PROCESSING OF MICROBIAL PROTEIN FOR FOOD USE.

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SUMMARY.

Processes were investigated for the production of protein fibres from Bakers' yeast, suitable for food use.

Cell disruption by sonication followed by protein extraction at pH 11 and precipitation at pH 3.8 resulted in high protein yield (70% for 100% disruption) but an unacceptably high nucleic acid content (13%w/w DM) in the isolate. Nucleic acid removal was attempted, the main emphasis being on endogenous nuclease methods.

Heat shock (70°C, 6 secs) followed by incubation (55°C, 2 hours, 3%w/v NaCl) of 10%w/v DM fresh whole yeast suspensions resulted in a 95% reduction in the nucleic acid content. However, when this process was followed by disruption, alkaline extraction and acid precipitiation the protein yield in the isolate was only 12% of that without heat treatment.

Incubation of 10%$^{w}/_{v}$ disrupted yeast suspensions at 50°C, 30 mins with 3%w/v NaCl led to an isolate with a protein to nucleic acid ratio (P/NA) of 14, but a protein yield only 37% of that without incubation.

Incubation at 50°C, 2 hours, pH 6 with 3%w/v NaCl, of the alkaline extracted material followed by acid precipitation resulted in an isolate with a P/NA of 25, without loss of yield. However the isolate was rather insoluble and did not yield good fibres on spinning.

Isolates produced by alkaline extraction and acid precipitation

from disrupted yeast suspensions were mixed with 3 N NaOH to give dopes which were spun through capillaries into an acid / salt coagulating bath. The strongest fibres were obtained using dopes of 20%w/w DM at pH 10.0. Spinning did not in itself lead to loss of nucleic acid.

Incubation of spun fibres (50°C, 2 hours, pH 6.0, 3%w/v NaCl) with the supernatant fluid separated following acid precipitation gave a product with a P/NA greater than 30. There was no loss in yield and fibre strength was not impaired.

By drying these fibres at room temperature and rehydrating in boiling water a texture similar to that of meat was obtained.

Dedication.

To Annie and Lizzy and to my Mum and Dad, for whom I thank God.

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CONTENTS.

1. INTRODUCTION.

The majority of the world's population today suffers from a shortage of dietary protein (Bender et al, 1970; Kapiotis, 1976). Over 300 million children are grossly retarded in their physical growth and mental development primarily owing to protein malnutrition (G. de Pontanel, 1972; Wickman, 1970). Arguments as to whether this is caused by a shortage of food in general rather than protein in particular do not mask the need for a greater effort to produce more high quality protein foodstuffs (Vogt, 1970). Despite technical advances, traditional methods of agriculture are increasingly unable to meet the needs of the rapidly growing populations of the poorer countries (Goldblith, 1970). Shortage of protein for animal feeding and for human food has led to dramatic rises in the prices of these commodities (Hammonds, 1975). Clearly cheap alternative sources of protein need to be developed. Nevertheless, the solution to the problems of worldwide food shortage is very complex and it is by no means certain that if new food supplies do become available in large quantities, they will reach those who are starving. At present over two thirds of the world's high quality protein supplies are consumed by only one third of the world's population; and there is no evidence to suggest that there is the necessary political will significantly to alter this imbalance (Levinson & Austin, 1975; Payne, 1974; Senez, 1972).

Despite this, considerable scientific and technical effort is being made to develop new sources of protein to supplement the more traditional feed and food products. These novel foods include oilseed protein, legumes, fishmeal, fortified cereals, leaf proteins, proteins recovered from various waste materials and the so called

'single cell protein' products with which this report is concerned (Altschul, 1974; Bender et al, 1970; Bieber, 1969; Birch et al, 1976; Pirie, 1975 (1 & 2); Tannenbaum and Wang, 1975).

Single cell protein is used to describe protein derived from various classes of microorganisms. Yeasts, bacteria, moulds and algae have been successfully grown on a variety of substrates. There are several characteristics of microorganisms which make them particularly attractive as a source of protein.

1. Microorganisms have a much more rapid growth rate than conventional agricultural products. For example, a 500 kg bullock can synthesize 0.4 kg protein/day. The same weight of soya can produce 40 kg protein/day. 500 kg of yeast, however, can produce over 50 metric tons of protein per day (Bunker, 1963).

2. Microorganisms which have been considered for protein production have a crude protein content of 40 - 80%w/w (N x 6.25) on a dry weight basis. This is higher than most plants and similar to some meat products, even when the 'non-protein' nitrogen has been subtracted (Lipinsky & Litchfield, 1974; Senez, 1972; Wang, 1968; Worgan, 1974).

3. Different microorganisms can synthesize protein from a very wide range of non-protein substrates, many of which are waste materials (e.g. Imrie, 1975).

4. Single cell protein (SCP) installations are not highly vulnerable to adverse weather conditions - a factor which has become increasingly important with climactic changes over recent years (Senez, 1972).

5. A SCP plant requires relatively small land area and may be built on a site unsuitable for agricultural use (Kihlberg, 1972; Process News, 1975).

At present the SCP which is being produced is almost entirely for animal feeding, being used to replace fishmeal, soyameal or milk powder in the animals' diet. Despite the increasing demand for animal protein, meat production is very inefficient. For example, four pounds of feed protein are required to produce one pound of meat protein from poultry (Senez, 1972). If SCP could be utilized directly as a human food, the protein utilization would be far more efficient. However, there are severe social and economic problems which prevent the manufacturers of SCP from advancing into the field of human foodstuffs. Products must first be subjected to rigorous and costly toxicological and nutritional testing. Moreover, the products must be presented in a form which is acceptable to the consumer. This would probably involve some form of texturing to simulate existing products, since it has been shown that there is great prejudice against unfamiliar forms of food (Goldblith, 1970; Yudkin, 1972). Such processing may lead, however, to the loss of the cost advantage of SCP.

Though the technology of SCP manufacture for animal feeding is well established there is, as yet, little information in the literature on the production of palatable protein products from microorganisms. The efforts that have been made have followed techniques used for textured vegetable protein (TVP) manufacture from soyameal and other oilseed meals. TVP products simulating meat and meat products have enjoyed considerable success in recent years, especially in the U.S.A. Whilst it is realised that such foodstuffs would be aimed initially at western markets, it is generally believed that in the long term a significant increase in the total world's food supply must be of benefit to the Third World. (Watson, 1973).

Clearly there are many advantages in producing food protein from

microorganisms as opposed to conventional agricultural methods. However much research is needed in the area of processing microbial protein to produce palatable and nutritious foodstuffs for human consumption. It is with this aspect of SCP manufacture that the present work is concerned.

2. SINGLE CELL PROTEIN - THE STATE OF THE ART.

2.1. The Current State of the Industrial Art.

2.1.1. Introduction.

Having stated briefly some of the reasons which make SCP production attractive, it is now worth looking at how far commercial interest has developed. It must be clearly stated here that the present concern is with the production of micro-organisms for use as protein rich feeds and foods. There are many examples of microbial products used for their specific functional, nutritional or flavouring properties (e.g. the use of yeasts in bread making or as yeast extract) but these are not considered here.

A recent article by Moo-Young (1976) lists 61 active or potential SCP plants (Table 2.1.1.). Of these 42 employ yeast as the microorganism. Six of the seven plants with capacities in excess of 50,000 tons/year use carbon substrates derived from oil - by far the most common being n-paraffins. In no case is the currently produced SCP material used commercially as a major protein replacer in human foods. Most of it is fed to livestock in place of soyameal, fishmeal or milk powder.

The following sections look more closely at the different types of processes in order to assess their relative merits.

2.1.2. Choice of Microorganism.

Bacteria, yeasts, moulds and algae have all been considered as

Table 2.1.1. Summary of Global SCP Production Facilities.

organization	substrate	organism	potential capacity by 1980 (tpa)
Western Europe			
BP, UK	paraffins	yeast	4,000
BP, UK	paraffins	yeast	100,000
BP, France	gas oil	yeast + bacteria	25,000
BP, Sardinia	paraffins	yeast	100,000
ICI, UK	methanol	bacteria	100,000
Shell, UK	methane	bacteria	1,000
RHM-DuPont, UK	carbohydrate	fungi	10,000
Tate & Lyle, UK	carob	fungi	4,000
Tate & Lyle, Belize	carob	fungi	pilot
Tate & Lyle + FAO, Cyprus		fungi	pilot
Liquichimica, Italy	paraffins	yeast	100,000
Nestlé, Switz.	paraffins, ethanol	yeast, bacteria	pilot
Biol. Carbon Res. W. Germany	carbon dioxide	algae	pilot
Pulp & Paper Co., Finland	sulphite liq	yeast	10,000
Sugar Co., Sweden	potato starch	yeast	10,000
Eastern Europe			
Kojetin, Czechoslovakia	paraffins	yeast	100,000
Chemopetrol, Czechoslovakia	ethanol	yeast	100,000
Chem. Ind. Ministry Romania	paraffins	yeast	60,000
USSR	nat. gas		commercial

Table 2.1.1. Summary of Global SCP Production Facilities (continued).

organization	substrate	organism	potential capacity by 1980 (tpa)
USSR	paraffins	yeast	200,000
USSR	sulphite liq	yeast	19,000
USSR	wood pulp	yeast	1,000,000
USA & Canada			
US Army	cellulose	bacteria	10,000
Amoco Foods Inc.	ethanol	yeast	10,000
RHM-DuPont, USA	carbohydrate	fungi	
Betchel-LSU, USA	cellulose	yeast, bacteria	
Milbrew, USA	whey	yeast	10,000
Anhauser-Busch, USA	molasses	yeast	2,000
Boise Cascade, USA	sulphite liq	yeast	10,000
Esso-Nestle,USA	ethanol	bacteria	
Gulf, USA	paraffins	yeast	pilot
General Electric, USA	manure	bacteria	pilot
Ontario Paper, Canada	sulphite liq	yeast	pilot
Silverwood Ind., Canada	whey	yeast	pilot
Central & South America			
Liquichimica, Brazil	paraffins	yeast	
ICAITI, El Salvador	coffee waste	fungi	pilot
Kojin, Cuba	molasses	yeast	large
IPF-UNIDO, Mexico	carbon dioxide	algae	pilot
Kyowa Hakko, Mexico	molasses	yeast	20,000

Table 2.1.1. Summary of Global SCP Production Facilities (continued).

organization	substrate	organism	potential capacity by 1980 (tpa)
Middle East			
Iran	methanol, paraffins	yeast	
Israel	methanol	yeast	pilot
Far East (excluding Japan)			
Chinese Petrol Co., Taiwan	paraffins	yeast	100,000
Chi-Yee, Taiwan	petroleum fractions	yeast, bacteria	9,000
Lemigas, Indonesia	paraffins		
N. Korea	gas oil		
Philippines	coconut	yeast	pilot
Rep. of China	paraffins	yeast	pilot
Japan			
Mitsubishi Gas	methanol	yeast	operations suspended by Japanese Government.
Mitsubishi Pet.	ethanol	yeast	
Indemitsu	ethanol		
Kanagafuchi & Takeda	ethanol, acetic acid	yeast	
Dainippon Ink	alcohols	yeast	
Kyowa Hakko Kogyo	methanol	yeast	
Mitsui Toatsu	alcohols	yeast	
Yamea Soy	acetic acid	yeast	
Aiinomoto	acetic acid	yeast	
Kojin	acetic acid	yeast	
Japan Pulp	sulphite liq	yeast	
Jujo Pulp & Paper	sulphite liq	yeast	
Osaka City	carbon dioxide	Chlorella	20,000

Compiled from: Moo Young,1976(primary ref.); Fineberg,1968; Imrie, 1975; Lipinsky & Litchfield,1974; Sherwood,1974; UN,1973(4).

protein sources.

2.1.2.1. Bacteria.

These have a very high protein content (up to 87%w/w crude protein - Worgan, 1974) and a rapid growth rate. However, due to their small size (1 - 5μm) and low density they are difficult to recover from the fermentation broth (Labuza, 1975).

2.1.2.2. Yeasts.

Yeasts generally contain more than 50%w/w crude protein and grow more slowly than bacteria (Peppler, 1967; Worgan, 1974). The cost of separation by centrifuging would be about one quarter of that for bacteria due to the larger size and higher density of the yeast cells (size 10 - 25μm) (Labuza, 1975). An added advantage of yeasts is their established use in a number of food products such as bread and yeast extract and their use in brewing. On the other hand bacteria are generally connected with infection and disease and are therefore unattractive to the consumer (Mateles and Tannenbaum, 1968).

2.1.2.3. Moulds.

Although the growth rate for moulds is less than that for yeasts and algae (Tannenbaum & Mateles, 1968) fungal mycellium is very easy to recover from the fermentation media. Separation costs could be ten to fifteen times lower than those for bacteria (Spicer, 1973).

2.1.2.4. Algae.

The major advantage of algae is their ability to photosynthesize carbon dioxide from the atmosphere as their sole carbon source. Surface culture on shallow lagoons of mineral salts has been

extensively studied (Bunker, 1963; Enebo, 1969; Oswald, 1969). Spirulina maxima, which can be grown at pH 9.5 - 10.0, thereby increasing carbon dioxide absorption efficiency and reducing contamination, yields 40 - 45 tons/hectare/year. The product can be easily dewatered and contains about 60%w/w crude protein. Its use seems limited by the large land area and warm climate which is required (Worgan, 1974). This blue-green algae has been traditionally eaten in Mexico and around Lake Chad in Africa. It is perhaps worth noting, however, that in Africa it was only eaten when alternative foods could not be obtained (United Nations, 1972 (1); 1973(5)).

Chlorella algae have also been grown in Japan using acetic acid or glucose as the carbon substrates. Artificial illumination is used when glucose is the substrate. These products, used mainly as flavouring for soy sauce, natto, soybean curd, rice-cake, fish paste, noodles, bread, sausages and whiskey sell for up to $30/kg. Hence they are of little current importance in the field of SCP production for use as high protein foods (Enebo, 1969; Tamiya, 1975).

2.1.3. Carbon Substrates.

The choice of carbon substrate is of great importance to the manufacture of SCP. Obviously substrate cost is important. Protein yield will also be important. Purity of the substrate will affect the cost of the recovery processes, since any toxic substances in the medium would have to be removed. The availability and estimated costs of various carbon substrates is shown in Table 2.1.2. They are split into two main groups, the first being carbohydrates ranging from the easily utilised mono saccharides to the cellulose and other highly polymerised carbohydrates which

Table 2.1.2. Potential World Production of SCP from Various Substrates.

substrate	approximate cost (p/lb, 1972)	amount potentially available for SCP prodn.(1000's tpa.)	possible protein prodn.(1000's tpa.)
carbohydrates:			
wheat straw	<0.2	300,000	60,000
wheat bran	<0.2	60,000	12,000
maize stalks	<0.2	120,000	24,000
maize cobs	<0.2	30,000	6,000
barley straw	<0.2	50,000	10,000
bagasse	<0.2	80,000	16,000
molasses	1.0	10,000	2,500
sulphite liq.	0.8	10,000	2,500
whey	<0.2	2,000	500
citrus pulp	<0.2	25,000	2,500
cattle manure	<0	100,000	
poultry manure	<0	2,000	
carbon dioxide	0		
hydrocarbons:			
petroleum fractions	0.8	100,000	50,000
natural gas	0.1	25,000	9,000
ethanol	1.2	80,000	30,000
methanol	0.6	45,000	9,000

Compiled from: Lipinsky & Litchfield, 1974; Senez,1972; United Nations,1973(4); Worgan,1974.

are not readily susceptible to microbial attack. The second group includes hydrocarbons ranging from methane to petroleum fractions. Their relative merits are discussed below.

2.1.3.1. Carbohydrates.

Molasses has been used for many years as the carbon substrate for Bakers' Yeast production (Harrison, 1967; Holloway, 1976; Peppler, 1967). Candida utilis and other organisms can be grown on spent sulphite liquors from the paper pulp industry to yield a feed or food grade SCP product (e.g. Romantschuk, 1975). S. Fragilis has been successfully grown on crude lactose obtained from cheese whey by ultrafiltration (Pace and Goldstein, 1975). Rank, Hovis, McDougall have developed a process for fungal fermentation of starch waste from field beans (Spicer, 1973). Tate and Lyle's process for growing moulds on vegetable waste is apparently suitable for small scale use in the underdeveloped countries (Imrie, 1975; United Nations, 1973 (1 & 4)). The Swedish 'Symba' process uses a mixed culture of yeasts grown on starch (Worgan, 1974). Straws - containing cellulose and hemi-cellulose material - are first hydrolysed by acid (Worgan, 1974) or alkali (United Nations, 1973 (4)) treatment prior to use as SCP substrates. Animal manures have also been considered (Brown D.E., 1975). All these substrates are essentially waste materials. Some would have a negative cost due to their potential as pollutants. As governments become increasingly aware of the need to control pollution by stricter legislation, the use of these materials as SCP substrates will become increasingly important - solving both an effluent treatment problem and a food shortage problem at the same time (Humphrey, 1975; Kihlberg, 1972; Senez, 1972).

2.1.3.2. Hydrocarbons.

Commercial interest has been largely centred on the use of hydrocarbons as SCP substrates (Fineberg, 1968), the major ones being:

1. Methane. Due to the small number of microorganisms which will grow on methane, it is possible to run non-aseptic fermentations (Tannenbaum & Mateles, 1968). This is the cheapest of the hydrocarbon substrates as in many oil producing areas it would simply be burnt off (Lipinsky & Litchfield, 1974). There is some risk of explosion however, due to the mixtures of methane with air during fermentation (Wargon, 1974).

2. Alcohols. Methanol, produced synthetically from methane has high potential as a substrate. It is miscible with water thus reducing mass transfer problems associated with other oil based substrates (Lipinsky & Litchfield, 1974; Pont, 1974; Wargon, 1974).

3. Gas oil. B.P. has developed a non-aseptic process for growing yeast on gas oil. Only the paraffins are utilized so the process includes dewaxing of the oil as well as SCP production (Shacklady, 1973; Sherwood, 1974). However the need to remove all traces of toxic substances (particularly aromatic carcinogenic compounds such as 3,4 benzpyrene) makes recovery of the yeast complex and expensive (Labuza, 1975). The B.P. plant at Lavera has now been shut down for conversion for n-paraffin substrates.

4. Purified n-paraffins. Aseptic culture of yeasts on n-paraffins has been favoured by many industrial concerns. Cell recovery is much easier as exhaustive extraction is unnecessary. B.P. has a 100,000 t.p.a. plant now commissioned in Sardinia (Laine et al, 1976).

Hydrocarbon processes usually involve sophisticated technology,

and need to be run on a large scale (about 100,000 t.p.a.) to be economically viable (Imrie, 1975). Plants are generally situated at or near an oil refinery close to an adequate supply of cooling water.

For the production of SCP from a hydrocarbon substrate the feedstock costs would be about a half of the total production cost. The selling price of the product would therefore, in general, be linked to the price of oil (Lipinsky & Litchfield, 1974).

2.1.3.3. Comparison of substrates.

Unlike hydrocarbons, carbohydrate substrates for SCP production are from renewable carbon sources. Fermentation and recovery technology is relatively straightforward and in some cases lends itself to small scale applications (Imrie, 1975).

Research on hydrocarbon substrates has powerful financial backing from the oil companies who see not just a profitable means of utilizing the less desirable petroleum fractions but also a useful public relations exercise as they present an image of 'seeking to resolve the world's food problems.'(Ebban, 1976).

The higher yields obtainable with hydrocarbons leads to higher oxygen demand so that the heat produced during fermentation may be two to three times greater than for carbohydrate substrates (Tannenbaum & Mateles, 1968). Carbon dioxide is only used for algae growth and has been adequately discussed in section 2.1.2.4.

2.1.4. Other Nutrients.

Other nutrients, in particular nitrogen and phosphorous and including

trace elements and growth factors are mostly supplied as water soluble, inorganic compounds. Where the value of the carbon substrate is high (e.g. n-paraffins) the cost of these nutrients is relatively small. Where a low value carbon substrate is used (e.g. carbon dioxide or manures) the cost of these nutrients may become the limiting economic factor (Tannenbaum & Mateles, 1968).

2.1.5. Scale of Operation.

A very important factor in determining SCP costs is the scale of operation. Humphrey (1975) estimates the selling price for yeast grown on n-paraffins as 17, 8.5 and 6.0 p/lb protein for plants of 10,000, 100,000 and 400,000 t.p.a. capacity. Size is limited by the supply of raw materials and at present the largest oil refinery could supply n-paraffins for a 400,000 t.p.a. yeast plant.

Scale of operation might not be so critical for processes which are less capital intensive than those using hydrocarbon substrates. For example the growth of moulds on vegetable wastes may be economically viable even at a village scale in the developing world (Imrie, 1975).

2.1.6. Product Recovery.

Wang (1969) and Labuza (1975) have written comprehensive reviews of the recovery of cells from the fermentation broth and their subsequent drying to produce a free flowing, odourless, stable powder. As has been mentioned previously, recovery from carbohydrate broths is straightforward. Hydrocarbon substrates present

problems of phase separation and, more particularly, removal of traces of toxic materials when crude oil fractions are used.

Initial dewatering (1 - 2% solids to 20 - 40% solids) of the broths is a function of cell size and density, so that bacteria are far more difficult to separate than yeasts, which are more difficult than filamentous fungi. Centrifuging is generally preferred on the grounds of cost. Other methods include filtration, flash evaporation, flotation and flocculation (Ganser & Wang, 1970). It may be possible by genetic manipulation to increase the size of microbial cells, making recovery easier (Thaysen & Morris, 1943; Wang, 1969).

Drum or spray drying are the preferred methods - spray drying giving the highest quality product, whilst drum drying is generally slightly cheaper. Some degree of pasteurization is achieved during the drying process (Labuza et al, 1970 (1 & 2); 1972). Unfortunately drying resulted in substantial loss of protein solubility, even after disintegration of the cells. Dried yeast protein, when used as a replacer for milk solids in bread making yielded loaves of a very poor quality. From these results it would appear that direct use of fresh SCP material to produce textured products would be a better appraach if the materials were to be used in foods, since the functional properties of the dried yeasts were very poor (Labuza et al, 1972; Labuza & Jones, 1973).

2.1.7. Marketing Considerations.

The economics of SCP processes are difficult to assess due largely to a lack of consistent data on production costs, as shown in

Table2.13.. One important point which does emerge however, is the dramatic increases in the prices of soyameal and fishmeal in 1972/73. By the late 1960's it appeared that SCP would not be able to compete in the animal feed market, but since 1972 there has been a resurgence of interest in SCP as fodder prices have soared (Hammonds, 1975; Lipinsky & Litchfield, 1974; Process News, 1975).

It is impossible, as yet, to assess the commercial potential of SCP products for human food use, since no such foods are currently on the market. Assuming that the development of SCP foods will follow a similar course to that of the TVP products, several important factors emerge. Firstly, nutritional properties alone will be insufficient to sell the product and, as Professor Spicer has rightly said, "The biological value of food that is not eaten is nil." (Spicer, 1973). It must first be processed into a palatable food. To achieve this some form of texturization will probably be necessary and this will be dependant upon functional properties, such as solubility, 'gelability', 'dispersability' etc. (Shelef & Morton, 1976; Tannenbaum, 1972). Secondly, it is difficult to persuade consumers to buy a completely new product, particularly when it is aimed at replacing meat which is so highly valued as a food. Hence the economics of marketing an SCP product for human consumption become very complex. Processing may lead to loss of cost advantage. If it does not the product must be priced very carefully. Too low a price suggests a low quality product which would be undesirable as a meat replacer. Too high a price will mean that the original meat products will be preferred. Textured soya foods are in fact priced just below the prices of the equivalent meat products (Goldblith, 1970; Yudkin 1975.)

Table 2.1.3. Cost Estimates for SCP, Soyameal and Fishmeal.

product	selling price (p/lb protein)							
	date of estimate							
	1967	1967	1968	1968	1972	1972	1973	1974
soyameal	4.3	4.3	5.0	4.3	5.2		12	12
fishmeal					13.2		12.5	18
Torula yeast:								
food				13				
feed		6		3.6				
yeast, molasses					24.5			9.5
bacteria, methanol					11			
algae, Chlor.	6	22						
algae, Scene.	2	22				10		
algae, Spirul.	9	7			1	17		
micro-fungi						4		
ref.	Abbott (69)	Enebo (70)	Wang (68)	Humph. (69)	Senez (72)	U.N. (72,2)	Humph. (75)	Lip. & Litch. (74)

There is considerable optimism about the impact of SCP on western markets. It is unlikely, however, that problems of food shortage in the Third World will be significantly affected by SCP for many years to come (Humphrey, 1975; United Nations, 1970 (1); 1973 (1)).

2.1.8. Overall Conclusions.

As has been already stated, the growth of yeast on n-paraffins has received by far the largest commercial attention. Apart from the role of yeast as a traditional food it is considerably cheaper to recover from the fermentation broth than bacteria and has a higher growth rate than moulds. Oil companies have been able to spend considerable sums of money on developing SCP processes using petroleum fractions of relatively low value. Purified n-paraffins have been preferred as they provide a well defined substrate free of toxic, aromatic compounds. The production of SCP from methane which is still burned off in many of the world's oilfields, is obviously attractive, but this is technically a more difficult process.

As pollution controls become more stringent, it is likely that SCP production from waste carbohydrates will increase in importance, since the substrate may have a negative cost which would make the process economically viable. There are problems in this area however, as many of the substrates are ill defined. Quality control will be of particular importance when SCP is used in human foods.

As already stated the technology for SCP production for animal feeding is well developed. It is the problems of SCP production in a form suitable for human consumption which must now be considered.

As shown in section 2.1.7. this may involve not just further processing on the feed product, but a revision of some processes used to make animal feeds - particularly where these lead to loss of desirable nutritional & funcional properties.

Before going on to assess the types of processing which may be required to produce attractive food products, the nutritional and toxicological properties of SCP products must be examined. Any new food product must be shown to be completely safe before being marketed. The cost of conducting such investigations is extremely high and this may explain, to some extent at least, the reluctance of SCP manufacturers to enter the human food market.

2.2. Nutritional and Toxicological Considerations.

2.2.1. Testing Procedures.

The United Nations Protein Advisory Group (PAG) has recommended procedures for the evaluation of new protein foods. PAG guideline no.6 (United Nations, 1969) suggests chemical analysis for the protein amino acid composition, nitrogen,nucleic acid, lipid, mineral, vitamin and contaminant contents, followed by biochemical digestability tests *in vitro* to determine the degree of hydrolysis with pepsin and trypsin. Products are examined for viable micro-organisms, both pathogenic and non-pathogenic. This is followed by safety evaluations on rats and other animals over a number of generations. Protein quality studies are carried out in young mammals to indicate the value of the protein product for promoting growth (Protein Efficiency Ratio) and nitrogen retention (Net Protein Utilization) when fed both as the sole source of protein

and as a supplement to other foods. PAG guidelines nos. 7 and 12 deal with testing of such products for human consumption (United Nations 1970 (2); 1972 (3)). After successful completion of the above programme acceptablity and tolerance tests are carried out in healthy human subjects followed by growth tests and nitrogen balance measurements. Special tests may be carried out according to the intended use of the product (e.g. PAG guideline no.8 on 'Protein Rich Mixtures for Use as Weaning Foods' - United Nations, 1971). Although these guidelines do not carry the force of law, they have received general acceptance (Oser, 1975).

2.2.2. Chemical and Biochemical Analyses.

Table2.2.1.shows the composition of a wide range of SCP and conventional feed and food products. Generally speaking SCP products have a high crude protein content but also a high nucleic acid content compared with conventional products. Crude protein is calculated as nitrogen content x 6.25. This considerably overestimates the true protein content of most microorganisms, mainly due to the nitrogen content of the nucleic acid. This may be corrected (United Nations, 1973(5)) but it is still not always clear in the literature just what 'protein content' refers to. In all cases the sulphur containing amino acids, methionine and cystine, are below the recommended FAO levels. Apart from their protein value SCP products may be useful sources of vitamins and minerals - especially the 'B' vitamins (Bunker, 1963; Scrimshaw, 1972; 1975; United Nations, 1972 (1)).

There has been concern over the content of carcinogenic aromatic hydrocarbons in SCP products grown on n-paraffins and gas oil. Although levels of, for example, 3,4 benzpyrene were reduced to

Table 2.2.1. Chemical Analysis of Selected SCP and Other Feed & Food Products (mean values).

essential amino acids (g/16gN)	FAO recommended values	C. utilis - sulphite liquor	BP yeast - gas oil	BP yeast - paraffins	Inst. Francais Petrol. yeast - paraffins	Bakers yeast - molasses	Esso-Nestlé bacteria - paraffins	ICI bacteria - methanol	bacteria IGT/10 methane	M. glutamicus bacteria		Chlorella algae - CO_2		Spirulina maxima algae - CO_2	RHM fungi - carbohydrates	fishmeal	soyameal	barley	maize	milk protein	egg	beef	chicken meat
										cells	isolate	cells	isolate										
leucine	4·8	9·1	7·8	7·4	7·8	7·0	5·6	6·6	8·2	6·8	8·3	6·8	7·1	8·0	7·1	7·3	7·7	6·7	14·6	10·1	8·9	7·8	6·6
isoleucine	4·2	6·0	5·3	5·1	5·3	5·2	3·6	4·2	4·6	4·3	5·4	3·1	3·9	6·0	3·1	4·6	5·4	3·7	4·2	6·6	6·6	5·2	4·1
valine	4·2	7·3	5·8	5·9	5·8	5·9	4·5	5·1	6·6	5·8	6·8	4·8	6·8	6·5	3·4	5·2	5·0	4·8	5·7	7·4	9·3	5·1	6·7
lysine	4·2	7·1	7·8	7·4	7·8	7·1	6·5	5·7	5·9	4·0	6·0	4·9	5·4	4·6	4·6	7·0	6·5	3·4	3·0	8·1	6·4	8·6	7·5
methionine	2·2	1·6	1·6	1·8	2·5	3·0	2·0	2·4	1·5	4·0	1·8	1·4	1·0	1·4	1·6	1·0	1·4	1·2		3·5	3·2	2·7	1·8
cystine	2·0	0·4	0·9	1·1			0·6	0·6	0·3	0·1	0·3	0·8	0·6	0·4	1·1	2·6	1·4	1·1	1·7		2·4	1·3	1·8
phenylalanine	2·8	5·3	4·8	4·3	4·8	4·1	2·9	3·4	4·8	3·4	4·5	3·5	4·2	5·0	3·1	4·0	5·1	5·9	5·8	5·4	5·8	3·9	4·0
threonine	2·8	6·1	5·4	4·9	5·4	5·1	4·0	4·5	4·6	4·4	4·4	3·9	4·4	4·6	3·9	4·2	4·0	3·4	4·1	4·3	5·1	4·5	4·0
tyrosine	2·8	4·3	4·0	3·6	4·0	2·5	3·1	3·0	3·9	2·9	2·9	2·6	3·5	4·0	2·6	2·9	2·7	2·8	5·2		4·2	3·0	2·5
tryptophan	1·4	1·5	1·3	1·4	1·3	1·5	0·9	0·9	3·8	1·4	1·4	1·8	1·3	1·4	1·7	1·2	1·5		0·7	1·3	1·6	1·0	0·8
protein (Nx6·25) %			69	60		55	68	85		80		55				61	44	16	9		35	86	
nucleic acid %		7	8	8		6		16				4		4		<1	<1	<1	<1	<1	<1	<1	<1
lipid of			2	9		2	13	7		2		7										25	5
carbohydrate dm						40		3		13		29											
ash			8	6		6	9			5		9										1	1

Compiled from: Brant, 1974; Bressani, 1968; Enebo, 1970; Evans, 1968; Gow et al, 1975; Kaul, 1975; Kelly, 1974; Klass et al, 1969; Mitsuda et al, 1969; Senez, 1972; Shacklady & Gatumel, 1972; Spicer, 1973; Young & Scrimshaw, 1975.

less than 1 ppb (the limit of the sensitivity of the test), SCP operations in Japan were suspended by the government in 1973 following pressure from consumer groups (Chem. Eng. News, 1973). This seems a rather startling decision when we compare these levels to those of such food products as lettuce - 12 ppb, spinnach - 12 ppb, endive - 50 ppb, leek - 7 ppb, mollusc - 55 ppb and cod - 15 ppb; the level for bakers' yeast, grown on molasses is 13 ppb (Ferrando, 1972; Shacklady, 1975).

In vitro digestability tests using trypsin and pepsin to hydrolyse protein from Chlorella and Scenedesmus algae showed that fresh or dried algae were poorly digested whereas disrupted cells were easily digested (Hedenskog et al, 1969). B.P.'s alkane and gas oil grown yeasts had pepsin digestabilities greater than 80% (Shacklady, 1973; 1975).

Nitrogen effeciency ratios, for disrupted and undisrupted samples of bakers' yeast, Scenedesmus and Spirulina algae, measured by protein synthesis in vitro in rat skeletal muscle, again showed that the protein from the disrupted cells was much more efficiently utilised. When the disrupted samples were supplemented with methionine nitrogen efficiency ratios approached that of casein. Separation of cell-wall-free protein concentrates gave further slight increases in NER values (Ormstedtet al, 1975).

2.2.3. Animal Testing.

Tate and Lyle have carried out toxicity tests on rats, chickens, pigs, veal calves and trout using their mould. Toxicological tests on rats and nutritional tests on broilers and pigs have been

carried out by I.C.I. using their bacterial product. Mould grown on sulphite liquors (PEKILO process) has been tested on pigs, calves and rainbow trout. R.H.M. have fed their fungal product to calves, swine, poultry and baboons. In the U.S.S.R. rats, dogs, rabbits, guinea pigs, cows, sheep, hens, turkeys, pond fish and monkeys have been fed yeast grown on n-alkanes. Animals fed on the yeasts were then fed to human subjects.

No abnormalities were reported following tissue examination of the animals. Products had a good nutritional value (United Nations, 1973). B.P. has made public a large amount of information on the results of their extensive animal feeding trials (Shacklady & Gatumel, 1972; Shacklady, 1973; 1975). Specific carcinogenicity and chronic toxicity studies with laboratory animals showed no tendency for the yeast to increase either the number or variations in the type of tumor by comparison with control diets. When the yeast was supplemented with 0.3% methionine values for Net Protein Utilization (NPU), True Digestability (TD) and Biological Value (BV) were similar to those for whole egg. Work on the nutrition of farm animals has been designed to evaluate SCP in terms of its ability to replace one or more of the high protein sources in existing feeds. The main interest has been in pigs and poultry with more recent concentration on calves and other young mammals. Experiments with broilers have shown that yeast supplemented with dl-methionine can form up to 20% of the total diet, replacing all other major protein sources (in particular soyameal and fishmeal) without sacrificing growth rate, feed conversion efficiency or carcass quality - even over five generations of birds. Similar results have been observed with pigs. B.P. yeast has also been

used to replace milk powder in liquid feeding systems of young animals. Twenty three generations of Japanese quail have been successfully reared on diets of up to 30% yeast.

Processing of SCP and in particular the breaking of the cell wall to free the cell contents has been shown to increase the digestability and the nutritional value of the products in rats and mice (Hedenskog et al, 1969; Hedenskog & Mogren, 1973).

A 50% mixture (on a protein content basis) of maize (NPU - 40) and yeast (NPU - 50) had an NPU of 70. Presumably the yeast supplements the lysine deficiency in the cereal whilst the cereal supplements the methionine deficiency in the yeast. The supplementing of high cereal diets in poorer countries with yeast seems at least a possibility (Shacklady & Gutamel, 1972).

2.2.4. Human Feeding.

Following successful animal feeding trials a number of organisations fed their SCP material to human subjects under controlled conditions. Two major problems arose - the inability of man to deal with large quantities of nucleic acid in the diet and gastrointestinal troubles and skin conditions which followed the consumption of certain products. These are discussed in the following sections.

2.2.4.1. Nucleic acid.

The high nucleic acid content of most SCP products has already been noted. Dietary nucleic acid is depolymerised by nucleases in the pancreatic juices. The purine bases are metabolised to

uric acid. Unfortunately man does not have the enzyme 'uricase' which oxidises uric acid to soluble and excretable allantoin. Uric acid is only sparingly soluble so that high levels of uric acid in the blood or the urine caused by a high intake of nucleic acid may lead to deposition of crystals in the joints, renal tracts and other tissues causing gout, kidney stones and related conditions (Kihlberg, 1972; Sinsky & Tannenbaum, 1975). In separate investigations Waslein et al (1970) and Endozien et al (1970) carried out tests for uric acid levels in the plasma and urine of healthy young male volunteers who were fed varying amounts of nucleic acid (0 - 10g/day) contained in Torula yeast and Chlorella algae as part of a controlled diet. The uric acid levels increased by approximately 100 - 150 mg/g RNA and 0.9mg/100ml plasma/g RNA in the urine and plasma respectively though there were no physiological disturbances in any of the subjects. Gout sufferers generally have uric acid levels well in excess of 7mg/100ml plasma. Not more than 2.0g/day nucleic acid intake would keep plasma uric acid levels below this in healthy male adults. It has therefore been recommended by the F.A.O. and the W.H.O. that not more than 2.0g/day of nucleic acid from SCP should be incorporated in the diet. This figure would be correspondingly lower for women and children of different ages (United Nations, 1975).

2.2.4.2. Toxic effects.

There have been a number of favourable reports on the consumption of yeast at levels of up to 20g/day (Scrimshaw, 1972). However recent tests with higher yeast levels and with other microorganisms have produced some disturbing results.

Waslein et al (1969) fed Hydrogenomonas eutropha (bacteria) to four

male adults who were confined to a metabolic ward. All suffered gastrointestinal problems akin to food poisoning. This was despite tests on mice, dogs, swine, rats, chickens, monkeys and chimpanzees at much higher levels of feeding, which had shown the product to have a good nutritional value and to have caused no ill effects in any of the animals.

Workers at the Massachusetts Institute of Technology investigated two types of yeast, a fungus and a bacterium (which had given good results in animal feed trials) but all produced unacceptable clinical reactions in the subjects to whom they were fed, even when the nucleic acid content had been substantially reduced. The toxic factors were not identified (Scrimshaw, 1975).

Dry Torula yeast (food grade) grown on sulphite liquor was fed at levels of 45, 90 and 135g/day to three groups of subjects for ten week periods. At 45g/day one subject developed a skin condition. At 90g/day eight out of eleven subjects developed skin conditions on the palms of the hands and the soles of the feet which disappeared spontaneously about three weeks after their onset. At 135g/day, out of ten subjects four developed skin conditions and two suffered nausea and abdominal discomfort. This product has received GRAS (Generally Recognised As Safe) status in the U.S.A. (Federal Register, 1974).Torula yeast grown on glucose, when fed at 90g/day gave no skin effects (Scrimshaw, 1972).

In another trial fifty persons were fed 20g/day of yeast grown on ethanol. Ten dropped out within the first few days because of the unpleasant 'yeasty' taste. Nine of the remainder experienced acute food poisoning; i.e. nausea, vomiting, severe diarrhoea, within

the first four weeks of the trial (Scrimshaw, 1972).
A bacterium grown on ethanol gave similar results but after acid washing the offending substance was completely removed and the product became quite acceptable (Scrimshaw, 1972).

The causes of these reactions have not been identified (Scrimshaw, 1975).

2.2.4.3. Nutritional value.
Studies on Chlorella algae and Torula yeast indicated that the biological values and true digestabilities of these products compared favourably to those of casein. BV was found to be dependant on the level of feeding and it was therefore suggested that a better method of assessment would be the amount of crude protein required to maintain a nitrogen balance in adults (reported as mg N/kg body wt/day) (Young & Scrimshaw, 1975).

2.2.5. Overall Assessment.

Chemical analysis suggests that many SCP products would be highly nutritious foods, especially when supplemented with methionine. In the case of animal feed trials this has been largely confirmed, some SCP products proving to be equally as good or better than soyameal, fishmeal or dried milk. Tests on animals suggested that disruption of the cell wall and its subsequent removal may be advantageous in improving the digestability and hence the nutritional value of the products. However SCP in the form of dry powdered whole cells would appear to be satisfactory for most animal feeding purposes.

Unfortunately when fed to human subjects SCP gave adverse effects. A major problem is that of nucleic acid content. Techniques have been investigated for removing nucleic acids from microorganisms and these are discussed in the following section.

The gastrointestinal and skin problems are particularly disturbing, especially as the toxic factors were not identified. Some were obviously contained in the growth media. There was a suggestion that it may be possible to remove the toxic materials by simple processing (e.g. acid washing - Scrimshaw, 1972). Toxic materials may be associated with the cell walls so that cell wall removal would be advantageous from this point of view as well as giving increased digestability and reducing the bulk in the diet (Scrimshaw, 1972; Tannenbaum et al,1966). Unfortunately no results from the feeding of cell wall free material to human subjects have yet been reported.

In the tests where large quantities of SCP were included in the diet (more than 50g/day) problems of acceptance arose. Many subjects could not tolerate the bitter, yeasty taste. It has been suggested that the processing of SCP to produce bland, textured products would be a great help in testing it at higher levels of feeding.

Clearly animal testing is insufficient to predict human reactions to SCP. Furthermore each product must be assessed independantly. Not only the species of microorganism but also growth and processing conditions are important in determining its food value (Scrimshaw, 1972; United Nations, 1975)

For food use, products of low nucleic acid content, preferrably free of the cell walls and processed to have a pleasing flavour and texture, are required. Considerable effort has been expended in producing a cell wall free, low nucleic acid content material and this work is reviewed in the next chapter. Relatively little information is available on the texturing of SCP materials and what there is has followed closely the techniques used for texturing vegetable proteins. These techniques are reviewed in a later section of this report.

2.3. Protein Processing.

2.3.1. Objectives.

Before reviewing the various stages of protein processing the objectives of the work must be considered. As already stated the product must be void of toxic substances, have a good nutritional value and a low nucleic acid content. It must be odourless and free from 'off' flavours (Enebo, 1970). Its functional properties will also be of great importance, whether it is to be used directly in a food product or textured to simulate meat and other foods. Hence the isolation processes must be mild, avoiding denaturation as far as possible in order to retain good functional properties, and in particular high protein solubility (Burke, 1971; Ebbon, 1975; Hey, 1975).

2.3.2. Cell Disruption.

As mentioned in section 2.2. disruption of the microorganisms increases their nutritional value by making the cell protein more

readily available. The cell walls themselves are probably indigestable by man and could be removed following disintegration (Ormstedt et al, 1975; Tannenbaum & Mateles, 1968).

Until recently, interest in cell disruption has come from biochemists seeking to isolate specific intracellular components. A large number of disruption techniques have been reported (Edebo, 1969; Edebo & Magnusson, 1973, Hughes et al, 1971; Wimpenny, 1967; Wiseman, 1969) but few are suitable for scaling up for commercial production (Dunnill & Lilley, 1975).

Assessment of the degree of disruption may be made by the following techniques (Hughes et al, 1971).

1. Cell counts. The number of undisrupted cells can be counted directly by cell viability tests, microscopic examination of stained or unstained smears or by Coulter Counter and similar techniques.

2. Enzyme activity. The activity of a particular enzyme system may be measured and related to degree of disruption. However, misleading results may be obtained due to the release of enzyme inhibitors or enzyme denaturation during processing.

3. Release of soluble protein. Hughes et al (1971) estimated that 100% breakage of yeast suspensions resulted in 80% of the cell nitrogen remaining in solution after centrifuging the treated sample at 6,000g. This will however, be dependant upon the extraction conditions. This method has been preferred by workers in the SCP field as it represents the desired aim - i.e. to

release as much of the protein from the cells as possible.

It is now possible to look at the various methods available, keeping in mind the need to be able to adapt them for industrial use.

2.3.2.1. Sonication.

Ultrasound was first used to disintegrate living cells as early as 1929. It is now a very widely used disruption technique (Hughes et al, 1971). An A.C. output (15 - 25kHz) from an electronic oscillator and amplifier is converted into mechanical waves by a magnetostrictive or piezoelectric transducer. This is coupled to the cell suspension by a half wave metal probe oscillating at the circuit frequency. Vibration of the probe tip causes streaming in the liquid suspension. Bubbles form, grow and coalesce until they reach their resonant size and then collapse violently producing shear stresses sufficient to rupture cell walls (Edebo, 1969; Hughes, 1961). It has been estimated that during cavitation pressures in excess of 1,000 atmospheres and temperatures of up to 10,000°K may occur locally (Hughes, 1961; Wimpenny, 1967).

The major factors which effect the degree of disruption caused by sonication are as follows.

1. Microbial species. Microorganisms vary considerably with regard to the ease with which they may be disrupted. In general ultrasound was found to be effective in breaking a very wide range of cells.

2. Amplitude. Disruption is improved by increasing the amplitude of vibration. However this leads to increased free radical formation which can cause denaturation of unstable enzymes (Edebo, 1969).

3. Viscosity. Increased viscosity inhibits cavitation and reduces disruption (Hughes et al, 1971).

4. Added nuclei. The addition of powdered glass to yeast suspensions undergoing sonication was reported to have doubled the rate of disruption (Hughes, 1961). It is thought that the effect of such material is to increase the sites for nucleation (Hughes et al, 1971).

5. Probe design. The geometry and the material of the probe are important. Amplitude is proportional to probe tip diameter. The preferred material for the tip is titanium. Softer metals (e.g. nickel) transmit greater power but pitting is rapid, leading to contamination of the product. Harder metals have a poor power transmission characteristics (Hughes, 1975).

6. Vessel design. The size and geometry of the vessel used are important. Shallow vessels are generally preferred over deep ones (Hughes, 1961).

Sonication lends itself to adaptation for continuous operation and a number of designs are available (Neppiras & Hughes, 1964).

James et al (1972) studied the kinetics of protein release from brewers' yeast and found them to be essentially a first order process. Batch processes are described by:-

$(1 - x) = e^{-kt}$ Where k = protein release constant

t = treatment time

x = fraction of the total releasable protein.

The continuous process at steady state was described by:-

$k_v V(1 - x_{eq}) = x_{eq} y$ Where k_v = protein release constant

V = reactor volume

x_{eq} = fraction of the releasable protein which is released at equilibrium.

y = flowrate.

k_v was found to be constant for yeast suspensions of 5 - 20% dry weight and for vessels of 75 - 450ml at constant power input.

Efficient cooling is required as considerable heat is generated during sonication. Cavitation may cause damage to material of molecular weight greater than 10^6 (e.g. DNA) but does not appear to affect most enzymes (Coakley et al, 1974).

2.3.2.2. High pressure liquid shear devices. There are several examples of cell disruption by high pressure liquid extrusion through narrow orifices.

