.0000	1.0000	95.900
H54	.79800	1400.0	1.0000	38.060	138.00	4.0000	1.0000	41.110
H54	.83400	4375.0	1.0000	31.400	168.00	3.0000	4.0000	128.48
H54	1.1390	3100.0	4.0000	39.970	361.00	6.0000	1.0000	122.38
H54	.84200	4935.0	1.0000	29.360	96.000	3.0000	2.0000	161.61
H54	1.1280	1950.0	1.0000	38.000	119.00	5.0000	1.0000	68.910
H54	.99900	2825.0	1.0000	31.430	186.00	4.0000	1.0000	72.500
H54	1.0330	950.00	1.0000	34.310	100.00	3.0000	1.0000	39.460
H54	1.0550	3545.0	1.0000	34.950	247.00	5.0000	1.0000	124.76
H54	1.0970	1275.0	1.0000	31.150	111.00	5.0000	1.0000	37.450
H54	.89000	1450.0	1.0000	35.330	214.00	8.0000	1.0000	65.570
H54	1.3190	750.00	1.0000	37.710	114.00	4.0000	2.0000	39.160
H54	.84700	1250.0	4.0000	41.310	72.000	4.0000	1.0000	45.000
♀10	.85600	2375.0	1.0000	36.410	80.000	7.0000	1.0000	53.690
♀10	.69300	4210.0	2.0000	36.700	205.00	5.0000	3.0000	222.75
♀10	.82900	2525.0	1.0000	39.910	150.00	7.0000	1.0000	67.910
♀10	.80800	4210.0	2.0000	35.640	205.00	5.0000	3.0000	222.75
♀10	.82300	1125.0	1.0000	38.290	35.000	6.0000	1.0000	25.250
♀11	1.0030	600.00	1.0000	25.340	255.00	10.000	1.0000	12.680
♂12	1.5080	420.00	2.0000	33.340	300.00	5.0000	1.0000	59.400
♂12	1.0070	625.00	1.0000	30.950	110.00	7.0000	1.0000	53.540
♂12	1.1620	370.00	5.0000	30.960	315.00	9.0000	2.0000	51.300
♂12	.88400	1525.0	1.0000	35.250	190.00	5.0000	1.0000	56.820
♂13	1.0850	1250.0	1.0000	36.020	330.00	4.0000	1.0000	37.130
♂13	1.1530	800.00	1.0000	40.860	235.00	8.0000	1.0000	23.680
♂13	1.1560	1050.0	1.0000	35.770	340.00	4.0000	1.0000	40.640

Parents

FAM.NO	N%DW	HILLWT	TUBERNO	DM%	SETTWT	WKEMERGE	STEMNO	VINEDW
♂2	1.0410	1510.0	3.0000	34.300	350.00	5.0000	4.0000	39.830
♂2	.98200	1075.0	2.0000	33.780	285.00	3.0000	3.0000	19.930
♂2	.86100	2050.0	1.0000	39.760	280.00	6.0000	1.0000	44.230
♂2	.90200	2050.0	3.0000	33.310	350.00	7.0000	3.0000	35.840
♂2	.75900	1500.0	3.0000	36.070	280.00	7.0000	3.0000	46.750
♂2	.77300	2150.0	2.0000	39.020	320.00	7.0000	1.0000	48.450
♂2	1.2720	1215.0	4.0000	33.240	300.00	3.0000	4.0000	
♂2	.70000	2150.0	2.0000	38.580	370.00	6.0000	2.0000	46.710
♂2	.88300	3150.0	1.0000	34.930	370.00	4.0000	1.0000	48.630
♂2	.98800	2050.0	3.0000	34.300	350.00	7.0000	3.0000	35.840
♀4	1.0650	210.00	1.0000	35.760	205.00	5.0000	2.0000	105.03
♀4	.74100	3800.0	1.0000	30.740	285.00	4.0000	1.0000	90.640
♀4	1.0860	3400.0	1.0000	35.100	310.00	8.0000	1.0000	108.39
♀5	1.1040	575.00	2.0000	36.470	185.00	4.0000	2.0000	31.460
♂6	.76000	1550.0	1.0000	35.840	190.00	7.0000	1.0000	50.160
♂9	1.2270	950.00	1.0000	30.340	325.00	6.0000	1.0000	76.070

Appendix 7(a)

Mean Values of Parents (Population 5)

Character / Parents[a]		% Nitrogen (d.w.)	Yield (g)	% Dry Matter
Male	2	0.9161 ± 0.1197 (10)	1890 ± 695 (10)	35.729 ± 1.690 (10)
Female	4	0.9640 ± 0.4158 (3)	2470 ± 2415 (3)	33.867 ± 5.872 (3)
Female	5	1.1040	575	36.470
Male	6	0.7600	1550	35.840
Male	9	1.2270	950	30.340
Female	10	0.8018 ± 0.2082 (5)	2889 ± 1209 (5)	37.390 ± 2.940 (5)
Female	11	1.0030	600	25.340
Male	12	1.1402 ± 0.2663 (4)	735 ± 1547 (4)	32.625 ± 3.761 (4)
Male	13	1.1313 ± 0.4158 (3)	1033.3 ± 2415 (3)	37.550 ± 5.872 (3)

Values are the mean ± 95% confidence limits
a numbers represent the code: data in Appendix 6

Appendix 7(b)

Mid-Parent Values (Population 5)

Mid-parent / Character[a]	% Nitrogen (d.w.)	Yield (g)	% Dry Matter
4 x 2	0.94005	2180	34.797833
5 x 6	0.93200	1062.5	36.155
10 x 13	0.9665665	1961.16	37.47
11 x 12	1.071625	667.5	28.9825

[a] numbers represent the code: data in Appendix 6

Appendix 8

Correlations between characters evaluated for plants of Population 5

CORRELATION MATRIX

N= 222 DF= 220 R@ .0500= .1317 R@ .0100= .1726

VARIABLE								
N%DW	1.0000							
HILLWT	-.1706	1.0000						
TUBERNO	-.0443	.1440	1.0000					
DM%	-.2232	.0250	.0401	1.0000				
SETTWT	.1447	.4176	.2658	-.0178	1.0000			
WKEMERGE	-.0502	-.3047	.0598	-.0201	-.0521	1.0000		
STEMNO	-.1587	.3061	.4070	.0046	.1404	-.0797	1.0000	
VINEDW	-.0661	.7673	.1452	.2152	.4560	-.2412	.2282	1.0000
	N%DW	HILLWT	TUBERNO	DM%	SETTWT	WKEMERGE	STEMNO	VINEDW