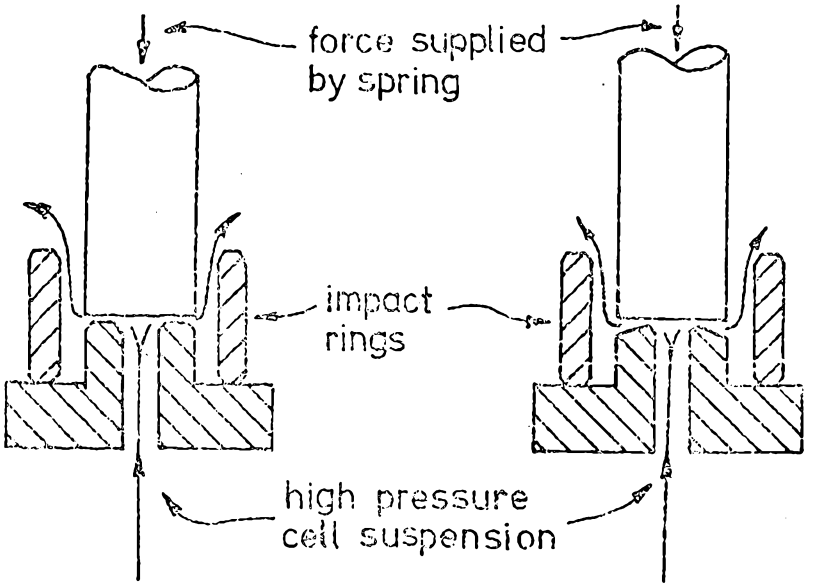
French Press. This was first used in 1950 to prepare extracts from Chlorella algae. Since then it has enjoyed considerable popularity as an effective method of breaking many microbial species. It consists of a steel cylinder having a small orifice and needle valve at its base and an 'O' ring sealed piston which is driven by a hydraulic press. Pressure is governed by the position of the needle valve and may be as high as 30,000psi. It is available in various degrees of automation, the most advanced being the Sorvall-Ribi Fractionator which is capable of continuous operation (Hughes et al, 1971). The other devices described below are variations on this original design.

Chaikoff Press. This is similar in design to the French Press

except that the disruption valve comprises a close fitting steel rod inside a steel cylinder, thus forming an annular orifice. Pressure depends on the clearance and length of the orifice. Pressures in excess of 3,000psi are rarely achieved and its use is limited to fragile organisms (Hughes et al, 1971).

Manton Gaulin Homogeniser. Because of the difficulty involved in scaling up laboratory machines, commercially available equipment used for other purposes (e.g. emulsification of paints) was investigated for cell disruption (Dunnill & Lilly, 1975; Lilly & Dunnill, 1969). Of these by far the most successful has been the Manton Gaulin Homogeniser. This was first used by Tannenbaum et al (1966) to disrupt Bacillus megaterium on a larger scale (15 galls/hour) than could be achieved with a French Press. Its performance was thoroughly investigated for disruption of Bakers' yeast, known to be difficult to disrupt, by Hetherington et al (1971). A high pressure positive displacement pump delivers cell suspension to the valve mechanism - shown in Figure 2.3.1. The pumping pressure is controlled by a hand screw acting as a compression spring which forces the valve onto its seat.

Fig. 2.3.1. Manton-Gaulin Homogenizer Valves.



(a). Flat valve seat. (b). Knife-edge valve seat.

Valves and seats are precision ground and are generally constructed of tungsten carbide. The knife edge valve was superior to the flat valve giving up to 30% greater protein release at the same operating pressure. Working at the maximum pressure of about 8,000psi it was possible to achieve 60% protein release in one pass at 30°C. Protein release was temperature and pressure dependant but independant of yeast concentration in the range 10 - 20%w/v dry matter. Working on the assumption of first order kinetics the following expression was developed:-

$$\log \frac{Rm}{Rm-R} = KNP^{n}$$

Where Rm = maximum protein available for release (mg/g pressed yeast)

R = actual protein released (mg/g pressed yeast)

K = rate constant (3.5×10^{-9} and 5.0×10^{-9} $cm^{5.8}\ kg_f^{-2.9}$ at 5°C and 30°C respectively)

N = equivalent number of discrete passes

P = pressure (kg_f/cm^2)

n = pressure exponent which was found to be 2.9 and to be independant of temperature.

The approximately third power relationship between pressure and disruption rate clearly demonstrates the desirability of higher pressure units. Although multiple passes through the homogeniser lead to increased disruption the homogenate becomes difficult to clarify due to the break-up of subcellular particles.

No loss in enzyme activity occurred though the rate of release of different enzymes varied depending upon their location in the cell. Yeast was used within two days of delivery as storage at 5°C

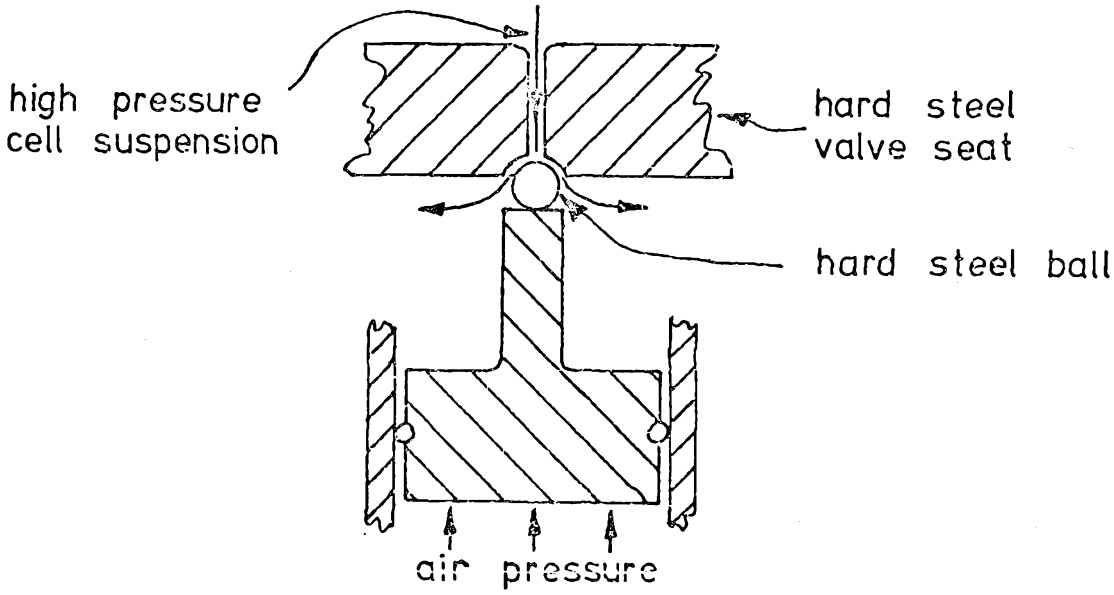
for seven days led to losses of up to 16% in the activity of some enzymes (Follows et al, 1971). There was no change in soluble protein release from yeast stored for one week.

Temperature rises of 5 - 8°C per pass were recorded and intermittant cooling was required for more than one pass (Lilly & Dunnill, 1969).

In an industrial process for isolating protein from yeast Anhauser - Busch Inc. cite the Manton Gaulin Homogeniser as their preferred method of cell disruption (Robbins et al, 1975 (1)).

Stansted Cell Disrupter. J.E. Sharpe (1976) describes a commercially available device (manufactured by Stansted Fluid Power Ltd.) in which an electrically or pneumatically driven high pressure pump delivers the cell suspension to a ball valve disrupter (Figure 2.3.2.).

Fig. 2.3.2. Stansted Ball Valve Disrupter.



The ball is held in place by an air operated piston. The back pressure on the piston governs the operating pressure of the pump.

In the pneumatic version of the pump air at 0 - 100psi is supplied to a large area piston connected to a small area piston which acts on the fluid. The pumping is therefore dependant on the ratio of the areas of the large and small pistons. Pressures up to 30,000psi can be achieved - sufficient to cause 100% disruption of Bakers' yeast in one pass. A temperature rise of about 1°C/1,000psi occurs during disruption so that pre-chilling is essential when working at high pressures.

Mechanism of Disruption. Considerable effort has been made by workers at University College, Cardiff to elucidate the mechanism of cell disruption by liquid shear devices. Brookman and Davies (1973) used a pneumatically operated high pressure pump similar to the one described above, capable of pressures of up to 45,000psi. This was superceded by an extreme pressure device capable of 75,000psi (Brookman, 1971 (1)). First experiments were with needle valves and then, in an attempt to produce a well defined flow channel, shallow grooves 12 μm deep, 3mm wide and 3cm long were machined between two flat plates bolted together. Up to 90% disruption of Bakers' yeast was attainable with one pass through the needle valve. Disruption was independant of yeast concentration above 4%w/v dry matter and increased exponentially with increasing pressure. The efficiency of the 'groove' valve fell with prolonged use due to excessive wear and stretching of the securing bolts (Brookman & Davies, 1973). Using the higher pressure pump and a needle valve complete disruption was achieved at 50,000psi. When the needle valve was replaced by capillary tubes of 0.152mm internal diameter and of various lengths it was found that at constant pressure the protein

release increased in inverse proportion to the tube length suggesting that protein release was a function of shear stress. Other results, however, indicated that the magnitude of the pressure rather than simply shear stress was the governing factor in cell disruption (Brookman, 1975 (1) & (2)).

Further investigations were undertaken using the Manton Gaulin Homogeniser (Brookman, 1974; Doulah et al, 1975). A detailed mathematical analysis suggested that small eddies (less than the cell diameter) gave rise to oscillations within the cell which, if the kinetic forces were great enough, caused the cell to break.

It seems logical that if shear force were an important mechanism in disruption then the shortest possible orifice would give the best results since shear stress is proportional to $1/L$. This is indeed the case - the best device being the knife edge valve in the Manton Gaulin Homogeniser which works very satisfactorily at pressures of only 8,000psi. Effective orifice length is probably longer in ball and needle valves which require much higher pressures. Considerably longer channels are used in the relatively inefficient Chaikoff Press and, of course, in the capillary tubes. It is concluded that both shear and absolute pressure are important for cell disruption. In all these devices wear of the disruption valves is a major problem.

2.3.2.3. Disruption with glass beads.

Agitation or grinding of cell suspensions has been used extensively for disruption of microorganisms (Zetalaki, 1969). Hand grinding using a mortar and pestle is slow and yields are poor (Hughes etal, 1971; Edebo, 1969). However, efficient laboratory devices do exist.

Rodgers and Hughes (1960) describe a 'Sonomec' wave pulse generator which is connected to a closed vessel containing the cell suspension and the glass beads. The height of the air column is a critical factor in producing resonance. Novotny (1964) used a specially designed stirrer which fitted into a centrifuge tube containing about 10ml of cell suspension and 25ml of 0.1mm diameter glass beads. Operating at 3,000 - 4,000 rpm 95% disintegration of Bakers' yeast was achieved in about five minutes. These devices were only useful for laboratory scale experiments and attempts were made to use existing ball mills as cell disrupters for large scale operation.

Using an industrial 'Perl Mill' Hedenskog et al (1970) investigated the effect of bead size and ratio of beads to suspension in the disintegration of several microbial species. Optimum bead size was about 0.2mm for yeasts and algae but less than 0.1mm for bacteria. Fresh Bakers' and Brewers' yeast was easier to disrupt than the spray dried Scenedesmus algae and lyophilized bacteria. This may have been due to the effects of processing on the algae and bacteria. With a cell suspension to bead ratio of 1.7, 5%w/v dry wt. yeast suspensions were completely disrupted in ten to twenty minutes. A larger quantity of beads increased the disruption rate.

Currie et al (1972) used a continuous operation agitator mill (Netzsch - Molinex KE5) which comprised a set of eccentrically mounted discs in a helical array which rotated inside a water cooled jacket to disrupt the Bakers' yeast. The rate constant as defined by Hetherington et al (1971) was dependant upon agitation speed, temperature, bead size, weight of beads, flowrate and yeast

concentration, assuming first order kinetics. Disruption increased with speed up to the maximum of 1,800rpm. This gave tip speeds which put the device on the border between systems acting by tangential shearing and systems acting by impaction. Low flowrates gave high disruption but also a high temperature rise - up to 40°C. Temperature could be easily controlled by pumping water through the cooling jacket. Increasing the weight of beads increased disruption. Small bead sizes (1mm) were most effective except at high yeast concentrations when the beads began to float and efficiency dropped rapidly. On a batch basis with 5 litres of 15%w/v dry weight yeast suspension 90% disruption was achieved in about three minutes.

A similar device, the Dyno Mill KD5 was used by Mogren et al (1974) and by Dunnill and Lilly (1975) for disruption of Bakers' yeast. The homogeniser was horizontally mounted with a series of rotating discs of special design. Speeds of up to 3,000rpm were possible - the highest speeds giving the highest disruption. Lead free glass beads (0.5 - 0.75mm diameter) occupied about half the working volume (5 litres). Disintegration was independant of concentration so that operation at high concentration was most economical, despite increased energy requirements. However high concentration and/or low flowrates led to high temperature rises (up to 40°C) which were difficult to control as the polyurethane coating of the cylinder acted as an excellent insulator. The efficiency of the machine was similar to that of the Manton Gaulin Homogeniser. Measurements for silica in the disrupted suspension showed that the glass content of the suspension after a single pass was about 0.01%, the fragments being about 1μm in size. After cell wall removal and protein precipitation there appeared to be no glass

in the product.

2.3.2.4. Other mechanical devices.

Fraser (1951) described a method of bursting bacteria (E. coli) by rapid release of gas pressure. A suspension of cells is placed in a thick walled container and gas pressure of 500 - 1,000psi is applied. The suspension is then rapidly expelled. 90% disruption was obtainable in two runs through the machine. It was believed that dissolved gases led to high internal pressure in the cells which caused them to burst on decompression. Speed of release of the suspension governed the disruption. If the suggested mechanism were true, cells should be disrupted by release of the gas rather than release of the suspension. This was not the case however and it would appear that shear in the outlet pipe was responsible for disruption. The device was ineffective with more robust organisms.

Hofsten and Tjeder (1961) disintegrated yeast by mixing equal volumes of pressed yeast and dry ice and blending the mixture in a laboratory homogeniser. Only 10 - 12% disruption occurred after 10 minutes with Bakers' yeast, compared with 10 - 20% disruption in 2 minutes for Brewers' yeast. Devices such as this one are not applicable to large scale use.

In 1951 D.E. Hughes reported a freeze press in which frozen cell suspensions at -10 --30°C or cell suspension mixed with powdered glass were forced through an annular slit ($\frac{1}{1000}$" - $\frac{15}{1000}$" wide) at 10 - 80,000psi using a fly press, hydraulic press or a weight dropping device. This was found to be very effective for treating various strains of yeast, algae, bacteria and fungi - complete disintegration being achieved in one pass when cell paste and

powdered glass in equal volumes were pressed at -10 --15°C and 80 - 90% disintegration being achieved without powdered glass at 35°C. The abrasive action of the crystals on the cells as they passed through the slit was thought to be responsible for disruption (Hughes et al, 1971).

A similar device to the Hughes press was described by Edebo (1960). The so called 'X' press used a small hole as the disruption valve instead of a slit. It was not so efficient as the Hughes press due, presumably, to the wider and longer orifice used (Hughes, 1971). A later version of the machine is capable of semi-continuous operation up to 10kg pressed yeast/hour operating at about 30,000psi. Cylindrical, frozen, pressed yeast samples at -35 - -20°C are fed automatically into the pressure chamber and are pushed through a small hole by a piston attached to a hydraulic pump. 90% disruption of Bakers' yeast was achieved with one pass. A mechanism of cell disruption due to phase changes at high pressure was suggested (Magnusson & Edebo, 1976).

2.3.2.5. Enzyme lysis.

Enzymes capable of decomposing the cell walls of yeast have been separated from, for example, snail gut. However, these are obviously not obtainable in large quantities. Exogenous lytic enzymes can be obtained by fermenting the enzyme producing organism under suitable conditions. However few microorganisms are susceptable to enzyme attack unless they have first been boiled. This denatures the protein in the cells and makes it difficult to extract (Carenberg & Heden, 1970; Wiseman, 1969). Enzyme lysis may become more economically feasible as immobilised enzyme systems are developed (Dunnill & Lilly, 1975).

2.3.2.6. Other techniques.

Autolysis has been used for the production of yeast extract from waste brewery yeast since 1902. Yeast is incubated at 40 - 60°C causing destruction of the cells' controlling mechanism and allowing proteolytic and nuclease enzymes to break up the protein and nucleic acid into small soluble fragments which can easily pass through the cell walls. This has been modified to include high temperature processes with addition of salts to give more rapid action. Most of the vitamins and amino acids remain intact but the final product is only suitable as a flavouring agent and vitamin supplement. The cell walls are separated by centrifugation and used as cattle fodder (Acraman, 1966; Bridson & Swaine, 1975; Ellison, 1973; Hough & Maddox, 1970). Other microorganisms grown specifically as SCP products have also been treated in this way, or by acid or alkali hydrolysis, to yield a low molecular weight, highly flavoured extract (Champagnat & Laine, 1966).

Various chemical extraction methods have been used. Wasthead and McLain (1964) used toluene lysis to extract enolase from Bakers' yeast. Aries (1952) extracted protein from yeast cells with 0.05 N NaOH at room temperature for one hour following extraction with ether or carbon tetrachloride. Product yield was less than 20%. Mitsuda et al (1969) investigated various extractants including NaCl, KCl, NaOH (0.5, 1 & 2.5%), phosphate buffer, detergent, butyl alcohol, urea and a number of other salts. Soaking in 8M urea led to 18 and 28% protein extraction yields for Chlorella algae and Torula yeast respectively. This increased to 50% for both organisms if the cells were pre-treated by boiling for five minutes with 1N NaOH. Freeze thawing and osmotic shock have also been used but are suitable for fragile cells only (Edebo,

1969; Edebo & Magnusson, 1973; Hughes et al, 1971; Mitsuda, 1969).

2.3.2.7. Comparison of methods.

Apart from autolysis which yields a denatured protein product chemical and enzymic methods are inefficient and/or expensive at present. The 'X' press has been successfully scaled up for semi-continuous use, but it is difficult to see the advantage of freeze pressing over liquid shearing as similar pressures are required and, in addition, operation must be carried out at very low temperatures. Little has been said about the problem of glass contamination from bead mill devices. Although Mogren et al (1974) found no glass particles in the final protein product these did exist in the cell wall fraction, presumably making it unsuitable for use as animal feed. With recent concern over glass contamination of food products it seems unlikely that such a process would find wide acceptance. Sonication does not lend itself to scale up beyond the pilot plant stage (Dunnill & Lilly, 1975) and so there remains the liquid shear devices, and in particular the Manton Gaulin Homogeniser. This is in fact the preferred method of disruption in a number of recent patents assigned to Anhauser-Busch Inc. dealing with the extraction of protein from yeasts (Robbins et al, 1975 (1)). The major disadvantage of this process is the high energy demand. Work is in progress to produce mutant strains of SCP organisms with weak cell walls which should make disruption easier (Dunnill & Lilly, 1975).

2.3.3. Protein Isolation.

2.3.3.1. Overall approach.

The need for cell disruption and nucleic acid removal has been

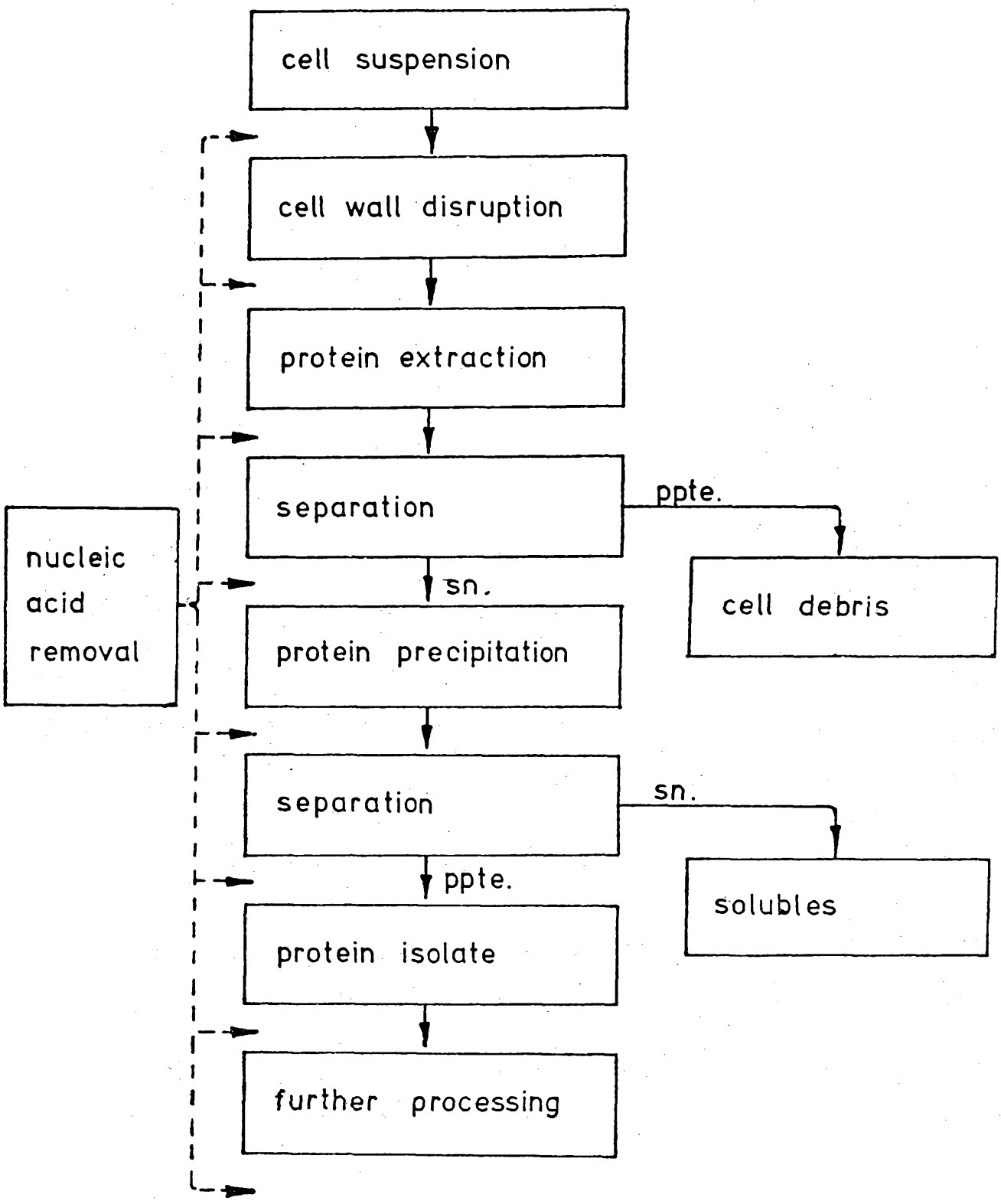
established and techniques for the achievement of the first of these have been discussed. A general procedure for the production of a high protein, low necleic acid, cell wall free product is shown in Figure 2.3.3. Cell disruption obviously occurs at the beginning of the process, prior to protein extraction. Nucleic acid removal on the other hand, may take place at any one of a number of positions along the extraction path. Strictly speaking protein extraction and isolation cannot be divorced from nucleic acid removal, but for clarity and to aid comparison between the various techniques which have been adopted, nucleic acid removal will be dealt with in a separate section.

2.3.3.3. Protein extraction and precipitation.

For the most part isolation of protein from microorganisms has followed a similar line to that of protein extraction from defatted oilseed meals - i.e. the meal is treated with dilute alkali, centrifuged or filtered to remove the insoluble residue and the protein is precipitated at the isoelectric point and collected by centrifugation (Ashton et al, 1970).

A considerable amount of work in this field has been carried out by Gudmund Hedenskog and his colleagues at the Royal Institute of Technology in Sweden. After disintegration of fresh Bakers' yeast with glass beads protein was extracted with NaOH at different pH's and separated by centrifugation. About 80% of the cell nitrogen was found to be soluble at pH 8 and about 90% at pH 11. Using spray or drum dried cells the solubility was only 20% and 40% at pH 8 and pH 11 respectively. Only 10% of the cell nitrogen was soluble even at pH 11 using fresh, undisrupted yeast

Fig. 2.3.3. Protein Isolation - General Approach.



(Hedenskog et al, 1970; Hedenskog & Mogren, 1973). Maximum precipitation of protein occurred at pH 4.0 following extraction at pH 11.5, even when separation of the cell walls was omitted (Hedenskog & Ebbinghaus, 1972). A protein yield of about 70% was obtained overall but the nucleic acid content of the isolate was very high - about 11%w/w of the dry matter. Different acids were tried as precipitants. Trichloracetic acid yielded a product containing 9%w/w nucleic acid, but this was still too high and the high cost compared with that of HCl was prohibitive (Hedenskog & Ebbinghaus, 1972) Heat precipitation from alkali solutions was used in an attempt to reduce the nucleic acid content and this is discussed in the next section of the report.

Vananuvat and Kinsella (1975) studied a number of parameters in attempting to isolate protein from freeze dried S. Fragilis grown on lactose. The cell walls were disrupted with glass beads. Extraction of protein at pH 6.2 increased with temperature up to a maximum of 50% at 40°C. Extraction with 2 - 8%w/v NaCl at pH 6.2 did not significantly increase the protein yield. Highest extraction of protein (about 70%) occurred at pH 11.5 at 40°C. Increasing the extraction time from half and hour to two hours at 40°C did not affect extraction efficiency at pH 11.5 but increased it at pH 6.2 from 50 - 65%, whereas with 2%w/v NaCl at pH 6.2 the protein extraction fell from 55 - 50%. Lower yeast concentrations gave the highest protein yields. For example, at pH 11.5 protein yield fell from 70 - 50% when the yeast concentration changed from 5 - 10% DM. (A concentration of about 5% is generally used for extraction of protein from leaves and oilseeds). Maximum protein precipitation occurred at pH 4 - 4.5 but it was again observed that a greater proportion of the protein extracted was precipitated

from the extract at pH 11.5 (about 90%) than from the extract at pH 6.2 (about 70%). The nucleic acid contents of the two products were 6%w/w and 2%w/w for extraction at pH 11.5 and 6.2 respectively. However the overall yield of the protein extracted at pH 6.2 was only 30% compared with 65% from cells extracted at pH 11.5. Heat precipitation of the extract at pH 11.5 in order to reduce the nucleic acid content was carried out at 80°C and pH 6, but this led to a drop in protein yield to only 54%.

Cunningham et al (1975) attempted to isolate protein from dried Candida tropicalis grown on n-paraffins by Gulf R.& D. Co. Several disintegration techniques were investigated, the best being homogenising (Manton Gaulin). However only 21% of the crude protein was water soluble even after four passes. This was probably due to reduced solubility caused by drying of the cells. 85% of the crude protein was soluble in 0.1 N NaOH after three passes through the homogeniser. Higher NaOH concentrations during extraction yielded dark brown isolates. The protein was precipitated at pH 3.8 and about 40% of the original cell nitrogen was recovered. The isolate contained 70% crude protein which was 25% soluble at pH 7 and 75% soluble at pH 8.

The influence of alkali and heat treatment on the protein from Bakers' yeast was studied in more detail by Marianne Lindblom (1974). Fresh yeast was disrupted using glass beads according to Novotny (1964). Protein solubility was a minimum (about 25%) at pH 4.0 and rose rapidly to about 90% at pH 8 and then levelled off to 90 - 95% up to pH 12. The addition of 1 M NaCl or 0.5 M $CaCl_2$ led to increased solubility between pH 4 and pH 6 but reduced solubility

at higher pH's (especially with $CaCl_2$) compared with NaOH only. It had already been shown that maximum protein precipitation occurred after extraction at pH 11.5. The following experiments were trying to understand this and similar phenomena. Disintegrated cells were extracted with water at pH 5.8, the solubles being referred to as the water extract. The cell debris were extracted at pH 11.5 and the solubles separated as the alkali extract. Both these solutions were split into high molecular weight (HMF) fractions and low molecular weight (LMF) fractions by dialysis giving samples with molecular weights above and below 5,000 (approximately). The following observations were made during tests on the above solutions:-

1. All of the low molecular weight material was present in the water extract.
2. None of this low molecular weight material could be precipitated at pH 4.
3. Half of the protein in the HMF of the water extract was precipitated at pH 4.
4. 95% of the protein extracted at pH 11.5 was precipitated at pH 4.0.

We can therefore conclude that low molecular weight protein can be extracted at low pH's but it is difficult to precipitate. High molecular weight protein is less soluble, requiring high extraction pH's but precipitating easily at pH 4.

Gel filtration of the water extract on Sephadex G200 showed that three protein peaks occur. About 20% appears in the void volume i.e. molecular weights greater than 10^6, 70% has a molecular weight of about 10^5 and 10% is low molecular weight peptides.

The influence of alkali treatment was investigated by increasing the pH of the water extract and then reducing it back to pH 7.0. The solubles were recovered by centrifugation. On increasing the pH to 11.0 and then reducing it back to pH 7 only 37% of the protein remained soluble. Gel filtration showed that all the low molecular weight material was retained in solution whereas most of the high molecular weight material had been precipitated. Structural changes appeared to be taking place which made the protein less soluble after alkali treatment.

Further tests were designed to see the effect of heating water extracts at pH 5.8, 7, 7.5 and 7.5 with 5% NaCl. At 20% all were 100% soluble. Solubility of the extract at pH 5.8 fell rapidly to only 25% at 70°C. At pH 7.5 98% of the protein was still soluble at 70°C, but at pH 7.5 with 5% NaCl solubility had fallen to only 39% at 70°C. Gel filtration showed that at pH 7.5 there was considerable loss of protein from the middle molecular weight peak (approximately 100,000) but an increase of protein in the high molecular weight peak as the temperature increased up to 70°C after which the protein content of both peaks fell rapidly as the solubility decreased. This apparent gain in molecular weight caused by heating or alkali and salt treatment is presumably due to protein interaction causing aggregation. When the aggregates reach a certain size they are precipitated. Similar properties have been observed for soya protein (Kelly & Pressy, 1966) and groundnut protein (Naismith & Thompson, 1955). This would explain why protein precipitation was so much better after alkaline extraction than after water extraction even in cases where the amount of soluble protein extracted by alkali and water was not significantly

different. Moore and Carter (1974) showed that aggregation due to non-covalent bonding occurred on heating soya protein and carbohydrate mixtures. Yeast contains large quantities of carbohydrate material as well as protein and it may be that such interactions are responsible for the effects described above.

2.3.3.4. Protein separation.

There has been relatively little work published on the separation of cell walls from soluble extracts or on the separation of protein precipitates from the soluble fraction.

Gray et al (1973) investigated the use of rotary vacuum precoat filtration for clarification of disrupted yeast suspensions. Various precoat materials were used, the best being standard 'Supercel'. Better clarification was obtained with centrifugation at 8,000g. However large amounts of the filter aid were required. Dunnill & Lilly (1975) suggested that such an approach would be too costly and would lead to problems of disposal of the cell debris which was contaminated with the filter aid. Flocculation had been tried but the flocculants used acted on the soluble protein as well as on the cell debris, causing loss of protein yield. Continuous centrifugation with intermittant solids discharge was thought to be the best approach.

Anhauser - Busch Inc. reported that ease of separation of protein extracted from disrupted yeast suspensions depended on a number of factors. Increasing pH increased the amount of protein extracted but also increased the viscosity and made separation more difficult. Extraction at pH 9.5 was considered an optimal balance. Increasing the temperature (e.g. to 50°C for five minutes) led to easier

separation. Disruption and hence protein extraction increased with the number of passes through the homogeniser. However this gave particles of a small size in the debris and made separation more difficult. Three passes were generally used.

The protein precipitate was easier to separate if it had first been heated rapidly to 70°C. This also led to higher protein yield and lower nucleic acid content. The addition of 0.3g $CaCl_2$/ 100g yeast solids doubled the sedimentation rate of the precipitate. This did not lead to a significant increase of Ca^{2+} in the product. Separations were carried out using centrifugation or filtration but process details were not given (Newell et al, 1975 (1) & (2); Robbins et al, 1975 (1)).

Protein precipitated by HCl from extracts of Bacillus megaterium was easier to separate after heating the suspension for five minutes at 100°C (Tannenbaum et al, 1966).

2.3.3.5. Conclusions.

From the above details of extraction, precipitation and separation methods the following conclusions may be drawn.

1. Cell disruption was required in order to obtain high yields of soluble protein. For effective cell disintegration fresh yeast was preferred to dried yeast.
2. Extraction at pH 11 - 11.5 gave the highest protein yield. Higher extraction pH's led to a brown concentration of the protein isolate.
3. Maximum protein precipitation occurred at close to pH 4. A greater percentage of the protein in the supernatent was precipitated following extraction at pH 11 rather than at pH 6 - 7.

4. The use of salts did not lead to improved protein extraction or precipitation.
5. Separation of the cell debris from the extracted protein was more difficult at high pH's and where disintegration had led to very small cell wall fragments. Separation was made easier by heating the cell suspension.
6. Separation of the protein isolate was made easier by heating the suspension and adding calcium salts.

Although isolates may be obtained (with a high protein yield) by straight forward extraction and precipitation these still contain unacceptable amounts of nucleic acid. A separate step in the process will therefore be required to reduce the nucleic acid content. Techniques for achieving this are discussed in the following section.

2.3.4. Nucleic Acid Removal.

As previously stated a limit of 2g/day of nucleic acid from SCP in the diet of a healthy male adult is currently recommended (United Nations, 1975). Assuming that SCP could form a major part of the diet - say 50g/day of protein - a protein to nucleic acid ratio of 25:1 or more would be a suitable target to aim for in products to be used as meat analogues. A lower ratio could still be acceptable if products were used, for example as meat extenders in products containing only 20 - 30% SCP. Although SCP products contain both RNA and DNA, the RNA content is generally many times more than the DNA content (e.g. RNA:DNA > 20:1 in Bakers' yeast (Kelly, 1974))so that the main problem becomes RNA rather than nucleic acid removal.

2.3.4.1. Enzymic methods.

Cell suspension - endogenous nucleases. In 1970 Tannenbaum et al filed a patent (U.S. patent no. 3,720,585) describing a process in which a suspension of Candida utilis was 'heat shocked' for a few seconds at 68°C and then incubated, first at 45 - 50°C and then at 55 - 60°C for a total of two hours. The cells were collected by centrifuging and it was found that about 80% of the nucleic acid had been removed due to the action of endogenous nucleases which reduced the nucleic acid to low molecular weight material capable of permeating the cell wall. The reason for two stages of incubation was not clear. It was stated that metal ions in solution led to more rapid nucleic acid hydrolysis though this was not further discussed. The optimum conditions were:-
heat shock at 68°C, 5 secs; incubation at 50°C for 1 hr and 55°C for 1 hr at pH 6.0 giving a product with a protein:nucleic acid ratio of 33-50:1.

This method is the basis for many of the processes described below. Similar results were claimed for Candida intermedia (Maul et al, 1970; Tannenbaum et al, 1973). Imada et al (1972 (1)) attempted to extract the enzyme responsible from C. lipolytica. Mechanical cell disruption gave the highest total enzyme activity , although solvent dehydration and buffer extraction gave the highest activity per gram of the protein extracted. Cell disintegration by sonication or toluene lysis yielded very little active enzyme. Ohta et al (1971), using C. utilis found that with no heat shock there was only a 10% reduction in nucleic acid content and without incubation no nucleic acid removal occurred at all. There was only a small reduction in nucleic acid when Bakers' yeast was used however (Sinskey & Tannenbaum, 1975). Canepa et al (1972), using Bakers' yeast, improved on this process by suspending the cells in

50mM Na_2HPO_4 and pretreating the suspension at pH 12.0 by the addition of caustic soda. Protein to nucleic acid ratios greater than 50:1 were obtained. Similar techniques have been applied to several species of bacteria with differing degrees of success (Abu Ruwaida et al, 1976; Abu Ruwaida &Schlegel, 1976).

Cell suspension - added nucleases. Castro et al (1971) tried a similar process using bovine pancreatic ribonuclease 'A' enzyme instead of the endogenous enzymes. The optimum conditions were:- heat shock at 80°C, 30 secs; addition of enzyme and incubation at 55°C, 1 hr, pH 7 - 7.5; enzyme/cell ratio of 1:10,000 (w/w). If the enzyme was added prior to heat shock some activity was lost. Addition of monovalent metal ions gave a slight increase in nucleic acid removal rate.

Cell homogenates - endogenous nucleases. Lindblom and Mogren (1974) attempted to reduce the nucleic acid level of Bakers' yeast following cell disruption by agitation with glass beads. After incubation of the disrupted cell suspension at 50°C for two hours followed by protein precipitation at pH 4.0 there was negligable nucleic acid removal. However on addition of 3%w/v NaCl a product containing only 1.4%w/w nucleic acid was obtained after 30 minutes incubation. The optimum pH was 5.6. Little reduction in RNA content occurred if undisrupted cells were incubated with 3%w/v NaCl. The process described above yielded a protein concentrate containing the cell walls. A cell wall free product was obtained by centrifuging the suspension after incubation and then precipitating the protein from the supernatant at pH 4.0. This gave a protein yield of only 14% due to heat and salt precipitation of the protein during incubation so that it was removed with the cell wall fraction. The yield was increased if

the cell walls were removed prior to incubation. However, a higher pH was required and this led to reduced nuclease activity. The nucleic acid content of the isolates without incubation was 12%.

Robbins et al (1975 (1)) outlined a process using food grade yeasts (C. utilis and Bakers' yeast) in order to produce a low RNA, cell wall free isolate. Yeast suspensions (12%w/v dry matter) were disrupted using a Manton Gaulin Homogeniser at 10,000psi. The suspension was diluted to 4%w/v and the pH adjusted to 9.5. The suspension was heated to aid separation (50°C, 5 mins.) and then rapidly centrifuged. Following pH adjustment to pH 6.0 the supernatant was incubated at 50°C for two hours. About 60% of the original protein was recovered by adjusting the pH to 4.5 and heating to 70°C prior to centrifuging. The product, which had a light colour and a bland flavour contained about 2%w/w RNA, giving a protein : RNA ratio of 40 (c/f without incubation protein/RNA = 5.5w/w). Mackeh et al (1974) described a similar process but no detailed information was given.

Cell homogenates - added nucleases. DNAase and RNAase from bovine pancreas have been used to reduce viscosity and hence aid protein recovery of E. coli suspension disrupted in a Manton Gaulin Homogeniser. The estimated costs of the enzymes was 10p/kg of dry bacterial cells (Melling & Atkinson, 1972). Bearing this in mind Newell et al (1975 (1)) searched for a cheap source of nuclease enzymes. Malt sprouts proved to be such a source. The enzymes were extracted by wet milling the sprouts (5%w/v solids) and extracting at 25°C, pH 4.5 - 5.5 for 15 hours. Following separation the supernatant fraction contained 1,000 nuclease units/gm sprouts.

Cell wall free yeast extracts were obtained from C. utilis or Bakers' yeast as described above (Robbins et al, 1975 (1)) and these were incubated with the malt sprout extract. Optimum conditions were 60°C, 1 hour, pH 7.0, 600 nuclease units/g yeast solids, 3 - 4%w/v yeast solids which gave a product with a protein to nucleic acid ratio of about 50. Overall protein yield was 50 - 70%.

Mechanism of enzyme processes. It is clear from the results of the heat shock / incubation experiments in whole cells that the heat shock was required to initiate enzyme action. Ohta et al (1972) suggested a number of possible mechanisms:-

a. Activation of the nucleases.
b. Thermal inactivation of inhibitors.
c. Release of nucleic acid from subcellular components, e.g. ribosomes.
d. Disorganisation of the cell control mechanisms.

Abu Ruwaida and Schelgel (1976) found that DNA was not degraded so the action was that of RNAase. Castro et al (1971) found that adding exogenous enzymes prior to heat shock at 80°C led to reduced enzyme activity whereas adding the enzymes after the heat shock step gave high activity. It would appear from this that the heat shock is required to inactivate some inhibitor rather than to activate the enzymes. It is likely that an optimal temperature of heat shock when using endogenous nucleases occurs due to the balance between inhibitor inactivation, requiring a high temperature, and enzyme inactivation, which, unfortunately, also increases the temperature. Imada et al (1972 (2)) showed that a macromolecular

inhibitor (not removed by dialysis) exists which is denatured by heat treatment. The inhibitor is promoted by ATP and purine nucleotides. This system probably serves as the nuclease repression mechanism in live cells.

Ohtaka and Uchida (1963) found that ribosomes are autolysed by 3% NaCl, even at 0°C. This was used by Lindblom and Mogren (1974) as an explanation for the increased enzyme action with the case of 3% NaCl for incubation of cell homogenates. However, the ribosomes do not contain sufficient of the cell RNA to explain the dramatic increase in activity of the enzymes following addition of salt. Barker et al (1975) noted that heating above 40°C with 3% NaCl led to considerable protein precipitation. It is therefore suggested that the macromolecule is a protein which is removed by heating in a NaCl solution. The process of Robbins et al (1975 (1)) requires no such activation. It seems that the hot extraction at pH 9.5 followed by reduction in pH to 6.0 prior to incubation denatures the inhibitor sufficiently to cause it to precipitate.

2.3.4.2. Alkaline hydrolysis.

Another approach to nucleic acid removal is to hydrolyse the RNA by treatment at an alkaline pH (Kihlberg, 1972). Following cell wall disruption using a Perl Mill Hedenskog and Ebbinghaus (1972) attempted to remove nucleic acid from Bakers' yeast by extracting the protein at a high pH, removing the cell walls by centrifuging and incubating the supernatant for two hours prior to acid precipitation at pH 4.0. Incubation at 25°C required a pH of more than 12.5 to give effective nucleic acid removal but this led to a considerable drop in protein yield. Incubation at

pH 11.5 required temperatures in excess of 80°C to produce a low nucleic acid product. This also led to reduced protein yield. Daly and Ruiz (1974), in an attempt to integrate protein extraction and texturisation, produced a protein concentrate by disruption of a 10%w/v dry solids suspension of Cellulomonas grown on bagasse in a Manton Gaulin Homogeniser, followed by precipitation of the protein and the cell debris at pH 4.0 using perchloric acid. At this stage the nucleic acid content was 10%w/w of the dry matter (DM). Spinning 'dopes' were prepared by slurrying the protein at 15 - 20%w/v DM in 0.5 - 0.1 N NaOH. The dope was incubated at 37°C and then pushed through a spinnerette (0.005" diameter holes) by nitrogen pressure into a bath containing 0.5N $HClO_4$. The fibres were then left to soak for five minutes and were then removed. Very weak fibres were produced and more than 16%w/w of the protein had to be replaced by casein in order to give satisfactory results. Incubation for 4 - 6 hours reduced the RNA content to about 1%w/w. The DNA content was 2 - 3%w/w so that the overall protein to nucleic acid ratio was greater than 20. It was suggested that nucleic acid hydrolysis took place during alkali incubation and that the hydrolysis products diffused out of the fibres into the co-agulation bath - the fibres acting as microdialysis tubes. Meanwhile, Anheuser-Busch Inc. had filed another patent describing the production of a cell wall free, low RNA, isolated yeast protein (IYP) using an alkali process. Extraction of the soluble protein followed the method described above of Robbins et al (1975 (1)). To this fraction NaOH was added. Two techniques were described: the high temperature, low alkali process (HTLA) using pH 10 - 10.5, 75 - 85°C for one to four hours and the low temperature, high alkali process (LTHA) using pH 11.5 - 12.5,

55 - 65°C for one to two hours. Following incubation the suspensions were cooled and the protein was separated by centrifugation at pH 4.5. The products contained less than 2%w/w nucleic acid giving a protein to nucleic acid ratio of about 40 in both cases. Without the alkali incubation step the product contained about 11.5%w/w nucleic acid.(Newell et al, 1975 (2)).

2.3.4.3. Acid hydrolysis.

Although acid hydrolysis of RNA has been used extensively in analytical work (Herbert et al, 1971) it is only recently that it has been studied for reducing the nucleic acid levels in SCP products. Tannenbaum et al (1966) extracted nucleic acid from a bacterial protein isolate with 5% trichloracetic acid at 90°C. Using a yeast, Rhodutorula glutinis, grown on domestic sewage Zee and Simard (1975) found that nucleic acid content could be reduced from 6.5% to less than 1%w/w by incubation with HCl at pH 2 and 90°C for two hours. This was preferred to an alkali process at pH 11.

2.3.4.4. Chemical extraction.

Nucleic acid may be precipitated from protein solutions using manganous chloride (Korkes et al, 1951). 10%w/v NaCl at 80°C has been used to extract RNA from yeasts (Peppler, 1970). 85%w/v phenol is reported to remove RNA from crude cell extracts (Sinskey &Tannenbaum, 1975).

2.3.4.5. Heat precipitation of protein.

Hedenskog and Ebbinghaus (1972) extracted protein from a 10% DM suspension of fresh Bakers' yeast by disruption in a Perl Mill followed by heat precipitation of the protein and cell debris at high pH. When the cell homogenate was heated to 80°C at pH 10.0

and then cooled to 25°C and centrifuged the resulting protein precipitate contained only 1 - 2%w/w nucleic acid and the protein yield was 70 - 80%. Lower temperatures and/or pH's could be used if 5 or 10% NaCl were added to the homogenate prior to heating. In a similar process protein was extracted from disintegrated suspensions of S. fragilis with 0.4%w/v NaOH, pH 11.5. After removal of the cell debris the pH was brought to 6.0 and the suspension was heated to 80°C, cooled rapidly and centrifuged. The resulting isolate contained only 1.4%w/w nucleic acid giving a protein to nucleic acid ratio of 60. The overall protein yield was 55%. No nucleic acid was detected in isolates extracted with water at pH 6.0 (instead of NaOH) and heat precipitated as above at 80°C. However the protein yield was only half that obtained by alkali extraction (Vananuvat and Kinsella, 1975). Unlike alkaline hydrolysis, in this process the nucleic acid remained in solution as high molecular weight material.

2.3.4.6. Ion exchange.

'Dowex' anion exchange resin (chloride form) has been used in analytical work to remove interfering components (especially proteins) from nucleic acid samples. Neutralised RNA fractions from Bakers' yeast were passed through a column of the resin which was then washed with dilute NaCl solution. The purified RNA samples were eluted with a solution of 1N HCl and 0.5 N NaCl. However only 1 or 2mg of RNA/g resin were retained (Deken-Grenson &Deken, 1959; Marcus & Feely, 1962; Smillie & Krotkov, 1960). This low capacity coupled with high resin costs has probably deterred workers in the SCP field from investigating the use of ion exchange resins. However less expensive resins with high

capacities have been developed and have been used for the recovery of protein from, for example, abbatoir wastes. These may be very attractive in the future as operating conditions are very much milder than the extremes of pH used at the moment (Grant, 1976 (1 & 2); Jones, 1976).

2.3.4.7. Cell physiology and growth.

It is well known that reducing the growth rate of microorganisms reduces their nucleic acid content. Phosphate limited media give similar effects. Unfortunately only a small nucleic acid reduction is possible using this type of approach (Sinskey & Tannenbaum, 1975).