Appendix 9

Data obtained for plants in Population 4

FAMILY	CODE	CODE	EM.DATE	FL.DATE	SEX	HEIGHT	BRANCHNO	HT1BRA	LEAFNO	LEAFSIZE	VIRUS
STEMCOL	STEMDIAM	THORN	STEMNO.	VIGOR	LEAFSPOT	TUBERNO	TUBSHAPE	TUSMOOTH	HARV.FW	S.LENDIA	NDW%
TUBERFW	DM%	LOC.	NFW%	TOTALWT	TUBERDW	N.HILLWT					
201.00	70.000	1.0000	3.0000	2.0000	2.0000	2.0000	2.0000	8.0000	3.0000	2.0000	4.0000
2.0000	2.0000	2.0000	2.0000	4.0000	2.0000	3.0000	4.0000	2.0000	4025.0	48.000	.89000
1450.0	26.400	2	.23496	3.4069	382.80	9.4571					
201.00	111.00	2.0000	4.0000	4.0000	3.0000	2.0000	1.0000	40.000	2.0000	3.0000	4.0000
3.0000	3.0000	2.0000	1.0000	4.0000	3.0000	1.0000	5.0000	3.0000	4750.0	105.00	.96000
4700.0	27.600	3	.26496	12.453	1297.2	12.586					
201.00	117.00	3.0000	1.0000	5.0000	4.0000	2.0000	2.0000	9.0000	2.0000	2.0000	3.0000
2.0000	2.0000	3.0000	1.0000	3.0000	2.0000	1.0000	4.0000	3.0000	3600.0	103.00	.86000
3575.0	37.800	1	.32508	11.622	1351.3	11.703					
201.00	132.00	2.0000	3.0000	1.0000	2.0000	3.0000	2.0000	53.000	2.0000	2.0000	3.0000
3.0000	2.0000	1.0000	2.0000	4.0000	2.0000	2.0000	5.0000	3.0000	5460.0	92.000	.81000
2475.0	30.700	3	.24867	6.1546	759.82	13.577					
201.00	137.00	2.0000	4.0000	5.0000	4.0000	1.0000	1.0000	55.000	1.0000	2.0000	3.0000
3.0000	2.0000	2.0000	1.0000	3.0000	2.0000	1.0000	5.0000	3.0000	1400.0	112.00	.86000
1400.0	29.000	3	.24940	3.4916	406.00	3.4916					
208.00	38.000	2.0000	2.0000	4.0000	4.0000	2.0000	1.0000	26.000	2.0000	2.0000	3.0000
2.0000	2.0000	1.0000	1.0000	3.0000	3.0000	1.0000	5.0000	2.0000	2200.0	94.000	.61000
2075.0	28.000	3	.17080	3.5441	581.00	3.7576					
208.00	308.00	1.0000	1.0000	1.0000	2.0000	3.0000	2.0000	15.000	3.0000	2.0000	4.0000
3.0000	2.0000	2.0000	1.0000	3.0000	4.0000	1.0000	5.0000	2.0000	2625.0	99.000	1.0300
3575.0	38.400	1	.39552	14.140	1372.8	10.382					
208.00	530.00	1.0000	1.0000	1.0000	2.0000	3.0000	2.0000	40.000	2.0000	2.0000	3.0000
3.0000	2.0000	1.0000	1.0000	3.0000	3.0000	2.0000	5.0000	2.0000	3725.0	154.00	.84000
3575.0	32.000	2	.26880	9.6096	1144.0	10.013					
208.00	544.00	2.0000	2.0000	2.0000	4.0000	1.0000	1.0000	19.000	1.0000	2.0000	3.0000
2.0000	2.0000	3.0000	1.0000	2.0000	2.0000	1.0000	5.0000	2.0000	800.00	212.00	.76000
800.00	29.400	1	.22344	1.7875	235.20	1.7875					
208.00	570.00	1.0000	1.0000	2.0000	4.0000	3.0000	2.0000	38.000	3.0000	2.0000	4.0000
4.0000	2.0000	3.0000	1.0000	3.0000	4.0000	1.0000	5.0000	2.0000	2900.0	169.00	1.0500
2875.0	38.600	4	.40530	11.652	1109.8	11.754					
208.00	715.00	1.0000	3.0000	5.0000	4.0000	3.0000	1.0000	17.000	2.0000	2.0000	2.0000
3.0000	2.0000	2.0000	1.0000	3.0000	3.0000	1.0000	5.0000	2.0000	1900.0	76.000	.77000
1850.0	34.400	2	.26488	4.9003	636.40	5.0327					
208.00	720.00	1.0000	4.0000	6.0000	4.0000	1.0000	1.0000	40.000	1.0000	2.0000	3.0000
3.0000	1.0000	3.0000	1.0000	3.0000	2.0000	1.0000	5.0000	3.0000	1650.0	-1.0000	.65000
1600.0	36.100	3	.23465	3.7544	577.60	3.8717					
213.00	73.000	2.0000	3.0000	2.0000	3.0000	2.0000	2.0000	5.0000	2.0000	2.0000	4.0000
3.0000	1.0000	3.0000	2.0000	3.0000	4.0000	2.0000	5.0000	2.0000	2050.0	139.00	1.0000
1550.0	25.000	1	.25000	3.8750	387.50	5.1250					
213.00	84.000	3.0000	3.0000	4.0000	3.0000	2.0000	1.0000	28.000	2.0000	2.0000	2.0000
2.0000	2.0000	3.0000	2.0000	3.0000	4.0000	2.0000	3.0000	3.0000	1225.0	63.000	.81000
975.00	33.300	3	.26973	2.6299	324.67	3.3042					
213.00	212.00	3.0000	1.0000	7.0000	4.0000	2.0000	2.0000	14.000	2.0000	2.0000	4.0000
1.0000	1.0000	3.0000	2.0000	4.0000	1.0000	2.0000	4.0000	2.0000	5460.0	48.000	.60000
2350.0	36.600	3	.21960	5.1606	860.10	11.990					
213.00	227.00	1.0000	3.0000	4.0000	3.0000	2.0000	1.0000	13.000	2.0000	2.0000	3.0000
3.0000	2.0000	3.0000	1.0000	3.0000	3.0000	2.0000	4.0000	2.0000	1625.0	37.000	.54000
1500.0	44.100	1	.23814	3.5721	661.50	3.8698					
213.00	335.00	1.0000	5.0000	4.0000	2.0000	1.0000	1.0000	29.000	1.0000	2.0000	4.0000
3.0000	1.0000	3.0000	1.0000	3.0000	1.0000	3.0000	4.0000	3.0000	2900.0	36.000	.49000
1325.0	36.700	4	.17983	2.3827	486.27	5.2151					
213.00	359.00	1.0000	4.0000	5.0000	4.0000	2.0000	1.0000	137.00	2.0000	3.0000	4.0000
2.0000	2.0000	2.0000	2.0000	3.0000	2.0000	1.0000	5.0000	2.0000	2200.0	99.000	.83500
2125.0	37.600	2	.31396	6.6716	799.00	6.9071					
213.00	372.00	2.0000	3.0000	10.000	1.0000	2.0000	1.0000	15.000	2.0000	2.0000	3.0000
2.0000	2.0000	2.0000	2.0000	4.0000	3.0000	3.0000	4.0000	2.0000	3850.0	71.000	.70000
1275.0	39.000	3	.27300	3.4807	497.25	10.510					
213.00	392.00	1.0000	3.0000	6.0000	4.0000	3.0000	2.0000	13.000	3.0000	2.0000	3.0000
2.0000	2.0000	3.0000	1.0000	4.0000	2.0000	1.0000	4.0000	2.0000	3750.0	76.000	.86000
3700.0	33.200	1	.28552	10.564	1228.4	10.707					
213.00	410.00	1.0000	4.0000	2.0000	3.0000	1.0000	1.0000	15.000	1.0000	1.0000	3.0000
2.0000	1.0000	2.0000	2.0000	2.0000	3.0000	1.0000	5.0000	3.0000	1100.0	159.00	.92000
1000.0	44.500	4	.40940	4.0940	445.00	4.5034					
213.00	552.00	1.0000	3.0000	2.0000	4.0000	1.0000	2.0000	21.000	2.0000	2.0000	4.0000
3.0000	2.0000	2.0000	1.0000	3.0000	4.0000	1.0000	4.0000	3.0000	850.00	209.00	.92000
850.00	39.000	3	.35880	3.0498	331.50	3.0498					
214.00	48.000	3.0000	2.0000	10.000	1.0000	3.0000	2.0000	35.000	2.0000	2.0000	4.0000
1.0000	3.0000	3.0000	2.0000	4.0000	4.0000	1.0000	4.0000	3.0000	3175.0	59.000	1.0100
3125.0	35.700	3	.36057	11.268	1115.6	11.448					
214.00	67.000	1.0000	2.0000	10.000	1.0000	3.0000	2.0000	15.000	2.0000	2.0000	4.0000
2.0000	2.0000	3.0000	3.0000	4.0000	4.0000	3.0000	4.0000	3.0000	3050.0	46.000	.85000
2025.0	38.000	3	.32300	6.5407	769.50	9.8515					
214.00	123.00	3.0000	2.0000	2.0000	2.0000	3.0000	2.0000	44.000	2.0000	3.0000	3.0000
1.0000	3.0000	4.0000	1.0000	4.0000	4.0000	3.0000	4.0000	3.0000	3600.0	47.000	.89000
1550.0	38.100	1	.33909	5.2559	590.55	12.207					
214.00	146.00	1.0000	2.0000	9.0000	4.0000	1.0000	1.0000	13.000	2.0000	3.0000	4.0000
2.0000	1.0000	2.0000	2.0000	3.0000	3.0000	1.0000	4.0000	3.0000	2750.0	123.00	.77000
2675.0	35.200	4	.27104	7.2503	941.60	7.4536					
214.00	175.00	3.0000	3.0000	2.0000	2.0000	2.0000	1.0000	24.000	2.0000	2.0000	4.0000
2.0000	3.0000	4.0000	2.0000	4.0000	3.0000	2.0000	4.0000	4.0000	3550.0	50.000	.96000
2000.0	30.400	3	.29184	5.8368	608.00	10.360					
214.00	181.00	3.0000	6.0000	2.0000	2.0000	1.0000	1.0000	13.000	2.0000	2.0000	4.0000
2.0000	2.0000	1.0000	2.0000	3.0000	3.0000	2.0000	4.0000	3.0000	2700.0	35.000	.50500
2300.0	32.500	4	.16412	3.7749	747.50	4.4314					
214.00	184.00	2.0000	2.0000	5.0000	4.0000	2.0000	1.0000	29.000	2.0000	2.0000	3.0000
2.0000	2.0000	2.0000	1.0000	3.0000	2.0000	1.0000	4.0000	1.0000	2050.0	44.000	.62000
2000.0	39.400	1	.24428	4.8856	788.00	5.0077					
215.00	12.000	2.0000	4.0000	2.0000	2.0000	1.0000	2.0000	14.000	3.0000	1.0000	4.0000
2.0000	1.0000	3.0000	4.0000	3.0000	4.0000	2.0000	2.0000	3.0000	1050.0	79.000	.75000
975.00	35.200	1	.26400	2.3100	308.00	2.7720					
215.00	13.000	3.0000	4.0000	10.000	1.0000	1.0000	1.0000	16.000	1.0000	2.0000	4.0000
2.0000	2.0000	2.0000	2.0000	2.0000	4.0000	1.0000	3.0000	3.0000	975.00	82.000	.69000
950.00	37.000	2	.25530	2.4253	351.50	2.4892					
215.00	19.000	2.0000	4.0000	2.0000	2.0000	2.0000	2.0000	25.000	2.0000	2.0000	4.0000
2.0000	2.0000	2.0000	2.0000	4.0000	3.0000	3.0000	4.0000	2.0000	2825.0	49.000	1.0500
1500.0	37.300	4	.39165	5.8747	559.50	11.064					
215.00	33.000	1.0000	3.0000	5.0000	4.0000	3.0000	2.0000	15.000	2.0000	3.0000	4.0000
2.0000	2.0000	3.0000	1.0000	4.0000	3.0000	2.0000	4.0000	3.0000	2350.0	92.000	.62000
1000.0	37.300	1	.23126	2.3126	373.00	5.4346					
215.00	38.000	2.0000	3.0000	5.0000	4.0000	2.0000	1.0000	13.000	2.0000	3.0000	4.0000
2.0000	2.0000	2.0000	1.0000	4.0000	3.0000	1.0000	4.0000	3.0000	2275.0	128.00	.81000
2175.0	41.500	1	.33615	7.3113	902.63	7.6474					
215.00	39.000	1.0000	2.0000	5.0000	4.0000	3.0000	2.0000	35.000	2.0000	3.0000	4.0000
1.0000	2.0000	2.0000	1.0000	4.0000	3.0000	1.0000	4.0000	2.0000	3350.0	51.000	.71000
3325.0	35.500	4	.25205	8.3807	1180.4	8.4437					
215.00	42.000	1.0000	1.0000	4.0000	3.0000	2.0000	2.0000	16.000	2.0000	2.0000	4.0000
2.0000	3.0000	3.0000	1.0000	3.0000	4.0000	1.0000	4.0000	3.0000	1400.0	47.000	.77000
1400.0	36.000	3	.27720	3.8808	504.00	3.8808					
215.00	49.000	1.0000	2.0000	6.0000	4.0000	2.0000	1.0000	17.000	2.0000	2.0000	4.0000
2.0000	2.0000	3.0000	2.0000	4.0000	3.0000	2.0000	4.0000	3.0000	2775.0	74.000	.58500
1050.0	40.100	1	.23458	2.4631	421.05	6.5097					
215.00	57.000	2.0000	1.0000	2.0000	3.0000	3.0000	2.0000	21.000	3.0000	3.0000	3.0000
2.0000	2.0000	3.0000	2.0000	4.0000	4.0000	3.0000	4.0000	3.0000	4325.0	84.000	.66500
3650.0	38.200	1	.25403	9.2721	1394.3	10.987					
215.00	64.000	2.0000	3.0000	5.0000	4.0000	1.0000	1.0000	23.000	1.0000	2.0000	4.0000
2.0000	2.0000	4.0000	3.0000	3.0000	2.0000	1.0000	3.0000	2.0000	1450.0	97.000	.77000
1425.0	38.000	2	.29260	4.1695	541.50	4.2427					
215.00	67.000	1.0000	1.0000	4.0000	4.0000	2.0000	2.0000	12.000	3.0000	3.0000	4.0000
2.0000	2.0000	2.0000	1.0000	4.0000	3.0000	1.0000	4.0000	3.0000	3600.0	57.000	.59000
3600.0	38.100	2	.22479	8.0924	1371.6	8.0924					
215.00	71.000	3.0000	4.0000	5.0000	4.0000	2.0000	2.0000	14.000	3.0000	2.0000	4.0000
1.0000	2.0000	2.0000	3.0000	5.0000	3.0000	4.0000	4.0000	2.0000	3825.0	75.000	1.0000
250.00	35.100	4	.35100	.87750	87.750	13.426					
215.00	73.000	1.0000	1.0000	6.0000	4.0000	3.0000	1.0000	28.000	1.0000	3.0000	3.0000
2.0000	2.0000	2.0000	1.0000	3.0000	3.0000	2.0000	4.0000	3.0000	2150.0	94.000	.68000
825.00	34.400	2	.23392	1.9298	283.80	5.0293					
215.00	87.000	2.0000	3.0000	10.000	1.0000	2.0000	1.0000	15.000	1.0000	2.0000	4.0000
2.0000	2.0000	2.0000	2.0000	3.0000	2.0000	2.0000	4.0000	3.0000	2025.0	101.00	.77500
1575.0	34.600	3	.26815	4.2234	544.95	5.4300					
215.00	89.000	1.0000	1.0000	5.0000	3.0000	3.0000	2.0000	9.0000	2.0000	3.0000	4.0000
4.0000	2.0000	2.0000	3.0000	4.0000	4.0000	1.0000	2.0000	2.0000	3000.0	30.000	.76000
2975.0	36.900	3	.28044	8.3431	1097.8	8.4132					
215.00	93.000	1.0000	3.0000	5.0000	4.0000	3.0000	2.0000	19.000	3.0000	3.0000	4.0000
2.0000	3.0000	2.0000	1.0000	4.0000	2.0000	2.0000	4.0000	3.0000	5040.0	68.000	.69000
4475.0	32.800	2	.22632	10.128	1467.8	11.407					
215.00	94.000	2.0000	4.0000	10.000	1.0000	2.0000	2.0000	25.000	2.0000	2.0000	4.0000
2.0000	2.0000	2.0000	1.0000	3.0000	3.0000	1.0000	4.0000	2.0000	2275.0	77.000	.74000
2250.0	31.600	1	.23384	5.2614	711.00	5.3199					
215.00	96.000	3.0000	3.0000	10.000	1.0000	1.0000	1.0000	23.000	1.0000	3.0000	4.0000
2.0000	2.0000	3.0000	1.0000	3.0000	3.0000	1.0000	4.0000	3.0000	1200.0	97.000	.61000
1175.0	37.400	1	.22814	2.6806	439.45	2.7377					
215.00	101.00	1.0000	1.0000	10.000	1.0000	1.0000	2.0000	11.000	2.0000	2.0000	4.0000
2.0000	1.0000	3.0000	4.0000	3.0000	3.0000	3.0000	4.0000	2.0000	2725.0	67.000	.83000
1850.0	34.500	4	.28635	5.2975	638.25	7.8030					
215.00	104.00	1.0000	1.0000	4.0000	2.0000	2.0000	1.0000	20.000	2.0000	3.0000	4.0000
2.0000	2.0000	3.0000	1.0000	4.0000	4.0000	2.0000	2.0000	4.0000	875.00	72.000	.86000
775.00	34.300	4	.29498	2.2861	265.82	2.5811					
215.00	105.00	1.0000	3.0000	5.0000	4.0000	2.0000	2.0000	12.000	2.0000	3.0000	3.0000
2.0000	2.0000	1.0000	1.0000	4.0000	2.0000	1.0000	4.0000	2.0000	3850.0	21.000	.64000
3825.0	37.400	2	.23936	9.1555	1430.5	9.2154					
215.00	108.00	1.0000	1.0000	5.0000	4.0000	2.0000	3.0000	21.000	2.0000	1.0000	4.0000
2.0000	2.0000	3.0000	1.0000	4.0000	3.0000	1.0000	4.0000	3.0000	3925.0	120.00	.67500
3875.0	35.000	4	.23625	9.1547	1356.3	9.2728					
215.00	112.00	1.0000	4.0000	9.0000	4.0000	2.0000	2.0000	15.000	2.0000	2.0000	4.0000
1.0000	2.0000	2.0000	3.0000	3.0000	3.0000	4.0000	4.0000	3.0000	2700.0	117.00	.83000
1325.0	34.400	4	.28552	3.7831	455.80	7.7090					
215.00	113.00	1.0000	4.0000	10.000	1.0000	2.0000	1.0000	15.000	2.0000	1.0000	4.0000
2.0000	1.0000	3.0000	2.0000	3.0000	4.0000	2.0000	4.0000	3.0000	2025.0	68.000	.83000
1250.0	38.400	2	.31872	3.9840	480.00	6.4541					
215.00	135.00	2.0000	1.0000	5.0000	4.0000	3.0000	2.0000	33.000	3.0000	3.0000	4.0000