2.3.4.8. Comparison of methods.

A problem with the heat shock / incubation methods on whole cells is that it is difficult to break the cell walls after treatment and hence to extract the protein (Newell et al, 1975 (1)). This will probably limit the use of these methods to production of SCP material for food supplements.

The expense of added enzymes, even using malt sprout extracts, is high and for this reason these processes are unlikely to make a significant commercial impact.

Ion exchange techniques may have some use in the future but no information on their use in SCP processing is currently available.

Chemical processes may be simple and rapid but problems of chemical residues, expense and loss of nutritional value may limit their use (Sinskey & Tannenbaum, 1975).

Altering the cell growth conditions gives only limited nucleic acid reduction and could be economically unsound since the growth rates are lowered (Sinskey & Tannenbaum, 1975).

The use of acid or alkali hydrolysis creates problems of loss of nutritional value of the protein (Kihlberg, 1972). It is known that treatment of edible soy protein with 0.1 N NaOH for eight hours at 60°C leads to the production of a toxic amino acid, lysinoalanine. In addition there is considerable loss of several essential amino acids (Woodard & Short, 1973). Hedenskog and Ebbinghaus (1972) found that alkali treatment at pH 12 and 25°C or at pH 11.5 and 80°C for two hours led to considerable loss of lysine. On the other hand Canepa et al (1972) found no loss of alkali sensitive threonine, serine or cystine after treatment of whole cells at pH 12 and 25°C. It would appear that the cell wall provides some protection against alkali attack. Zee (1975) found that acid hydrolysis at pH 2, 90°C, 2 hours, led to losses of lysine, histidine and argenine. These were not so great however as losses due to alkali hydrolysis at pH 11, 90°C, 2 hours, after which there was also a considerable loss of methionine. The alkali hydrolysis processes of Newell et al (1975 (2)) led to loss of several amino acids, particularly cystine, methionine and lysine from C. utilis. The protein efficiency ratios (PER) for the LTHA and HTLA products were only 0.41 and 0.71 respectively when fed to rats. Supplementation of the LTHA product increased this to 1.87 (c/f casein, 2.5). Untreated cells, homogenised cells and protein concentrates with a high nucleic acid content all had PER's of about 1.6 without supplementation.

Heat precipitation of Bakers' yeast protein from alakline solutions

caused slight loss of lysine. The product had a PER of 2.09 compared with whole cells, 1.66 and casein, 2.26, when fed to mice (Hedenskog & Ebbinghaus, 1972; Lindblom & Mogren, 1974). However the product had a solubility of only 10% at pH 12 which would considerably limit its usefulness (Lindblom, 1974).

Endogenous nuclease processes following cell disruption seem to afford the best solution at present. Incubation at 50°C with 3%w/v NaCl gave a product from Bakers' yeast with a PER of 2.17 when fed to mice (c/f casein, 2.26) (Lindblom & Mogren, 1974). However the product was again rather insoluble due to the combined effects of heat and salt treatment. The process described by Robbins et al (1975 (1)) using endogenous nucleases following cell wall disruption and removal yields a product with a good amino acid spectrum and a PER of 2.1 when fed to rats (c/f untreated Bakers' yeast, 1.8; casein, 2.5). Furthermore the product had a bland flavour, was light cream in colour, dispersed easily and absorbed water and fat. It could be textured by thermal extrusion, gel coagulation or fibre formation. Extruded products were used in recipes for beef patties, chili con carne, pizza, sugar cookies, vanilla breakfast drink and a protein fortified caramel candy.

It is generally thought that the use of high proportions of SCP in human foods will not be a reality until the mid 1980's. However the publication of Anheuser-Busch patents has led others to follow suit since a number of other companies have recently filed patents in this field. Published investigations on the processing of SCP into textured food products probably represent

only a small fraction of the total research under way, since for the most part, only the results of academic research are openly available (Lipinsky & Litchfield, 1974). What is available is reviewed in the following section.

2.4. Textured Products.

An obvious outlet for high protein products such as soya or SCP is as a substitute for meat. Techniques have been developed by which less expensive vegetable proteins can be converted into textured products suitable for use as meat extenders or even replacers. Products must have meat-like texture and appearance, have good organoleptic properties (i.e. taste and smell), retain their texture after cooking, have a high nutritional value and, of course, they must be considerably cheaper than meat to make them attractive (Bender, 1975; Gutcho, 1973; United Nations, 1972 (4); 1973 (2)).

There is some dispute as to whether such processing is really necessary. The two points of view were put very forcibly in recent issues of the 'New Scientist.' Firstly, the attitude of a large food producing company:-

"if we are to encourage the consumer to use a higher proportion of proteins from vegetable sources, these must be processed into a range of attractive, nutritionally sound products." (Leslie & Sutton, 1975)

In a letter to the editor a few weeks later a Mr. Brunner replied:-

"There is an excellent low-energy technique for using soya beans. It runs: soak, season, boil, eat." (Brunner, 1976).

2.4.1. Textured Vegetable Proteins (TVP).

Many vegetable proteins have been used for producing textured protein products. Among these are soya, groundnut, rapeseed, sunflower, cottonseed, fababean, field pea, wheat gluten and zein. Animal proteins such as whey protein, casein and proteins from the wastes of the meat industry have also been investigated (Balmaceda & Rha, 1974; Boyer, 1953; Fleming et al, 1974; 1975; Naismith & Thompson, 1955; United Nations, 1973 (3); Young & Laurie, 1974). However by far the most commonly used material is soy bean meal remaining after the extraction of oil for the production of margarine, cooking oils and soap. Even so most of this meal is still used for animal fodder.

It is likely that in the near future SCP products will be textured using techniques borrowed from the TVP field. An understanding of such techniques will therefore be essential before attempts are made to produce textured microbial products.

2.4.1.1. Protein properties.

At this stage a brief look at some protein properties which are of particular importance in texturising processes is necessary. These may also help to explain some of the phenomena observed in protein processing which have already been discussed.

Proteins are amphoteric in nature, having in particular carboxyl and amino groups. At high pH's they display a net negative charge and at low pH's a net positive charge. In between there is a specific pH at which the net charge is zero. This is the isoelectric

point where the protein is generally least soluble and may often be precipitated out of solution. The actual pH at which this occurs will vary from protein to protein (Meyer, 1960; Taylor, 1964).

Primary protein structure consists of α-amino acids linked together by covalent peptide bonds to give high molecular weight polypeptide chains. Secondary structure is caused mainly by hydrogen bonding giving coiled or other protein configurations. This secondary structure is stabilised by electrostatic and Van der Waals forces. Finally two or more subunits may be linked together to give the final protein structure as is the case in many enzyme systems (Nozaki & Huyaishi, 1971). Different combinations of amino acids and different configurations lead to widely differing protein properties. Globular proteins (e.g. albumins) have a symmetrical structure and are generally quite soluble. Fibrous proteins such at keratin occur as asymmetric unfolded chains linking together and forming the large, insoluble aggregates of hair, bone , feathers, skin and other muscle tissue. Conjugated proteins which are complexes of globular proteins with non-proteinaceous material may also occur. For example, glycoproteins or mucoproteins are combinations of proteins with carbohydrates, nucleoproteins with nucleic acid and lipoproteins with lipids (Bate-Smith & Morris, 1952; Fox & Foster, 1957; Lundgren, 1949; Taylor, 1964).

These so called 'native' proteins may be denatured (i.e. their secondary and tertiary structures may be altered) by heating, extremes of pH, high salt concentrations, chemical agents, high shear forces, film formation and ultraviolet or ultrasonic treatment. This is particularly true of globular proteins where

the molecules unfold causing enhanced activity of their chemical groups. This may lead to aggregation of molecules by hydrogen disulphide and ionic bonds to form a stable gel lattice, to loss of biological (e.g. enzymic) activity and to loss of solubility. Such denaturation is generally irreversible (Bate-Smith & Morris, 1952; Taylor, 1953). Severe heat, salt or alkali treatment may lead to break-up of peptide bonds and to destruction or alteration of amino acids causing loss of nutritional value of the protein (Bender, 1972: Meyer, 1960). These denaturation processes in particular are of great importance during texturing.

2.4.1.2. Protein extraction.

Many processes have been described for extracting and concentrating protein from oilseeds but these are generally variations on the basic process which is described below.

1. Beans are cracked, dehulled and flaked.
2. Oil is removed by a combination of heating, milling and solvent extraction. Solvent extraction using hexane is the preferred method of producing high quality, undenatured protein isloates.
3. The defatted meal is extracted with dilute alkali at pH's greater than 7.0 and the insolubles are removed.
4. Protein is precipitated at the isoelectric point (generally pH 4.5 - 5.0), washed and centrifuged to give a light coloured protein isolate.

Further processing is aimed at reducing bitter 'off' flavours and producing better functional properties without suffering reduced protein yield. For some purposes protein concentrates (i.e. defatted meal plus slight further processing) will prove sufficient. For others and in particular those to be used in spun

products for meat analogues, bland, high protein isolates will be required. (Note that there is no clear boundary between concentrates and isolates but these terms are used extensively within this field). (Ashton et al, 1970; Burke, 1971; Leslie & Sutton, 1975).

Techniques for imparting texture to such products have been well documented, particularly in the patent literature. This has been reviewed by Ashton et al (1970), Burke (1971) and Gutcho (1973). Unless otherwise stated the information given below is drawn directly from these sources.

2.4.1.3. Gelation.

Beginning in 1956 a series of patents by M.L. Anson and M. Pader assigned to Unilever Ltd., were published relating to the production of 'chewy protein gels.' Heat stable gels were formed by heating a protein slurry at the appropriate pH, temperature and protein concentration. Gel formation involved an unfolding of the protein molecules to give an asymmetric and reactive structure followed by cross linking to form a '3D' network (Fleming et al, 1975). Disulphide bonds were thought to be of particular importance since gelation was inhibited by, for example sodium sulphite (Cirle et al, 1964). Heat denaturing the protein during extraction led to the production of more stable gels. Gel precursors (in this case protein mixtures which could be gelled on heating) of 20 - 30%w/w protein at pH 7.0 could be pushed through a die using a 'spaghetti' type extruder. The fibres produced were coated with binders and other agents and heat set by autoclaving.

Although this method of texturing has had little commercial impact

gelation is of great importance in other texturing processes. For convenience the methods have been catagorised but in practice there is a great deal of overlap between the processes.

2.4.1.4. Thermoplastic extrusion.

A plasticised mix of defatted oilseed flour, water and flavourings is subjected to a high temperature and pressure extruded through dies. On emergence steam flashes off causing expansion of the product and partial drying. The resulting material has an open, foam-like texture consisting of interconnected ribbons of varying widths and thicknesses. Early patents covered extrusion processes but it was not until the patent of Arnold et al (1966) assigned to Archer Daniels Midland Co. that a high pressure and temperature process resulting in an expanded product was described. Temperatures of 240 - 350^{o}F were used with pressures around 1,000psi. This led to high shear rates through the die causing some molecular orientation. Further orientation occurred during expansion. The pH was in the range 6.5 - 7.5. Up to 3% NaCl was added as this increased the firmness of the product on rehydration. Calcium chloride caused cross linking which improved the texture. Flavouring or colouring compounds could be added prior to extrusion. Solvent extracted soya meal was the preferred protein source. After extrusion the expanded product was cut into chunks and dried.

Such products are now widely used, especially in the U.S.A., as meat extenders in stews, sausages, pies, burgers etc., generally replacing 20 - 30% of the meat content (Shelef & Morton, 1976).

2.4.1.5. Spinning.

This rather misleading title describes those processes in which

a protein slurry at an alkaline pH (generally known as a dope) is extruded through a spinnerette containing many small holes into a coagulating solution, usually an acid salt bath. Fibres are drawn and stretched to give an elastic tow which, following further treatment, is made to resemble meat products. This is the most sophisticated texturising technology and has been the subject of a large number of patents. The products are considered to be superior in texture to those produced by extrusion or other methods.

In 1949 Lundgren wrote a comprehensive review of protein fibre spinning. His work still forms a valuable basis for understanding the processes involved even though it was again aimed at textile manufacture from protein fibres. The length of the protein chain is particularly important, a molecular weight in excess of 10,000 being required for good fibre formation. Fibre tenacity increases linearly up to a molecular weight of about 25,000 after which it ceases to have a great effect on fibre strength. Very high molecular weight material may be difficult to dissolve and therefore be unsuitable for fibre spinning (Lundgren, 1949; Moncrieff, 1975). Alkaline agents have been used widely to dissolve proteins prior to spinning. Too high a pH however may degrade the protein. Other dispersing agents such as urea, detergents, guanidine salts, formamide, sodium salicylate and lithium chloride have been used. Globular proteins are unfolded at high pH's causing an increase in viscosity of the solution. This dope is then passed through a spinnerette where partial alignment of the molecules into the coagulation bath occurs. The fibres are stretched causing further molecule alignment and allowing close contact between adjacent chains so that micelles (areas of crystalline structure) are formed. Chemical hardening agents such as formaldehyde have been

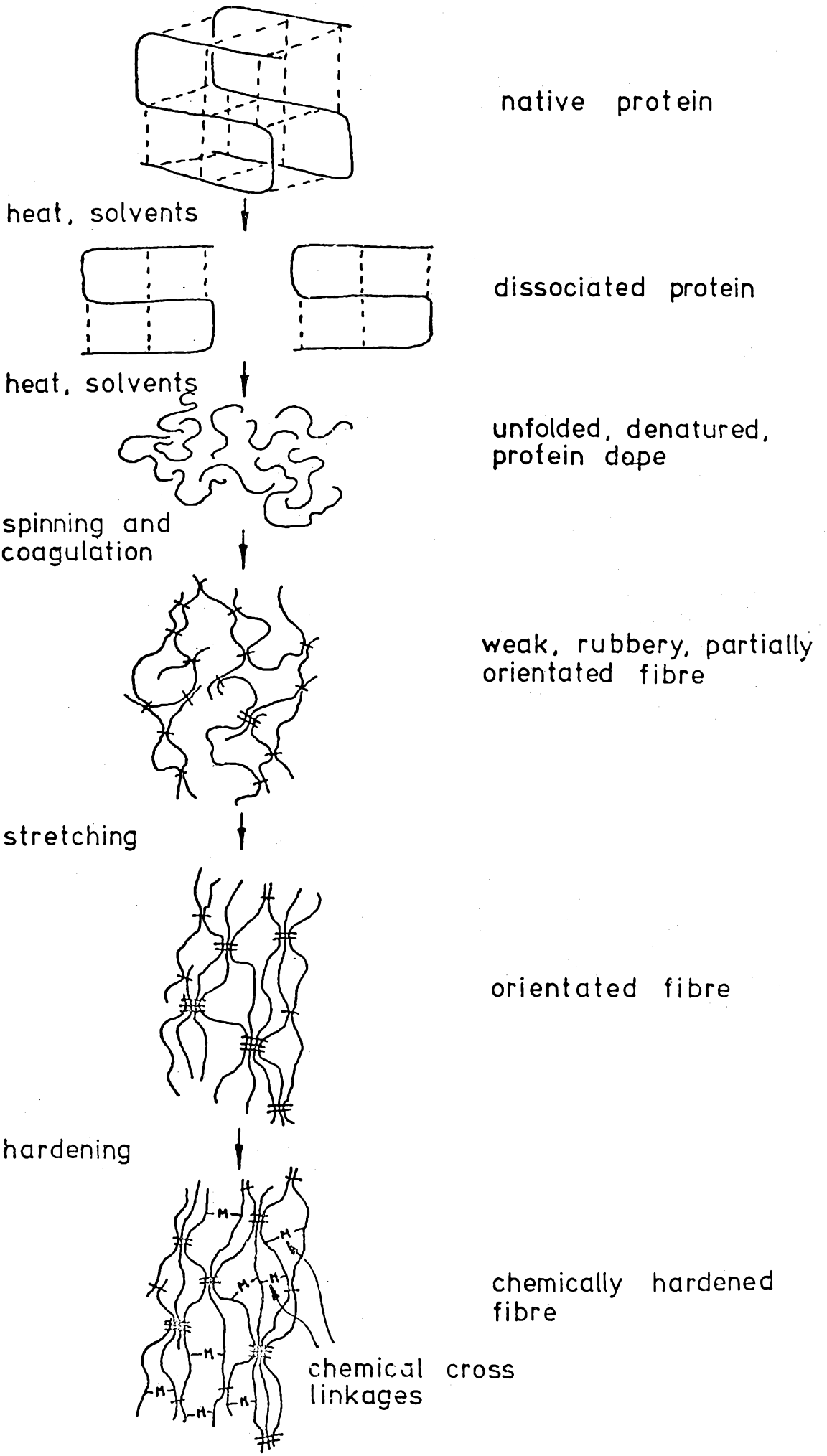
used to render the fibres less susceptible to alkali or detergent attack. Several bond types are formed during spinning including covalent bonds, the most important being disulphide bonds as well as the secondary hydrogen, ionic and Van der Waals bondings which serve to stabilise the structure. A diagramatic representation of this process is shown in Figure 2.4.1. The fibres show varying degrees of anisotropy (i.e. their properties are different in different directions) depending on the degree of molecular orientation. Highly orientated fibres are in general strong and brittle, have a low elongation at break, a high lustre, a low moisture absorbing capacity, high chemical resistance and a low dyeing affinity. Poorly orientated fibres are weak and pliable, have a high elongation at break, a low lustre, high moisture absorption, low chemical stability, low wet strength, a high dying affinity and are warm and soft to handle. Synthetic protein fibres are typical of the latter category (Lundgren, 1949; Moncrieff, 1975, Treloar, 1971; Zwick, 1967).

There are a large number of variables in the spinning process which determine the fibre properties. These include:-

1. Dope rheology. Viscosity increases exponentially with the protein concentration. It is also a function of pH, temperature and the concentrations of other additives. Dope viscosity rises to a maximum and then falls with time (dope 'ageing'). This increase is due to cross linking in solution and this is followed at a later stage by alkaline hydrolysis causing a drop in viscosity. The highest viscosity, short of gel formation, is reported to give the best fibres (Circle et al, 1964; Kelly & Pressy, 1966; Lundgren, 1949; Thompson and Johnston, 1947).

2. Shear forces. These will be governed by the rate of extrusion

Fig. 2.4.1. The Molecular Basis Of Protein Spinning.



through the spinnerette, spinnerette dimensions, drawing velocity and the length of the bath (Balmaceda and Rha, 1974; Watson & Hadley, 1973). Shear causes the molecules to be aligned in the direction of flow (Lundgren, 1949; Moncrieff, 1975). The shear history of the dope prior to spinning is also of importance and this may be considerably affected by the passage of the dope through filters (Ashton, 1974; Kaufman, 1969; Pearson, 1969).
3. Coagulating bath conditions. These include bath composition, temperature, fibre residence time and the diffusivity of the coagulating or solvent species. Rate of diffusion, which is also dependant upon fibre diameter, may be the rate limiting step in fibre spinning (Balmaceda & Rha, 1974; Booth, 1967; Paul, 1968).

Fibres may be catagorised according to tensile strength, elasticity, elongation at break and a numberof other parameters. Balmaceda and Rha (1974) suggested using spinnability curves to define the region in which fibre spinning was possible. Graphs of maximum drawing velocity at break versus bath length were drawn for a range of different spinning conditions. For textile use protein fibres must be strong, resistant to chemical attack and have a reasonably high wet strength. However for food product manufacture the desired fibre characteristics are not so easy to define. In this case strength must be balanced with elasticity, juiciness, succulence, bite, mouth feel, chewiness, flavour, colour and many other properties which are exceedingly difficult to define (Society of Chemical Industry, 1960). Although considerable advances have been made in quantifying some of these parameters, the only true test for such properties is the human taste panel (Leslie, 1975).

In 1953 Boyer's patent dealing with the manufacture of protein

fibres and their incorporation into food products was published. Textured food products resembling various kinds of meat were made from soy, groundnut, casein, cereal and fish isolates, generally up to 90% protein). An alkaline dope at high pH (up to pH 12) was pushed through a spinnerette similar to those used for rayon manufacture. The fibres were coagulated in an acid/salt bath and subjected to about 100% stretch. Excess water was removed by squeezing and the white, elastic fibres were neutralised to pH 6. Binders such as starch, flour, dextrins, proteins, gums, alginates or carboxymethyl cellulose (CMC) were applied and the fibres were treated with a fat, usually derived from the appropriate animal. Fibre tows were assembled, pressed together and cut into chunks or slices. Sodium chloride or Aluminium sulphate in the coagulating bath helped to stabilise the fibres and prevented their redissolution on cooking. Acids used in the bath included lactic, acetic, hydrochloric, citric or phosphoric, used at 0.5 - 10%w/w concentrations. There have been a large number of subsequent patents describing additives and techniques by which the Boyer process is modified. For example sulphites were used to improve texture, reduce off flavours and prevent bacterial contamination of the food. The addition of colours and flavours before spinning ensured even distribution rather than surface coverage of the fibre. Toughness could be regulated by pH control of the product. Spinning dopes at lower pH's prevented off flavours. The development of a continuous process which automatically mixed the protein slurry and the alkali allowed it to 'age' and to be pumped through the filter to the spinnerette has been achieved using a screw mixing device (Ashton et al, 1970; Boyer, 1953; Burke, 1971; Gutcho, 1973; Rosenfield, 1974; Westeen & Kuramoto, 1965).

A significantly different process was described by Tombs (1972) in a patent assigned to Unilever Ltd. High concentration protein Mesophase (or liquid crystal) solutions were prepared by dissolving relatively undenatured vegetable protein isolates in salt solutions of about 0.5 ionic strength at a pH close to the isoelectric point (Shaw et al, 1944). The protein solutions were fluid in the range 15 - 50%w/v though sodium sulphite was required to prevent gelation at high concentrations. This mesophase dope was heat settable so that it could be used as a binder or for production of 'chewy' gels. It could also be spun into a cold water bath in which the salt diffused out of the fibres causing them to coagulate. The fibres were then heat set at more than 90°C. If the spinnerette head was suitably cooled the fibres could be spun directly into a hot water bath. No stretching was required. This process had the advantage that high pH's, known to cause off flavours and loss of nutritional value, were not used and the dopes were not subject to any 'ageing' process, making control easier (Leslie, 1975; Leslie & Sutton, 1975; Tombs, 1972).

2.4.1.6. Comparison of processes.

Several processes other than those described here have been patented but had little commercial impact as yet. Thermoplastic extrusion is by far the most widely used method of texturisation, mainly because the process is simple, requires a relatively impure protein concentrate and the products are cheap being only one quarter or one third the price of meat. These are used as meat extenders, to give a low cost product, or food supplements to increase the nutritional value and improve functional properties but not as analogues since the texture and taste are not sufficiently convincing (utret & Roux, 1975; Martin, 1975; Ziemba, 1969).

Spinning produces a very high quality product but the processing is more expensive due to the need to use higher quality protein isolates and the more elaborate techniques required for texture and flavour production. The products cost about 25% less than meat but can be sold as meat analogues. 'Kesp' produced by Courtoulds Ltd. is such a product, being used to replace meat in pies and stews etc. (Watson & Hadley, 1973). Spinning is a useful guide to other functional properties, particularly solubility, as a soluble, relatively undenatured isolate is required. Furthermore it is easier to quantify the spinning process in terms of dope rheology and fibre properties and this makes it a useful research tool as well as a most effective texturing method.

One other point should be made at this stage. Much has been said about the use of TVP in meat products, but problems arise since for example, a steak pie in which the meat is replaced by TVP can no longer be called either steak or meat. Most meat products have a minimum meat content which is defined by law. Beefburgers for example must be 80% meat, beef curry, 35%, cottage pie, 25%, cornish pasty, 12½%, pork sausage, 65%, corned beef, 100%, Lancashire hot-pot, 25%. In products where meat has been replaced albeit by a highly nutritious TVP to a greater extent than the law requires, the above names may no longer be used. Furthermore terms such as 'Rancho', 'Chickful' or even 'Burger' may not be allowed as they suggest meat content. Spun products produced by Worthington Foods Inc. in the U.S.A. have trade names such as Fry sticks, Infasoy, Vegetarian burger, Veja-links and Wham slices (Ashton et al, 1970; Martin, 1975; Rank, Hovis, McDougal).

2.4.2. Textured SCP Products.

2.4.2.1. Spun products.

The first report of protein spinning using single cell protein was made by Heden et al in 1971. Bacteria grown on methane or methanol was disrupted by freeze pressing. The biomass was suspended in carbonate / bicarbonate buffer at pH's between 9.5 and 11 at concentrations of 0.1 - 0.7g Cells/ml buffer. After being stirred for two hours at 0°C the supernatant fluid was separated by centrifuging and used for spinning. The apparatus which had been developed for DNA spinning consisted of a syringe pusher which forced the dope vertically through a capillary into a coagulating bath containing a 0.7% propionic acid. The fibres were wound onto a teflon coated drum with 400 - 800% stretch. Capillaries of various sizes gave better results than the spinnerettes. Fibre formation was improved when the capillaries were heated to 80°C during spinning using a microwave heater. "Good" fibres were also produced if the cell walls were retained in the spinning dope. Bacterial fibres were superior to those produced from mixtures of bacterial and soya proteins. The nucleic acid content of the fibres was very high however, being 2o - 30% of the dry matter. It would have been interesting to see how the fibre formation was affected if the nucleic acid had been removed prior to spinning.

Huang and Rha (1971; 1972) extracted protein from dried Torula yeast using 0.05 N NaOH (Aries, 1952). Cell debris were removed by centrifuging and the protein was precipitated at pH 3.2 using 1 N HCl. It was found that the protein was soluble in 0.05 N NaOH

at temperatures up to 60°C after which heat coagulation occurred. This temperature was therefore used for spinning and for measurement of rheological properties. Dopes of 10, 15, 20 and 25%w/w protein at pH 8, 9, 10 and 12 were prepared and spun into air at 25°C using an Instron capillary rheometer (dry spinning). Fibres were stretched as they left the capillary but cooled and dried rapidly, after which no further stretch could be applied. Dried fibres exhibited quite a high tensile strength but lacked elasticity and resistance to shear. The best fibres were formed using a 20%w/w protein dope at pH 9 with a shear rate of 700 sec^{-1}. Capillaries of 0.1 to 0.6mm diameter were again found to give better fibres than spinnerettes. Dopes of less than 10%w/w protein were too thin for spinning, while at concentrations greater than 25%w/w the dopes were very difficult to handle, particularly due to air bubbles in the dope and the formation of rigid gels.
A Brookfield LVT viscometer with cylindrical spindles was used for rheological measurements. All the samples tested except for concentrations of 10%w/w protein or less and for 15%w/w protein at pH 10 and 12 showed yields stress values which generally speaking, increased in proportion to the apparent viscosities of the dopes. Apparent viscosities increased with dope concentration from less than 5 centipoise at 10%w/w to more than 30 poise at 25%w/w protein (pH 9). Highest viscosities were at pH 9 for 15, 20 and 25%w/w protein dopes. The dopes were time dependant and pseudo-plastic in nature, the apparent viscosity decreasing with increasing shear rate. Low shear rates of 1 - 10 sec^{-1} were used. It was postulated that increasing protein solubility occurred up to pH 9 followed by protein hydrolysis at higher pH's causing reduced viscosity. Gelation generally occurred at pH 12. The

apparent viscosities were much lower than those reported for other protein dopes used in spinning. Fibre strength was improved by the addition of carboxymethyl cellulose to the dope at levels of 0.5 - 2.0%w/w. Fibres spun into acid/salt baths (wet spinning) could not be stretched and lacked strength (Rha, 1975; 1976). The work of Daly and Ruiz (1974) has already been described (section 2.3.3.2). They attempted to combine fibre formation with nucleic acid removal using protein from Cellulomonas bacteria. However, fibres were very weak, although capillaries again gave better results than spinnerettes.

2.4.2.2. Extrusion and other processes.

Balmaceda and Rha (1973) investigated the coagulation rates of the protein dopes described above in a solution containing 14%w/v Na_2SO_4, 8%w/v $Al2(SO_4)_3$ and 10%w/v H_2SO_4 at 0, 24, 36 and 60°C. Dopes were held between glass plates and the coagulating medium diffused in from the edges allowing the position of the coagulation interface to be measured. The process was diffusion limited, the diffusion rate increasing with temperature and decreasing with increasing protein concentration in the dope. This work was carried out with a view to producing 'chewy gel' type products.

Hoer (1972) described a process for texturising vegetable or yeast proteins by pumping suspensions at pH 8 - 12 and concentrations of up to 35%w/w through a heat exchanger at 240 - 315°F and 50 - 5,000psi. The suspension was then forced through a narrow orifice and the textured product was collected. A protein content of greater than 70% was required in the material used.

Standard Oil (1973) patented a process for texturing a wide variety of bacteria and yeast products, although S. cerevisiae, S. fagilis, S. carlsbergensis and C. utilis were the preferred organisms. An aqueous paste of whole or partially broken cells at pH 6.0 was subjected to heating (212 - 275°F) and shearing in a screw extruder working at 50 - 1,000psi before being forced through a die into the atmosphere. The fibres produced were dried directly or autoclaved and then dried. The quantity of water used was just sufficient to produce a smooth paste, generally about 30%w/w. A ratio of whole to disrupted cells of 10:1 gave excellent results.

The process of Robbins et al (1975(1)) has already been described (section 2.3.3.8). They claimed that their product had fibre forming, thermal extrusion and gel coagulation properties, though the only details of texturisation were for a simple extrusion process in which a 40%w/w dry matter paste of the isolated protein was extruded through a meat mincer. The product was cut to the desired length and dried. The rehydrated product was used as a meat extender in several recipes.

2.5. Overall Process Analysis.

Looking at the complete process from biomass production to protein texturisation one clear fact emerges from the literature that research at an academic level and as far as is known at an industrial level has been very compartmentalised. Some workers report on SCP fermentation, others on drying, others on disintegration, protein extraction, nucleic acid removal or texturisation but with only one exception not an overall process. Hence most of the

successful nucleic acid removal processes involve heat treatment which reduced the protein solubility making texturisation more difficult. The exception is the research carried out at Anheuser-Busch Inc. They have produced a low nucleic acid product at high protein yield using simple techniques which had at least some functional properties. In addition the cell wall debris were not wasted but had a 'fat-like' feel and could be used as a fat replacer in salad cream, ice cream and dietary products. It contained mostly cell wall glycan, being about 90%w/w carbohydrate, 6%w/w protein, 2%w/w nucleic acid and 0.3%w/w lipid (Sucher et al, 1975). The supernatant remaining after protein precipitation contained low molecular weight vitamins, peptides and free amino acids, carbohydrates and the nucleic acid fragments. This could be evaporated to give a neutral flavoured paste suitable as a vitamin supplement or heat treated to give the characteristic yeast extract colour and flavour for use in spreads, stews and sauces (Robbins et al, 1975 (2)).

Clearly the development of integrated processes such as the one described above will be of prime importance as the producers of SCP seek to move from the animal feed market to the production of human foods containing a high proportion of microbial protein.

3. OBJECTIVES OF THE EXPERIMENTAL WORK.

As stated in section 2.5 there is a marked absence of literature available describing an integrated approach to extraction and texturisation of protein from microbial sources. It was therefore the primary aim of the present work to attempt to develop a complete process beginning with fresh microbial material and ending with a textured, high protein, low nucleic acid, cell wall free product suitable for incorporation in foods. Maintenance of a high protein yield was of obvious importance. Having established the primary objective it was possible to define more closely the scope of the work.

It was decided at an early stage to use a commercial fresh yeast as the starting material. Such a material would be of consistent quality and of food grade. Furthermore yeasts have in general been preferred to other microorganisms for SCP production (section 2.1.). In deciding to use fresh yeast there was already a departure from the current practice in the animal feed SCP field where the product is dried after fermentation. The reasons for using fresh, as opposed to dried, yeast have already been discussed in section 2.3.2.

At the other end of the processing operation it was decided to attempt spinning as a method of texturing, at least in the first instance. Spinning is an established and well documented technique and can be quantified more easily than other texturing processes in terms of dope rheology and fibre characteristics which could be related to previous processing operations. Continuation of the

process from fibre production to incorporation in foods was beyond the scope of the work.

The decisions to use fresh yeast and the spinning technique are discussed further in subsequent sections of this report.

In all experiments the aim was to use simple techniques and inexpensive chemicals. Although no detailed economic evaluation was planned processes which would obviously be uneconomical were not seriously considered.

The importance of retaining good functional properties in the protein and in particular of retaining high solubility prior to texturising, imposes a need to use mild processing conditions to avoid excessive denaturion of the protein.

Most workers in the SCP field have used stepwise extraction and precipitation techniques as outlined in Figure 2.3.3. (section 2.3.3.3.). It was decided to follow this general approach.

Finally it was hoped to gain a deeper understanding of the nature of some of the isolation and texturisation processes. In particular gel filtration studies were planned so that changes in molecular weight patterns could be monitered.

4. MATERIALS AND ANALYTICAL METHODS.

4.1. Materials.

4.1.1. Bakers' Yeast.

Fresh Bakers' yeast was used as the single cell protein source in all the experiments. It was chosen for the following reasons:-

1. It was a food grade product.
2. It was produced as a fresh yeast cake and had not therefore suffered any processing damage.
3. Many other workers in this field have used Bakers' yeast so that comparison with published date was possible.
4. It could be supplied locally on a regular basis by the Distillers Company Ltd.

4.1.2. Chemicals.

The chemicals used in this work are listed in Table 4.1.1.

4.1.3. Instruments.

Optical density measurements were made using a Unicam SP500 series 2 ultraviolet and visible spectrophotometer. In later experiments this was coupled with a Unicam SP40P automatic sample changer and a Unicam A45 linear/log Decade recorder. This gave a very satisfactory performance on batches of up to 40 samples, providing that a sample of high optical density was not immediately followed by a sample of much lower optical density, in which case there

Table 4.1.1. Chemicals.

chemical	grade	supplier
acetaldehyde	LR	BDH
acetic acid	AR	Fisons
acetone	LR	Fisons
alcohol dehydrogenase from yeast		Sigma
albumen, bovine serum	fraction V	Sigma
ammonium sulphate	AR	BDH
blue dextran	2000	Pharmacia
catalase, bovine liver	purified powder	Sigma
copper sulphate	AR	Fisons
deoxyribonuclease-1, bovine pancreas		BDH
deoxyribonucleic acid, calf thymus	sodium salt	Sigma
diphenylamine	AR	BDH
disodium hydrogen orthophosphate	AR	BDH
ethanol, 95%	LR	Fisons
ferric chloride	AR	BDH
Folin & Ciocalteu's phenol reagent	LR	BDH
hydrochloric acid, conc.	AR	Fisons
lauryl sulphate (SDS)		Sigma
lysozyme, egg white	grade 1	Sigma
manganous chloride	AR	Hopkin & Williams
2-mercaptoethanol	type 1	Sigma
orcinol	monohydrate	Sigma
pepsin, hog stomach		Sigma
perchloric acid	AR	Sigma

Table 4.11. Chemicals (continued).

chemicals	grade	supplier
phenol	AR	Fisons
ribonuclease, bovine pancreas		BDH
ribonucleic acid, yeast	type XI	Sigma
sephadex G50	fine	Pharmacia
sephadex G200		Pharmacia
sepharose 6B		Pharmacia
sodium azide	LR	Fisons
sodium bicarbonate	AR	Fisons
sodium carbonate	AR	Fisons
sodium chloride	AR	Fisons
sodium dihydrogen orthophosphate	AR	BDH
sodium hydroxide	AR	Fisons
sulphuric acid, conc.	AR	Fisons
thyroglobulin, bovine	type 1	Sigma
trypsin, bovine pancreas	type III	Sigma
uracil	LR	Fisons

abbreviations: AR - analytical grade reagent
LR - laboratory grade reagent
BDH - British Drug Houses Ltd.

was considerable contamination of the latter. Glass or silica cells of 1cm lightpath were used as appropriate.

A Pye Dynocap pH meter was used for all pH determinations.

Weighings for dry matter determinations and analytical use were performed on a Unimatic LLX4D balance. Other weighings were carried out using a Microwa 7720 torsion balance.

The following centrifuges were used as appropriate:-
The MSE Super Minor with swing out head; the MSE Major with swing out or angle heads; the MSE Refridgerated High Speed 18 with angle heads.

Finnpipette 1 - 5ml adjustable and Centaur 0.1 and 0.5ml single volume pipettes were used in later experiments. The Finnpipette was accurate and reproducible to within 0.5% over the range 0.5 - 5.0mls and the Centaur pipettes to within 1% using distilled water. Also in later experiments reagents used in analysis were dispensed by attaching Gordon Keeble polycarbonate syringe valves to 1, 5 or 10ml syringes and using these to deliver the required amounts of solutions into tubes for analysis. This could be done to within 1% accuracy and was a very much less expensive technique than using reagent dispensing bottles. The use of such equipment saved a considerable amount of time in the performance of routine analyses.

Other instruments used are described in the text.

4.1.4. Glass and Polypropylene Ware.

All glass and polypropylene ware, as well as other equipment, was thoroughly cleaned by soaking in detergent, washing and rinsing in tap and distilled water.

4.1.5. Water.

Distilled water was used throughout the experiments.

4.2. Analytical Methods.

4.2.1. Protein Determination.

The usual method for estimation of protein in food products is the Kjeldahl nitrogen test. The sample is digested with a catalyst and concentrated sulphuric acid, causing the conversion of nitrogen in the sample to ammonium salts. Ammonia is liberated by the addition of alkali and estimated titrimetrically after distillation into a known quantity of acid. It is commonly assumed that :-protein = total nitrogen x 6.25. However in microbial cells this can lead to considerable overestimation of protein since the nucleic acid in particular contains substantial amounts of nitrogen (Herbert et al, 1971; Meyer, 1960).

Ultraviolet absorbtion at 280nm by proteins has been used for analysis but is subject to gross errors due to the prescence of nucleic acid which absorbs 20 - 60 times more strongly than most proteins. Also absorption is dependant on the nature of the protein

and other factors (Herbert et al, 1971; Layne, 1957).

The biuret reaction provides a useful method of protein analysis. Cupric ions react in alkaline solutions with the peptide linkages of proteins giving an intense violet colour which can be measured spectrophotometrically, the optical density being proportional to the concentration of protein in solution. The results are similar for different proteins and there is no significant interference from nucleic acids or other cellular macromolecules. The test cannot be used in the presence of ammonium salts (Herbert et al, 1971; Layne, 1957).

Initial experiments were carried out using a 'Total Protein Biuret Kit' manufactured by Boehringer Mannheim G.M.B.H. The test solution contained 0.1 M NaOH, 16mM K-Na-tartrate, 15mM KI and 6mM $CuSO_4$. The potassium sodium tartrate and potassium iodide form soluble complexes with copper ions which are released on addition of protein (Gornall et al, 1949). Standard curves were prepared using bovine serum albumen solution. There were two major problems with this method. Firstly it was only suitable for use with protein concentrations greater than 20mg/ml although only 0.1ml of solution was required. Secondly and more seriously the alkaline extracted protein solutions obtained from disintegrated yeast suspensions, even after centrifuging at 40,000g for 30 minutes, were not optically clear, probably due to the presence of cell membranes and other colloidal material. Impossibly high protein concentrations were recorded due to turbidity. For this reason the method was abandoned.

The biuret method recommended by Herbert et al (1971) for whole

microbial cells was tried and found to be completely satisfactory. The protein solution or cell suspension is boiled in 1 N NaOH for five minutes to disolve protein. The solution is cooled and copper sulphate solution is added. The copper ions react with the peptide linkages giving the characteristic biuret colour. Excess copper ions are precipitated so that the blue copper sulphate colour does not interfere. The samples are centrifuged to remove the copper precipitate and the optical density is measured at 555nm. (Complete details are given in Herbert et al, 1971, p.247). Protein concentrations of 0.5mg/ml can be measured. The major advantage of this method is that during centrifuging the turbidity, still present after alkaline digestion, disappears, leaving an optically clear solution. Presumably the copper hydroxide precipitate interacts with the suspended, insoluble yeast particles causing them to be removed from the solution. Furthermore it was possible to use the same technique for whole yeast suspensions, solutions or precipitate suspensions. Results for whole cells were in excellent agreement with data supplied by the Distillers Company Ltd. (Grylls, 1974; Kelly, 1974). This method was used in all cases where the protein was available in sufficient concentrations.

The method described by Lowry et al (1951) involving the formation of copper complexes with protein followed by reduction of Folin and Ciocalteu's Phenol reagent to give a blue colour, is suitable for use in the range 20 - 300µg/ml protein. A linear relationship between optical density and protein concentration does not exist however and care must be taken in preparing standard curves. It is much more sensitive than the Biuret method but subject to

interference from a wide variety of compounds which also reduce the Folin reagent. Different proteins may give widely differing colour values due to the difference in their tyrosine and trypotophan contents on which the reactions largely depend. This method, as described by Herbert et al, was used to analyse fractions collected during molecular weight determinations by gel filtration where the protein concentrations were too low to be measured by Biuret (Herbert et al, 1971; Lowry et al, 1951). There was close agreement between the Lowry and Biuret methods for duplicate yeast protein samples. Bovine serum albumen was used as the standard protein in all cases.

4.2.2. Nucleic Acid.

Nucleic acids are generally estimated in one of three ways:-

1. Determination of phosphorous. This requires separation of RNA and DNA from each other and from other phosphorous containing material (e.g. phospholipids).
2. Determination of the ribose and deoxyribose of RNA and DNA by, for example, the orcinol and the diphenylamine reactions respectively. In this case interfering carbohydrates and sugars must be removed. These particularly affect the orcinol reaction which may lead to gross errors in estimating, for example, yeast RNA.
3. Determination of purine and pyrimidine bases by ultraviolet absorption.

In all cases extraction prior to analysis is critical. The following steps are of the greatest importance:-

1. Removal of low molecular weight acid soluble material, in

particular free sugars (these affect the orcinol reaction) and protein fragments (these affect ultraviolet absorption).

2. Removal of lipids. This is only necessary if phosphorous analysis is used.
3. Extraction. Extraction under varying conditions with alkalis or acids has been used to separate RNA and DNA. Too severe an extraction procedure may also extract unwanted, interfering material. Too mild conditions give incomplete extraction of nucleic acids - especially of DNA. In some cases it may be necessary to remove interfering material by ion exchange or other techniques (Deken-Grenson & Deken, 1959; Marcus & Feeley, 1962; Smillie & Krotkov, 1960).

No method has been found to be completely satisfactory and methods are chosen to suit the particular materials being analysed (Herbert et al, 1971; Munro & Fleck, 1966 (1);(2)).

Trevelyan outlines a method of estimating RNA in microorganisms by complexing the purine bases with silver. However his recommended procedure is rather lengthy, taking three days in all (Trevelyan,1975).

It was decided to adopt the basic extraction procedure recommended for microorganisms by Herbert et al (1971). Their procedure involved the following steps. Acid soluble material was extracted with 0.25 N $HClO_4$ at 0°C for 30 minutes. Perchloric acid was preferred to trichloracetic acid as it did not absorb ultraviolet light to any significant extent. The cells were then digested with 0.5 N $HClO_4$ at 70°C for 15 minutes, three digestion periods being sufficient to extract all the RNA and more than 98% of the DNA.

This procedure was modified as follows:-

1. The cold acid wash for the removal of low molecular weight material was omitted since this had already been substantially achieved by acid precipitation. Furthermore there was interest in the total nucleic acid content (including nucleotides etc.), not just in highly polymerised nucleic acid.
2. Only one period of extraction (15 minutes at 70°C) was used as this was found to give complete extraction of RNA if small samples were used for analysis (to give less than 50µg/ml nucleic acid in solution after extraction).

The standard procedure used for extraction was therefore as follows:-

1. A sample of washed cells or protein isolate was suspended in distilled water to give a dry solids concentration of 5 - 50mg/ml depending on the amount of nucleic acid in the sample.
2. 0.1ml of this suspension was pipetted into a 15ml glass centrifuge tube and to it was added 9.9ml 0.5 N $HClO_4$. After mixing this was put into a water shaker bath at 70°C and left for 15 minutes.
3. The tube was cooled to room temperature by immersing in cold water and then centrifuged at 2,000g for 10 minutes. The supernatant was used for analysis. Care was taken in separating the supernatant as the precipitate was very loose and easily resuspended.

RNA was measured by the orcinol reaction (Herbert et al, 1971, p.285), DNA by the diphenylamine reaction (Herbert et al, 1971, p.317) and total nucleic acid by ultraviolet absorption at 260nm and 280nm to allow for the absorption of protein fragments (Layne, 1957)

(see Figure 4.2.1.). Yeast RNA and calf thymus DNA were used as standards and these were treated in the same way as the samples to counter the hyperchromic effect (Munro & Fleck, 1966 (1)).

Results for the analyses of material precipitated at pH 3.8 following extraction of disintegrated yeast cells at various pH's are shown in Figure 4.2.2. Two points emerge:-

1. The DNA content of the isolates is negligable.
2. The values for total nucleic acid by UV are less than those for RNA by orcinol. This difference is particularly marked at pH 11 and 11.5. It is interesting to note that maximum carbohydrate extraction occurs at this pH so that it seems likely that the orcinol method gives readings which are too high due to interference from carbohydrates.

The UV spectra of yeast RNA, calf thymus, DNA and extracted isolates were measured over the range 220 - 280nm. Yeast RNA and the isolates had the maximum and minimum absorbancies at 260 and 230nm respectively compared with 268 and 230nm for DNA.

On the basis of these results it was decided to use the method of ultraviolet absorption, making allowance for protein in solution by measuring the optical densities at 260nm (absorption maximum for RNA) and 280nm (absorption for yeast proteins). A solution containing 40µg/ml of pure yeast RNA gave an optical density of 1.0 at 260nm after treatment as described above. It was found that where the ratio optical density at 280nm / optical density at 260nm $\frac{E280}{E260}$ was less than 0.6, the nucleic acid content of the isolate calculated according to Layne (1957) was in close agreement with that calculated from the optical density at 260nm alone.