2.0000	2.0000	3.0000	1.0000	4.0000	3.0000	3.0000	3.0000	3.0000	3450.0	74.000	.48500
2050.0	39.900	1	.18866	3.8676	797.45	6.5089					
215.00	137.00	3.0000	4.0000	4.0000	3.0000	2.0000	2.0000	18.000	3.0000	2.0000	4.0000
2.0000	1.0000	3.0000	3.0000	4.0000	3.0000	3.0000	4.0000	3.0000	3300.0	114.00	.70500
1275.0	35.400	2	.24957	3.1820	451.35	8.2358					
215.00	143.00	1.0000	2.0000	7.0000	4.0000	2.0000	2.0000	23.000	3.0000	2.0000	4.0000
2.0000	3.0000	3.0000	2.0000	4.0000	2.0000	1.0000	4.0000	3.0000	4300.0	63.000	.77000
4275.0	31.500	1	.24255	10.369	1346.6	10.430					
215.00	154.00	2.0000	6.0000	6.0000	2.0000	1.0000	1.0000	33.000	1.0000	3.0000	4.0000
2.0000	2.0000	3.0000	1.0000	3.0000	1.0000	1.0000	4.0000	2.0000	1475.0	72.000	.53500
1450.0	35.300	2	.18985	2.7384	511.85	2.7856					
215.00	159.00	1.0000	1.0000	5.0000	4.0000	2.0000	2.0000	18.000	2.0000	2.0000	4.0000
1.0000	2.0000	2.0000	1.0000	3.0000	2.0000	2.0000	4.0000	2.0000	2175.0	59.000	.70500
2000.0	40.400	4	.28482	5.6964	808.00	6.1948					
215.00	162.00	2.0000	1.0000	5.0000	4.0000	2.0000	2.0000	8.0000	2.0000	2.0000	4.0000
2.0000	2.0000	2.0000	1.0000	4.0000	2.0000	2.0000	4.0000	3.0000	3475.0	66.000	.64500
1575.0	38.800	1	.25026	3.9416	611.10	8.6965					
215.00	168.00	1.0000	3.0000	2.0000	4.0000	3.0000	2.0000	23.000	2.0000	2.0000	3.0000
2.0000	2.0000	2.0000	1.0000	3.0000	3.0000	1.0000	4.0000	2.0000	1950.0	65.000	.64000
1950.0	38.000	3	.24320	4.7424	741.00	4.7424					
215.00	171.00	3.0000	1.0000	5.0000	4.0000	3.0000	2.0000	32.000	2.0000	1.0000	3.0000
2.0000	2.0000	3.0000	1.0000	3.0000	4.0000	1.0000	4.0000	2.0000	1550.0	62.000	.77000
1550.0	37.600	3	.28952	4.4876	582.80	4.4876					
215.00	182.00	3.0000	3.0000	2.0000	3.0000	2.0000	2.0000	25.000	2.0000	2.0000	3.0000
2.0000	1.0000	3.0000	3.0000	4.0000	4.0000	2.0000	4.0000	3.0000	3875.0	53.000	.81000
1750.0	38.900	1	.31509	5.5141	680.75	12.210					
215.00	187.00	3.0000	2.0000	5.0000	4.0000	2.0000	2.0000	22.000	2.0000	2.0000	3.0000
2.0000	3.0000	3.0000	2.0000	3.0000	2.0000	2.0000	4.0000	3.0000	2625.0	57.000	.90000
2025.0	35.700	2	.32130	6.5063	722.92	8.4341					
215.00	195.00	1.0000	1.0000	5.0000	4.0000	3.0000	2.0000	23.000	3.0000	2.0000	3.0000
2.0000	2.0000	3.0000	1.0000	4.0000	1.0000	2.0000	4.0000	2.0000	3725.0	81.000	.85000
1800.0	36.500	2	.31025	5.5845	657.00	11.557					
215.00	197.00	1.0000	4.0000	4.0000	2.0000	3.0000	2.0000	19.000	2.0000	2.0000	3.0000
3.0000	2.0000	3.0000	1.0000	4.0000	1.0000	1.0000	4.0000	2.0000	3175.0	66.000	.59000
3125.0	34.800	2	.20532	6.4162	1087.5	6.5189					
215.00	203.00	2.0000	4.0000	6.0000	4.0000	2.0000	1.0000	37.000	2.0000	2.0000	3.0000
2.0000	2.0000	2.0000	2.0000	3.0000	3.0000	2.0000	3.0000	3.0000	1600.0	64.000	.77000
800.00	38.300	1	.29491	2.3593	306.40	4.7186					
215.00	216.00	1.0000	2.0000	10.000	1.0000	2.0000	1.0000	26.000	1.0000	2.0000	4.0000
3.0000	2.0000	3.0000	1.0000	3.0000	2.0000	1.0000	4.0000	3.0000	2150.0	90.000	.63000
2100.0	39.500	3	.24885	5.2258	829.50	5.3503					
215.00	224.00	1.0000	4.0000	10.000	1.0000	2.0000	1.0000	53.000	1.0000	2.0000	3.0000
2.0000	2.0000	2.0000	1.0000	4.0000	2.0000	2.0000	4.0000	3.0000	3300.0	67.000	.89000
2825.0	33.400	3	.29726	8.3976	943.55	9.8096					
215.00	226.00	1.0000	4.0000	5.0000	4.0000	2.0000	1.0000	46.000	2.0000	3.0000	3.0000
3.0000	2.0000	2.0000	1.0000	4.0000	3.0000	1.0000	5.0000	3.0000	3700.0	78.000	.92000
3650.0	36.800	3	.33856	12.357	1343.2	12.527					
215.00	232.00	2.0000	1.0000	5.0000	4.0000	3.0000	2.0000	19.000	2.0000	2.0000	3.0000
2.0000	2.0000	2.0000	2.0000	3.0000	2.0000	3.0000	4.0000	3.0000	1800.0	63.000	.82500
1275.0	31.200	4	.25740	3.2818	397.80	4.6332					
215.00	247.00	1.0000	2.0000	2.0000	2.0000	2.0000	2.0000	11.000	3.0000	2.0000	4.0000
2.0000	2.0000	2.0000	3.0000	4.0000	3.0000	3.0000	4.0000	3.0000	4150.0	52.000	.90500
1700.0	32.400	2	.29322	4.9847	550.80	12.169					
215.00	258.00	3.0000	4.0000	6.0000	4.0000	2.0000	2.0000	32.000	2.0000	3.0000	4.0000
2.0000	3.0000	2.0000	1.0000	3.0000	2.0000	2.0000	4.0000	3.0000	4600.0	78.000	.98000
1375.0	34.500	2	.33810	4.6489	474.38	15.553					
215.00	264.00	3.0000	2.0000	4.0000	4.0000	2.0000	2.0000	9.0000	3.0000	3.0000	4.0000
3.0000	2.0000	3.0000	1.0000	4.0000	2.0000	1.0000	4.0000	2.0000	5400.0	76.000	.59000
5140.0	35.600	1	.21004	10.796	1829.8	11.342					
215.00	284.00	1.0000	1.0000	5.0000	2.0000	3.0000	2.0000	49.000	3.0000	3.0000	3.0000
2.0000	3.0000	2.0000	1.0000	5.0000	3.0000	1.0000	3.0000	3.0000	5500.0	61.000	.85000
4725.0	37.800	3	.32130	15.181	1786.0	17.671					
215.00	288.00	3.0000	3.0000	5.0000	3.0000	2.0000	1.0000	21.000	3.0000	3.0000	4.0000
3.0000	2.0000	3.0000	1.0000	4.0000	2.0000	1.0000	5.0000	2.0000	3650.0	77.000	1.0400
3650.0	37.400	1	.38896	14.197	1365.1	14.197					
215.00	291.00	2.0000	3.0000	4.0000	3.0000	3.0000	2.0000	29.000	3.0000	2.0000	4.0000
2.0000	2.0000	2.0000	2.0000	5.0000	2.0000	3.0000	1.0000	2.0000	4600.0	157.00	.69500
1850.0	30.800	1	.21406	3.9601	569.80	9.8468					
215.00	304.00	2.0000	4.0000	2.0000	4.0000	1.0000	1.0000	15.000	2.0000	2.0000	3.0000
1.0000	2.0000	3.0000	3.0000	3.0000	3.0000	3.0000	4.0000	2.0000	2200.0	78.000	.79000
725.00	34.500	3	.27255	1.9760	250.13	5.9961					
215.00	307.00	2.0000	5.0000	5.0000	2.0000	1.0000	1.0000	34.000	1.0000	3.0000	4.0000
3.0000	2.0000	3.0000	2.0000	4.0000	3.0000	2.0000	4.0000	3.0000	5000.0	91.000	.77000
2350.0	37.500	4	.28875	6.7856	881.25	14.437					
215.00	310.00	3.0000	3.0000	5.0000	4.0000	2.0000	2.0000	25.000	2.0000	2.0000	4.0000
1.0000	2.0000	3.0000	1.0000	4.0000	3.0000	2.0000	4.0000	3.0000	2100.0	75.000	.85000
1350.0	36.600	4	.31110	4.1998	494.10	6.5331					
215.00	314.00	1.0000	1.0000	5.0000	4.0000	3.0000	2.0000	21.000	2.0000	2.0000	4.0000
2.0000	1.0000	3.0000	2.0000	4.0000	3.0000	2.0000	5.0000	2.0000	4225.0	63.000	.59000
2450.0	34.900	4	.20591	5.0448	855.05	8.6997					
215.00	315.00	2.0000	4.0000	2.0000	4.0000	2.0000	1.0000	15.000	2.0000	2.0000	4.0000
2.0000	2.0000	3.0000	2.0000	3.0000	2.0000	4.0000	4.0000	3.0000	1775.0	106.00	.84000
1000.0	34.800	1	.29232	2.9232	348.00	5.1887					