Fig. 4.2.1. Calculation Of Nucleic Acid And Protein Contents By Absorption At 260nm And 280nm. (Constructed from data for yeast RNA and yeast enolase given by Layne, 1957)

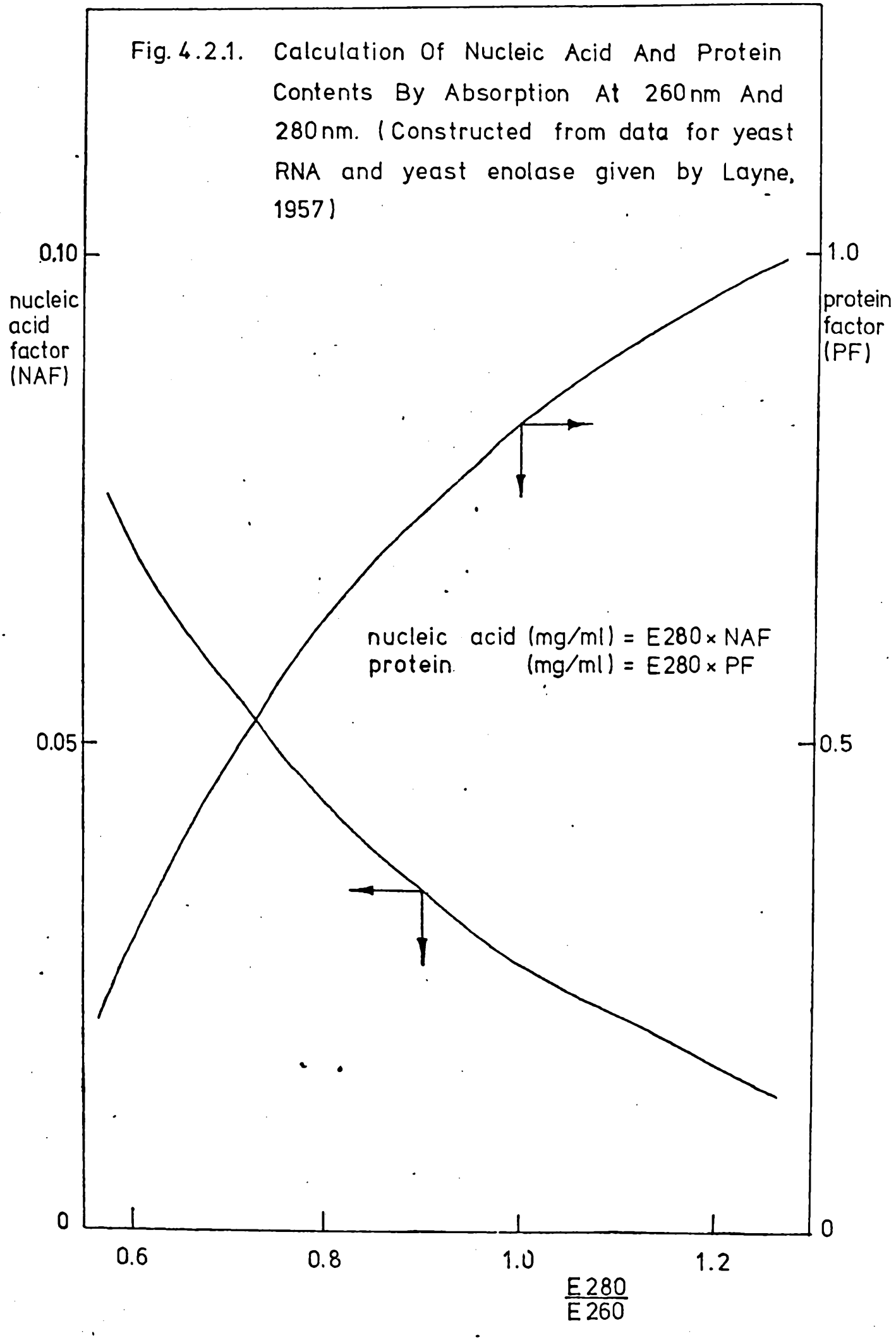
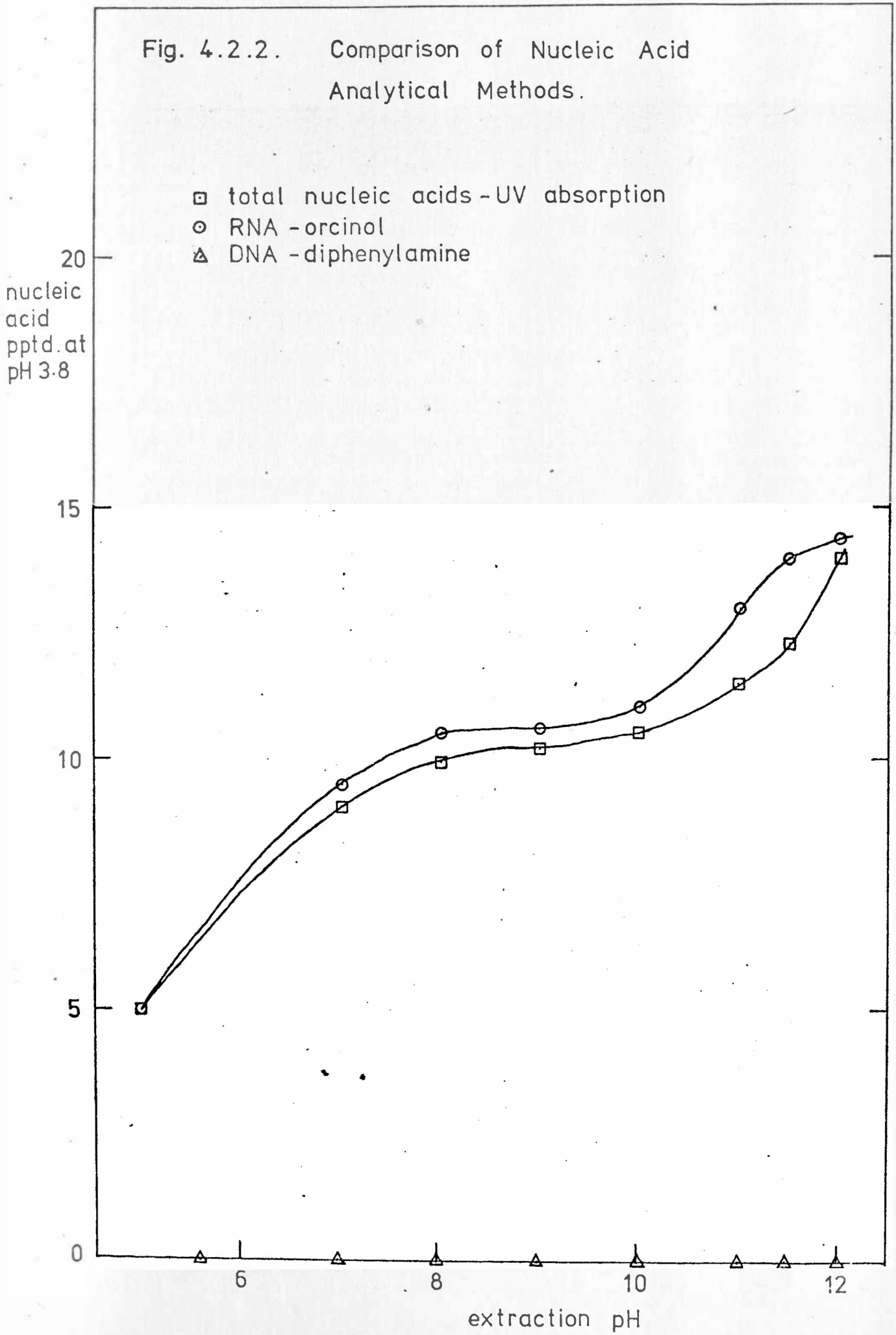


Fig. 4.2.2. Comparison of Nucleic Acid Analytical Methods.



4.2.3. Total Carbohydrate.

The phenol method described by Herbert et al (1971, p.272) was used for determination of total carbohydrate. This gave higher readings than the Anthrone test since it measured not only hexoses but also nucleic acid sugars. However unlike the Anthrone test it gave almost identical colours for all hexoses and was also much simpler to use. 1ml samples containing 20 - 100µg glocose equivalent were mixed with 1ml of 5%w/v phenol in a thick walled test tube. 5ml of concentrated sulphuric acid were added rapidly and mixed with the other solutions. Considerable heat of mixing occurred which was sufficient to complete the reaction. The tubes were cooled and the optical density was read at 488nm. Cells or protein isolates were completely dissolved giving optically clear solutions.

In practice this method did not give very reproducible results - the optical densities of duplicate samples differing by up to 20%. The temperatures of the solutions in the tubes were measured. Some reached only 90°C whereas others exceeded 110°C after H_2SO_4 addition, even in similar tubes. The following method was therefore investigated:-

1. Prepare the sample and add the phenol solution to it using pyrex tubes as recommended by Herbert et al (1971).
2. Immerse the tubes in cold water, about 20°C, to prevent temperature rises greater than 100°C. In practice the temperature did not rise to more than 80°C using this method.
3. Slowly add the 5ml H_2SO_4, shaking carefully in the water bath.
4. Allow all tubes to cool and then immerse in a boiling water bath. Heat for 15 minutes.

5. Cool in a water bath to room temperature and leave for 20 minutes. Measure the optical densities at 488nm.

Using this method the results were highly reproducible, presumably due to the uniform heating of all the samples. Glucose was added as the carbohydrate standard.

4.2.4. Dry Matter.

Samples were dried to constant weight (usually overnight) at 105°C using glass weighing bottles. Prior to weighing the samples were stoppered and desiccated at room temperature. Unless otherwise stated protein, nucleic acid and carbohydrate contents are given as percentages of the dry matter content.

4.2.5. Molecular Weight Determination.

Molecular weight determinations were carried out using Sephadex G50 and G200 and Sepharose 6B gels packed in Pharmacia K26/40 column. Samples were eluted with 0.1 M carbonate / bicarbonate buffer at pH 10.0 and 0.02% sodium azide was used to prevent bacterial contamination. Columns were initially run in a cold room at 5 °C but it was found that clogging of the gel, due to the presence of suspended particles in the samples even after centrifuging at 40,000g, occurred long before bacterial growth became a problem. Therefore most experiments were performed at room temperature. The high pH was necessary to dissolve the protein in the samples. 1%w/v sodium dodecyl sulphate (SDS) in 0.1 M phosphate buffer at pH 7.0 was used in some experiments in order to dissociate non-covalent bonds (Andrews, 1965; Pharmacia,

1972 (1);(2); Weber & Osborn, 1969; Weber et al, 1972). 5ml fractions were then collected and these were analysed for nucleic acid by absorption at 260nm and 280nm (Layne, 1957) and for protein by the Lowry method (Lowry et al, 1951). In some cases a crude estimate of the protein content of the samples was made using the method of Layne (1957) by simply measuring the optical densities at 260 and 280nm. Gross errors occurred due to the high nucleic acid content of the samples but the shape of the protein elution curves were still in good agreement with those determined by the Lowry method. In general 80 - 85% of the protein applied to the column was accounted for by summation of the protein contents of the fractions between the void volume (v_o) and the elution volume for uracil (v_e). *

4.2.6. Protein Solubility.

The solubility of protein at room temperature was measured at different pH's. Generally 1g of wet protein isolate was suspended in distilled water to give 10ml. 1N NaOH solution was added until the required pH was obtained. A sample of the suspension was taken for protein analysis and the remainder was centrifuged at 40,000g for 15 minutes. A sample of the supernatant was analysed for protein. Solubility was defined as

$$\text{Percentage solubility} = \frac{\text{protein concentration in supernatant}}{\text{protein concentration in suspension}} \times 100$$

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* Most of these determinations were carried out by Miss R. Sawicka of the Institute of Fermentation Industries, Warsaw, Poland, whilst on a U.N. scholarship in Birmingham. Some determinations were subsequently made by Mr. N. Tongue of the Biochemical Engineering Section of this Department.

Protein solubility is dependant on concentration so that 10%w/v (wet isolate basis) suspensions were generally used (about 20mg protein/ml). This gave approximately the same protein concentration as occurred during alkaline extraction.

4.2.7. Measurement of Fibre Strength.

It was found on starting the work on fibre spinning that fibres were generally very weak and difficult to handle. This made measurement of fibre tension by standard methods, as outlined by Moncrieff (1975), impracticable. A more simple and rapid method was therefore devised, suitable for use with even the weakest fibres. This is described below.

A single fibre strand was taken from the coagulating bath by hooking it round a spatula and drawing it up vertically into the air in the wet state and measuring the maximum length of fibre which could be held under its own weight before breakage occurred. In practice this proved to be a most useful technique since the maximum height at which a fibre could be held above the bath (250cm - the height of a man standing above the bath with arm outstretched vertically) was approximately the same as the height to which the strongest fibres encountered could be raised. As this was a rapid technique, easy to perform, it was used throughout the experiments. In general three measurements were taken and the mean value was recorded. For a particular set of conditions on any spinning run the technique proved to be very reproducible.

The force per unit area acting on fibres of the same length but different diameters, assuming that their densities were

similar, was theoretically the same. Hence it was possible to compare the strengths of fibres spun from different diameter capillaries.

Immediately after emergence from the capillary into the bath the fibres were very weak, since they had not had the time to coagulate. In practice fibres gained their full strength after about 1 - 1½ minutes, after which no noticeable increase in strength took place. During this period the colour changed from the brown colour of the dope to the light cream colour of the fully coagulated fibres (see section 5.5). For this reason the test for fibre strength was not carried out until fibres had been in the coagulating bath for two minutes.

5. EXPERIMENTAL PROCEDURE AND RESULTS.

In this section the experimental procedures and results are shown. The nature of the work was such that experiments often followed directly as a result of previous experiments. Therefore a considerable amount of discussion of the results is also included so that the final discussion, section 6, is for the assessment of the overall process rather than individual experimental details.

5.1. Yeast Suspensions.

A weighed amount of fresh yeast was crumbled into a beaker and suspended in sufficient distilled water to give a suspension of approximately 30%w/v wet yeast cake. This was centrifuged at 2,000g for 10 minutes and the supernatant was discarded. The yeast cake was covered in a thin layer of brown precipitate, presumably products of autolysis since the amount of this substance increased with yeast age. The cake surface was washed to remove this substance and resuspended in distilled water to a similar concentration. This was centrifuged again, the supernatant discarded and the cake surface washed. The washed cake was suspended in distilled water to the required concentration. The loss of dry matter during washing was negligible. The analysis of such a suspension is shown in Table 5.1.1., overleaf.

The dry matter content of the fresh pressed yeast cake was always between 28.5 and 29.5%. However, on storage for five days at 4°C this increased to 31.5% and the cake became cracked and brown on the outside. The yeast was therefore loosely wrapped in

Table 5.1.1. Typical Analysis of Bakers' Yeast.

component		analyses (section 3.2) -mean values	data from the Distillers Co. Ltd. (Kelly, 1974)
dry matter content (pressed yeast cake)		29%	28 - 29%
% of dm	protein	45	44 - 45
	nucleic acid	7.5	5.5 - 6.5
	carbohydrate	44	40
	lipid	not determined	2

aluminium foil to prevent dehydration and stored at 4°C until used - usually within three days of delivery. Protein content was within the region 43 - 46%. The carbohydrate content was about 44%, 4% higher than the data supplied by the Distillers Co. Ltd. suggested (Kelly, 1974). This is presumably due to the nucleic acid and ribose. Herbert et al (1971) report that yeast RNA absorbs about half as strongly as glucose at 488nm following phenol / H_2SO_4 treatment. At an RNA level of 7% this would give the impression of an extra 3.5% carbohydrate, which accounts for the above discrepancy. The nucleic acid content varied widely between 5.5 and 8.5% for different batches of yeast. This variation in protein and in nucleic acid contents in particular was also found during protein processing. Trends were always the same but absolute values varied considerably from batch to batch.

5.2. Cell Disruption.

Of the methods described in section 2.3.2. only sonication was available in the laboratory as a method of cell disruption capable of producing sufficient quantities of relatively undenatured protein to be used in texturising experiments. It was appreciated that this method was not entirely satisfactory as it was known to denature some enzymes and other proteins and was rather slow compared with liquid shear methods. The instrument used was a Dawes 125 watt Soniprobe 7530 - 1A and generator 7530 - A with a $\frac{1}{2}$" titanium tip. It will be appreciated that this series of experiments was designed to find the conditions for maximum protein release per unit time rather than rigorously to examine the sonication process.

5.2.1. Assessment of Disruption.

During initial trials rather qualitative microscopic tests of cell disruption were made using smears stained with Gram's stain (without counterstaining by Carbol Fushin). Intact cells were stained whereas broken cells were not. This was presumably because the stain was held by the viable cells whereas it was easily washed out of disrupted cells during the acid / alcohol washing stage.

Protein release was estimated by taking a small sample of sonicated cell suspension diluting with sufficient distilled water to give a solids concentration of about 5mg/ml and centrifuging for 10 minutes at 2,000g. The protein content of the supernatant

was measured.

There was good agreement between the two methods. In later trials protein release alone was used to assess disruption. Prolonged sonication led to release of about 95% of the total protein in the cell, after which no intact cells were observed. It was therefore concluded that about 95% of the cell protein was water soluble at a solids concentration of 5mg/ml (i.e. about 2.5mg/ml protein)

5.2.2. Batch Sonication.

5.2.2.1. Procedure.

Initial tests using 50ml of 10%w/v yeast suspension in a glass beaker held in an ice bath proved unsatisfactory due both to the poor disruption efficiency and the high rise in temperature ($10^{\circ}C$ - $44^{\circ}C$ in 30 minutes).

A Dawes 'Rosett' type glass cooling cell, suitable for continuous operation, was tested and found to give a much more satisfactory performance (Rosett, 1965) (Figure 5.2.1.). Cooling water was passed through the outer jacket and the inner jacket was filled with yeast suspension to the overflow level, 45ml. The sonicator tip was immersed in the suspension to a depth of about 1cm (this was not found to be critical). Full power gave the highest disruption rates and was used in all subsequent experiments. The whole apparatus was enclosed in a soundproofed box.

5.2.2.2. Use of glass beads.

As already stated the use of glass beads during sonication is

Fig. 5.2.1.(a). Sonication Apparatus Using The Rosett Cooling Cell

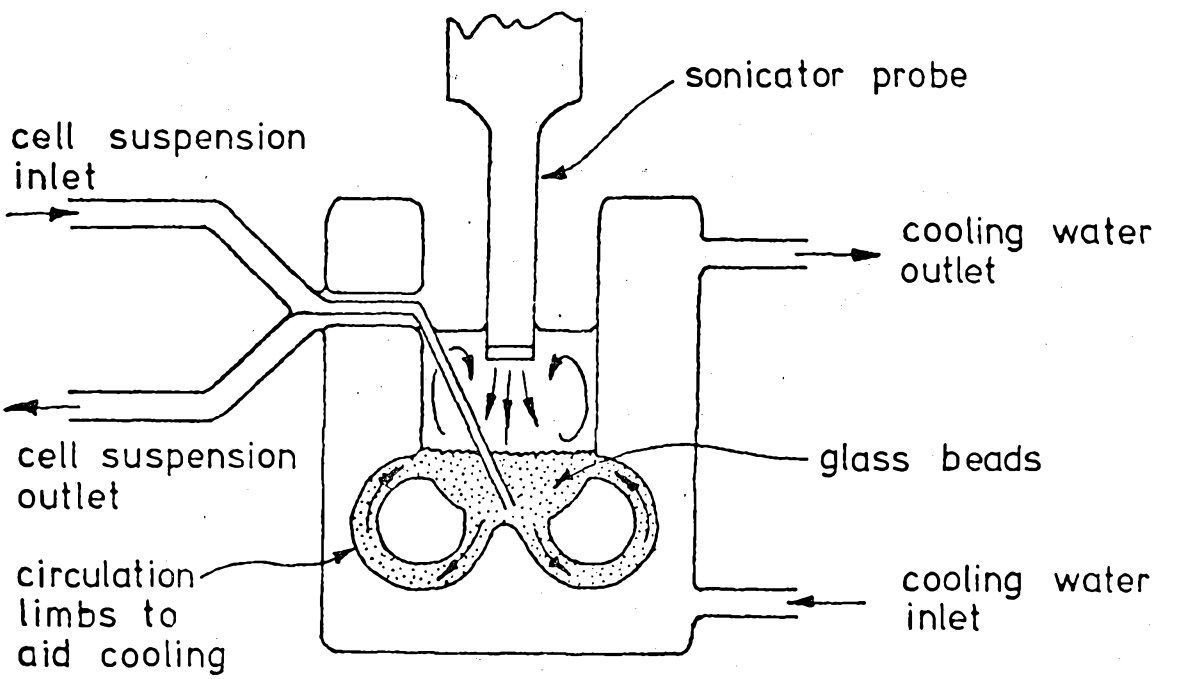
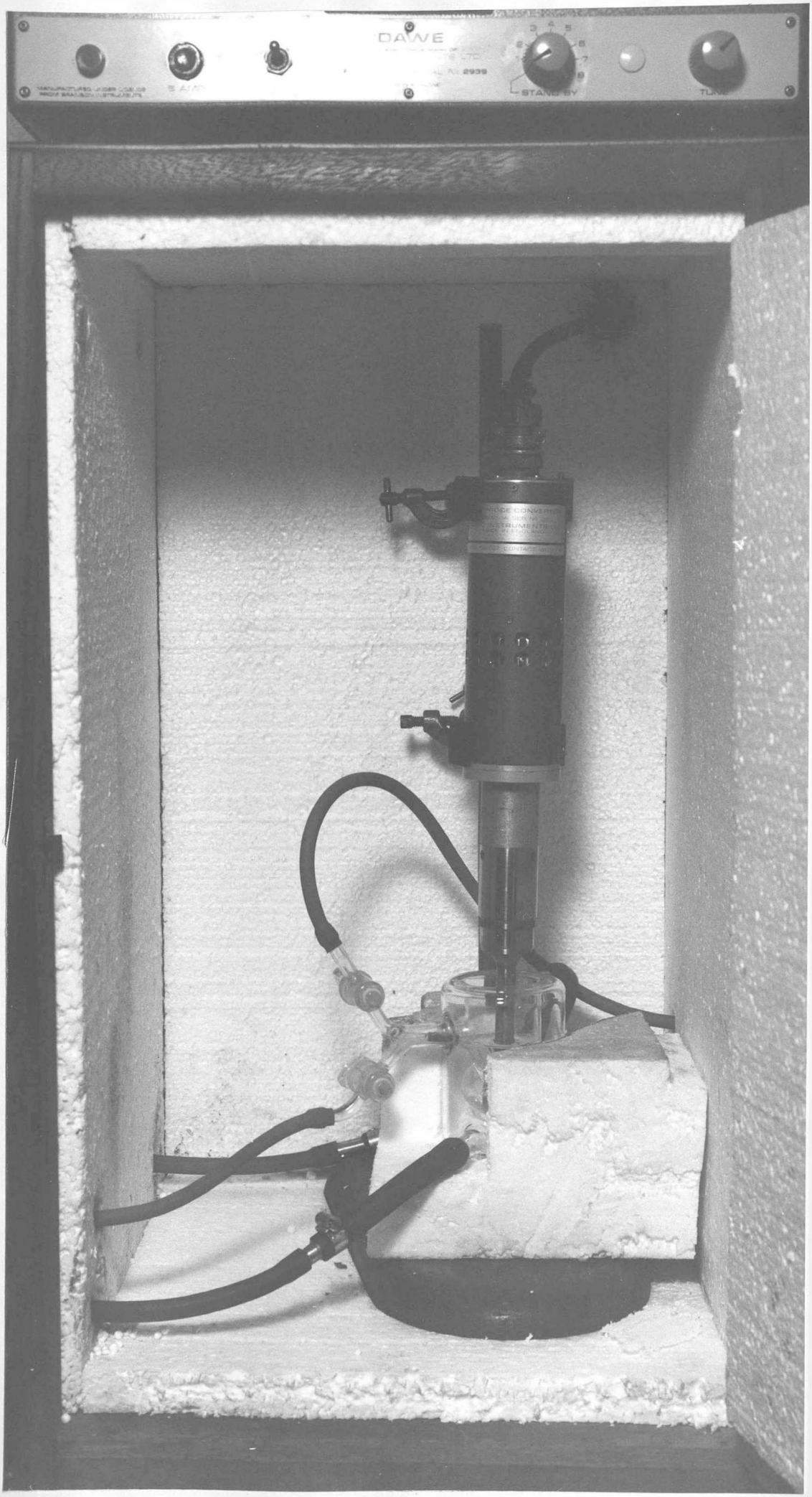


Figure 5.2.1.(b). Sonication Apparatus - Rosett Cell.



reported to increase the rate of cell disruption (section 2.3.2.1.). Disruption rates increased with increasing bead to suspension ratios but this also lowered the effective chamber volume and led to large numbers of beads being lost in the overflow during continuous operation. 10ml of beads was considered optimal. The voidage of the beads was approximately 0.5 so that the effective chamber capacity was reduced from 45 to 40mls. The results for using bead sizes of 0.05mm ø to 1.4mm ø (glass ballotini grades 20 - 3) are shown in Figures 5.2.2. and 5.2.3. Larger beads were violently agitated and circulated at speed through the cooling tubes. Smaller beads appeared to form an "emulsion" with the cell suspension so that there was little vibration of the beads as they flowed around the cell. Cooling water at 15°C was used and a temperature of 18°C in the suspension could be maintained with beads greater than 0.2mm ø. Cooling was less efficient with smaller beads and with no beads the suspension reached 24°C. It would appear that the beads, if large enough to prevent "emulsification" scoured the surface of the glass breaking up the laminar boundary layer and causing a 'scraped' heat exchanger effect which gave greater cooling efficiency.

From Figure 5.2.2. and Figure 5.2.3. it is clear that of the grades tested, grade 6 ballotini gave the most rapid protein release. This is particularly interesting since Hughes et al (1971) suggest that glass beads increase sonication efficiency as a result of increased numbers of nucleation sites. If this were the case the disruption rates should be highest with the smallest beads since these have the highest surface area / unit volume. Powdered glass (results not shown) gave slightly worse disruption than operation with grade 20 glass beads. The optimum bead size, about

Fig. 5.2.2. Effect of Glass Bead Size on Protein Release - 1. (6% w/v dm. yeast susp'n.).

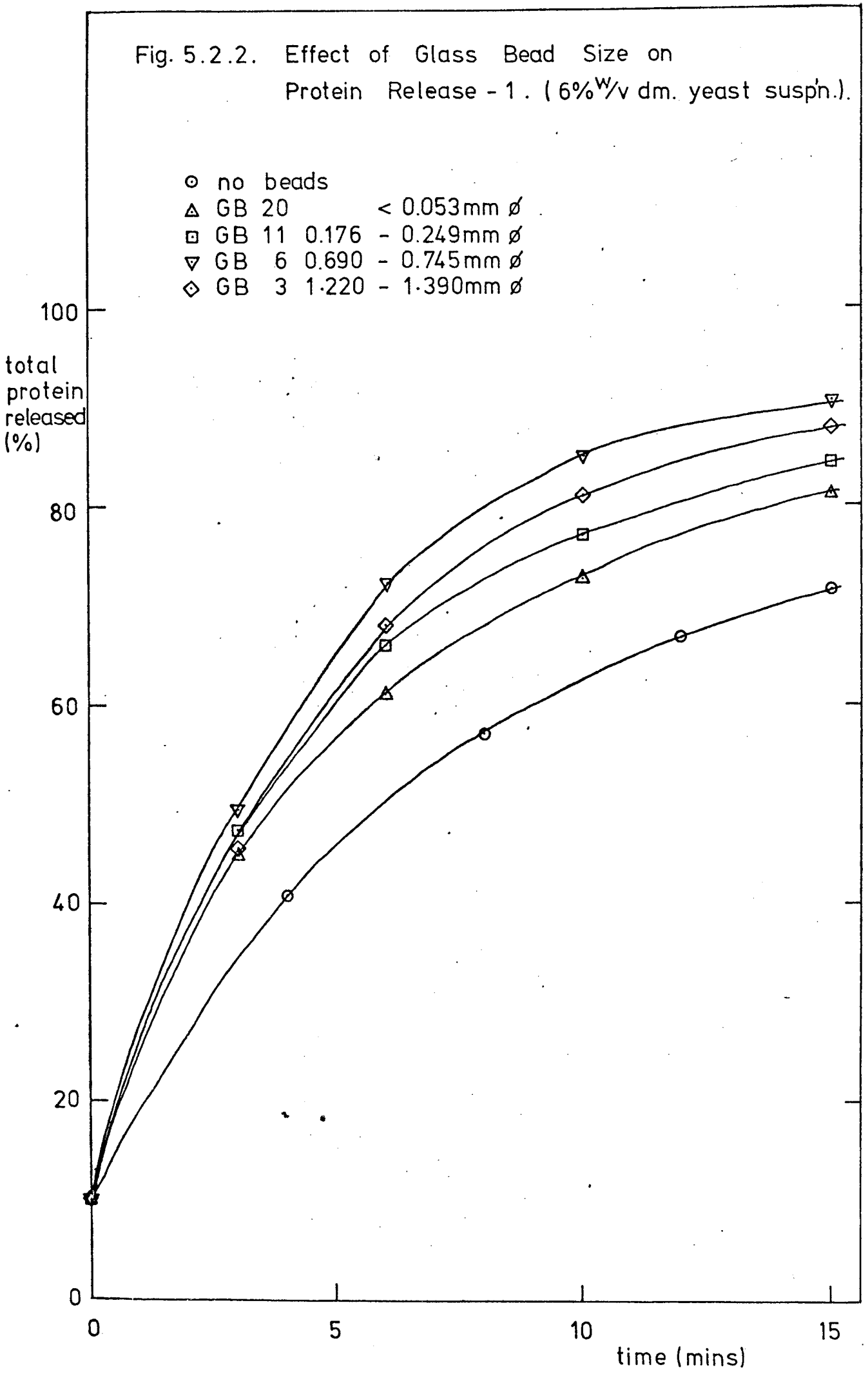
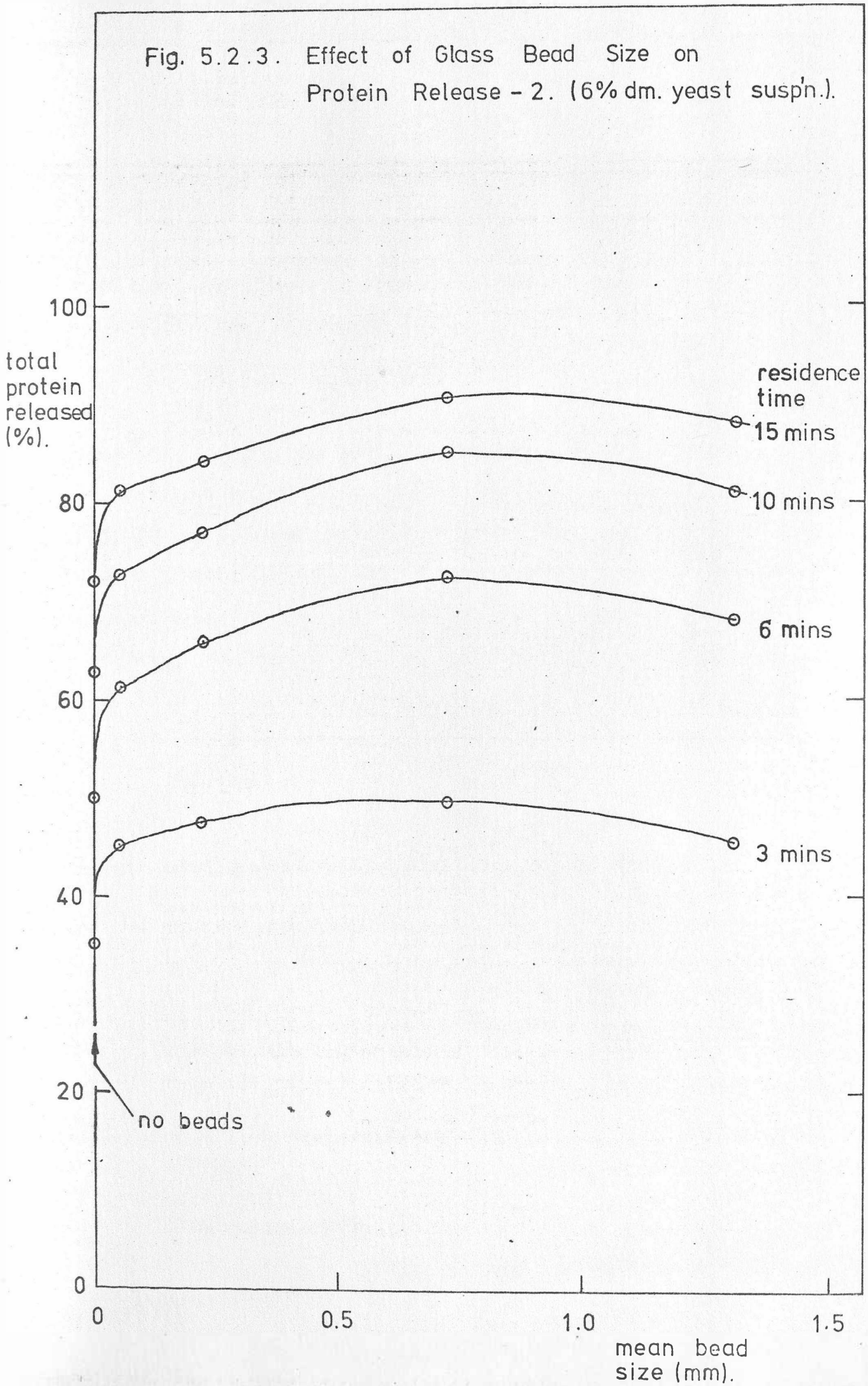


Fig. 5.2.3. Effect of Glass Bead Size on Protein Release - 2. (6% dm. yeast susp'n.).



0.7mm ø is similar to that used in the bead mills described in section 2.3.2.3. in which the cavitation does not occur and cells are disrupted by the high shear forces present. With the above apparatus increased liquid shear forces rather than increased cavitation would therefore seem to be the reason for higher efficiency with glass beads.

5.2.2.3. Effect of yeast concentration.

Variation of yeast concentrations in the range 6 - 15%w/v did not result in any very significant difference in the disruption rate. 3% yeast suspensions gave slightly lower rates of disruption (Figure 5.2.4.). This concentration independance seems to be true for most types of disruptors in the range of 5 - 20%w/v (Brookman & Davies, 1973; Dunnill & Lilly, 1975; Hetherington et al, 1971: James et al, 1972; Mogren et al, 1974).

5.2.2.4. Kinetics.

As already stated James et al (1972) showed that for a batch process yeast disruption by sonication was essentially a first order process and could be described by:-

$$(1 - x) = e^{-kt}$$

where k = protein release constant

x = fraction of the total releasable protein.

t = time of treatment.

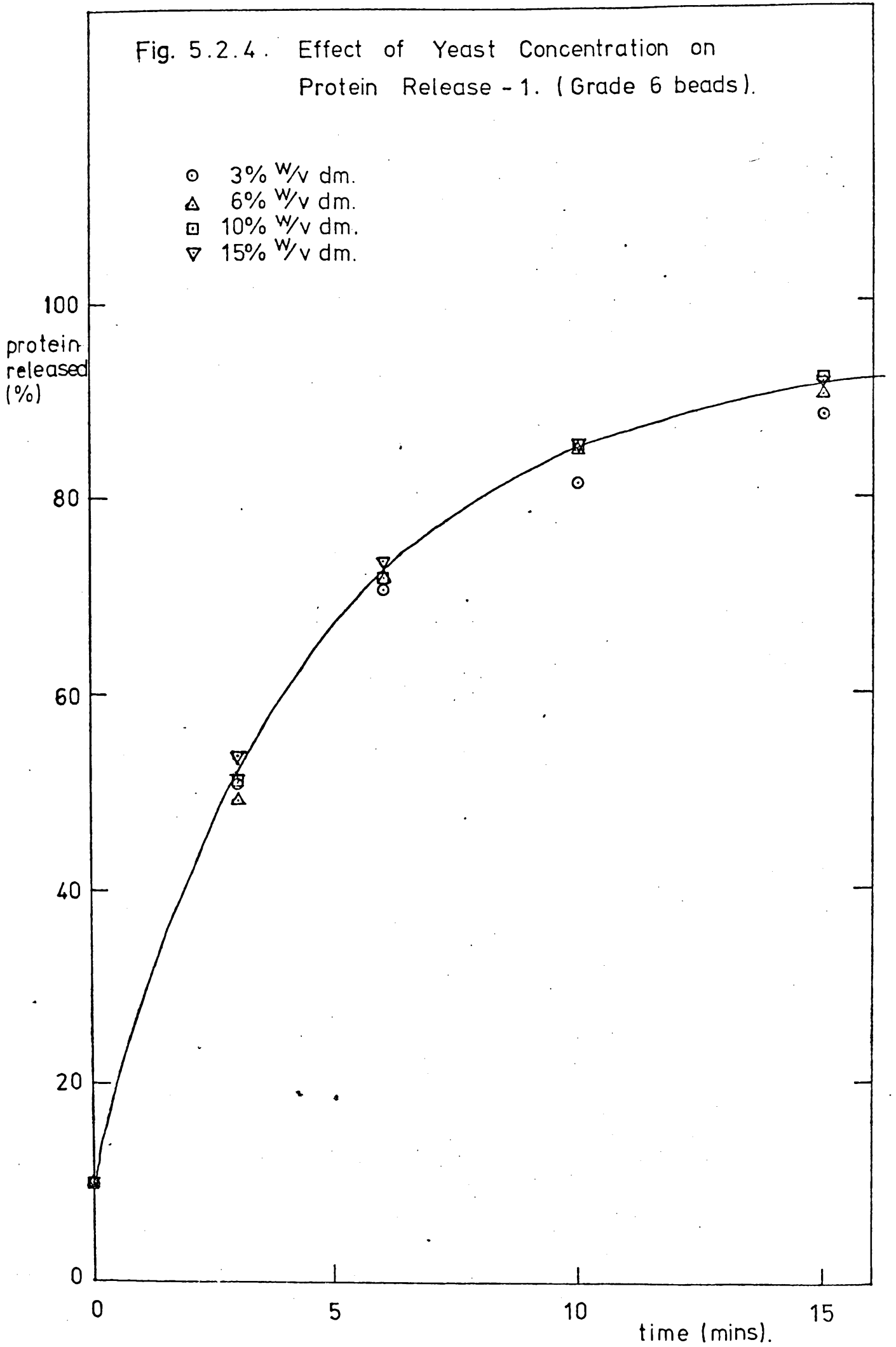
Applying this to our work we have:-

$$\ln(1 - x) = - kt \quad \text{or:-}$$

$$\log (1 - x) = - k't$$

A plot of log (1 - x) vs. t should yield a straight line, slope - k'. Total releasable protein was taken as 95% of the total protein in

Fig. 5.2.4. Effect of Yeast Concentration on Protein Release - 1. (Grade 6 beads).



the cells. Such graphs for different bead sizes and different concentrations are shown in Figure 5.2.5. and 5.2.6. There is obviously considerable departure from linearity as disruption proceeds particularly in the case of small bead sizes and for low concentrations of yeast. The major reason for this would appear to be the changing flow characteristics due to release of high molecular weight cell material on disruption, causing increased viscosity. High viscosity is known to inhibit cavitation and hence reduce disruption efficiency (Hughes et al, 1971). It may be that for large bead sizes disruption by cavitation is less significant than that by liquid shear forces which is not so viscosity dependant. At low yeast concentrations viscous effects may be more marked since the initial suspension viscosity is low (only about 2cp for 3%w/v yeast suspensions) so that viscosity increases may be proportionally much greater than for higher concentrations where the initial suspension viscosity may be 100cp or more (15%w/v yeast suspension) (Labuza et al, 1970 (1)).

5.2.2.5. Soniprobe tips.

Titanium tips were found to be very susceptible to pitting, particularly when used with glass beads. Disruption efficiency decreased with increased pitting. Stainless steel and hard steel tips were tried in an effort to prevent rapid erosion and to reduce cost (new titanium tips were about £8 each (1976)) but these gave much lower disruption rates, particularly in the case of hard steel tips. The best solution was found to be to remove the titanium tips which simply screwed into the sonicator horn, and machine them flat when the pitting reached a depth of about 0.5mm, usually after 20 hours use (Hughes, 1975). In this way high efficiency was maintained and the tips had a longer life.

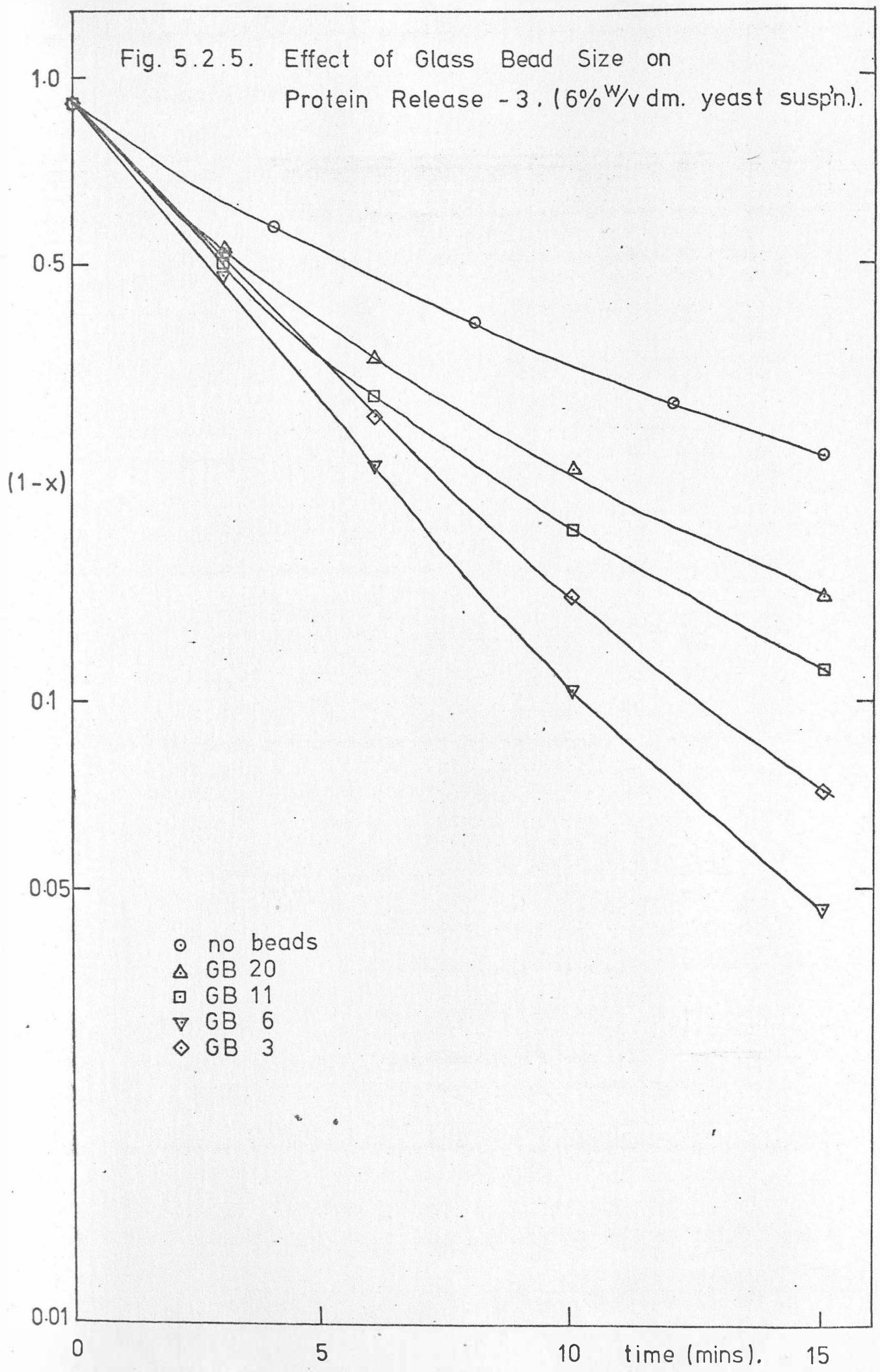


Fig. 5.2.5. Effect of Glass Bead Size on
Protein Release - 3. (6% w/v dm. yeast susp'n.).
1·0
0·5
(1-x)
0·1
0·05
0·01
no beads
GB 20
GB 11
GB 6
GB 3
0
5
10
time (mins).
15

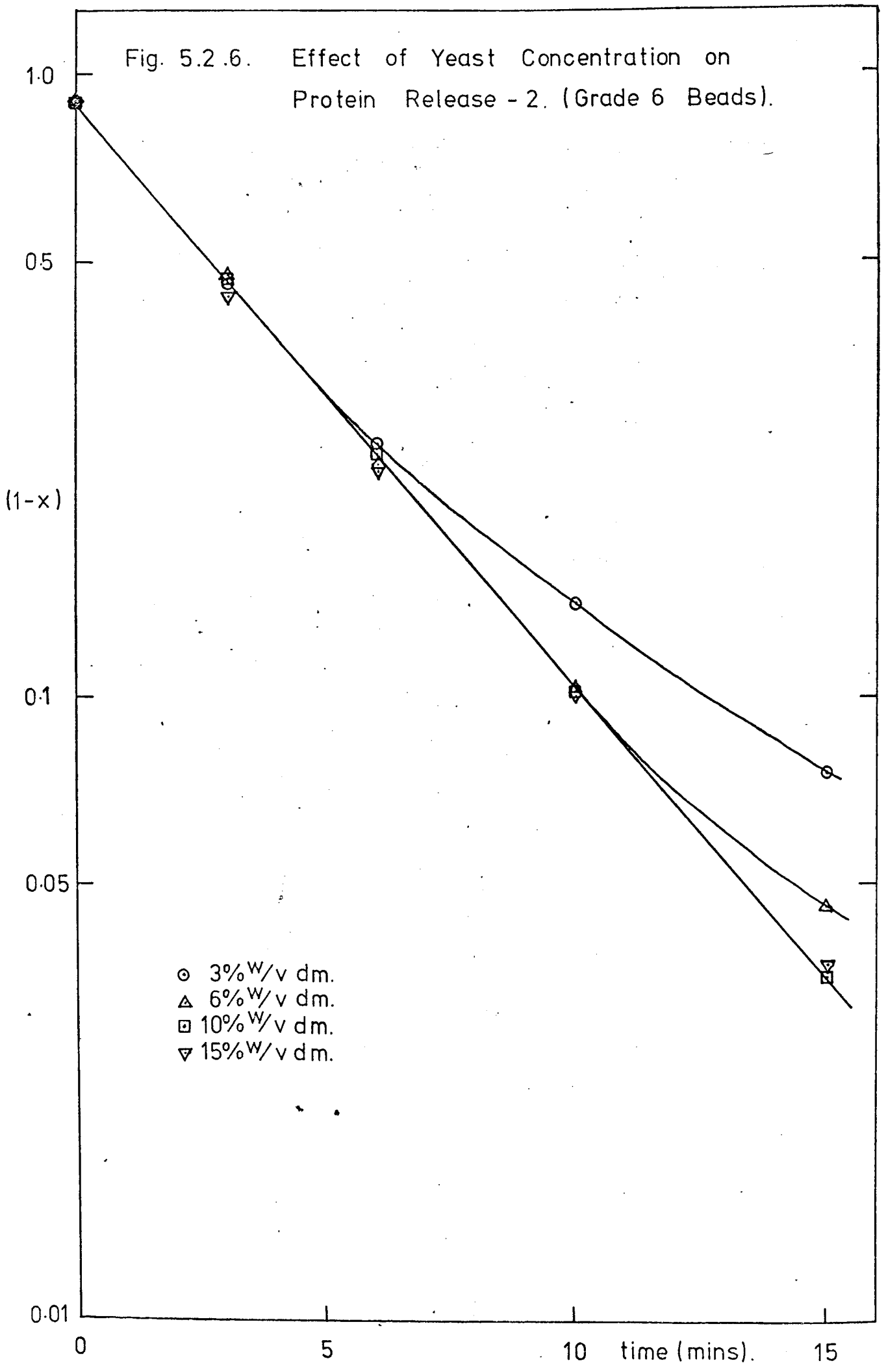


Fig. 5.2.6. Effect of Yeast Concentration on Protein Release - 2. (Grade 6 Beads).
1·0
0·5
(1-x)
0·1
0·05
0·01
0
5
10
time (mins).
15
3%W/v dm.
6%W/v dm.
10%W/v dm.
15%W/v dm.

5.2.3. Continuous Sonication.

5.2.3.1. Rosett Cell.

Based on the results of the batch tests it was decided to use 10ml of grade 6 glass ballotini and a yeast suspension concentration of 10%w/v. Higher yeast concentrations gave a higher rate of soluble protein production since the degree of disruption is independant of concentration. However concentrations much higher than 10%w/v were difficult to handle as yeast sedimentation occurred in the connecting tubes and in the overflow pipe of the cell causing blockage. Beads smaller than 0.5mm ø were carried out of the sonication chamber in the overflow. The system used is shown schematically in Figure 5.2.7. Fresh 10%w/v yeast suspension was pumped from a stirred aspirator standing in an ice/water bath to the Rosett cell which acted as a C.S.T.R. and overflowed into another aspirator also held in an ice/water bath. The Rosett cell was cooled by tap water at 15°C. The suspension temperature did not exceed 20°C at any time. Disruption was tested at different flowrates as described in section 5.2.1. The results shown in Figure 5.2.8. are expressed in terms of percentage total protein released and total protein release rate (mg/min). Also shown are the theoretical lines for percentage protein release and protein release rates based on the batch results for 10%w/v yeast suspension and grade 6 beads. It is known that:-

$$\text{mean residence time} = \frac{\text{reactor volume}}{\text{flowrate}}$$

so the mean residence times can be calculated for a series of flowrates and the percentage protein release which could be expected for a perfect plug flow reactor can be determined from Figure 5.2.4. Hence the theoretical protein release rate can also

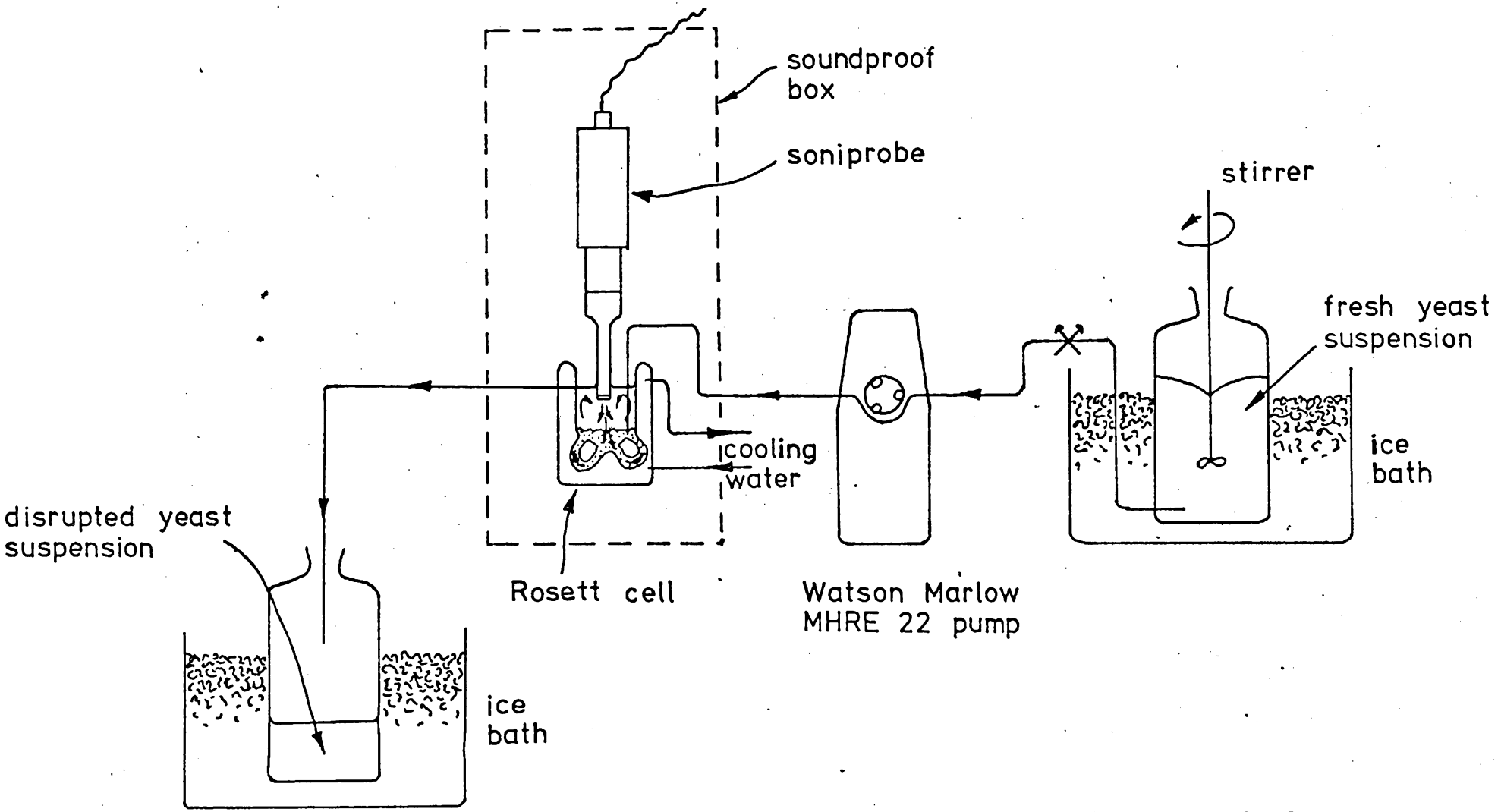


Fig. 5.2.7. Schematic Diagram of Sonicator in Continuous Operation.

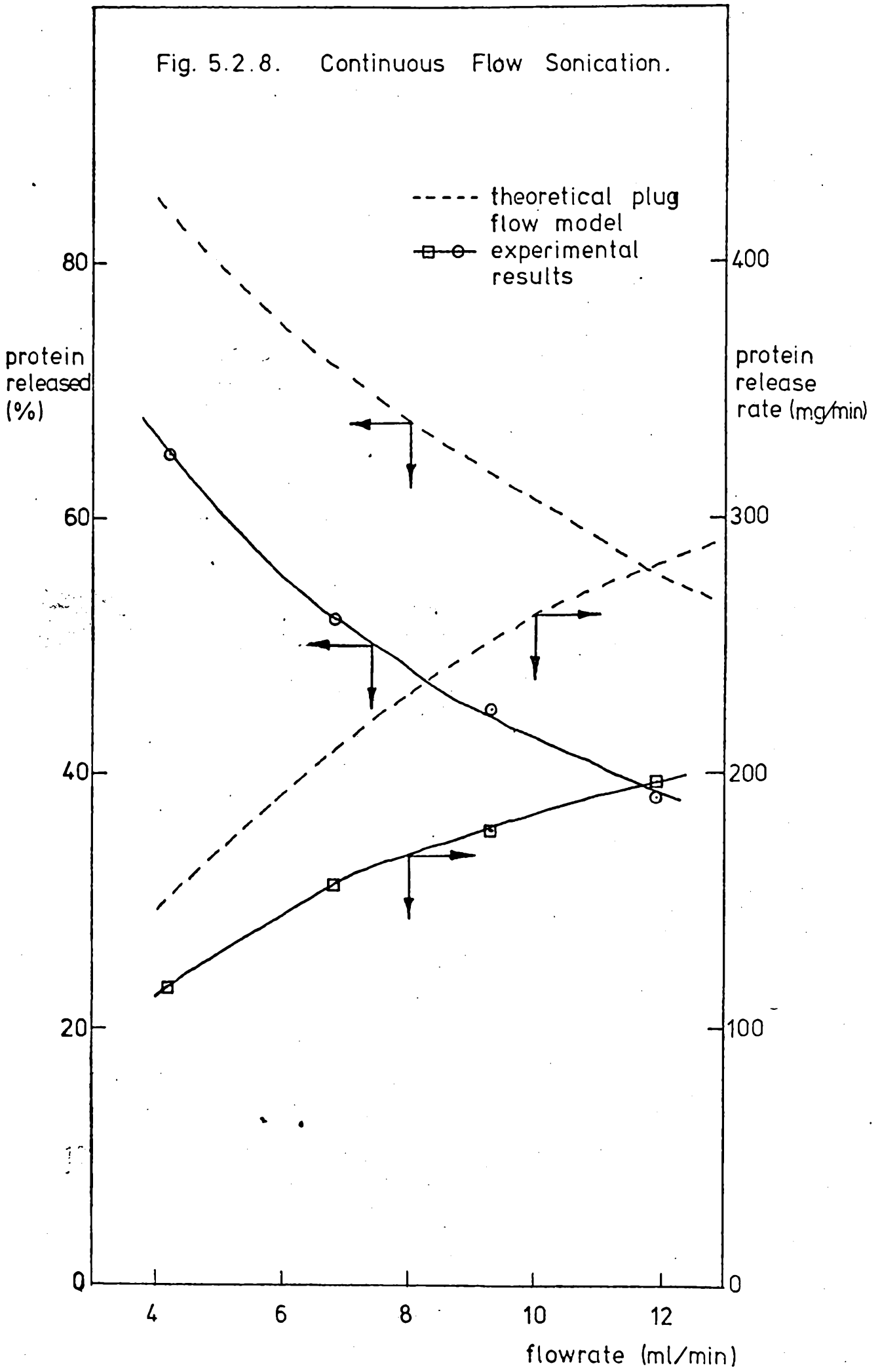


Fig. 5.2.8. Continuous Flow Sonication.
theoretical plug flow model
experimental results
protein released (%)
protein release rate (mg/min)
80
60
40
20
0
400
300
200
100
0
4
6
8
10
12
flowrate (ml/min)

be calculated. The actual protein released is well below the plug flow model due to the spread of residence times of individual cells so that some cells are washed out without disruption after only a short time in the reaction vessel whereas others remain for long periods after disruption. Departure from the theorectical model increases with flowrate.

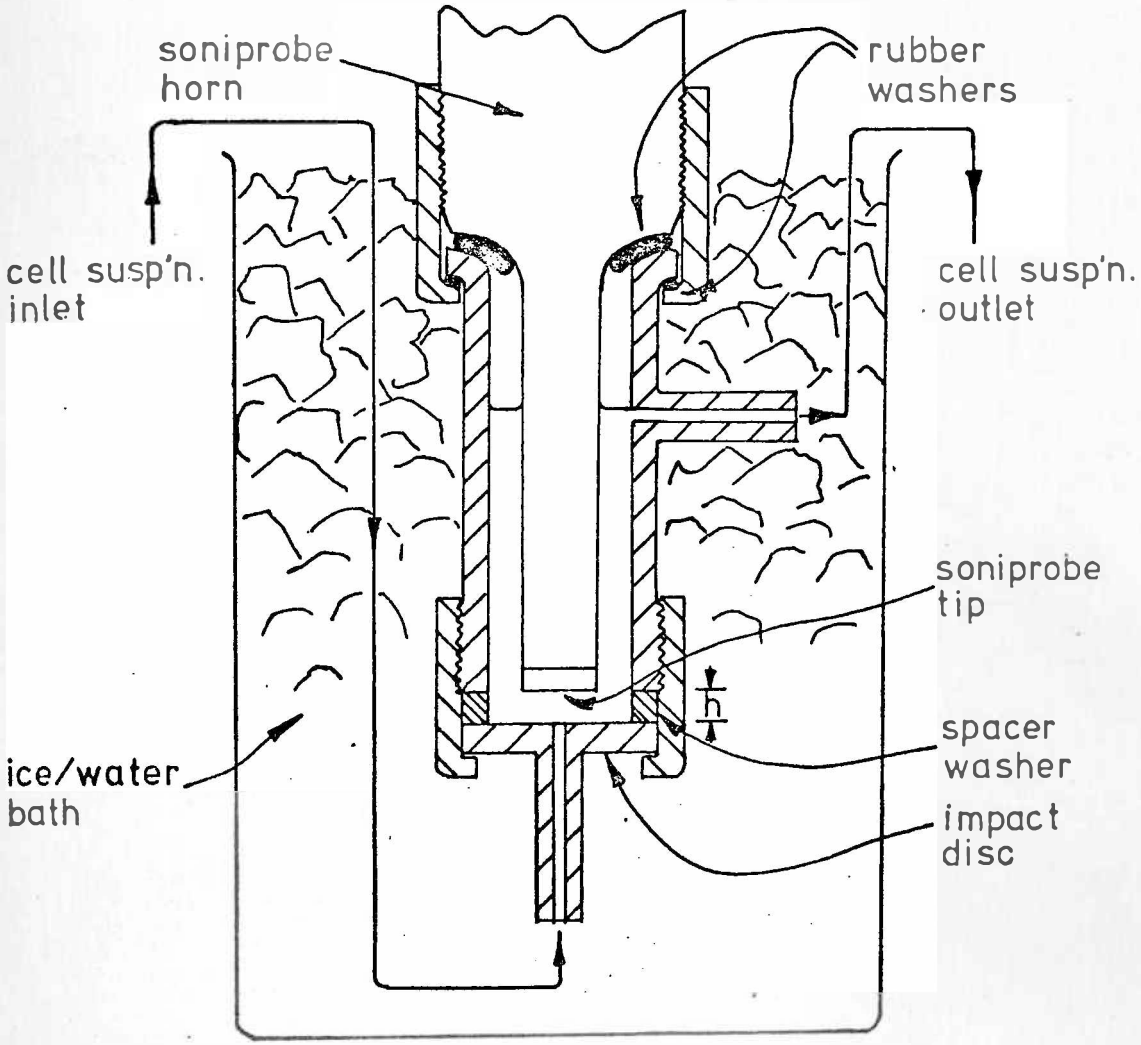
Based on these results it was decided to use a flowrate of 10ml/min for future work, giving about 45% disruption. Higher rates did not give substantially increased protein release rates and led to considerable yeast wastage.

5.2.3.2. Neppiras and Hughes cell.

In later experiments it was suspected that some of the endogenous nucleases were being denatured by sonication. A device based on a design by Neppiras and Hughes (1964) was constructed. This is shown in Figure 5.2.9. In this design the cell suspension passes through a region of small volume but high sonic intensity. The effective sonication chamber volume could be altered by using spacers of different widths. It was hoped that the shorter residence time in such a device might result in reduced enzyme denaturation. In some experiments the sonication chamber was filled with glass beads grade 6, to the level of the top of the spacer. This was not practicable with spacers less than 2mm wide. From the results for protein release shown in Figure 5.2.10 the following conclusions may be drawn:-

1. Protein release increases slightly as the gap width is decreased. Presumably there are two factors here - sonic intensity which increases with decreasing gap width, and residence time which decreases with decreasing gap width.

Fig. 5.2.9. Neppiras and Hughes Cell.



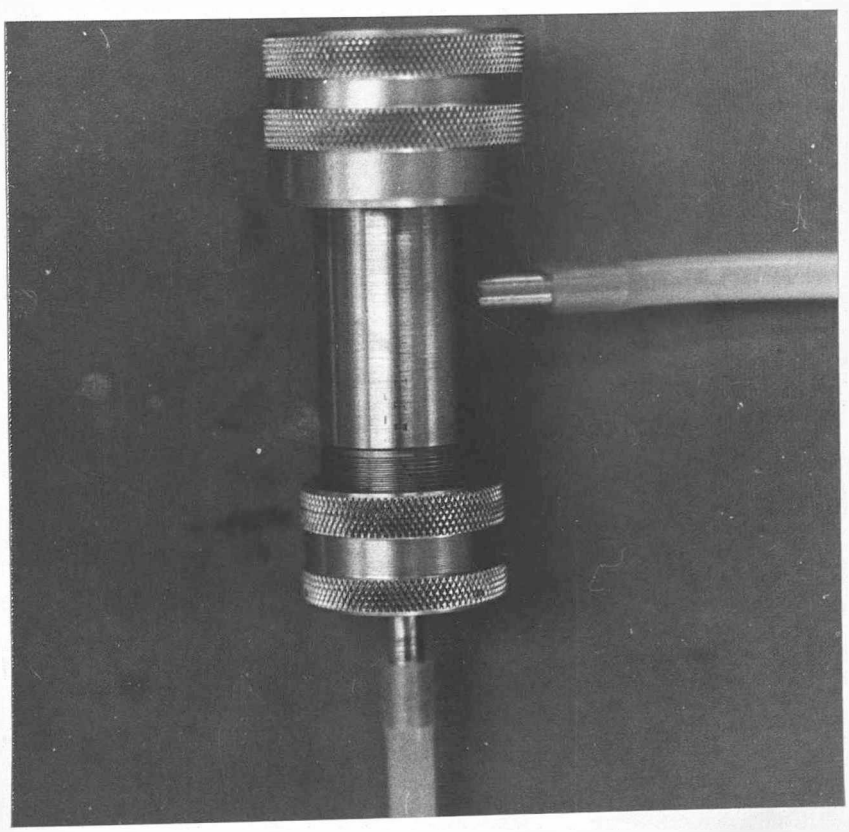
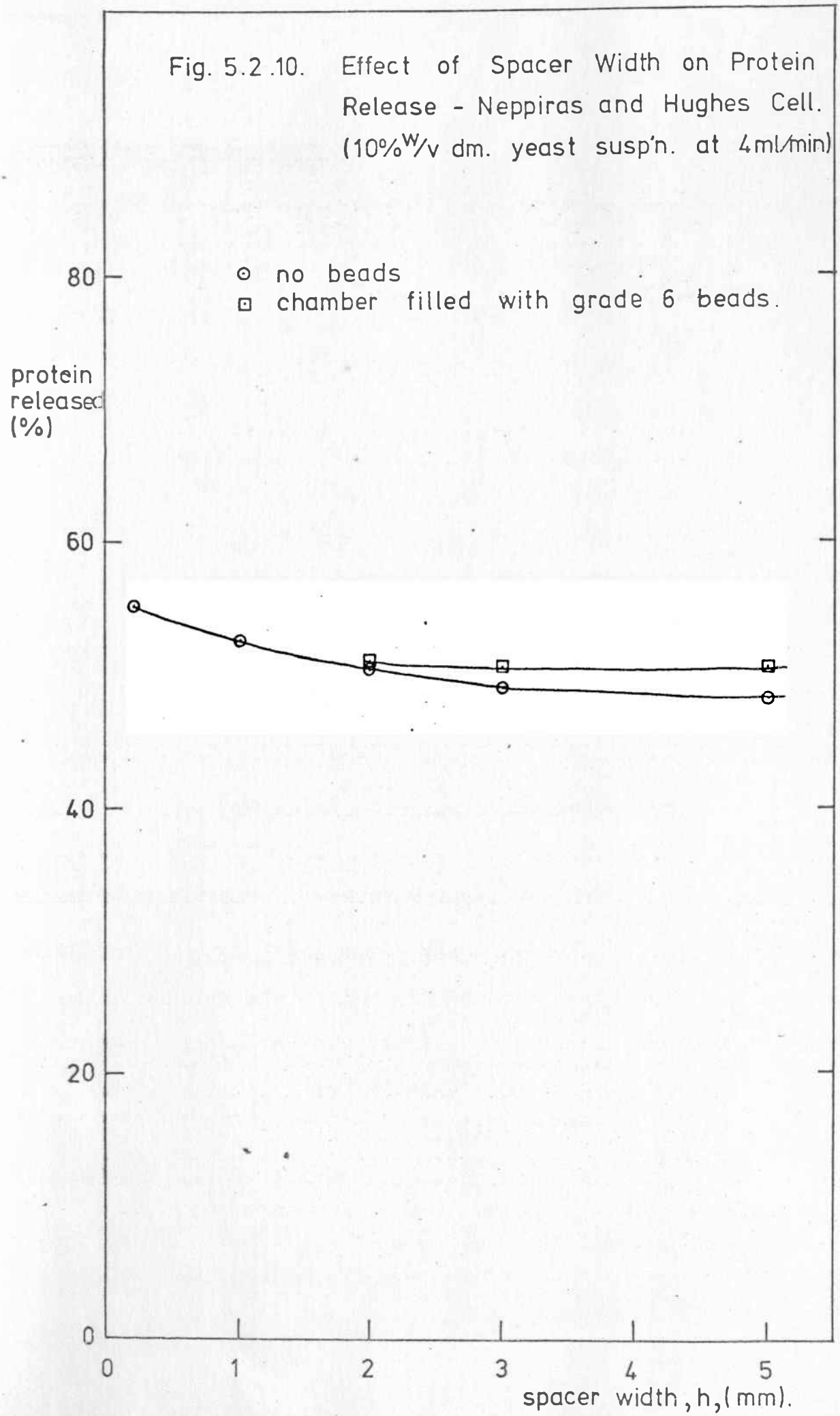


Fig. 5.2.10. Effect of Spacer Width on Protein Release - Neppiras and Hughes Cell. (10%$^{w}/_{v}$ dm. yeast susp'n. at 4ml/min)



2. Glass beads give a significant but slight increase in protein release when compared with the results using similar spacers at the same flowrate.

Further experiments were conducted using the smallest spacer, 0.2mm, and different flowrates. The results appear in Figure 5.2.11. Clearly the disruption rates and hence the protein release rates were considerably poorer than those obtained using the Rosett cell. Furthermore cooling was less efficient, temperature rises of more than 15°C being recorded for flowrates about 5ml/min. The cell was held in an ice/water bath to prevent temperatures exceeding 20°C. Rapid pitting of the impact disc made from stainless steel, occurred during sonication even without glass beads.

5.2.3.3. Enzyme activity.

A qualitative assessment of enzyme denaturation by sonication was made by disrupting yeast cells using prolonged batch sonication (3o mins) (1), continuous flow sonication (10ml/min) in the Rosett cell (2) and by continuous flow sonication (10ml/min, 0.2mm spacer) in the Hughes and Neppiras cell (3). Duplicate 10ml samples of disrupted yeast suspension were alkali extracted and centrifuged to remove the cell walls and incubated at pH 6.0 and 50°C for two hours to allow nucleic acid hydrolysis by the endogenous nucleases. The protein was precipitated at pH 3.8, washed and analysed for protein and nucleic acid. The ratio of protein to nucleic acid in the product should give an indication of nuclease activity. (Details of this method are given in section 5.4.4.). The results are shown in Table 5.2.1. overleaf.

Fig. 5.2.11. Effect of Flowrate on Protein Release - Neppiras and Hughes Cell. (10%$^{W}/_{v}$ dm. yeast susp'n, spacer width 0.2mm, no glass beads).

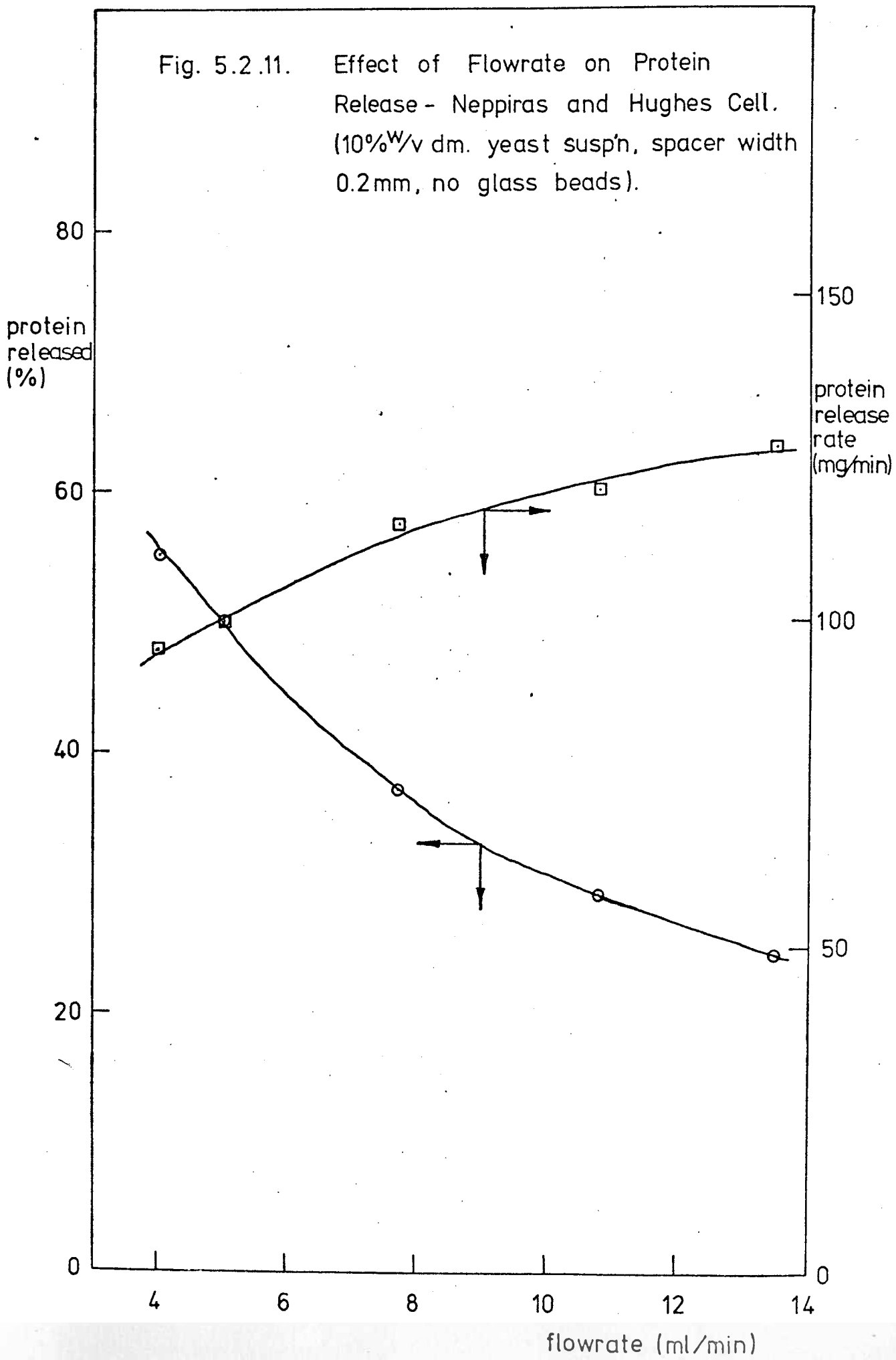


Table 5.2.1. Nuclease Inactivation by Sonication.

disruption process	protein/nucleic acid
batch sonication, 30mins, Rosett Cell	8·3
continuous sonication, 10ml/min, Rosett Cell (mean residence time, 4mins).	22·1
continuous sonication, 10ml/min, Hughes and Neppiras Cell (mean residence time, 1·5secs).	17·1

Assuming that the intensity of sonication is the same in (1) and (2) and that it is greater in (3) where the residence time is shorter, we can draw the following conclusions:-

1. Comparing (1) and (2) it is clear that nucleases are denatured following prolonged treatment.
2. Comparing (2) with (3) it seems that intensity of sonication as well as residence time is important in that even with a residence time of only 1.5secs the nuclease action following disruption in the Neppiras and Highes cell was less than that with the Rosett cell at 10ml/min (mean residence time - 4 minutes).

No method known to give undenatured material was available at this time so it was not possible to calculate the extent of nuclease denaturation. However nucleases are quite stable enzymes and if they were denatured, as they obviously were by sonication, it is likely that other, more sensitive protein material would

also have been affected.

Sonication was not entirely satisfactory as a disruption technique for our purposes but proved to be the best method available during the course of this work. The standard conditions used in further work were 10%w/v yeast suspension flowing through the Rosett cell (with tap water cooling) at 10ml/min using 10ml grade 6 glass ballotini (0.7mm ø) in the sonication chamber as in Figure 5.2.7.

5.2.4. Stansted Disrupter.

At a late stage in the work a Stansted Cell Disrupter, described in section 2.3.2.2., became available for a short trial period. With this machine 85% of the total protein was released after one pass at 25ml/min (yeast concentration 10%w/v dry matter). No viable cells were observed on microscopic examination. Furthermore the cell debris proved easier to separate, probably because the cell walls were not broken into such small fragments as with sonication. Such a machine is now being purchased.

5.3. Protein Isolation.

5.3.1. Precipitation with Acetone and Aluminium Sulphate.

First attempts at producing a protein isolate of high purity using alkaline extraction and acid precipitation did not yield isolates containing more than 70%w/w protein. It was therefore decided to investigate techniques used for enzyme separation in order to try and produce a high purity protein product for use

as a standard of comparison with protein isolates prepared using simpler techniques. Several combinations of extraction and precipitation conditions were investigated but the highest purity samples were produced by fractional precipitation with acetone and ammonium sulphate.

5.3.1.1. Experimental procedures.

Acetone precipitation. Yeast suspensions were prepared and disrupted by the standard techniques described above. The suspension pH varied from 5.6 to 6.4 depending upon the degree of disruption achieved and also on the particular yeast batch. It was usually pH 5.8. A 1.0 N NaOH solution was added to adjust the pH as required and the suspension was centrifuged at 2,000g for 20 minutes to remove the cell debris. The supernatant was cooled to 0°C and acetone at -10°C was added to give the required acetone to supernatant ratio (v/v). The mixture was left for one hour in an ice / salt bath at -5°C and then centrifuged. The supernatant was poured off into another centrifuge tube and the procedure above was repeated to increase the acetone to supernatant ratio. The precipitate was washed with HCl solution at pH 3.8 or resuspended in distilled water and dialysed against distilled water to remove the acetone. The precipitate was analysed for protein and dry matter contents.

Ammonium sulphate precipitation. The protein was extracted from the disrupted yeast suspension as above and cooled to 5°C. Sufficient $(NH_4)_2SO_4$ was added to the supernatant to give a solution at 10% of the saturation level for $(NH_4)_2SO_4$. Samples were left in the refri gerator for one hour and then centrifuged as above. The procedure was repeated on the supernatant to give

higher concentrations of $(NH_4)_2SO_4$. The precipitate was washed or dialysed and analysed as above.

5.3.1.2. Results.

Both acetone and ammonium ions were found to interfere with the biuret protein test making the washing or dialysis step essential. Considerable amounts of protein were lost using dialysis so that washing with HCl at pH 3.8 was preferred.

Extraction at pH 11.0 gave the best results in terms of protein yields although it did not significantly affect the protein content of the isolates. Results for extraction at this pH followed by fractional precipitation with acetone and ammonium sulphate are shown in Tables 5.3.1. and 5.3.2. respectively. It was possible both by fractional precipitation with acetone or with $(NH_4)_2SO_4$ to obtain an isolate of high purity (>90%w/w protein) but the yields were very low. For example, increasing the acetone to supernatant ratio from 1.0:1.0 to 1.2:1.0 or the $(NH_4)_2SO_4$ level from 40 - 50% saturation yielded precipitates containing 98 and 99%w/w protein respectively. However the yields were only about 1% and 6% respectively compared with a yield of 82% of the extracted protein for precipitation with HCl at pH 3.8. The pH 3.8, HCl precipitate was only 72%w/w protein but it was decided to use this approach for further work due to the much higher yields.

5.3.2. Alkaline extraction and acid precipitation.

Because of the high yields obtainable by alkaline extraction and

TABLE 5.3.1. PRECIPITATION OF PROTEIN WITH ACETONE AND HCl.

FRACTION	% PROTEIN IN DRY MATTER	% PROTEIN PRECIPITATED
ALKALI EXTRACTED SUPERNATANT (pH 11.0)	53.1	100.0
ACETONE : SUPERNATANT RATIO 0.2 : 1.0	48.1	~5
0.4 : 1.0	53.7	~10
0.8 : 1.0	61.5	~15
1.0 : 1.0	72.9	~2
1.2 : 1.0	97.6	~1
PRECIPITATION WITH HCl AT pH 3.8	72.0	82.0

TABLE 5.3.2. FRACTIONAL PRECIPITATION OF PROTEIN WITH AMMONIUM SULPHATE.

FRACTION	% PROTEIN IN DRY MATTER	% PROTEIN PRECIPITATED
ALKALI EXTRACTED SUPERNATANT (pH 11.0)	55.0	100.0
10% SATURATION WITH $(NH_4)_2SO_4$ (pH 9.0)	73.4	33.4
20%	79.0	7.5
30%	55.0	5.9
40%	80.0	6.6
50%	98.7	6.2
60%	91.1	2.3
80%	74.0	1.0
100%	—	0

acid precipitation coupled with the economic consideration that this technique could be suitable for commercial use, this method was investigated at some depth.

5.3.2.1. Washing of insolubles.

It was found that after alkaline extraction and centrifugation a considerable amount of the protein remained in the cell debris which could be extracted on washing the debris with distilled water. For example, a 10ml sample of disrupted yeast suspension at pH 11.0 was centrifuged, after which 255mg protein were present in the supernatant. The precipitate was resuspended in 5ml of distilled water and centrifuged again. The second supernatant contained 39mg protein. Further washings yielded only an extra 6mg protein. Hence 85% of the protein was extracted without washing and 98% was extracted after one wash. The standard technique of washing once with half the original volume of distilled water was therefore adopted.

Protein isolates were also washed in the same manner to remove low molecular weight components.

Because of the high buffering capacity of the protein it was found to be unnecessary to readjust the pH after washing since the original pH was retained.

5.3.2.2. Cell debris separation.

When the experiments on cell disruption were performed the 10%w/v yeast suspensions were at a pH of about 6.0. These were considerably diluted prior to centrifuging for cell debris separation. However, as has already been noted, at higher concentrations and higher pH's

the cell debris proved difficult to separate (section 2.3.3.4.). On centrifugation two distinct regions of precipitate were observed. Microscopic examination revealed that the lower layer was made up of undisrupted cells, cream in colour, compact and easy to separate. The upper region contained the insoluble cell debris. This was grey in colour and proved difficult to separate, particularly at higher pH's where the viscosity of the protein solution is increased. However for a 10%w/v dry matter yeast suspension, even after complete cell disruption, centrifugation at 2,000g for 20 minutes was found to give a firm precipitate (i.e. one which did not pour off with the supernatant) and a fairly clear supernatant. (Note that the supernatant was never optically clear even after centrifuging for 30 minutes at 40,000g). Filtration of the supernatant was also attempted but filter papers of various grades were rapidly screened by the slimy precipitate. Filter aids were not used.

The protein isolate, after precipitation, did not present any separation difficulties. In fact the protein 'curd' settled out on its own to a large extent and it was possible to achieve a suspension containing up to 10%w/w protein simply by leaving the acidified supernatant overnight in the refrigerator. However, for convenience the protein was usually separated by centrifuging at 2,000g for five minutes. If it was required for use in spinning it was centrifuged at 40,000g for 30 minutes to give a protein concentration of more than 20%w/w (about 30%w/w dry matter.

5.3.2.3. Optimum pH's for extraction and precipitation.

Procedure. To sonicated yeast suspensions at 5°C sufficient

3 N NaOH solution was added to raise the pH to the desired level for protein extraction. The suspension was stirred rapidly during NaOH addition to prevent local extremes of pH occurring. The suspension was centrifuged, washed and recentrifuged as described above. To the combined supernatants, known as the 'alkali extract', 3 N HCl was added until the required precipitation pH was achieved. The suspension was left for one hour in the refrigerator and then centrifuged and washed to yield a precipitate hereafter referred to as the 'protein isolate'. This was resuspended in distilled water and analysed for protein, dry matter and nucleic acid contents.

Results. As shown in Figure 5.3.1., protein extraction increased slightly with pH up to a maximum of pH 11. However there was a very considerable increase in protein precipitation at pH 3.8 from the alkali extract at pH 11 compared with that at pH 6. Presumably at the higher pH less soluble higher molecular weight material is extracted which is easier to precipitate, but this alone is insufficient to explain the increase in protein precipitation. For example at pH 11 20% more protein was extracted than at pH 6 but 35% more of the protein extracted at pH 11 was precipitated. Clearly there was an effect on the nature of the protein after extraction at high pH which affects its solubility (Lindblom,1974). Extraction and precipitation fell at pH's greater than 11 presumably due to hydrolysis and amino acid destruction.

Maximum precipitation of protein occurred at pH 3.8 regardless of the extraction pH (Figure 5.3.2). These results are in good agreement with those of other workers who have studied protein extraction from Bakers' yeast (Hedenskog & Ebbinghaus, 1972;

Fig. 5.3.1. Extraction and Precipitation of Yeast Protein. (Results are for a number of different yeast batches).

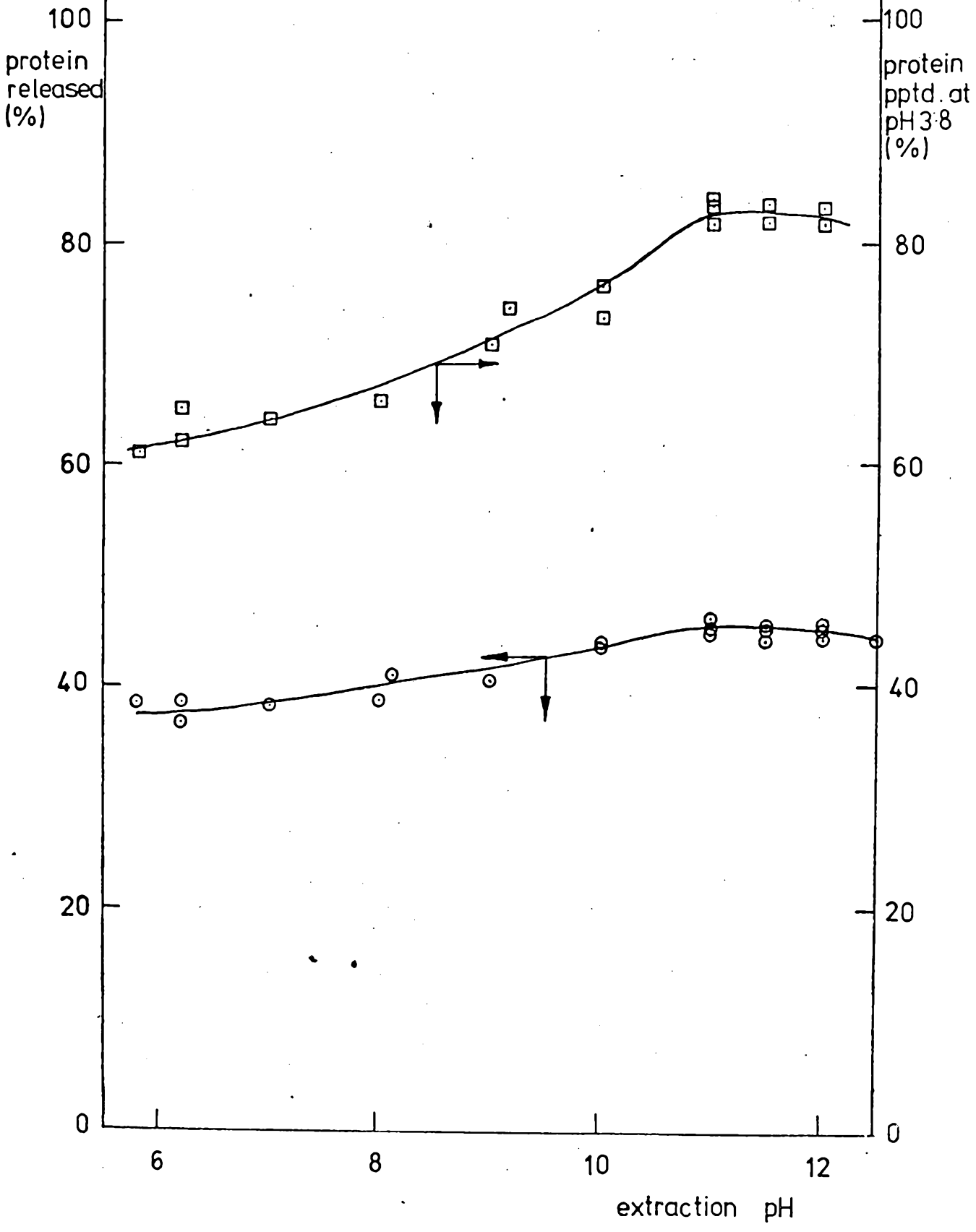
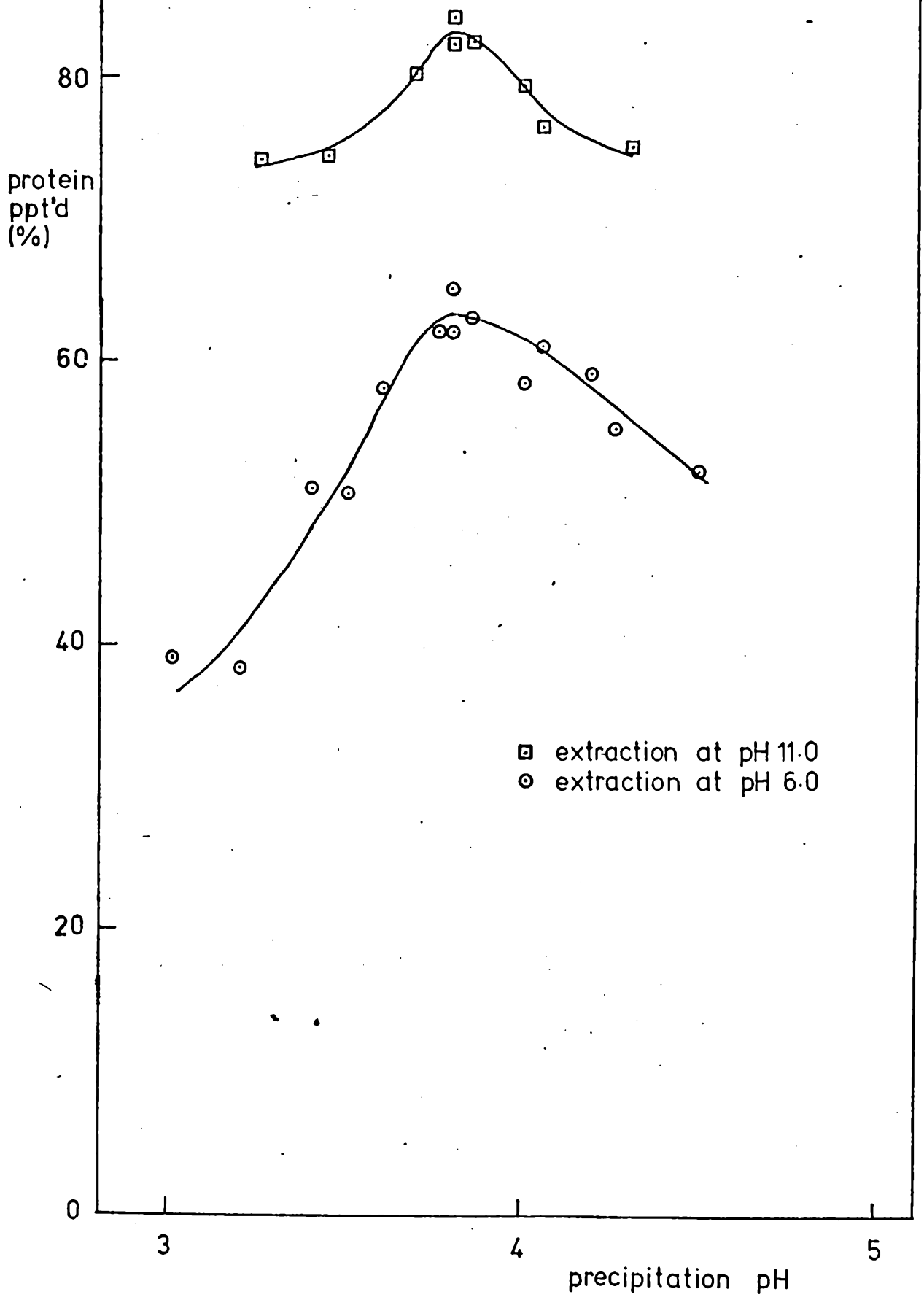


Fig. 5.3.2. Precipitation of Yeast Protein at Different pH's. (Results are for a number of yeast batches).



Lindblom, 1974).

The protein content of the isolate reached a maximum of about 70% after extraction at pH 11 followed by precipitation at pH 3.8.

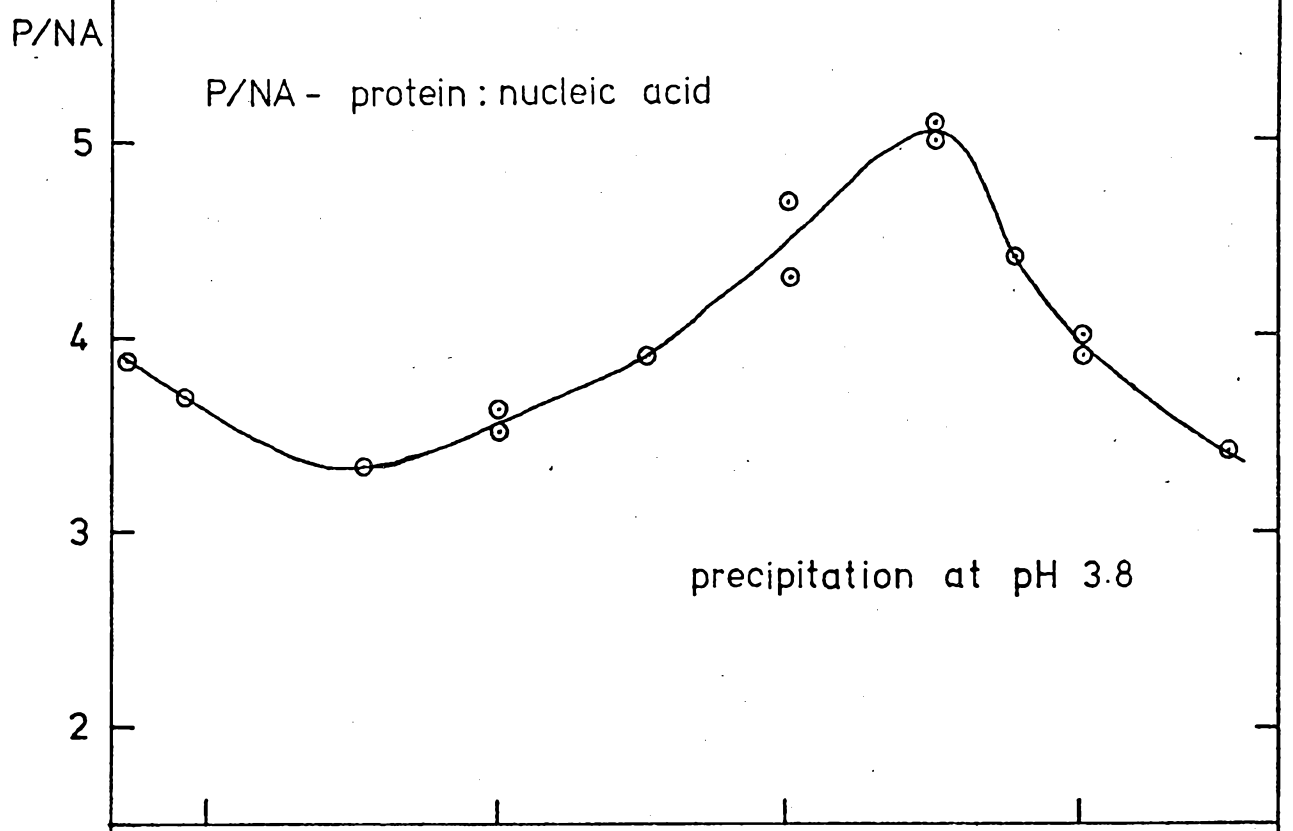
Fortunately the protein to nucleic acid ratio was also a maximum under these conditions (Figure 5.3.3.) but was still only 5.0 (i.e. the nucleic acid content of the isolate was about 14%w/w) (c/f Hedenskog & Ebbinghaus, 1972 - 11%w/w and Robins et al, 1975 (1) - 14%w/w).