CORRELATION MATRIX

Correlations between characters evaluated for plants of Population 4

N= 81 DF= 79 R@ .0500= .2185 R@ .0100= .2847

VARIABLE												
EM.DATE	1.0000											
FL.DATE	.0285	1.0000										
SEX	-.2103	-.3340	1.0000									
HEIGHT	-.4995	-.1098	.1045	1.0000								
BRANCHNO	-.4548	-.1900	.1415	.4905	1.0000							
HT1BRA	.1728	-.0124	.0192	.0796	-.1939	1.0000						
LEAFNO	-.3154	-.3139	.2074	.4702	.5588	-.1592	1.0000					
LEAFSIZE	-.0389	.0284	.1236	.1376	-.1172	.2172	.0764	1.0000				
VIRUS	.0485	.1754	-.1727	-.1930	.1766	-.1419	.1314	.1314	1.0000			
STEMCOL	.0431	-.2121	-.0802	.0229	-.1560	.0802	-.0498	.0839	-.0824	1.0000		
STEMDIAM	-.1217	-.0279	.0232	.3135	.1005	.1863	.1551	.3243	-.0349	-.1017	1.0000	
THORN	-.1021	.0466	-.0325	-.0783	.0106	-.1231	-.0287	-.0979	.1307	-.0856	-.0359	1.0000
STEMNO.	.1476	.0593	-.2187	-.2264	.0728	-.1742	.1146	-.2944	.1993	-.2113	-.3279	.1258
VIGOR	-.1536	-.0419	.0004	.4514	.3905	.0083	.5316	.3275	.1750	-.1218	.2251	.0365
LEAFSPOT	-.1779	-.0864	-.2097	.1212	.1037	-.1164	.1371	-.0804	.0513	.0154	-.0125	.0630
TUBERNO	.1063	-.0741	-.1306	.0762	.1418	-.1810	.2033	-.1118	.1150	-.3538	-.1224	.0731
TUBSHAPE	.0566	-.1252	.1504	-.0268	-.1180	.2051	-.1636	-.0381	-.1769	.2110	-.0643	-.1392
TUSMOOTH	.0806	.0910	-.1657	-.1535	-.1299	.0126	-.1556	.0856	.1380	-.0790	.1784	.1584
HARV.FW	-.1643	-.0689	.0160	.3935	.4294	.0448	.4859	.3066	.1122	.0096	.2176	-.1111
S.LENDIA	-.0162	-.1772	.1421	-.1324	-.0344	.0506	-.0602	-.1089	.0774	.1863	-.0969	-.0784
NDW%	-.0077	-.1446	-.0977	.0916	.1093	.1327	.1816	-.0979	.0126	.0510	.2339	.0121
TUBERFW	-.2680	-.0203	.0601	.3566	.3128	.0964	.3880	.3423	.0220	.2620	.3006	-.1443
DM%	-.1168	.1335	.0557	-.0104	-.0661	-.0284	-.0719	.0266	-.0016	-.0483	-.1336	.1751
LOC.	.0364	.1048	-.0746	-.1294	.0008	.0825	-.2568	-.1442	.0954	-.0830	-.1105	-.1782
NFW%	-.0641	-.0680	-.0602	.0799	.0710	.1171	.1335	-.0871	.0051	.0127	.1451	.0839
TOTALNT	-.2660	-.0299	.0032	.3711	.2741	.1546	.3918	.3017	.0109	.2800	.3271	-.0868
TUBERDW	-.3115	-.0048	.0737	.3611	.3142	.0851	.3856	.3586	.0139	.2501	.2630	-.1066
N.HILLWT	-.1584	-.0668	-.0381	.3912	.3963	.1124	.4840	.2819	.0788	.0124	.3001	-.0568

	EM.DATE	FL.DATE	SEX	HEIGHT	BRANCHNO	HT1BRA	LEAFNO	LEAFSIZE	VIRUS	STEMCOL	STEMDIAM	THORN
STEMNO.	1.0000											
VIGOR	.0329	1.0000										
LEAFSPOT	.2456	-.0488	1.0000									
TUBERNO	.5088	.2375	.0208	1.0000								
TUBSHAPE	-.2963	-.2550	-.1357	-.2135	1.0000							
TUSMOOTH	.0497	.0630	.2318	.1361	-.1698	1.0000						
HARV.FW	.0073	.7252	-.2406	.1725	.0500	-.0338	1.0000					
S.LENDIA	-.0735	-.2725	.0885	-.1157	.1696	-.0138	-.1840	1.0000				
NDW%	.1673	.0533	.2838	.0691	.2022	.0851	.0397	.2684	1.0000			
TUBERFW	-.3093	.3929	-.1303	-.4180	.1774	-.1061	.6443	-.0900	.0174	1.0000		
DM%	-.0787	-.0491	.1395	-.1093	-.1130	.0065	-.1530	-.1300	-.1903	-.0779	1.0000	
LOC.	.1310	-.0140	.0797	.0994	.1001	.1113	-.0151	-.0708	.1310	-.0695	-.0457	1.0000
NFW%	.1172	.0152	.3408	-.0077	.1312	.0818	-.0472	.2008	.8435	-.0188	.3581	.1210
TOTALNT	-.2672	.3474	.0035	-.4126	.2248	-.0768	.5545	-.0112	.3300	.9137	.0504	-.0505
TUBERDW	-.3222	.3853	-.1046	-.4323	.1486	-.1178	.6117	-.1170	-.0140	.9793	.1027	-.0819
N.HILLWT	.0444	.6708	-.0779	.1527	.1148	-.0009	.8898	-.1191	.4124	.5713	-.0125	.0202

	STEMNO.	VIGOR	LEAFSPOT	TUBERNO	TUBSHAPE	TUSMOOTH	HARV.FW	S.LENDIA	NDW%	TUBERFW	DM%	LOC.
NFW%	1.0000											
TOTALNT	.3477	1.0000										
TUBERDW	.0496	.9242	1.0000									
N.HILLWT	.3829	.6624	.5708	1.0000								

	NFW%	TOTALNT	TUBERDW	N.HILLWT

Correlations between characters evaluated for plants of Population 4 in Location 1

CORRELATION MATRIX <1> LOC.:1

N= 24 DF= 22 R@ .0500= .4044 R@ .0100= .5151

VARIABLE	EM.DATE	FL.DATE	SEX	HEIGHT	BRANCHNO	HT1BRA	LEAFNO	LEAFSIZE	VIRUS	STEMCOL	STEMDIAM	THORN
EM.DATE	1.0000											
FL.DATE	.2043	1.0000										
SEX	-.2582	-.1606	1.0000									
HEIGHT	-.3341	-.1947	.1380	1.0000								
BRANCHNO	-.3012	-.1789	-.1556	.4830	1.0000							
HT1BRA	.0683	.1322	-.2174	.2462	-.0808	1.0000						
LEAFNO	-.1705	-.2097	.0880	.5286	.5107	-.0183	1.0000					
LEAFSIZE	-.2708	.1581	.0200	.3516	-.0599	.2166	-.0053	1.0000				
VIRUS	.1291	.2248	-.1556	-.0690	.1111	-.2903	.2289	.0999	1.0000			
STEMCOL	0.	-.1445	.0000	-.0945	-.0913	-.5627	.2652	-.0274	.2739	1.0000		
STEMDIAM	-.3208	.2916	.1183	.3185	-.0710	.4150	.0438	.3901	-.0710	-.3565	1.0000	
THORN	-.0801	-.2475	-.0413	0.	.1240	.0572	-.0328	.2602	-.2067	-.1698	.1321	1.0000
STEMNO.	.3819	-.3374	-.1127	-.2624	.1690	.0087	.2232	-.5572	.0563	-.1157	-.5401	.1048
VIGOR	-.1617	.0563	.1837	.6051	.3841	.1623	.5514	.3854	.2505	-.1601	.2194	.0311
LEAFSPOT	.0521	-.3594	-.5384	.1393	.2692	.1553	.0142	.0161	-.0538	-.0369	-.3632	.2003
TUBERNO	.0723	-.4254	.0622	.3733	.1119	.2903	.1150	.0410	.0124	-.3236	-.0221	.2545
TUBSHAPE	-.2601	-.0731	.0366	-.1138	-.2076	-.3284	-.3193	.2525	-.1099	.3512	.0911	.2045
TUSMOOTH	-.0362	.0321	.0560	-.0193	.1308	.0820	-.0938	.1624	.1308	-.4861	.0862	.3128
HARV.FW	-.4473	-.0495	.2234	.5676	.5662	.0498	.6326	.3357	.0324	.0248	.2874	.1208
S.LENDIA	.0217	-.2158	.0831	-.1970	-.0954	-.2537	-.2554	-.0475	.1718	.0623	-.1734	-.1191
NDW%	.1427	-.3410	-.1891	.0575	.0984	-.1099	.1240	-.1492	-.0178	.1864	-.0932	.0412
TUBERFW	-.4233	.0485	.1107	.3139	.3883	-.2154	.6369	.2644	.0258	.3678	.2864	.0296
DM%	-.2254	.0378	.0927	.1221	-.3204	.1337	-.0302	.2777	-.2171	-.0612	.1500	-.0533
NFW%	.0030	-.3080	-.1459	.1390	-.0810	.0093	.1282	.0320	-.1425	.1038	.0382	-.0147
TOTALNT	-.3862	-.0607	.0145	.3183	.2740	-.2047	.5769	.2210	-.0082	.3920	.2370	-.0061
TUBERDW	-.4802	.0128	.1183	.3294	.3250	-.2049	.6172	.3224	-.0203	.3721	.2814	.0152
N.HILLWT	-.3953	-.1725	.0969	.5328	.4390	.0620	.5576	.3150	-.0910	.0460	.2688	.1585

VARIABLE	STEMNO.	VIGOR	LEAFSPOT	TUBERNO	TUBSHAPE	TUSMOOTH	HARV.FW	S.LENDIA	NDW%	TUBERFW	DM%	NFW%
STEMNO.	1.0000											
VIGOR	.0423	1.0000										
LEAFSPOT	.4095	-.0674	1.0000									
TUBERNO	.3469	.2773	.2511	1.0000								
TUBSHAPE	-.4645	-.3947	.0296	-.4169	1.0000							
TUSMOOTH	.3316	.2200	.2867	.3938	-.1951	1.0000						
HARV.FW	-.0866	.7802	-.1930	.0746	-.1282	.0306	1.0000					
S.LENDIA	-.0456	-.2338	-.1700	-.0446	.0315	-.1218	-.2297	1.0000				
NDW%	.0746	-.1492	.1627	-.1717	.3952	-.1230	-.0022	.2273	1.0000			
TUBERFW	-.2645	.3169	-.2487	-.4028	.2306	-.2324	.7326	-.1723	.2114	1.0000		
DM%	-.1800	.1479	.1299	.0463	-.0095	.2644	-.0028	-.5884	-.3534	-.0440	1.0000	
NFW%	-.0356	-.0348	.2130	-.1629	.3619	.0560	.0291	-.1142	.8055	.2163	.2581	1.0000
TOTALNT	-.2748	.2233	-.1414	-.4387	.3606	-.2129	.5842	-.1400	.5046	.9078	.0564	.5785
TUBERDW	-.3032	.3144	-.2092	-.3889	.2477	-.2002	.7137	-.2316	.1729	.9824	.1264	.2824
N.HILLWT	-.1112	.6362	-.0646	-.0375	.1129	.0487	.8622	-.2600	.3993	.7182	.1173	.5152

VARIABLE	TOTALNT	TUBERDW	N.HILLWT
TOTALNT	1.0000		
TUBERDW	.9214	1.0000	
N.HILLWT	.7785	.7337	1.0000

Appendix 10(c)

Correlations between characters evaluated for plants of Population 4 in Location 2