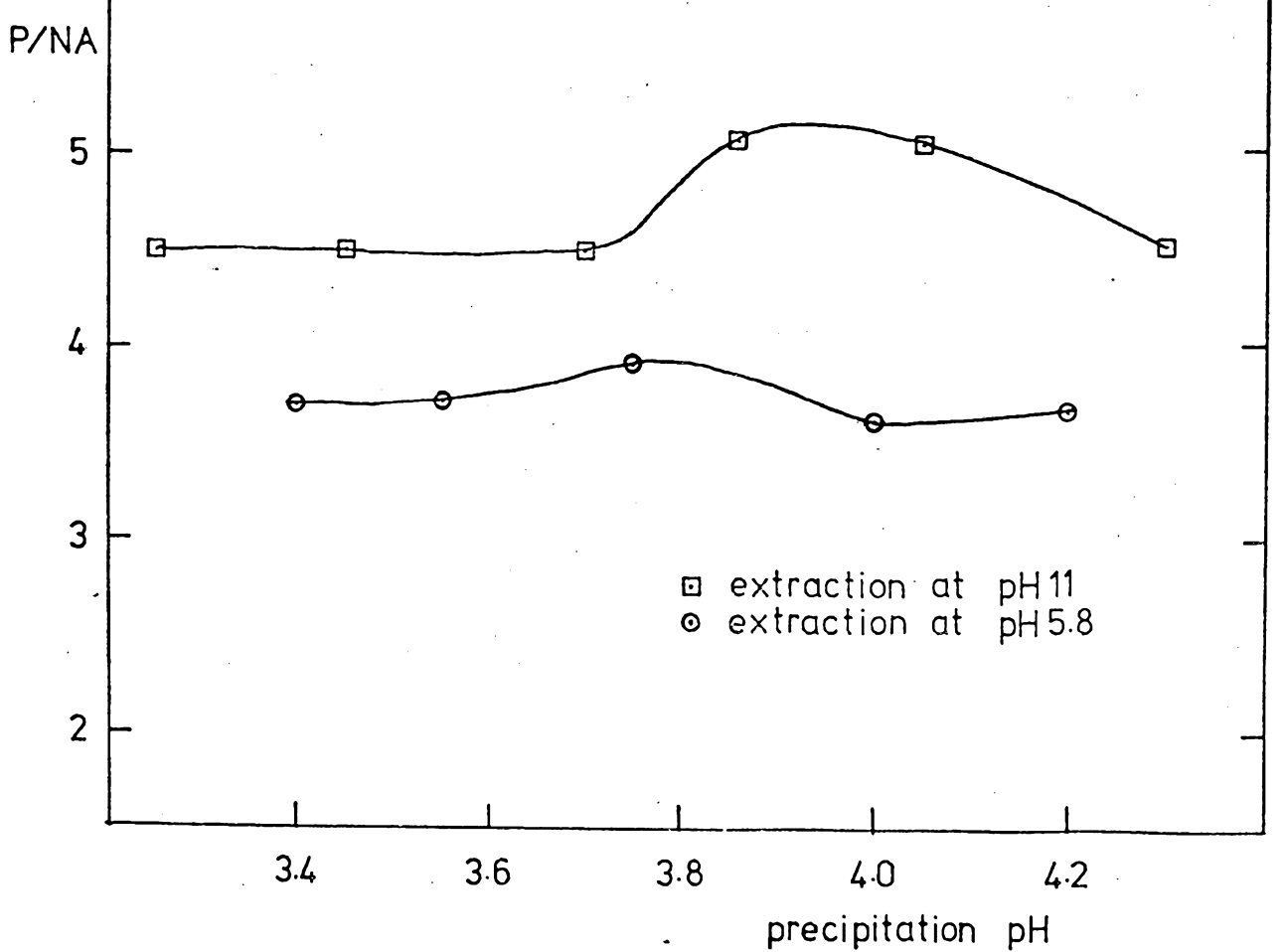
Approximately 6.0 m moles of NaOH are required to extract about 1g of protein at pH 11.0 and about 8 m moles of HCl are required to precipitate at pH 3.8 from a solution containing 1g of protein at pH 11.0.

5.3.2.4. Effects of time and temperature on precipitation. Protein precipitation was carried out at 0°C to 50°C for one half to 20 hours following extraction at pH 11.0. No significant differences in protein yield or protein to nucleic acid ratios were observed over the entire range of conditions investigated. It was found however that there was a slight decrease in the protein content, expressed as percentage of the dry matter, of the isolates after prolonged precipitation. For example, the protein content of isolates precipitated at 25°C were 69% and 66% respectively after precipitation for one half and 20 hours. This was presumably due to increased precipitation of non protein material, probably carbohydrates.

The effects of time and temperature on protein extraction were

Fig. 5.3.3. Protein : Nucleic Acid Ratio of Isolates Extracted and Precipitated at Different pH's .





not investigated. As stated in section 2.3.4.8. there is considerable danger of protein damage at high pH's and high temperatures. This work has shown that extraction at pH's greater than 12 has led to brown coloured isolates. Hedenskog (1975) suggested that treatment at pH 11 and ambient temperatures did not lead to protein damage. As there were no facilities for nutritional and toxicological testing it was decided to err on the side of safety and to extract as rapidly as possible at 5°C.

5.3.2.5. Solubility.

It was found that solubility was dependant upon protein concentration. At pH 10.0 the solubilities of the samples of the same isolate were 98%, 85% and 80% for protein concentrations of 10, 21 and 39mg/ml. The concentration for solubility testing was therefore standardised at 20mg/ml since this was approximately the same as the protein extraction concentration thus allowing useful comparison between solubility before and after protein isolation.

Figure 5.3.4. shows the solubility of isolates at pH 7 and pH 11 following precipitation at different temperatures from 0°C to 50°C. All the samples were approximately 100% soluble at pH 11. The spread of points above the 100% line is probably due to small errors in sampling. Solubility at pH 7 was a maximum at 25°C. Raising the precipitation temperature lowered the protein solubility due to heat denaturation. The solubility at pH 7 was also slightly lower at temperatures less than 20°C.

Figure 5.3.5. shows the solubility of fresh protein isolate between pH 4 and pH 11.5. The isolate was slightly less soluble over a range of pH's following storage for three days at 4°C.

Fig. 5.3.4. Solubility of Protein Precipitated at Different Temperatures.

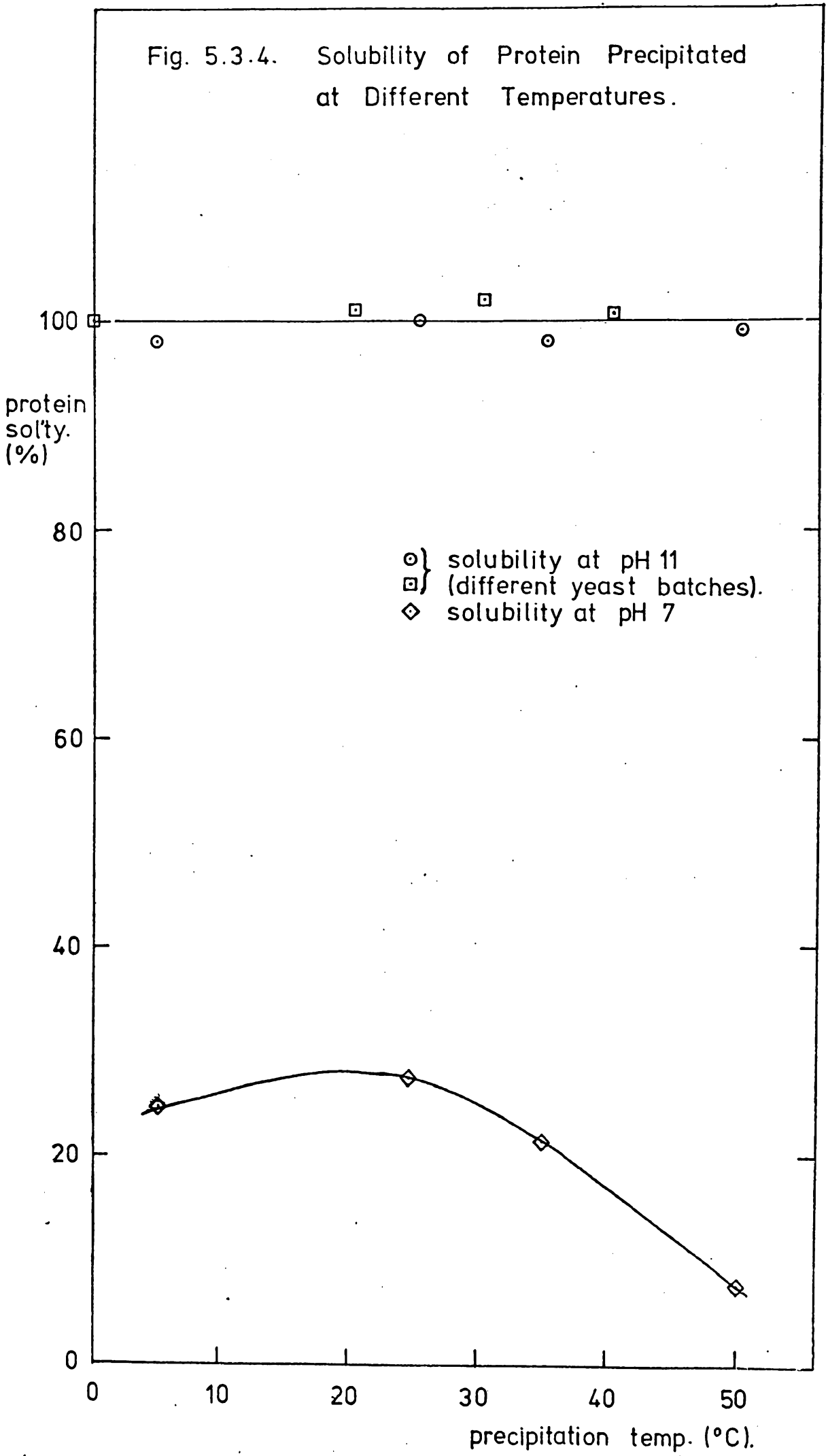
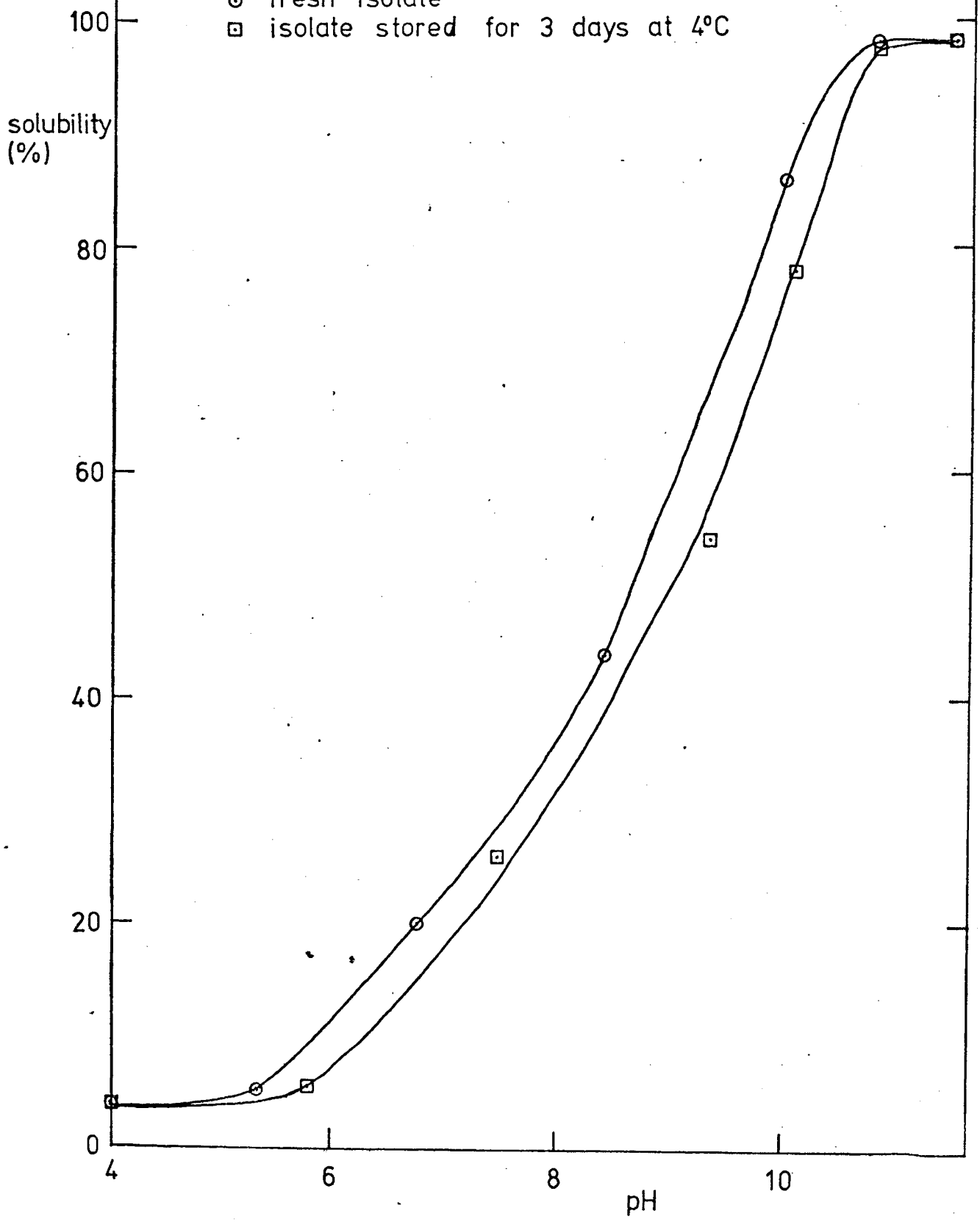


Fig. 5.3.5. Solubility of Protein Isolates Prepared Under Standard Conditions. (Extraction at pH 11, 5°C; precipitation at pH 3·8, 20°C).



The striking fact about Figures 5.3.4. and 5.3.5. is that the solubility of the protein at neutral pH's is much lower than it was prior to protein isolation. On extraction 83% of the protein extracted at pH 11 was also soluble at pH 6 (Figure 5.3.1.). After precipitation only 11% of the protein extracted at pH 11 was soluble at pH 6. Clearly the denaturation occurring during extraction, which serves to increase the protein yield, also serves to reduce solubility.

5.3.2.6. Molecular weight distribution.

Molecular weight distribution was of interest for two reasons. Firstly it made possible observation on a molecular basis some of the results of processing on the protein and nucleic acid. Secondly it gave some indication of the overall molecular weight distribution which was possibly of value in explaining the texturising properties of the protein (Lundgren, 1949; Young & Lawrie, 1975).

Initial experiments were performed on Sephadex G50 columns. It was found that the bulk of the extracted material was eluted in the void volume. All the protein in the isolate appeared in the void volume suggesting that even the smallest protein molecules in the isolate had molecular weights greater than about 30,000 (Pharmacia, (1)).

Sephadex G200 gave a better picture but the bulk of the protein in the alkali extract and in the protein isolate still appeared in the region of the void volume (Figure 5.3.6.). The elution of known molecular weight standards showed that about 50% of the

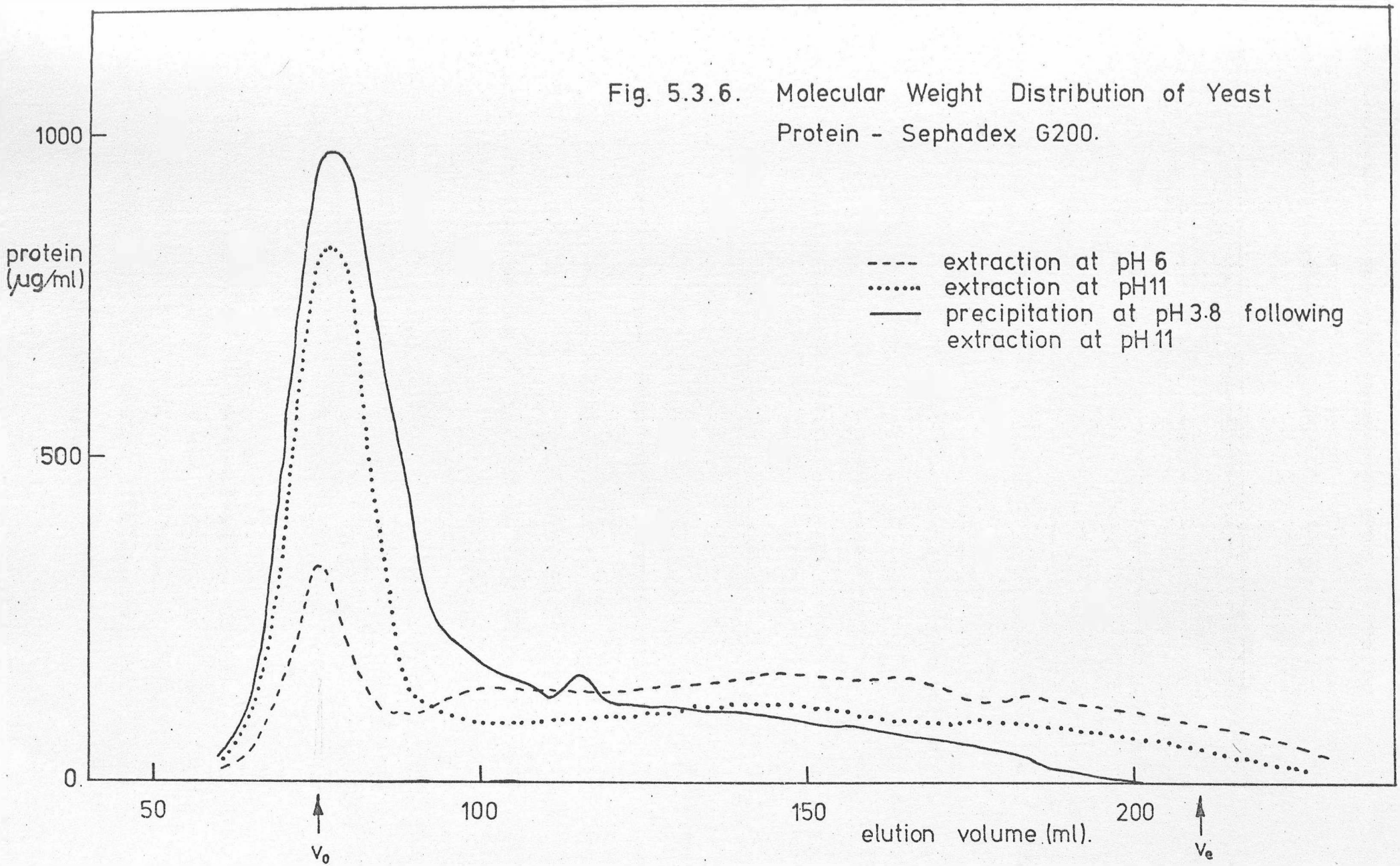


Fig. 5.3.6. Molecular Weight Distribution of Yeast Protein - Sephadex G200.

protein in the alkali extract had molecular weights greater than 100,000 (Figure 5.3.7. and 5.3.8.). 70% of the protein in the isolate had molecular weights greater than 100,000. In the water extract on the other hand only 14% of the protein had molecular weights greater than 100,000. Results for the water extract are similar to those obtained by Lindblom (1974) but she did not elute samples at high pH's. It is interesting that little aggregation appears to take place as a result of elution with buffer at pH 10.0. It is therefore tentatively suggested that the aggregation is also a function of protein concentration, since the concentration during gel filtration is considerably less than that used during protein extraction.

Sepharose 6B was found to give a much wider elution pattern and was used for all further work. Figure 5.3.9. shows the elution patterns for alkali extracted protein, water extracted protein and alkali extracted protein which had been heated at 100°C for five minutes in 1%w/v sodium dodecyl sulphate (SDS) and eluted with 1% w/v SDS in the buffer. The SDS served to disrupt the non-covalent bonds and, in particular, disulphide bonds. Using SDS the alkali extract had a reasonably similar molecular weight spectrum to that of the water extracted protein. Alkali extraction appears therefore to cause protein aggregation by non-covalent bonding and it is this, rather than the extraction of material of higher molecular weight, which accounts for the increased protein. precipitation after alkali extraction. Hence it must be concluded that the bulk of the protein (about 70%) is made up of covalently bonded units with molecular weights in the range 10,000 - 100,000 although alkali and other treatment causes aggregation to give apparent molecular weights as high as 4,000,000 or more (Figures 5.3.16. and 5.3.17.).

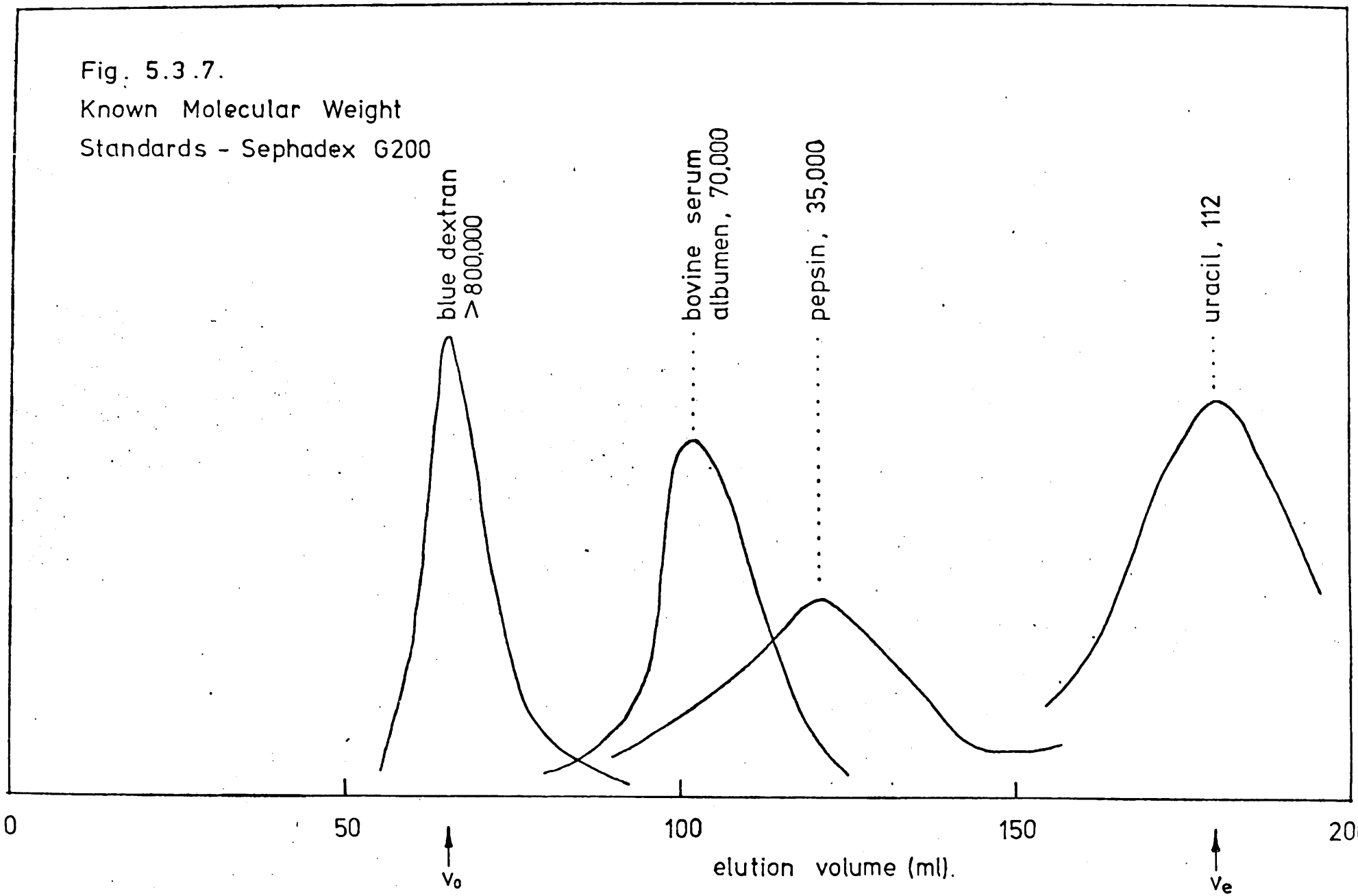


Fig. 5.3.7.
Known Molecular Weight
Standards - Sephadex G200

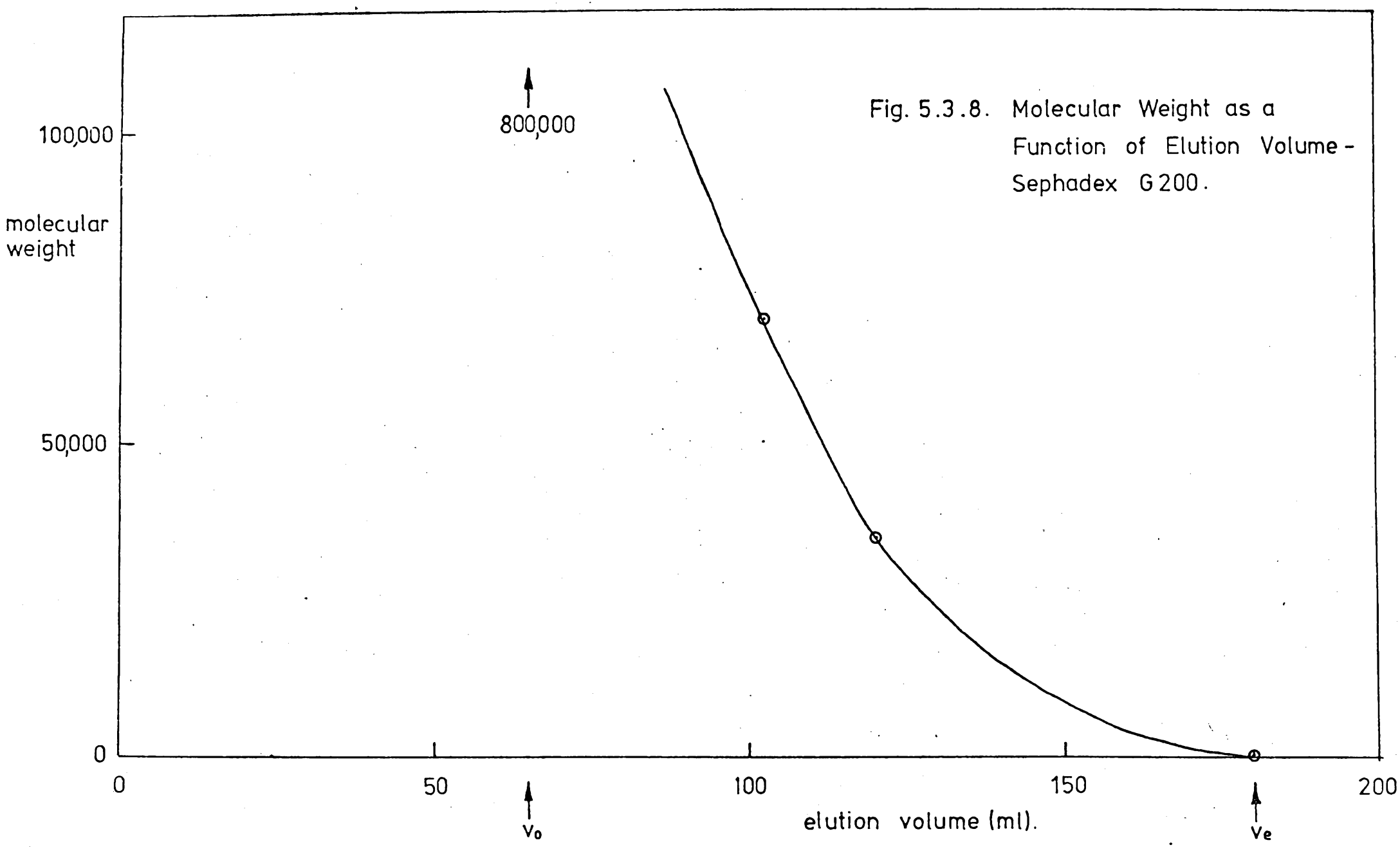


Fig. 5.3.8. Molecular Weight as a Function of Elution Volume - Sephadex G 200.

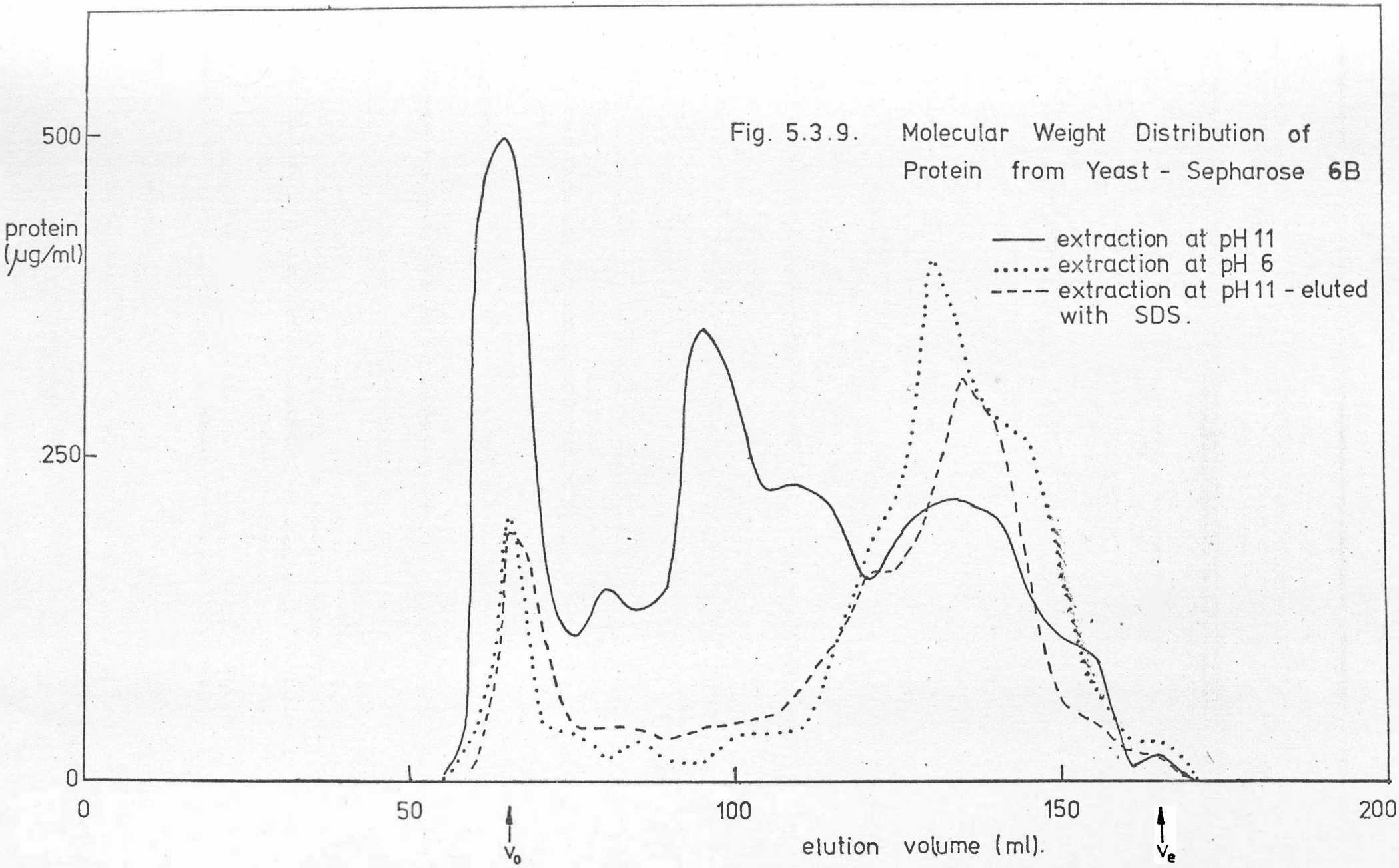


Fig. 5.3.9. Molecular Weight Distribution of Protein from Yeast - Sepharose 6B

Figure 5.3.10. shows the elution patterns of protein precipitated from the alkali and water extracts. Although the alkali extract precipitate has a higher molecular weight average the molecular weight pattern for the water extract precipitate is significantly different from that of the water extract itself suggesting that further aggregation takes place on precipitation.

Figures 5.3.11. and 5.3.12. show the elution patterns for protein and nucleic acid following fractional precipitation with HCl at pH 8.7, 6.5 and 3.8 following alkali extraction at pH 11. As expected it is largely the high molecular weight material which is least soluble. This is particularly true of nucleic acids. Low molecular weight material is precipitated however, even at quite high pH's. Because the protein from yeast is such a mixture of different components no single pH at which all the protein is least soluble exists and pH 3.8 represents the optimum pH in terms of maximum protein precipitation.

Protein denaturation and aggregation take place at low as well as high pH's (Fox, 1957). It may be that pH 3.8 is low enough to cause denaturation and aggregation to relatively low molecular weight material making it less soluble and more easily precipitated. Hence the precipitation is not simply isoelectric focussing but denaturation and isoelectric focussing combined. The optimum precipitation pH is certainly much lower than that used for soya protein isolation (about pH 5.0. Ashton et al, 1971). This would also explain the high molecular weight range of the isolate from the water extract shown in Figure 5.3.10.

Because of the reports of protein damage following alkali treatment

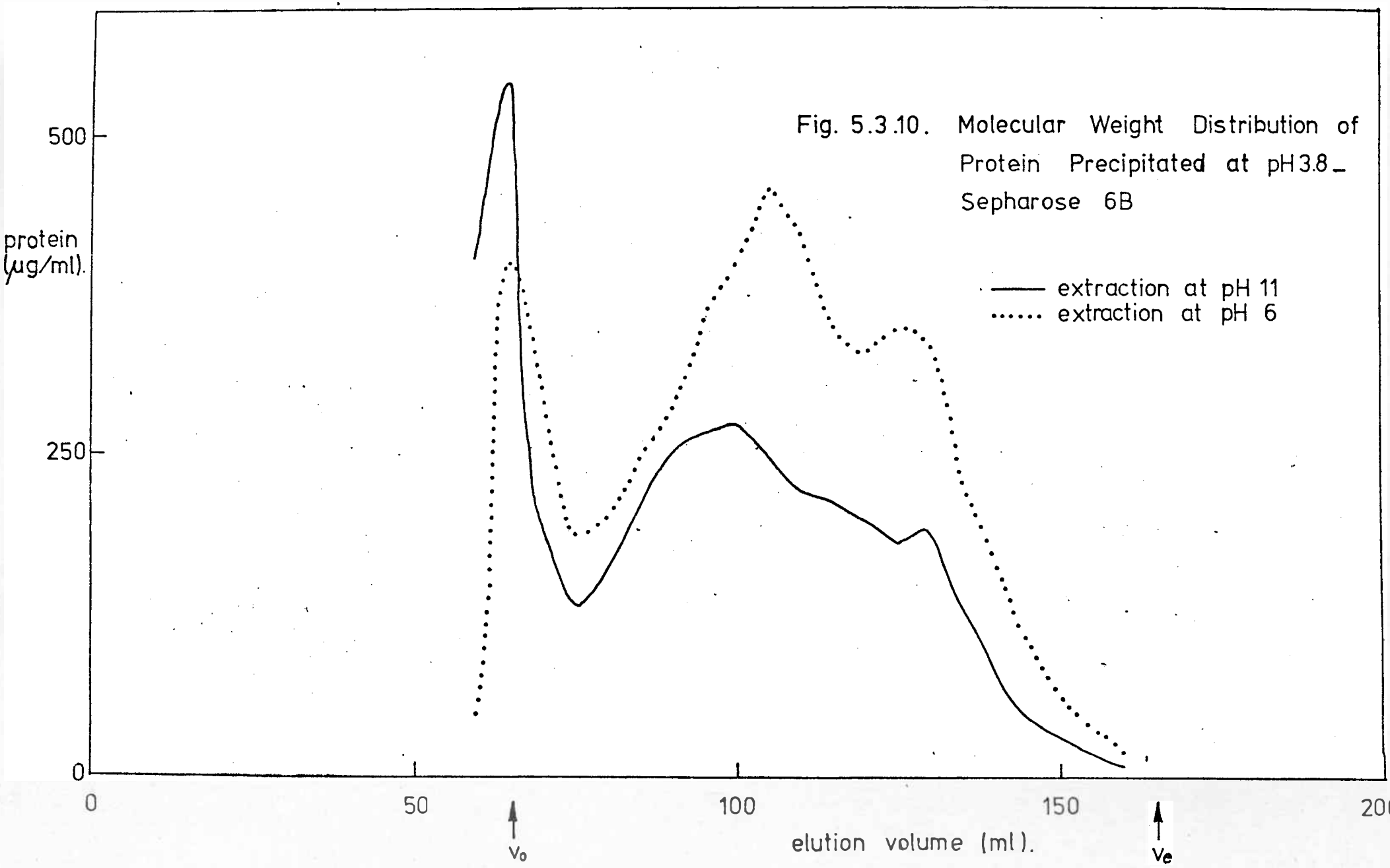


Fig. 5.3.10. Molecular Weight Distribution of Protein Precipitated at pH 3.8 – Sepharose 6B

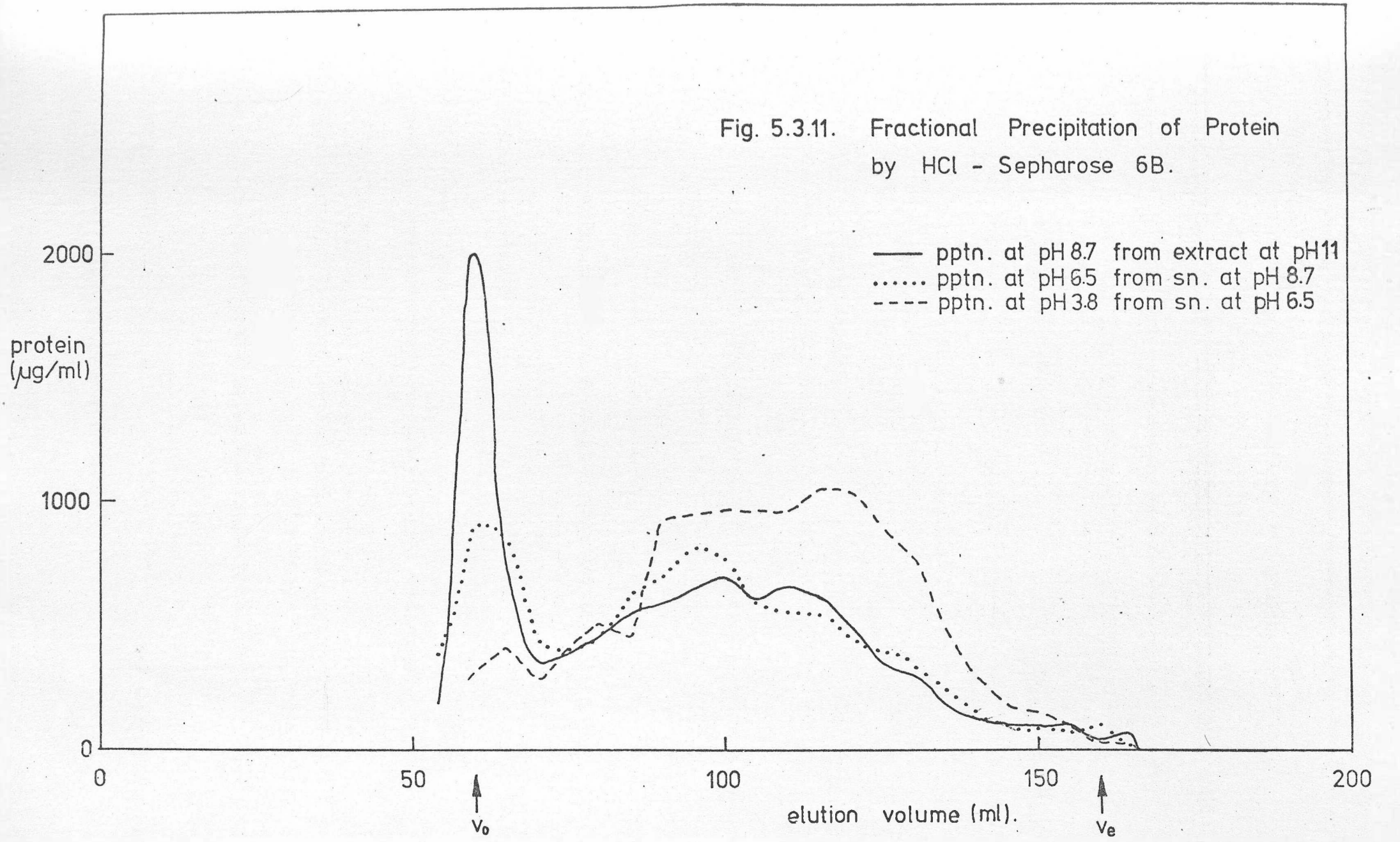


Fig. 5.3.11. Fractional Precipitation of Protein by HCl - Sepharose 6B.

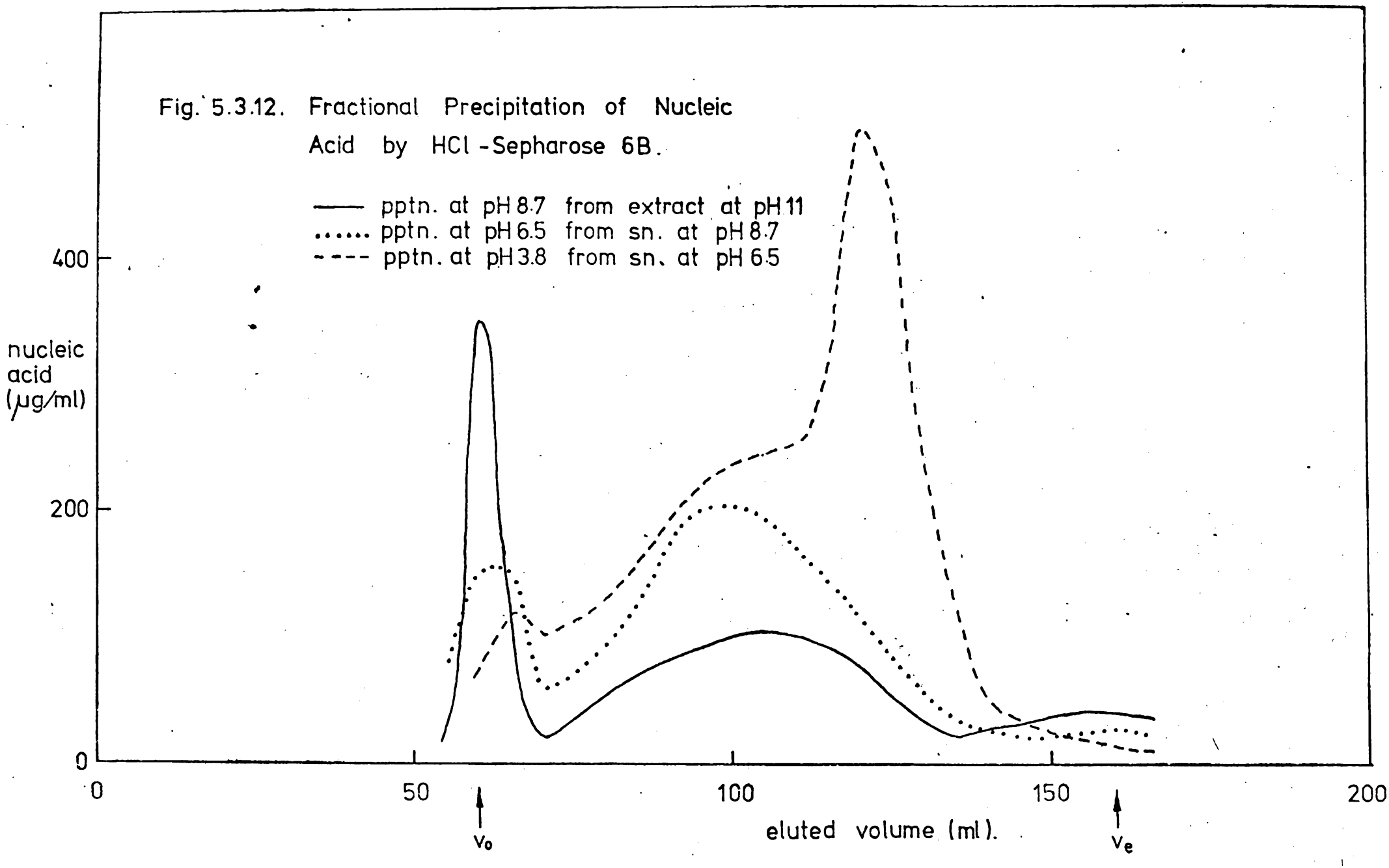


Fig. 5.3.12. Fractional Precipitation of Nucleic Acid by HCl-Sepharose 6B.
pptn. at pH 8.7 from extract at pH 11
pptn. at pH 6.5 from sn. at pH 8.7
pptn. at pH 3.8 from sn. at pH 6.5
nucleic acid (µg/ml)
400
200
0
0
50
100
150
200
V_o
eluted volume (ml).
V_e

(Canepa et al, 1972; Hedenskog & Ebbinghaus, 1972; Woodard & Short, 1973) alkali extracted protein was incubated at 4°C, 25°C and at 37°C for 24 hours to see what changes occurred in the molecular weight pattern. Storage of the extract at 4°C did not alter the protein or nucleic acid spectra significantly. Incubation at 25°C for 24 hours caused some further protein aggregation and this effect was quite marked at 37°C. The nucleic acid on the other hand, suffered considerable hydrolysis at 25°C and almost complete hydrolysis at 37°C after incubation for 24 hours (Figures 5.3.13. & 5.3.14.).