CORRELATION MATRIX <2> LOC.:2

N= 18 DF= 16 R@ .0500= .4683 R@ .0100= .5897

VARIABLE												
EM.DATE	1.0000											
FL.DATE	.4224	1.0000										
SEX	-.3617	-.1981	1.0000									
HEIGHT	-.5279	-.3616	.2745	1.0000								
BRANCHNO	-.3697	-.6116	.1402	.3593	1.0000							
HT1BRA	.1823	-.0160	.2204	-.0198	-.3282	1.0000						
LEAFNO	-.3281	-.5056	.1261	.4022	.7387	-.1698	1.0000					
LEAFSIZE	.0237	-.1291	.5682	-.0000	.0658	.3320	-.0870	1.0000				
VIRUS	.3813	.1661	-.2924	-.6305	-.0212	.1249	.1120	.1072	1.0000			
STEMCOL	-.0918	-.3333	-.1540	.5423	.0510	-.0258	-.0674	-.2582	-.6644	1.0000		
STEMDIAM	-.1524	-.1422	.4642	.1285	.3020	.0496	-.0160	.4896	-.0984	-.0474	1.0000	
THORN	.2938	.2667	-.0220	-.3254	-.2548	-.1006	-.1685	-.3873	.2076	-.2000	-.1896	1.0000
STEMNO.	.1419	-.0329	-.2352	-.5171	-.1089	.0148	.0776	-.4669	.4778	-.3617	-.3663	.4274
VIGOR	-.2511	-.5890	.2176	.3879	.7183	-.2784	.7942	.1086	.0175	-.0421	-.0698	-.0421
LEAFSPOT	-.1665	.3348	-.3530	-.1059	-.2915	-.1689	-.0658	-.3601	.1158	-.0279	-.4033	-.2790
TUBERNO	-.2404	-.3740	-.1833	.2028	.4765	-.2649	.5731	-.3073	.2259	-.2380	-.1048	-.0340
TUBSHAPE	-.1524	-.4266	.1826	.5913	.0846	.4862	.2717	.1224	-.4527	.5215	-.0112	-.4740
TUSMOOTH	-.1232	.3478	-.0492	-.0808	.1140	-.2766	.1508	-.0000	.3714	-.4472	.1060	0.
HARV.FW	-.3381	-.5672	.2390	.4788	.8735	-.1910	.7723	.2419	.0763	-.0257	.3629	-.4110
S.LENDIA	-.0909	-.1507	-.0889	.1422	-.1786	.3847	-.1830	-.1623	-.0144	.3584	-.2105	.0508
NDW%	-.2431	-.1684	.0577	.0941	.1979	.1561	.2655	-.3463	.0335	-.0988	.2361	-.0565
TUBERFW	-.2595	-.3973	.2535	.4139	.5266	-.0123	.3818	.3352	-.1686	.3067	.3205	-.4409
DM%	.1238	.5993	.2190	-.3013	-.3881	.2141	-.3509	.0452	.0291	-.2091	-.2188	.3237
NFW%	-.1704	.1698	.1854	-.0454	-.0247	.2910	.0659	-.3313	.0345	-.2017	.1289	.1357
TOTALNT	-.3528	-.4078	.3303	.4256	.5385	.1199	.4078	.2584	-.1988	.2759	.3764	-.4551
TUBERDW	-.2474	-.3210	.3014	.3541	.4804	.0117	.3427	.3609	-.1648	.2552	.2797	-.4021
N.HILLWT	-.3373	-.4431	.2744	.3891	.7797	-.0681	.6850	.1343	.0828	-.1054	.4030	-.3256

	EM.DATE	FL.DATE	SEX	HEIGHT	BRANCHNO	HT1BRA	LEAFNO	LEAFSIZE	VIRUS	STEMCOL	STEMDIAM	THORN
STEMNO.	1.0000											
VIGOR	-.0346	1.0000										
LEAFSPOT	.3073	-.3932	1.0000									
TUBERNO	.4081	.3791	.1660	1.0000								
TUBSHAPE	-.3663	.1097	-.0463	.0403	1.0000							
TUSMOOTH	.2206	-.0941	.5614	.3801	-.3180	1.0000						
HARV.FW	-.2136	.7270	-.2464	.5184	.2211	.1419	1.0000					
S.LENDIA	.1064	-.3691	.1873	.0700	.2802	-.1077	-.1847	1.0000				
NDW%	.3216	-.1175	.1170	.5448	.1931	.1127	.2852	.1386	1.0000			
TUBERFW	-.4595	.4824	-.2314	-.2376	.3032	-.1636	.5539	-.1194	-.3023	1.0000		
DM%	.0266	-.2858	.1202	-.6182	-.2262	.1232	-.4732	.0053	-.3119	-.0248	1.0000	
NFW%	.3340	-.2878	.1752	.2149	.0849	.1853	.0240	.1649	.8474	-.3225	.2374	1.0000
TOTALNT	-.3791	.3945	-.1933	-.1757	.3969	-.1546	.5726	-.0312	-.0377	.9486	.0260	-.0231
TUBERDW	-.4658	.4648	-.2231	-.3311	.2663	-.1531	.4884	-.1623	-.3549	.9866	.1217	-.2978
N.HILLWT	-.1036	.5149	-.1909	.5557	.2294	.2014	.9115	-.0946	.5895	.3179	-.3496	.4049

	STEMNO.	VIGOR	LEAFSPOT	TUBERNO	TUBSHAPE	TUSMOOTH	HARV.FW	S.LENDIA	NDW%	TUBERFW	DM%	NFW%
TOTALNT	1.0000											
TUBERDW	.9389	1.0000										
N.HILLWT	.4481	.2677	1.0000									

	TOTALNT	TUBERDW	N.HILLWT

Correlations between characters evaluated for plants of Population 4 in Location 3

CORRELATION MATRIX <3> LOC.:3

N= 22 DF= 20 R@ .0500= .4227 R@ .0100= .5368

VARIABLE	EM.DATE	FL.DATE	SEX	HEIGHT	BRANCHNO	HT1BRA	LEAFNO	LEAFSIZE	VIRUS	STEMCOL	STEMDIAM	THORN
EM.DATE	1.0000											
FL.DATE	-.1249	1.0000										
SEX	.0924	-.6754	1.0000									
HEIGHT	-.5850	.1342	-.3765	1.0000								
BRANCHNO	-.6880	-.1474	.0531	.6100	1.0000							
HT1BRA	.4097	-.0961	.0134	-.0166	-.1812	1.0000						
LEAFNO	-.4564	-.4165	.1418	.4744	.5255	-.1413	1.0000					
LEAFSIZE	.0978	-.0908	-.0145	.0847	-.0727	.1622	.3141	1.0000				
VIRUS	-.3357	.2510	-.2866	.0878	.3022	-.4339	-.0585	.1333	1.0000			
STEMCOL	.1739	-.1904	.1279	-.0715	-.0886	.2442	-.2364	.4223	.0573	1.0000		
STEMDIAM	-.1429	-.1304	-.3443	.3101	.1061	.1238	.4318	.2892	.2553	-.0906	1.0000	
THORN	-.1558	.1393	-.0784	-.1121	-.0116	-.3356	-.0471	-.3090	.2547	-.3766	.0895	1.0000
STEMNO.	-.1298	.1109	-.2766	.2034	.1388	-.5398	.1765	.0315	.1848	-.2152	-.0895	.2369
VIGOR	-.1855	.1775	-.4203	.4911	.2424	.2585	.5075	.5750	.1477	-.0284	.3372	-.0990
LEAFSPOT	-.2969	-.1773	.0301	.3087	.3592	-.3051	.5096	.0155	.0367	.0188	.4859	.1739
TUBERNO	.1909	.2261	-.3895	-.0578	-.1840	-.3408	.0234	-.2257	-.0993	-.4431	-.1883	.1836
TUBSHAPE	.5436	-.1057	.2256	-.3819	-.3623	.4583	-.3340	-.1728	-.0880	.0376	-.1442	-.2437
TUSMOOTH	.2908	.0152	-.3812	-.1440	-.2160	.3967	-.1883	.1514	.2131	.1845	.4352	.2892
HARV.FW	-.1263	.0615	-.2934	.4160	.1672	.2538	.4287	.4774	.1260	-.0932	.1545	-.2086
S.LENDIA	.2579	-.2220	.1097	-.3440	-.0481	.0868	-.0290	-.0252	.1139	.1807	.1131	-.4216
NDW%	.2930	-.0703	-.2421	.0437	.0074	.3743	.1830	.2765	.1676	.0450	.6379	.1106
TUBERFW	-.1082	.0934	-.1927	.4649	.1234	.4499	.3450	.7291	.1720	.1879	.4162	-.2624
DM%	-.3906	.3457	-.1433	.2068	.4329	-.3947	.1278	-.0916	.1456	-.0659	-.2318	.2657
NFW%	-.0028	.1516	-.2996	.1776	.3087	.0887	.2797	.1893	.2284	-.0168	.4336	.2358
TOTALNT	-.1007	.1488	-.2635	.4771	.1775	.4517	.3881	.7080	.1695	.1302	.4940	-.1572
TUBERDW	-.2260	.1758	-.2267	.5301	.2297	.3841	.3828	.7124	.1658	.1546	.3556	-.1987
N.HILLWT	-.1093	.1291	-.3932	.4741	.2012	.3136	.4907	.5490	.1344	-.0912	.3245	-.1306