The molecular weight pattern of the protein isolate following digestion in 1 N NaOH for five minutes at 100°C not surprisingly showed considerable protein hydrolysis and complete nucleic acid hydrolysis. Herbert et al (1971), however, reported that this did not affect the formation of the biuret colour (Figure 5.3.15.).

Figures 5.3.16. and 5.3.17. show the elution patterns of known molecular weight standards. The results were not entirely consistent (compare the positions of bovine serum albumen and alcohol dehydogenase) even after SDS treatment. The reasons for this are not fully understood.

5.3.3. Overall Assessment.

To summarise what has been found: firstly it was possible to produce protein samples of high purity only with very low yields: secondly extraction at pH 11 followed by precipitation at pH 3.8 gave a high protein yield but an even higher nucleic acid yield so that the protein to nucleic acid ratio in the product actually

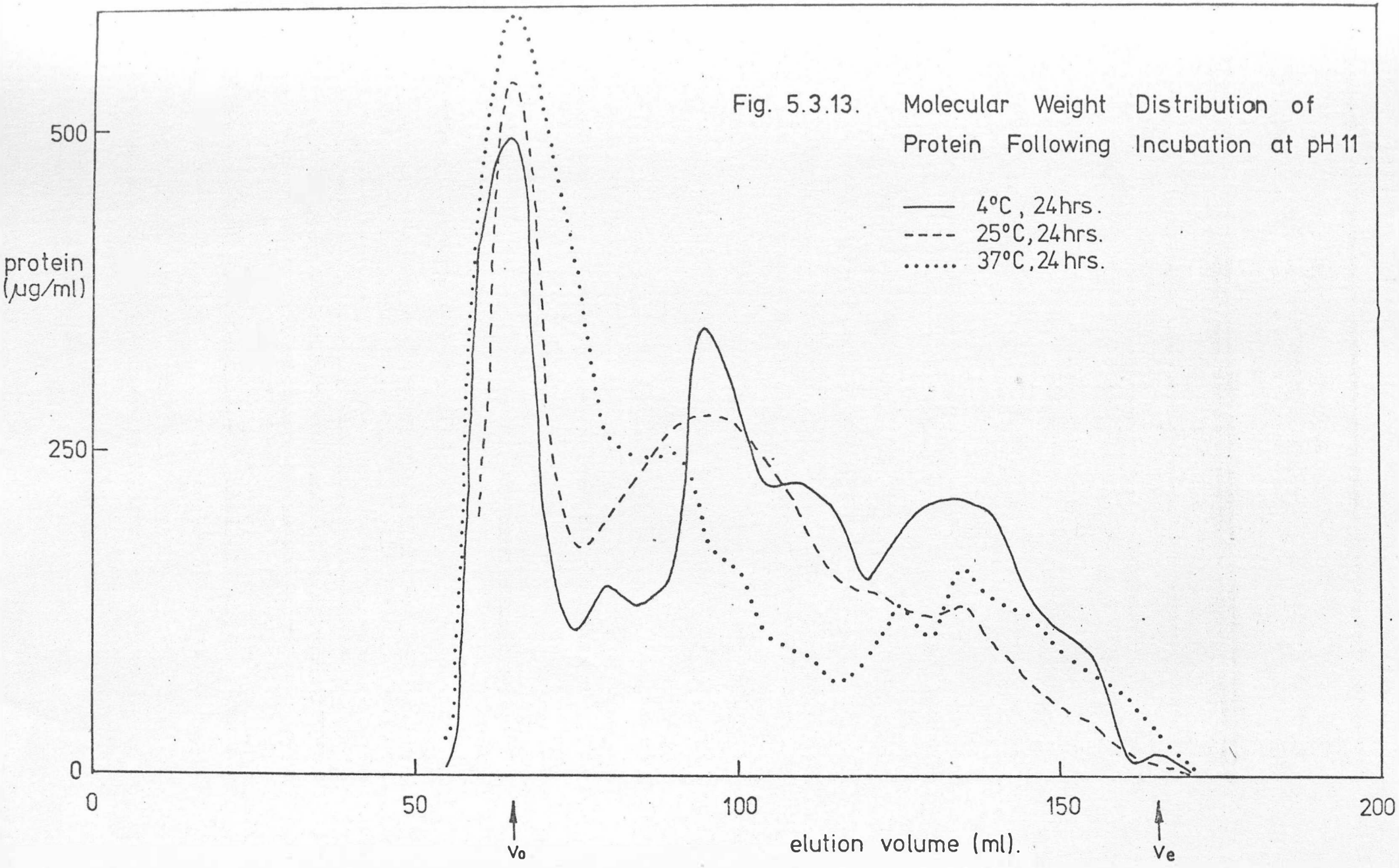


Fig. 5.3.13. Molecular Weight Distribution of Protein Following Incubation at pH 11

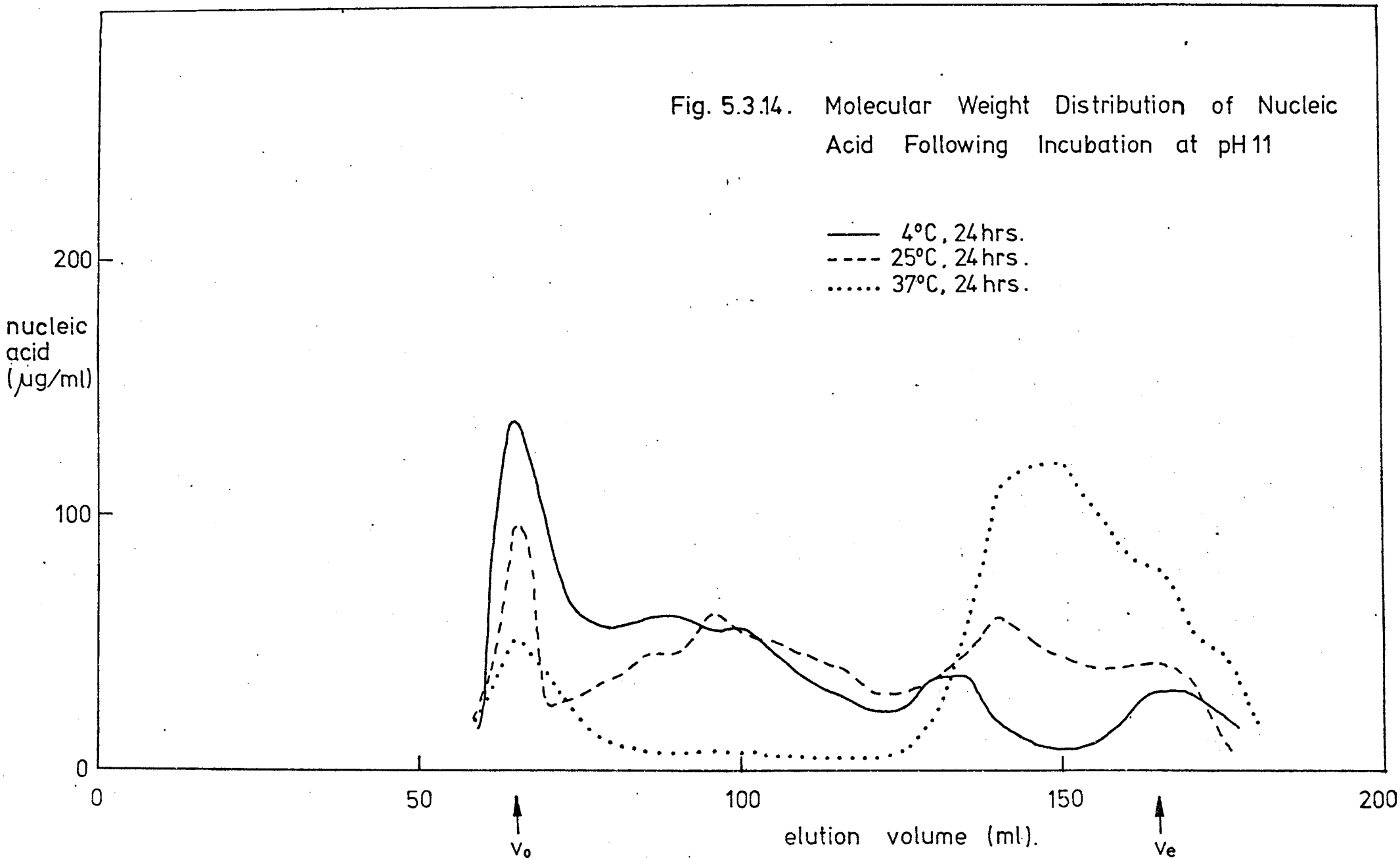


Fig. 5.3.14. Molecular Weight Distribution of Nucleic Acid Following Incubation at pH 11
4°C, 24hrs.
25°C, 24hrs.
37°C, 24hrs.
nucleic acid (µg/ml)
200
100
0
0
50
100
150
200
elution volume (ml).
V_o
V_e

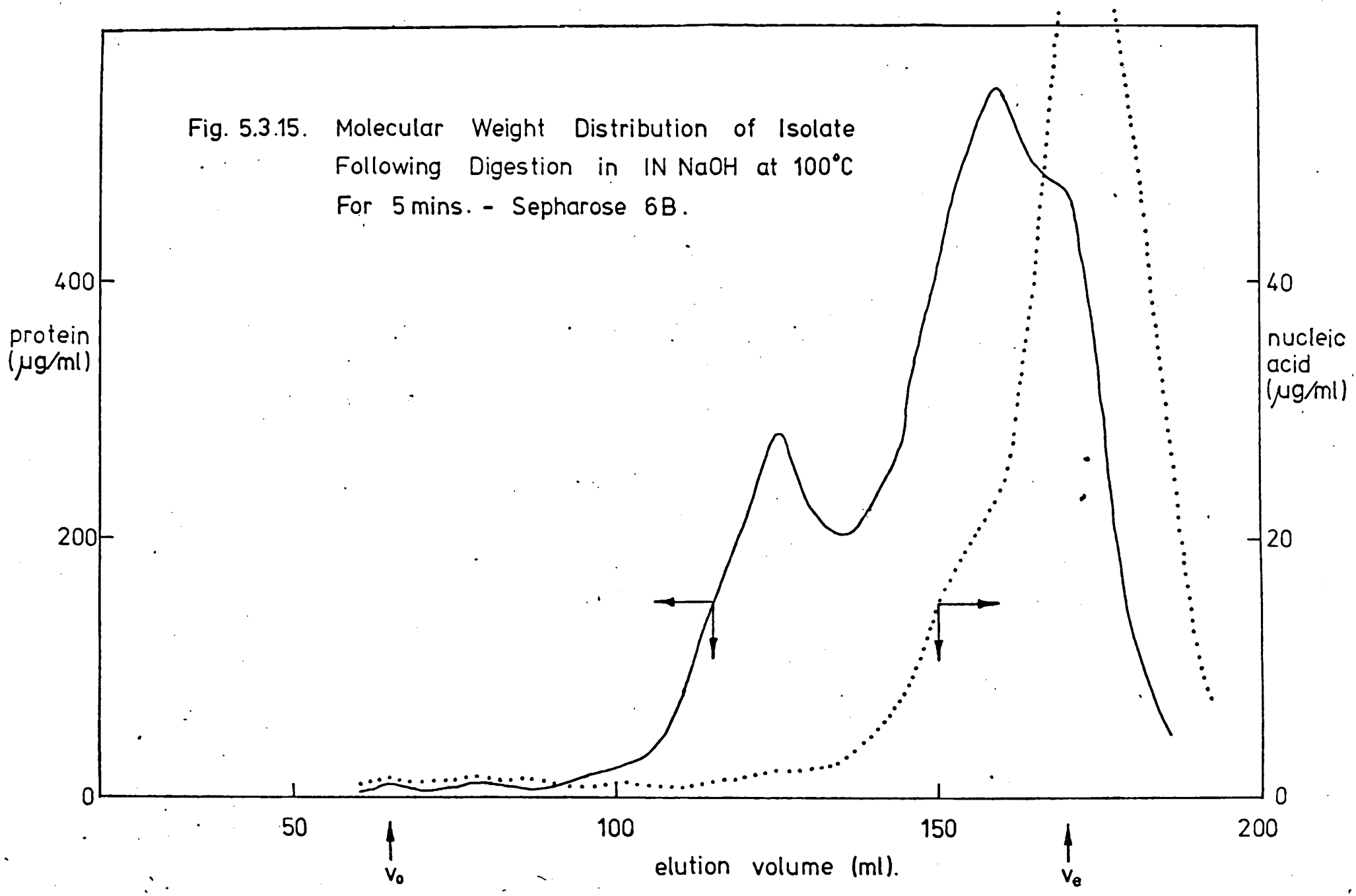
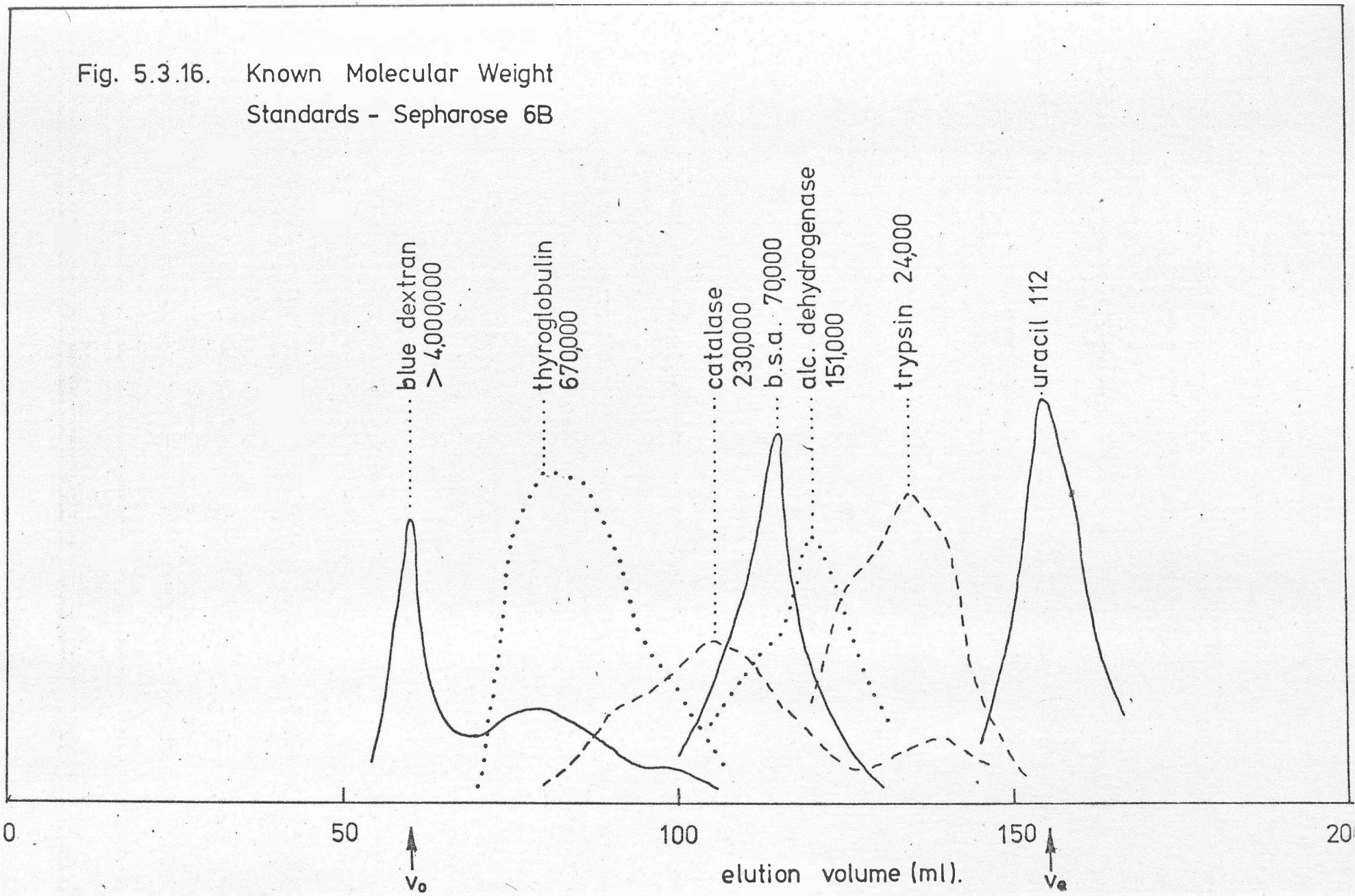


Fig. 5.3.15. Molecular Weight Distribution of Isolate Following Digestion in IN NaOH at 100°C For 5 mins. - Sepharose 6B.

Fig. 5.3.16. Known Molecular Weight Standards - Sepharose 6B



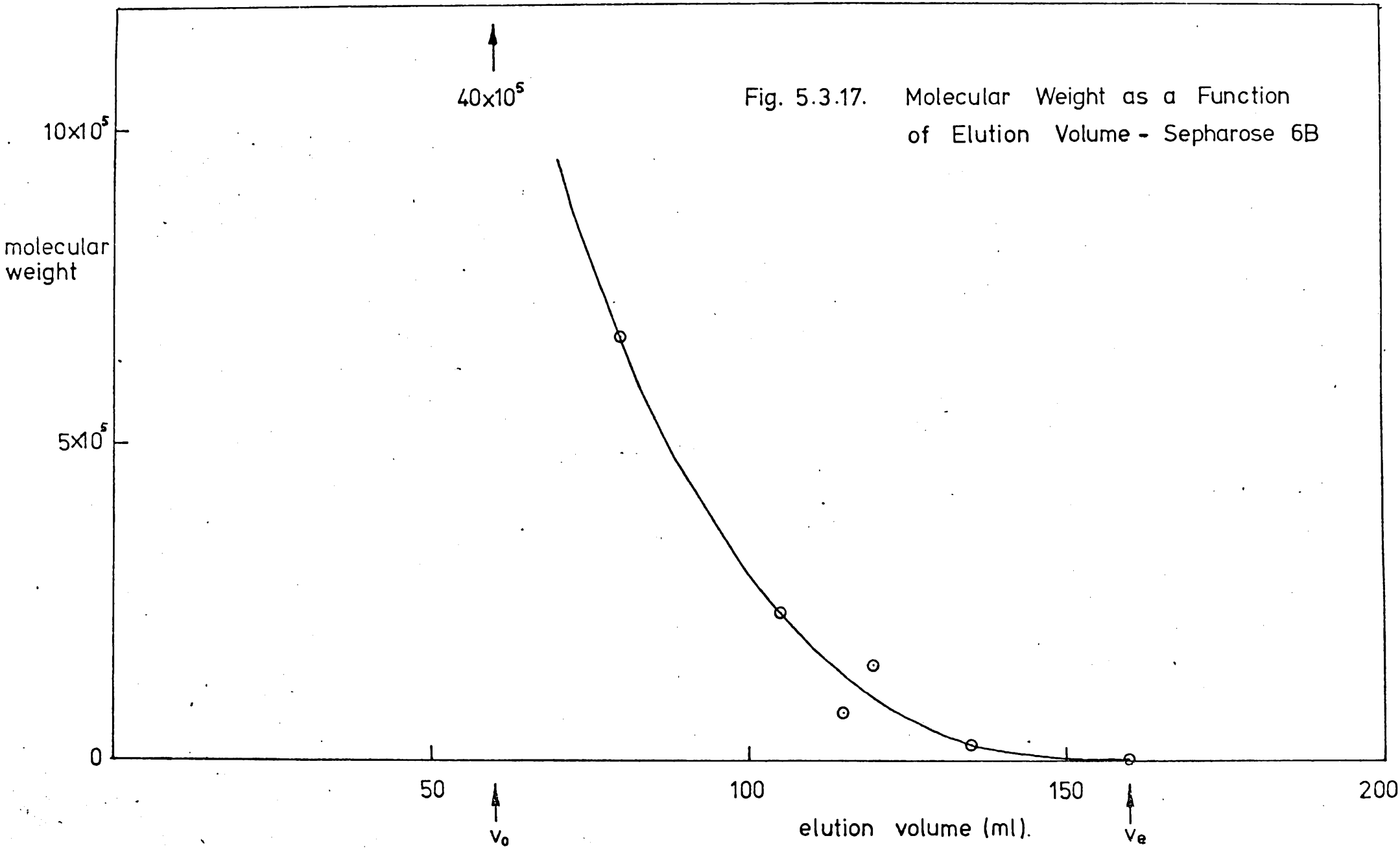


Fig. 5.3.17. Molecular Weight as a Function of Elution Volume - Sepharose 6B

decreased from about 6.0 in the fresh yeast to about 5.0 in the isolate: thirdly, processing in this way has a considerable effect on the protein structure, particularly with regard to the formation of large, non-covalently bonded aggregates.

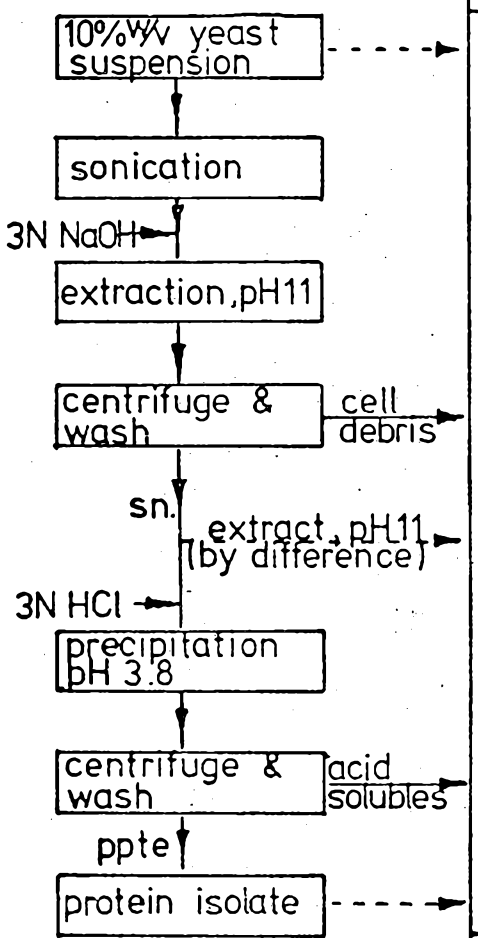
It was decided to adopt the standpoint that high protein yield was the primary target coupled with the highest possible functional properties of the protein. For this reason rapid extraction and washing at pH 11 and 5°C followed by precipitation and washing at pH 3.8 at room temperature was adopted as the standard technique for protein isolate preparation. A material balance on this procedure is shown in Figure 5.3.18. The results are based on 100% yeast disruption.

Due to the high nucleic acid content of the product it was necessary to investigate methods of reducing this to a low level. These are described in the next section.

5.4. Removal of Nucleic Acid.

As suggested in section 2.3.4., for SCP material intended for use as meat analogues a protein to nucleic acid ratio of 25:1 or more would be required in order to keep the level of SCP nucleic acid in the diet down to less than 2g/day. Although the ratio 25:1 is a somewhat arbitary figure it nevertheless provides a useful basis by which to assess the different techniques. The results of experiments to increase the protein to nucleic acid ratio are reported below. Following the conclusions of section 2.3.4.8. major emphasis was put on endogenous enzyme methods.

Fig. 5.3.18. Typical Material Balance for Yeast Protein Isolation. (For 100% cell disruption).



	yield (%)				composition (% of dm.)			protein
	protein	nucleic acid	carbo-hydrate	dry matter	protein	nucleic acid	carbo-hydrate	nucleic acid
10%w/v yeast suspension	100	100	100	100	45	7.5	44	5.9
cell debris	10	3	34	20	22	1.1	75	20
extract, pH11 (by difference)	(90)	(97)	(66)	(80)	(50)	(9.2)	(36)	(5.5)
acid solubles	17	19	nd.	34	22	4.3	nd.	5.1
protein isolate	72	83	19	47	68	13	18	5.2
unaccounted for	-1	+5	—	-1				

sn. - supernatant
ppte.- precipitation
nd. - not determined

5.4.1. Preliminary Investigations.

Several early attempts to remove nucleic acid were unsuccessful.

Precipitation of protein with acetic acid instead of hydrochloric acid did not give an increased protein to nucleic acid ratio (P/NA). Precipitation with perchloric acid at room temperature and pH 3.8 gave a P/NA of 20:1 with no loss of protein yield but this method was rejected due to the high acid cost, about fourteen times the cost of HCl on an equimolar basis.

Precipitation of nucleic acid from water or alkali extracts with 0 - 20%w/v manganous chloride was attempted but high nucleic acid removal also resulted in considerable loss of protein. The method has been used for purification of some enzymes and other proteins (Korkes et al, 1951) but proved unsuitable for this work.

Following the observation that alkaline hydrolysis of yeast nucleic acid occurred at pH 11, even at ambient temperatures, the effect of pH, time and temperature of extraction was studied with a view to using this process for nucleic acid removal (section 5.3.2.6., Figure 5.3.14.). Extracts from disrupted yeast suspensions were incubated at temperatures of 25,30,37 and 45°C at pH 11 or pH 12. Incubation times from one to 24 hours were investigated. In no case did the P/NA level exceed 7.0. More severe conditions were not investigated for fear of toxic effects and loss of nutritional value. Clearly nucleic acid hydrolysis does occur (Figure 5.3.14) but it is insufficient to prevent precipitation of the nucleic acid fragments.

The use of NaCl to change the ionic strength during protein extraction and precipitation did not lead to significant changes in P/NA levels. Concentrations of up to 5% NaCl were used.

5.4.2. Heat Shock and Incubation of Yeast Cells.

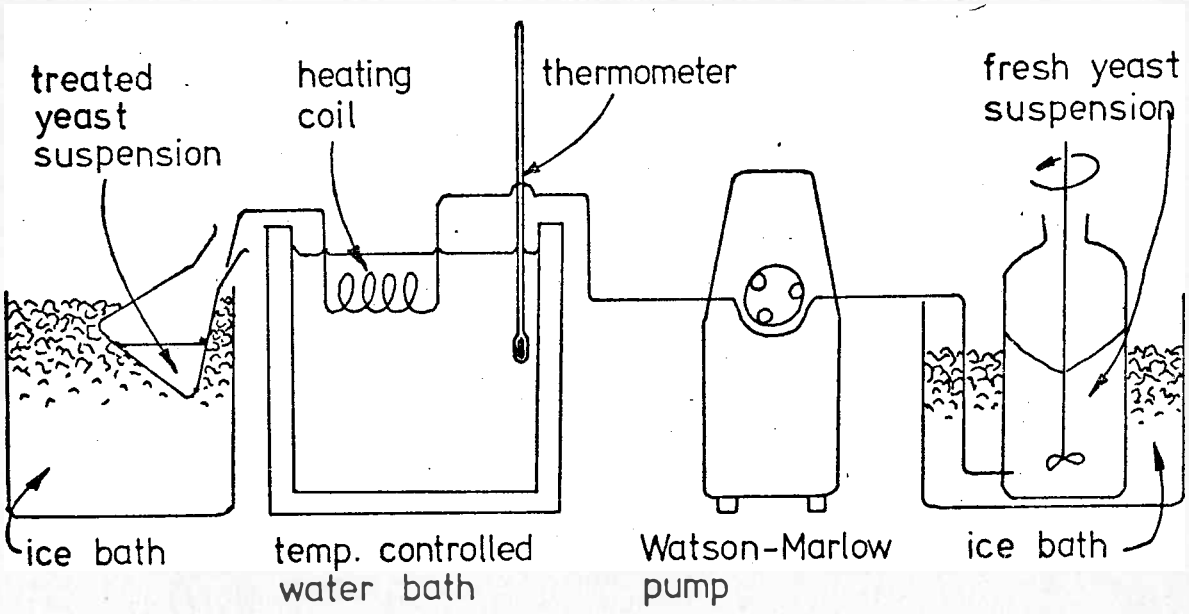
Most of the methods of nucleic acid removal referred to in the literature are based on that described by Maul et al (1970) (section 2.3.4.1.). This method had not previously been thoroughly investigated for Bakers' yeast and it was therefore decided to study it in some detail.

5.4.2.1. Apparatus.

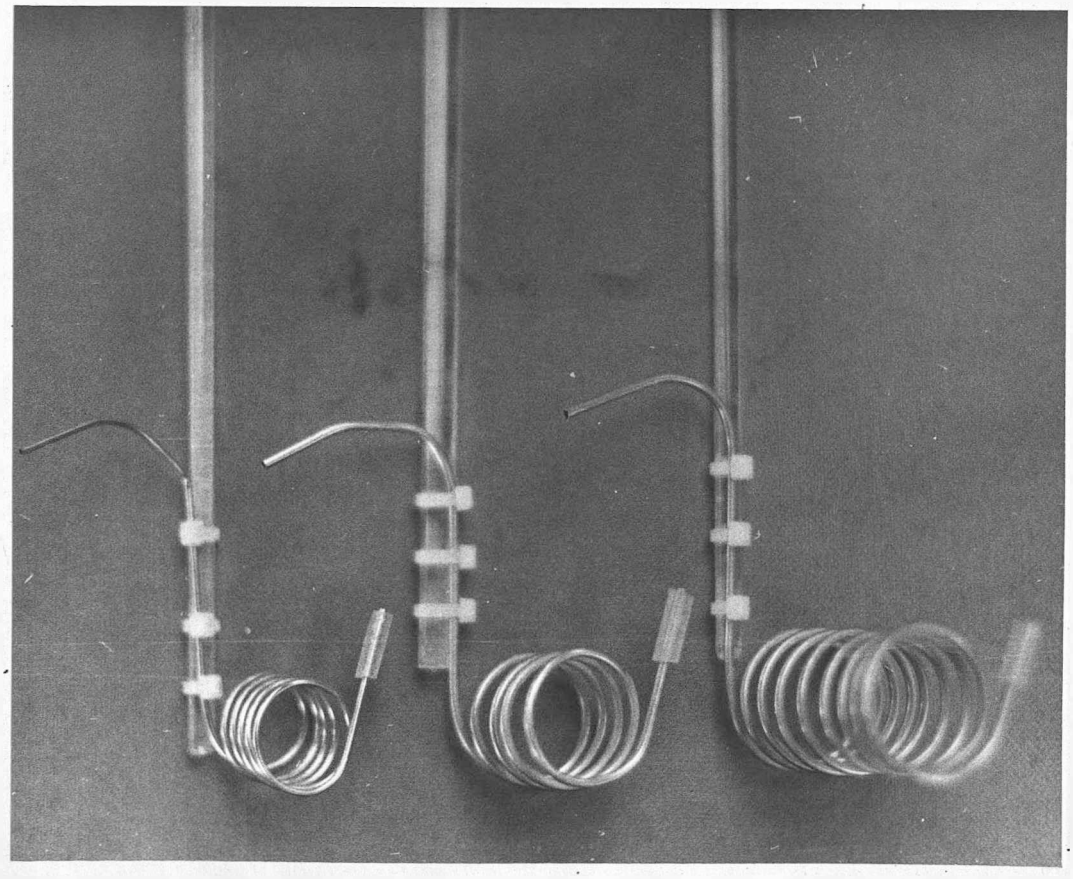
The apparatus used for heat shocking is shown in Figure 5.4.1. A fresh yeast suspension at 0°C was pumped to a thin walled, stainless steel tube immersed in a water bath held at the required temperature. The suspension passed from the tube into a collecting vessel.

In Tannenbaum's patent on this process a tube 56cm long and 0.795mm in diameter was used. They reported that for a heat shock time of 6.0 seconds the temperature of the suspension in the tube was within 1°C of the bath temperature after 1 second. Our tubes were designed to give Reynolds numbers not less than those achieved by Tannenbaum et al (1973). A standard flowrate of 5ml/min and tubes of different lengths and diameters were used to give residence times of 2 - 20 seconds. It was envisaged that, if successful, this process would operate continuously in conjunction with sonication.

Fig. 5.4.1.(a). Apparatus for Heat Shock.



(b) Heat Shock Coils.



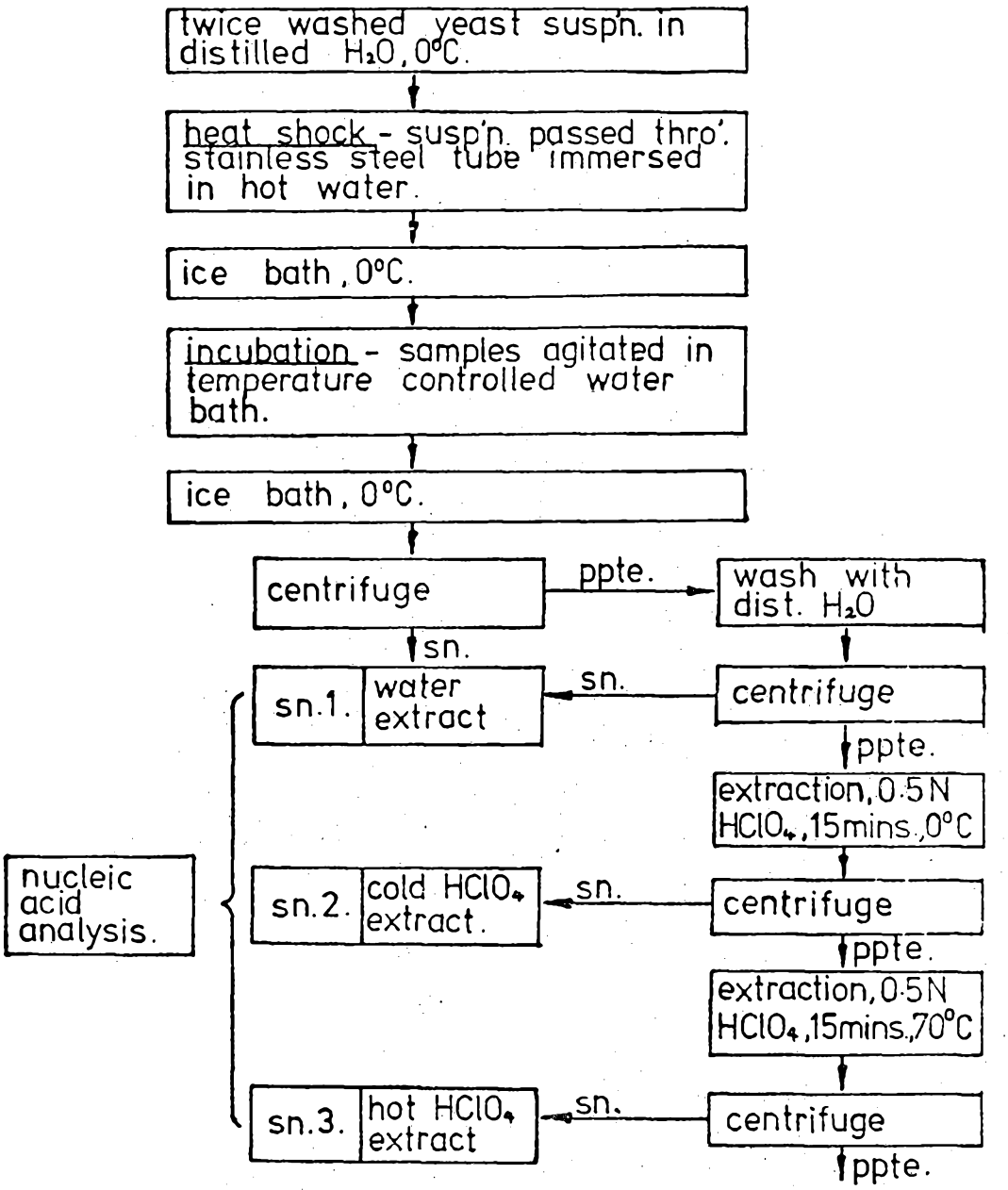
5.4.2.2. Procedure.

A series of preliminary experiments was aimed at finding the optimum nucleic acid removal conditions without having to resort to the lengthy procedure of sonication and protein extraction from each sample treated (Figure 5.4.2.). The temperatures quoted for heat shocking are the water bath temperatures. The heat shock times are the mean residence times for fluid in the steel coils. Fluid was collected by touching the tube outlet onto the side of a conical flask or test tube in an ice bath. This prevented 'drop' formation at the end of the tube which would have increased the effective residence time. Fresh yeast suspension was passed through the tubes, collected on ice and 10ml samples were incubated at different temperatures. These were cooled and the cells were centrifuged, washed with distilled water and re-centrifuged. The combined supernatants were called supernatant (SN) 1. The cell pellet was extracted with 10mls 0.5 N $HClO_4$ for 15 minutes at $0^{\circ}C$. This served to extract any acid soluble nucleic acid fragments which would not have been precipitated on protein isolation. The tubes were centrifuged again. The cold $HClO_4$ extract was known as SN 2. Finally the remaining cell pellet was extracted with 10mls 0.5 N $HClO_4$ at $70^{\circ}C$ for 15 minutes to hydrolyse and extract the remaining, highly polymerised nucleic acid. The supernatant after centrifuging was called SN 3. The three supernatants were analysed for nucleic acid by UV as described in section 4.2.2. The effectiveness of the treatment was described by:

$$\% \text{ nucleic acid removed} = \left\{\frac{SN1 + SN2}{SN1 + SN2 + SN3}\right\} \times 100.$$

$$= \left\{\frac{\text{acid soluble fragments}}{\text{total nucleic acid}}\right\} \times 100$$

Fig. 5.4.2. Heat Shock and Incubation - General Procedure.



5.4.2.3. Results.

Heat shock. Using incubation periods of one hour at 50°C and one hour at 55°C, the optimum heat shock temperature was found to be 72°C. This was quite critical and may explain the poor results obtained for Bakers' yeast using heat shock temperatures of 68°C (Sinskey & Tannenbaum, 1975) which is less than one third as effective as heat shock at 72°C for a residence time of 2.5 seconds. Residence times of 2.5 or 6 seconds gave the best results, 6 seconds being preferred as the temperature optimum was less critical (Figures 5.4.3. and 5.4.4.). In this way nucleic acid removal of more than 50% was achieved.

Incubation. The optimum incubation temperature following heat shock was 55°C. Incubation at 60°C gave better results over a short period (one hour) but prolonged incubation at this temperature appeared to denature the enzyme (Figure 5.4.5.). No advantage was found in using combinations of two periods of incubation as described by Maul et al (1970).

A linear relationship exists between incubation time and nucleic acid release up to 50°C. At 55°C and particularly at 60°C there is a fall off in rate with time as the enzymes are heat denatured and hence inactivated (Figure 5.4.6.).

Cell suspension concentration. Figure 5.4.7. shows that nucleic acid removal is much greater at lower yeast concentrations. As nucleotides are produced they diffuse through the cell wall into the suspending fluid. If the cell concentration is high the concentration gradient of nucleotides across the cell walls rapidly decreases as nucleodtides are released into the suspending

Fig. 5.4.3. Effect of Heat Shock Temperature on Nucleic Acid Removal. (Incubation : 50°C, 1hr + 55°C, 1hr).

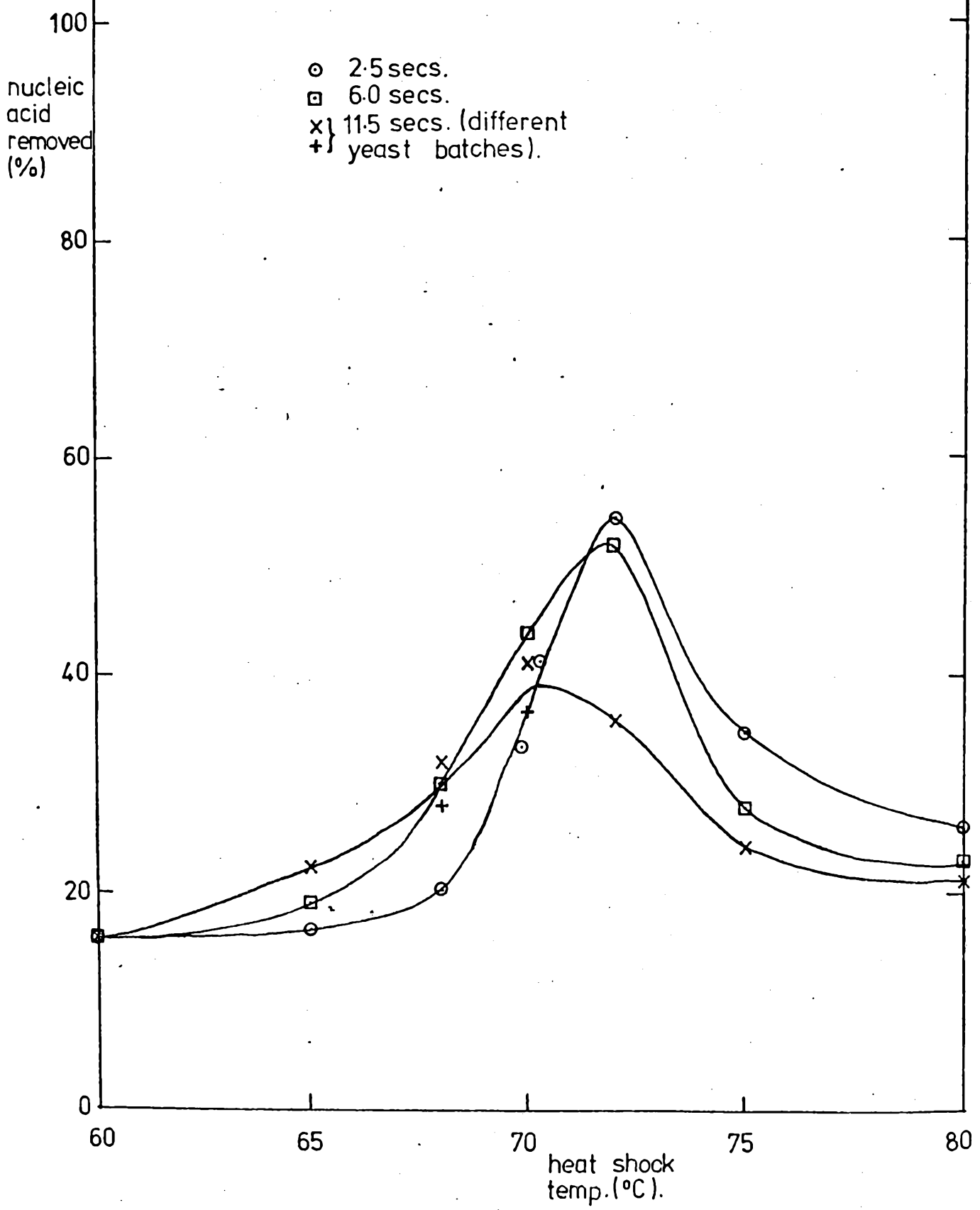


Fig. 5.4.4. Effect of Heat Shock Time on Nucleic Acid Removal. (Incubation: 50°C, 1hr + 55°C, 1hr.).

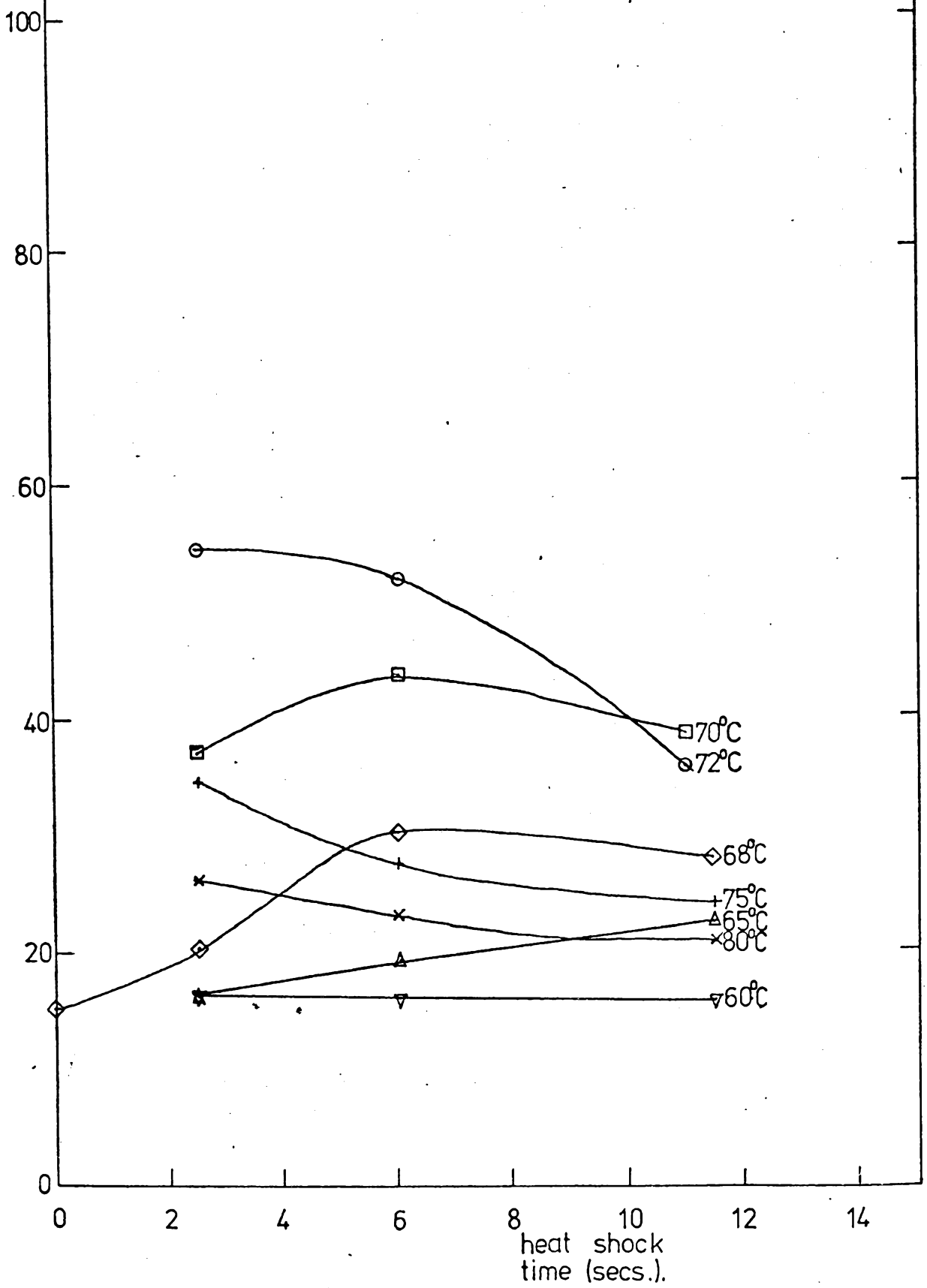


Fig. 5.4.5. Effect of Incubation Temperature on Nucleic Acid Removal. (Heat shock: 70°C, 6 secs.).