	STEMNO.	VIGOR	LEAFSPOT	TUBERNO	TUBSHAPE	TUSMOOTH	HARV.FW	S.LENDIA	NDW%	TUBERFW	DM%	NFW%
STEMNO.	1.0000											
VIGOR	.2078	1.0000										
LEAFSPOT	.1870	-.0404	1.0000									
TUBERNO	.6946	.1574	-.1044	1.0000								
TUBSHAPE	-.4480	-.2228	-.4174	-.0938	1.0000							
TUSMOOTH	-.1714	.1848	-.0263	-.0852	.1782	1.0000						
HARV.FW	.1703	.8715	-.3594	.2076	.0296	.0378	1.0000					
S.LENDIA	-.2751	-.2391	.0437	-.1234	.2354	.0990	-.1956	1.0000				
NDW%	.0554	.3930	.3816	-.0211	.0188	.6230	.1370	.2776	1.0000			
TUBERFW	-.1955	.7588	-.0757	-.3565	-.0082	.1785	.7120	-.1486	.3439	1.0000		
DM%	.0882	.0331	.2159	.0496	-.4600	-.2504	-.1286	-.1184	-.2493	-.1524	1.0000	
NFW%	.1121	.3768	.5305	.0057	-.2816	.3978	.0199	.2032	.7681	.2052	.4196	1.0000
TOTALNT	-.1426	.7929	.0703	-.3171	-.0894	.2674	.6432	-.1307	.5092	.9577	-.0093	.4595
TUBERDW	-.1628	.7842	-.0411	-.3500	-.1444	.1290	.6844	-.2141	.2758	.9647	.0844	.3014
N.HILLWT	.1707	.9560	-.1645	.1705	-.0603	.1745	.9435	-.1891	.3649	.7818	-.0117	.3147

	TOTALNT	TUBERDW	N.HILLWT
TOTALNT	1.0000		
TUBERDW	.9648	1.0000	
N.HILLWT	.7984	.7898	1.0000

Appendix 10(e)

Correlations between characters evaluated for plants of Population 4 in Location 4

CORRELATION MATRIX <4> LOC.:4

N= 17 DF= 15 R@ .0500= .4821 R@ .0100= .6055

VARIABLE												
EM.DATE	1.0000											
FL.DATE	-.2814	1.0000										
SEX	-.3676	.0759	1.0000									
HEIGHT	-.5724	-.1681	.6220	1.0000								
BRANCHNO	-.5118	.1617	.4831	.6004	1.0000							
HTIBRA	-.0100	-.4115	.0798	.3947	.0594	1.0000						
LEAFNO	-.4023	.0435	.4372	.5589	.5177	-.0604	1.0000					
LEAFSIZE	-.0244	.2615	-.1311	.0314	-.4272	.2870	.0220	1.0000				
VIRUS	.0440	.2227	-.1133	-.0567	.1318	.2048	.2988	.3873	1.0000			
STEMCOL	.0303	-.3431	-.3393	-.1081	-.2949	.4861	-.1440	.0148	-.0268	1.0000		
STEMDIAM	.0400	-.2892	.2331	.4108	.3462	.2982	.4085	.1304	.1650	-.2083	1.0000	
THORN	-.3988	.1416	-.1162	.1066	.1821	.4286	-.1070	.0297	.2506	.4170	-.2025	1.0000
STEMNO.	.1657	.5471	-.2946	-.3038	.0142	-.5989	.1049	-.0763	-.0752	-.1863	-.2837	-.2020
VIGOR	-.0320	-.0189	.1288	.3280	.3487	.2278	.3873	.2988	.5092	-.2665	.4364	.2403
LEAFSPOT	-.2340	-.0780	.0295	.2027	.0628	.0653	.4653	.1846	.2066	.0957	.2697	.1195
TUBERNO	.3110	.2826	-.2024	-.0510	.0925	-.3300	.1304	-.0362	.0655	-.2848	.1157	-.1231
TUBSHAPE	.0698	-.1535	.3377	.1358	.2089	.1422	.0100	-.4929	-.2521	.2362	-.3447	-.0632
TUSMOOTH	.1895	-.0149	-.1862	-.3570	-.4848	-.1588	-.4364	.1212	-.2191	.0807	.0516	.0784
HARV.FW	.1701	.1425	.0851	.0552	.2686	.3350	.0574	.1293	.4847	.1686	.0478	.2131
S.LENDIA	-.1952	.0241	.3565	.0028	.0597	.0550	.1141	-.2839	-.2561	.3197	-.0977	.1790
NDW%	-.1624	-.0897	.1043	.2247	.1970	.0711	.4436	-.0404	-.1856	.0248	.2783	.0483
TUBERFW	-.3106	.0550	.2272	.1892	.3364	.3747	.0428	.0467	.3064	.2453	.0362	.1241
DM%	.1085	-.3578	.0230	-.2207	-.1756	.1991	-.3261	-.3151	-.2117	.1366	-.2241	.0890
NFW%	-.0810	-.2288	.0873	.1054	.0918	.1259	.2582	-.1627	-.2439	.0856	.1507	.0517
TOTALNT	-.3863	-.0464	.2503	.2731	.3496	.5350	.1871	.0338	.2195	.4094	.1142	.2233
TUBERDW	-.3151	.0100	.2402	.1838	.3232	.4277	.0244	.0276	.2962	.2780	.0305	.1539
N.HILLWT	.1178	.0349	.1248	.1320	.3243	.3988	.3026	.1055	.3774	.1994	.2322	.2020

	EM.DATE	FL.DATE	SEX	HEIGHT	BRANCHNO	HTIBRA	LEAFNO	LEAFSIZE	VIRUS	STEMCOL	STEMDIAM	THORN

STEMNO.	1.0000											
VIGOR	-.0580	1.0000										
LEAFSPOT	.0658	.2428	1.0000									
TUBERNO	.5951	.2222	-.3225	1.0000								
TUBSHAPE	.1265	-.3268	-.1202	-.2036	1.0000							
TUSMOOTH	-.2404	-.1690	.0435	-.0956	-.5708	1.0000						
HARV.FW	.1467	.5190	-.0311	.0480	.3272	-.4132	1.0000					
S.LENDIA	-.0069	-.3423	.4772	-.4193	.3838	.1085	-.0868	1.0000				
NDW%	.2200	.1380	.5254	.1325	.0284	-.2064	-.2259	.4785	1.0000			
TUBERFW	-.3361	-.0155	.1461	-.6727	.2857	-.2173	.4795	.1772	-.3528	1.0000		
DM%	-.2363	-.3484	.0047	-.3722	.4116	-.1760	-.1761	.4676	.2844	-.0687	1.0000	
NFW%	.1036	-.0255	.4640	-.0395	.1898	-.2312	-.2578	.5802	.9384	-.3098	.5940	1.0000
TOTALNT	-.3272	-.0917	.3529	-.6805	.3793	-.3329	.3308	.4754	.1196	.8558	.2106	.1843
TUBERDW	-.3779	-.0481	.1518	-.7126	.3321	-.2548	.4636	.2407	-.2960	.9901	.0607	-.2177
N.HILLWT	.2226	.5323	.2399	.1094	.3408	-.5429	.7986	.2036	.3854	.2347	.0928	.3486

	STEMNO.	VIGOR	LEAFSPOT	TUBERNO	TUBSHAPE	TUSMOOTH	HARV.FW	S.LENDIA	NDW%	TUBERFW	DM%	NFW%

TOTALNT	1.0000		
TUBERDW	.8977	1.0000	
N.HILLWT	.4027	.2683	1.0000

	TOTALNT	TUBERDW	N.HILLWT

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ABBREVIATIONS

The abbreviations used in this thesis are those recommended in the "Policy of the Journal and Instructions to Authors", Biochem. J. (1978) 169, 1-27, with the following additions:

bis	N,N'-methylene-bis-acrylamide
BSA	bovine serum albumin
con-A	concanavalin-A
DIECA	diethyldithiocarbamate
DTT	dithiothreitol
GHCl	guanidine hydrochloride
IEF	isoelectric focussing
2-ME	2-mercaptoethanol
PAG(E)	polyacrylamide gel (electrophoresis)
PAS	periodic acid-Schiff
PVP	polyvinylpyrrolidone
TCA	trichloroacetic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	tris (hydroxymethyl) aminomethane
% T	total acrylamide gel concentration (acrylamide plus bis) (g/100 ml)
% C	crosslinking agent (g/100 ml) x 100/%T

Abbreviations used in appendices 1-10 are listed in table 7. Abbreviations used in the figures are given in the corresponding legends.

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