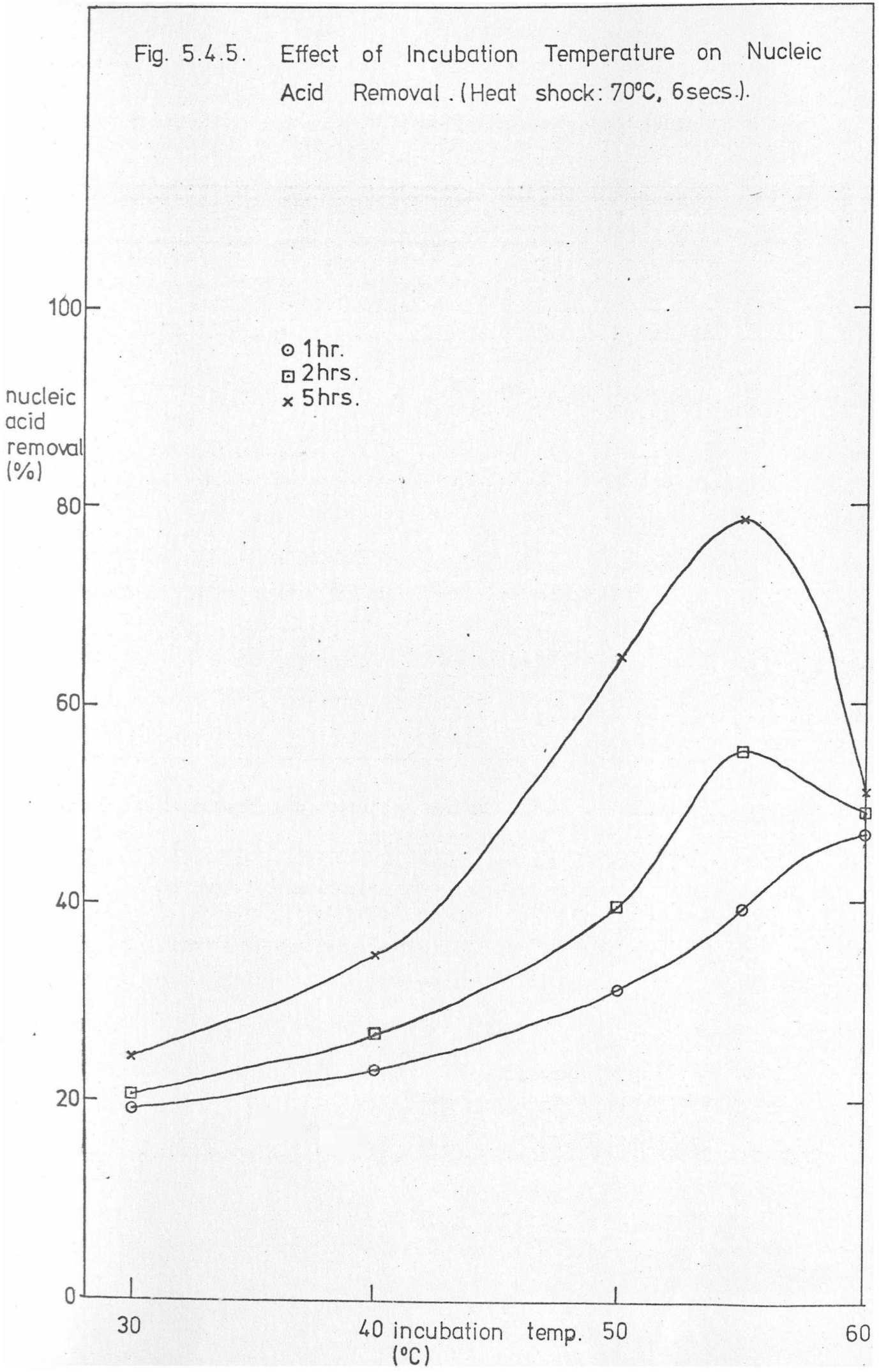


Fig. 5.4.6. Effect of Incubation Time on Nucleic Acid Removal. (Heat shock: 70°C, 6 secs.).

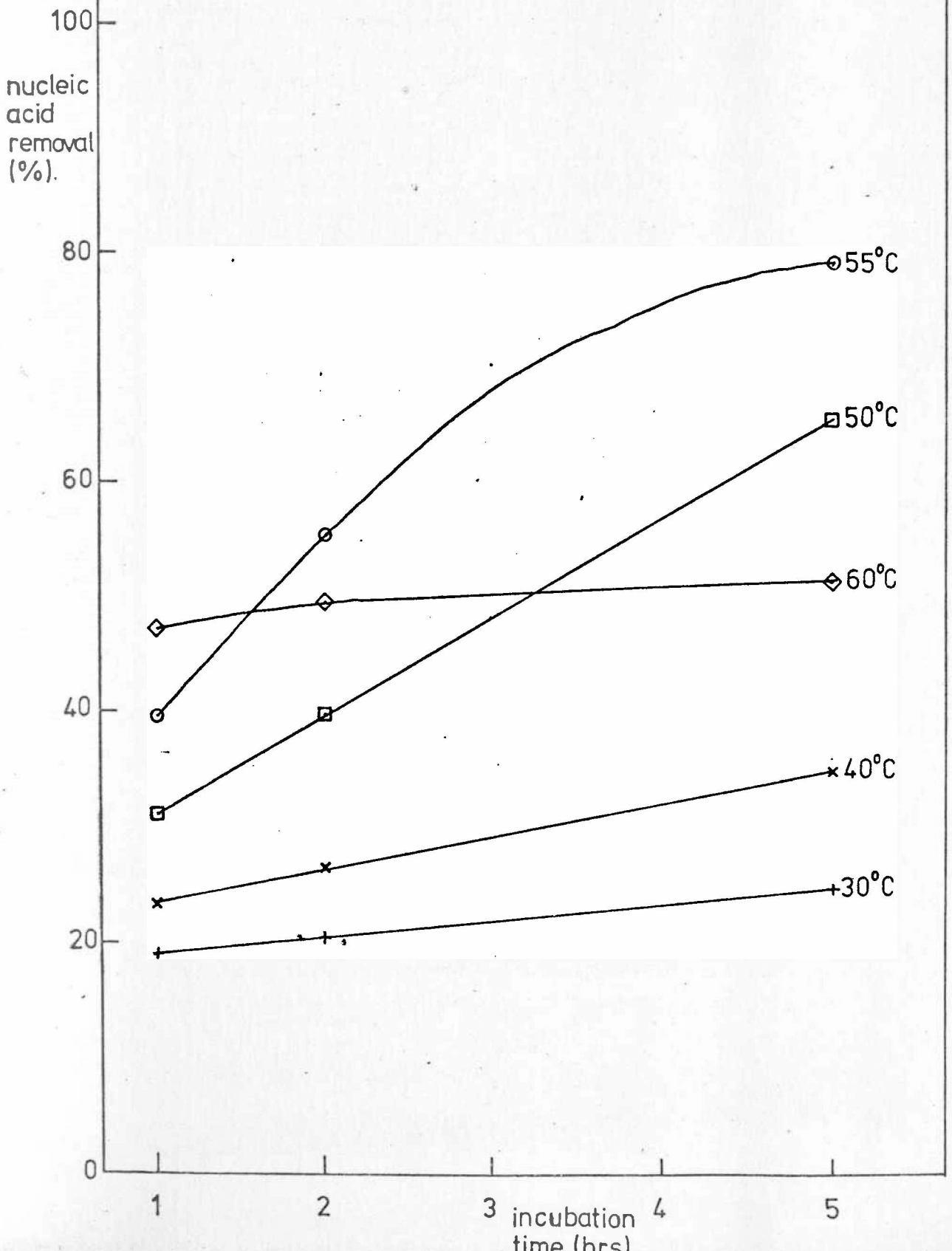
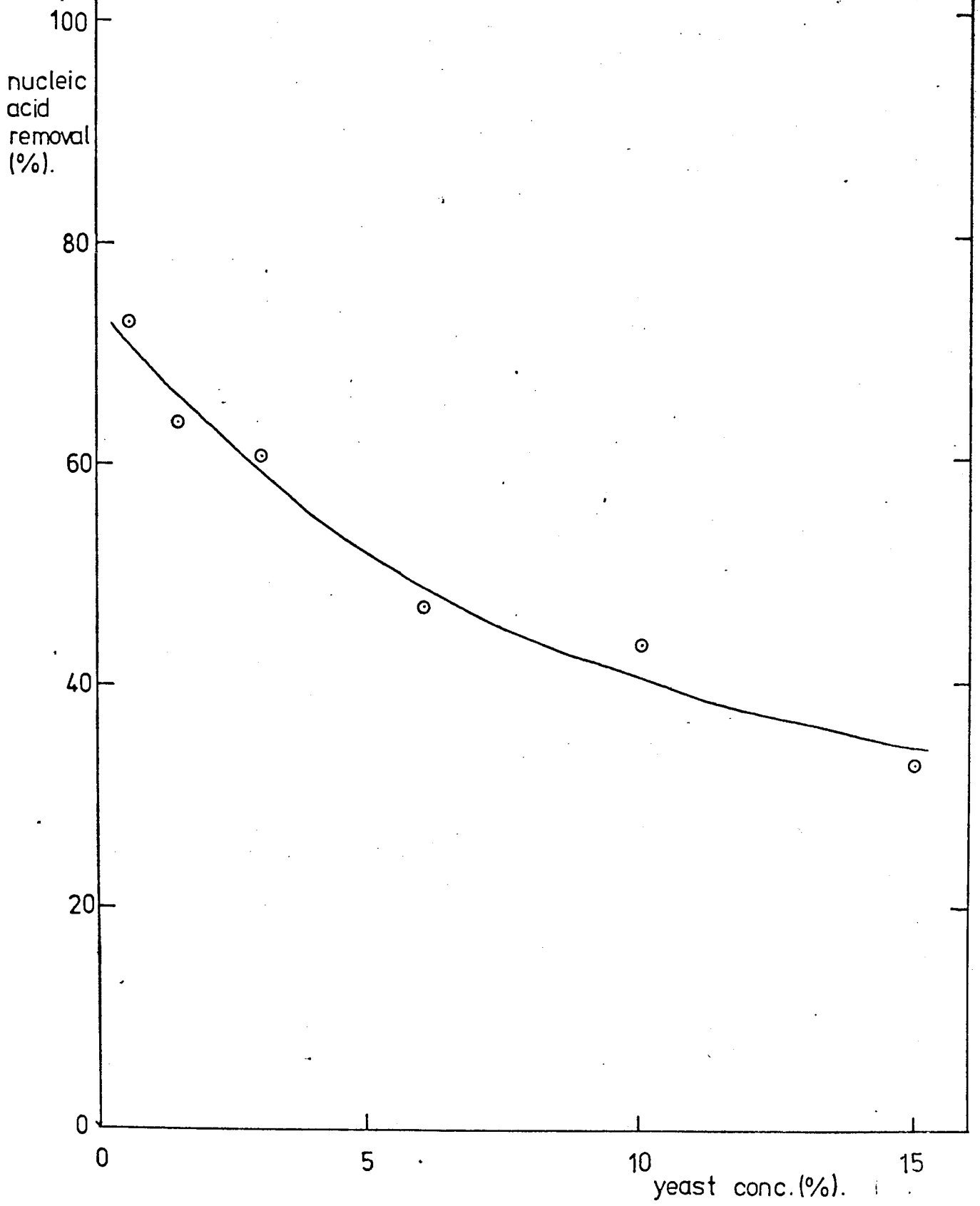


Fig. 5.4.7. Effect of Yeast Concentration on Nucleic Acid Removal. (Heat shock: 70°C, 6secs.; incubation: 50°C, 1hr.; 55°C, 1hr..)



fluid. Imada et al (1972 (2)) reported that inhibition of nuclease action was by a macromolecular inhibitor promoted by ATP and purine nucleotides. Hence it would seem that high nucleotide concentrations inhibit the reaction. This is borne out by the fact that the ratio SN 1 (nucleotides released into the suspending fluid) to SN 2 (nucleotides remaining in the cells) increases with increasing concentration and leads to decreased nucleic acid removal (Table 5.4.1.)

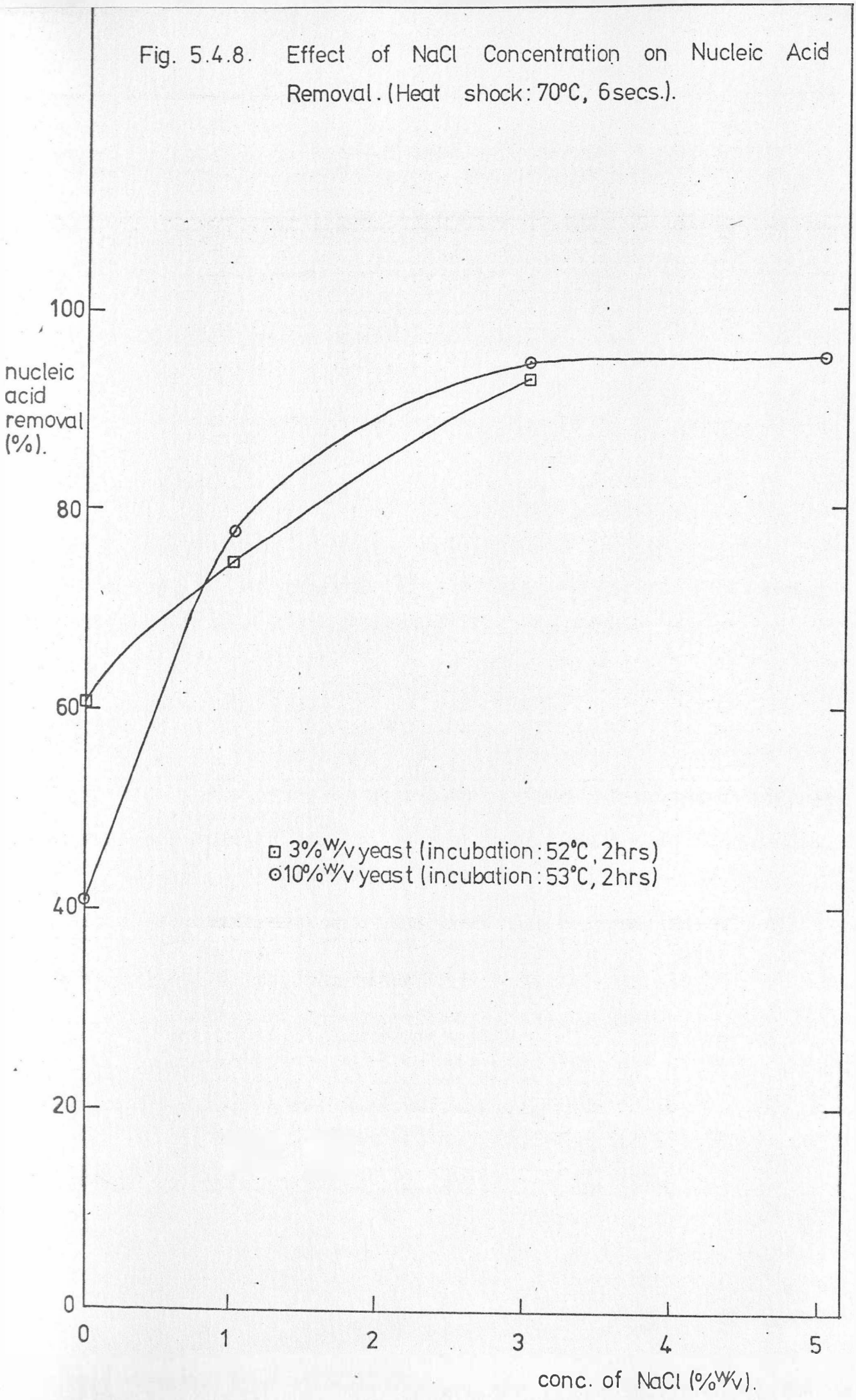
Table 5.4.1. Effect of Yeast Concentration on Nucleic Acid Removal.

yeast concentration (%w/v dm.)	SN 2 / SN 1	% nucleic acid removed
0.6	0·13	72·9
1.5	0.30	63·6
3·0	0.37	60·6
6·0	0·38	46·7
15·0	0·47	32·5

Yeast age. There was a 17% drop in nucleic acid removal after storage of the pressed yeast for one week at 4°C probably due to inactivation of the nucleases as the cells begin to autolyse.

Incubation with NaCl. As shown in Figure 5.4.8. incubation with NaCl gave a dramatic increase in nucleic acid removal, the optimum being 3% NaCl at which over 90% of the nucleic acid was removed following heat shock at 70°C, 6 seconds and incubation for 2 hours at 53°C. As shown by the curves for different yeast

Fig. 5.4.8. Effect of NaCl Concentration on Nucleic Acid Removal. (Heat shock: 70°C, 6 secs.).



concentrations, it was the actual salt concentration rather than the salt to cell ratio which was important. As previously reported (Barker et al, 1975) it appears that salt is effective in inactivating the inhibiting mechanism. With salt the effect of yeast concentration appears to be less important (Figure 5.4.8.).

Because of the dramatic changes brought about by the use of NaCl the heat shock and incubation conditions were reinvestigated (Figures 5.4.9. - 5.4.12). The optimum conditions for heat shock and incubation are similar to those obtained without salt but incubation for much shorter times led to high nucleic acid removal (e.g. ½hr at 60°C gave 92% nucleic acid removal).

5.4.2.4. Complete extraction.

A selection of the most promising conditions was made on which to carry out the complete extraction process. Samples were sonicated on a batch basis, after heat shock and incubation, for 20 minutes (usually quite sufficient to effect complete disruption). The standard extraction and precipitation procedure was then performed and the protein isolates were analysed for protein, nucleic acid and dry matter contents.

Results.

Table 5.4.2. Extraction of Protein from Heat Treated Cells.

heat treatment		protein yield cf. control. (%).	protein / nucleic acid
control - no heat treatment		100	4·9
no NaCl	heat shock 70°C, 6sec, no incub'n.	68	8·9
	heat shock 70°C, 6sec, incub'n. 55°C 2hrs.	44	7·8
3%w/v NaCl	heat shock 70°C, 6sec, no incub'n.	31	4·9
	heat shock 70°C, 6sec, incub'n. 55°C 2hrs.	12	17·1

Fig. 5.4.9. Effect of Heat Shock Temperature on Nucleic Acid Removal in Yeast Suspensions Containing 3% W/v NaCl. (Incubation : 52°C, 2hrs.).

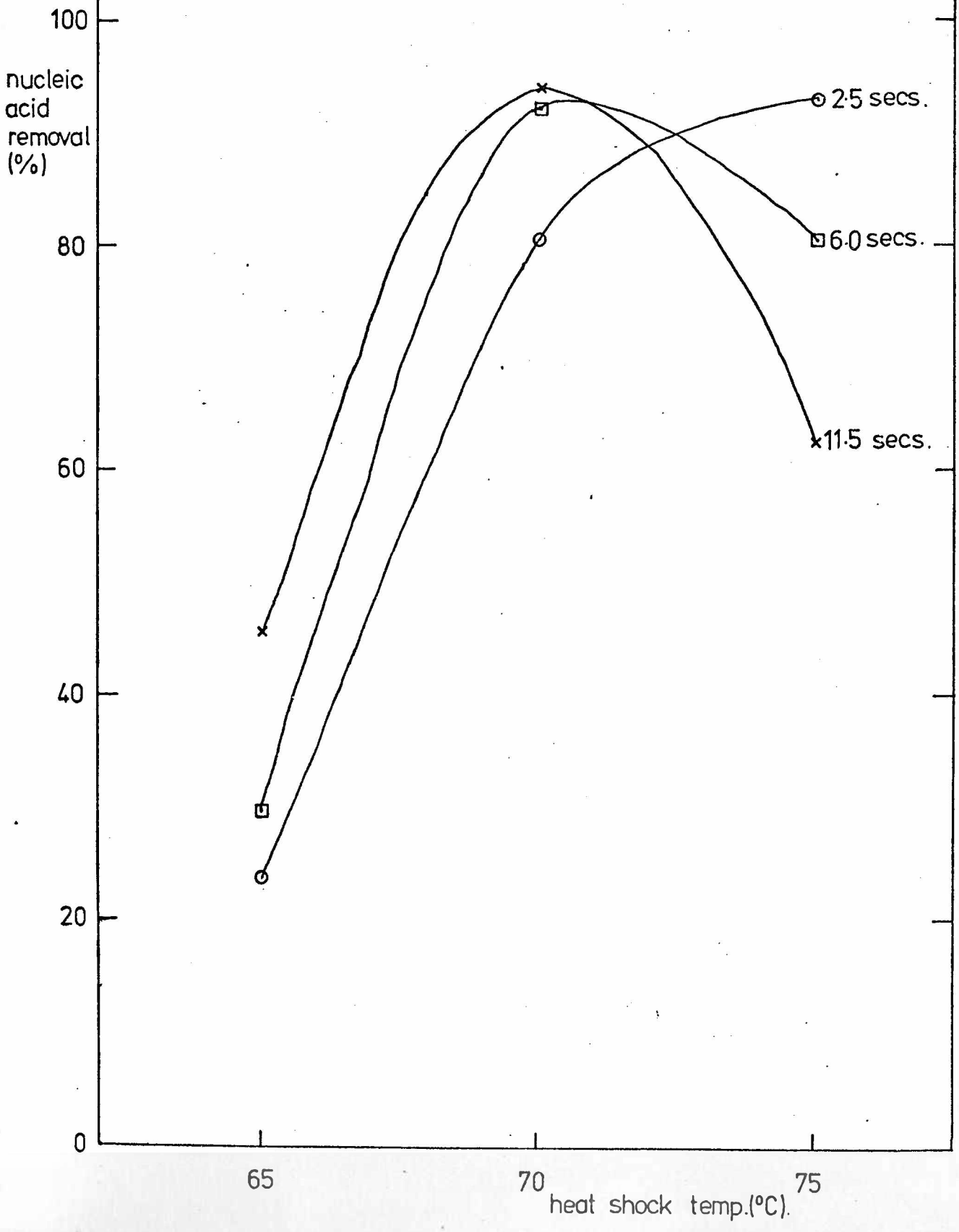


Fig. 5.4.10. Effect of Heat Shock Time on Nucleic Acid Removal in Yeast Suspensions Containing 3% W/v NaCl. (Incubation: 52°C, 2hrs.).

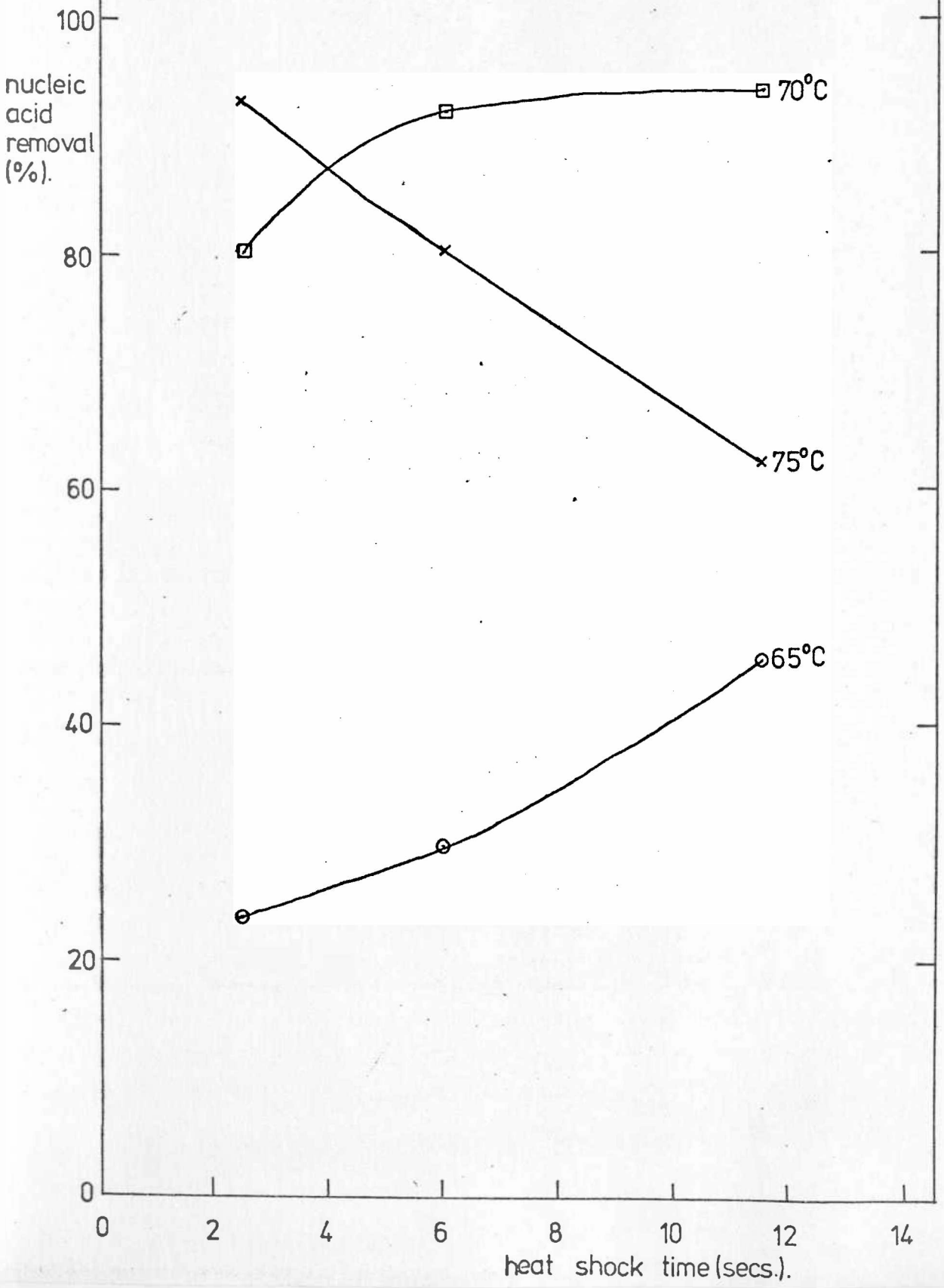


Fig. 5.4.11. Effect of Incubation Temperature on Nucleic Acid Removal in Yeast Suspensions Containing 3% NaCl. (Heat shock: 70°C, 6 secs.).

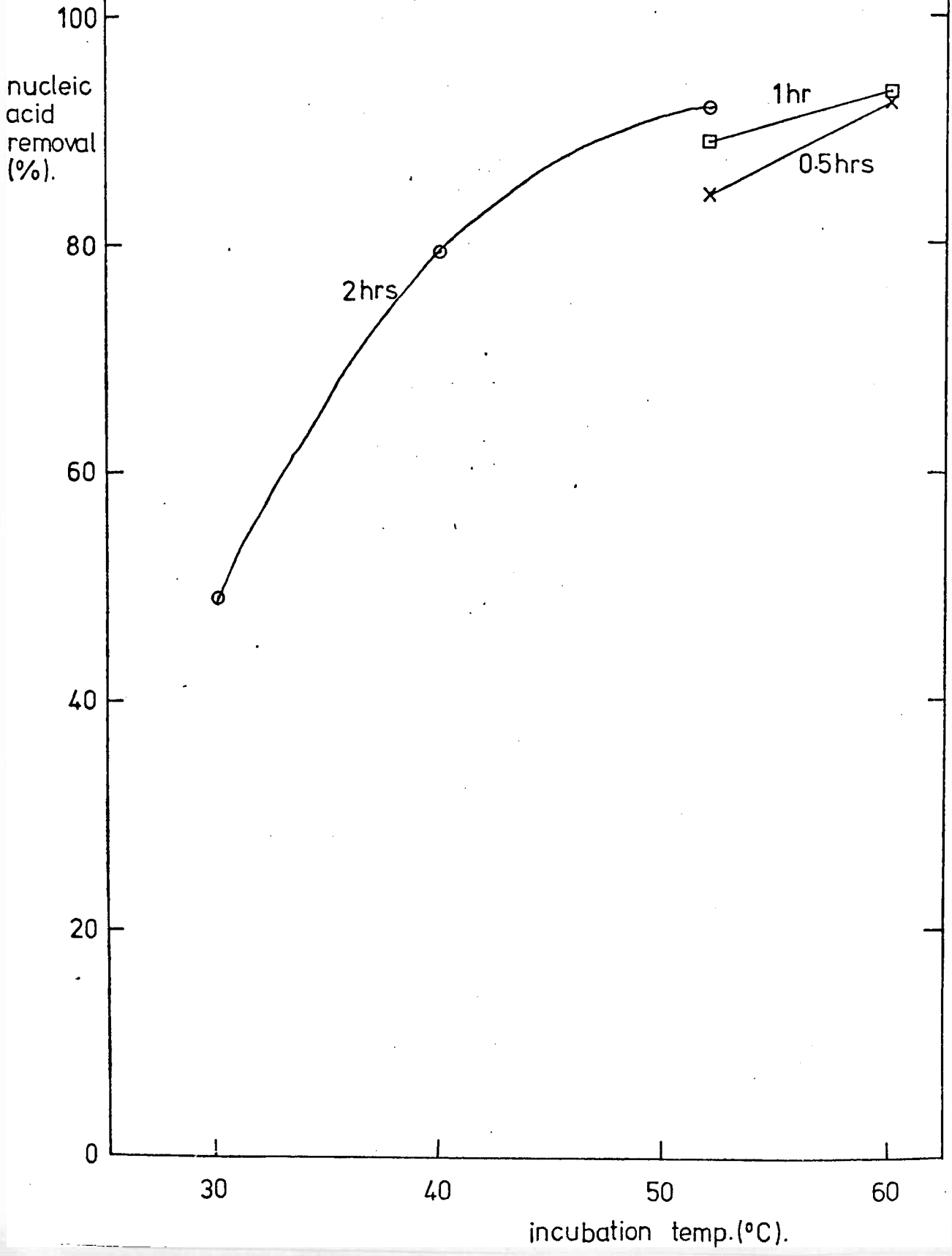
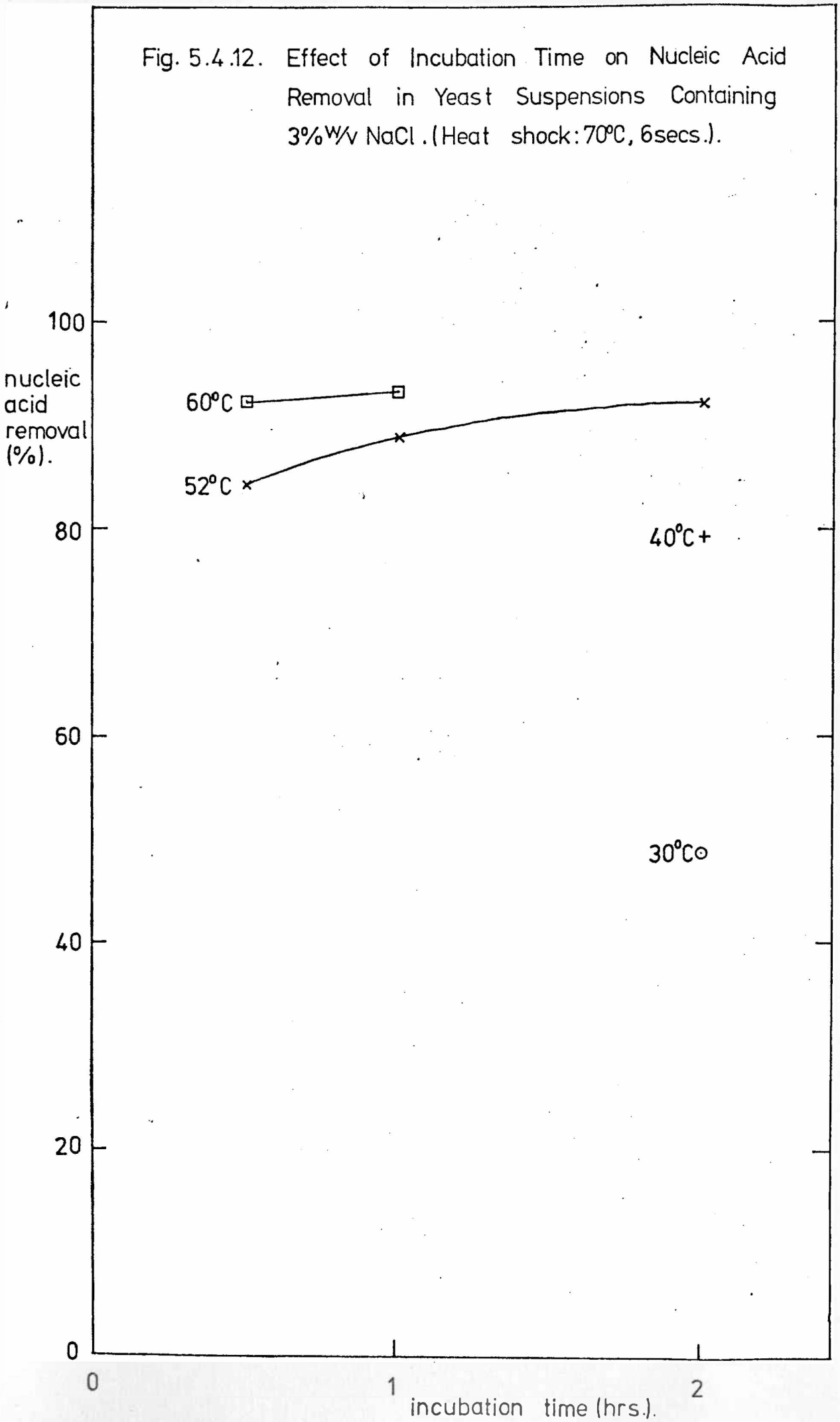


Fig. 5.4.12. Effect of Incubation Time on Nucleic Acid Removal in Yeast Suspensions Containing 3% W/v NaCl. (Heat shock: 70°C, 6secs.).



The results were disappointing. In general conditions which led to high nucleic acid removal also led to low protein yields (Table 5.4.2.). Reduction in protein yield was due both to heat and salt denaturation of the protein so that it was not solublised at the alkaline extraction stage and to the increased difficulty of rupturing the cells following heat treatment (Newell et al, 1975 (1)). Absolute levels of nucleic acid removal were consistent with the results for preliminary tests. Low protein to nucleic acid levels were therefore due to low protein yield rather than low nucleic acid removal. Loss of protein by denaturisation was almost equally due to the heat shock and the incubation treatment.

Incubation at lower temperatures, although giving higher protein yields, did not lead to significant nucleic acid removal.

It was concluded that this method would be extremely effective for removal of nucleic acid in cases where further extraction was not to be performed and functional properties were not important. However, the process did not meet the criteria already defined in section 3.

5.4.3. Incubation of Disrupted Cell Suspensions.

5.4.3.1. Endogenous nucleases.

Lindblom and Mogren (1974) had been successful in removing nucleic acid from Bakers' yeast by incubation of the disrupted suspension with 3%w/v NaCl at 50°C. As they had thoroughly investigated the process their optimum conditions were used. After incubation the suspension was cooled and extracted at pH 11.0. Protein was precipitated at pH 3.8 and the isolate analysed for protein, nucleic

acid and dry matter. The results are summarised in Table 5.4.3.

Table 5.4.3. Nucleic Acid Removal by Incubation of Disrupted Yeast Suspensions.

incubation conditions	protein yield in isolate cf. control	protein/nucleic acid
control – no incubation	100	4.5
incubation at :		
50°C, 20 mins, no NaCl	104	4.7
50°C, 10 mins, 3%w/w NaCl	58	8.3
50°C, 20 mins, 3%w/w NaCl	39	11.8
50°C, 30 mins, 3%w/w NaCl	37	13.8

Yet again a similar pattern is seen; heating with NaCl leads to a reduced nucleic acid content but the protein is insolublised and not, therefore, extracted at pH 11. Without salt protein yield is good but there is no nucleic acid removal.

5.4.3.2. Exogenous nucleases.

Incubation with other nucleases was attempted to see whether the inhibiting mecha nisms were only effective against the endogenous nucleases. Bovine pancreatic ribonuclease was used at 0.1 - 20µg/ml, pH 5 - 8, 25 - 50°C, with 0 - 3%NaCl, incubating for periods of 5 minutes to one hour. In some experiments 0.4 - 2.0µg/ml of bovine pancreatic deoxyribonuclease was also used.

In no case did the protein to nucleic acid ratio exceed 10.0. The most effective conditions were incubation at high temperatures with 3%NaCl but this again led to reduced yield, even at 40°C

(e.g. incubation at 40°C with 3% NaCl, 0.4µg/ml RNase, pH 6.0 for 20 mins gave a protein to nucleic acid ratio of 8.3 but the protein yield was only 55% of the control without heat treatment). Adding DNase had no effect on P/NA levels.

5.4.3.3. Assessment of results.

Clearly the results show that after cell disruption the inhibitary mechanism was still effective. Furthermore it was effective against exogenous as well as endogenous nucleases. Although heating with 3% NaCl at 50°C helped to inactivate the inhibitors much of the protein was insolublised and could not be extracted at pH 11. This method would be useful for preparation of a product including the cell walls where solubility was unimportant.

5.4.4. Incubation of Protein Suspensions after Alkaline Extraction.

Robbins et al (1975) described a process for extraction of protein from disrupted yeast and incubation, following cell wall removal, at pH 6.0 to remove nucleic acids by endogenous nuclease action (section 2.3.4.1.). It was sought to emulate this using variations on the standard extraction and precipitation conditions outlined in section 5.3.3.

5.4.4.1. Procedure.

Yeast suspensions were disrupted by sonication in the usual way and protein was extracted at pH 9, 10 or 11. The cell walls were separated by centrifuging and 3 N HCl was added to the alkali extract to adjust the pH to the required level, usually pH 6. The suspensions were then incubated at 40 - 60°C for ½ - 2 hours,

some with NaCl, then cooled in an ice bath. Protein was precipitated at pH 3.8 and the isolate was analysed for protein, nucleic acid and dry matter.

5.4.4.2. Results.

Incubation pH. Incubation at pH 6.0 gave the highest protein to nucleic acid ratio. Different pH's did not affect the protein yield which was about 5% greater than for the control samples with no incubation (Figure 5.4.13).

Incubation temperature. 50°C was the optimum incubation temperature. Again the protein yield did not vary within the range 40 - 60°C (Figure 5.4.14).

Incubation time. Protein to nucleic acid ratio reached a maximum of about 20:1 after incubation for two hours at 50°C. Again the protein yield did not vary significantly with time (Figure 5.4.15).

Yeast concentration. Unlike previous experiments the protein to nucleic acid ratio was not at a maximum at lower yeast concentrations. It reached an optimum value (about 23:1) at a concentration of 5%w/v (Figure 5.4.16). It is known that protein solubility is greater at lower concentrations and the following explanation is therefore suggested.

1. After cell disruption the inhibitary mechanism was still active but this was denatured following extraction at pH 11.0.
2. When the pH was lowered to 6.0 for incubation the denatured inhibitor (probably a protein as already suggested) was less soluble than before and was precipitated. (This ties in with

Fig. 5.4.13. Incubation of Extracted Protein - Incubation pH. (10%W/v yeast conc.; extraction, pH11; incubation, 50°C, 2hrs.)

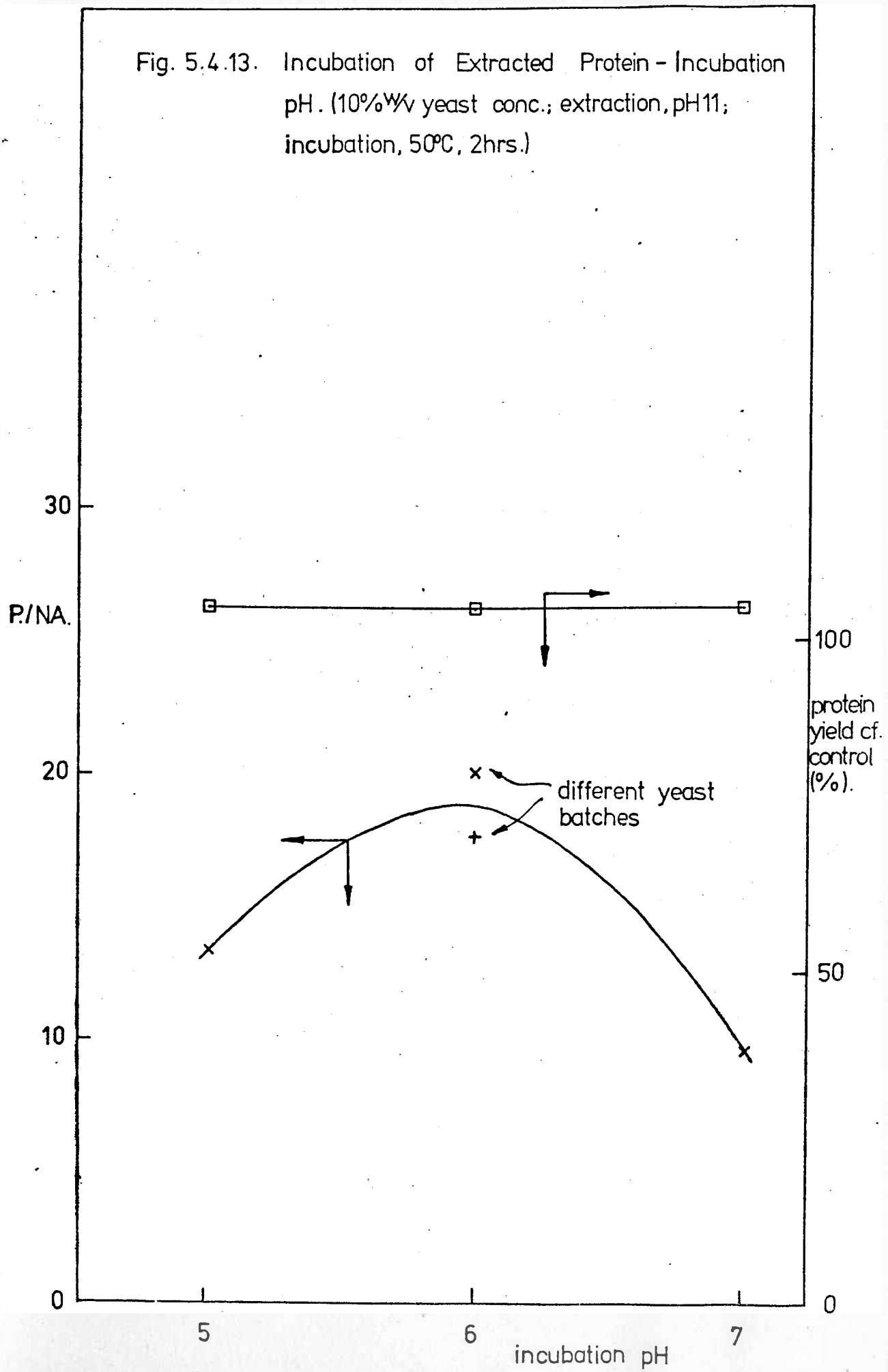


Fig. 5.4.14. Incubation Following Extraction- Incubation Temperature. (Yeast conc.,10%W/v; extraction, pH 11; incubation, pH 6, 2hrs.).

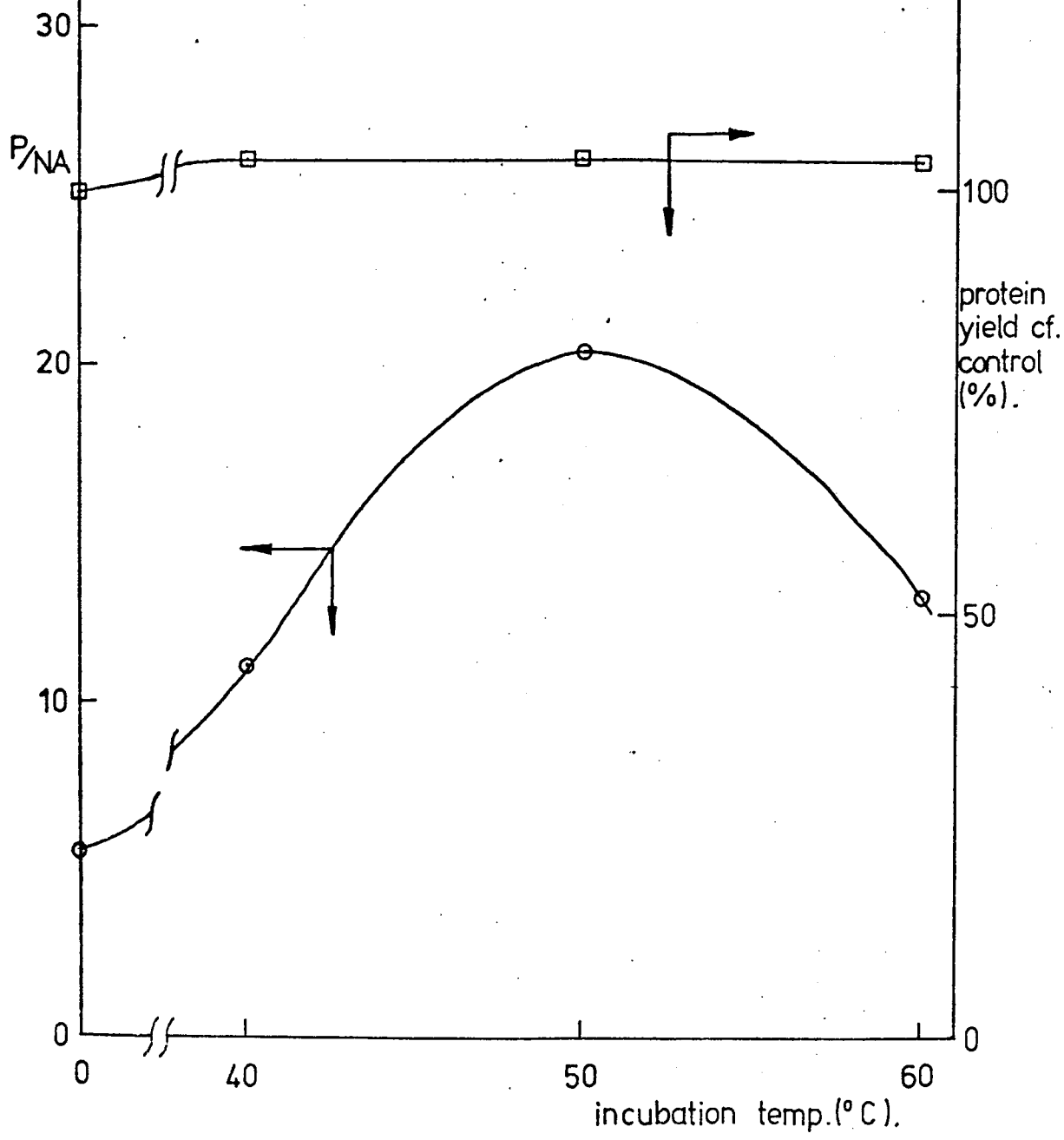


Fig. 5.4.15. Incubation Following Extraction - Incubation Time. (Yeast conc., 10% W/V; extraction, pH11; incubation, 50°C, pH6.)

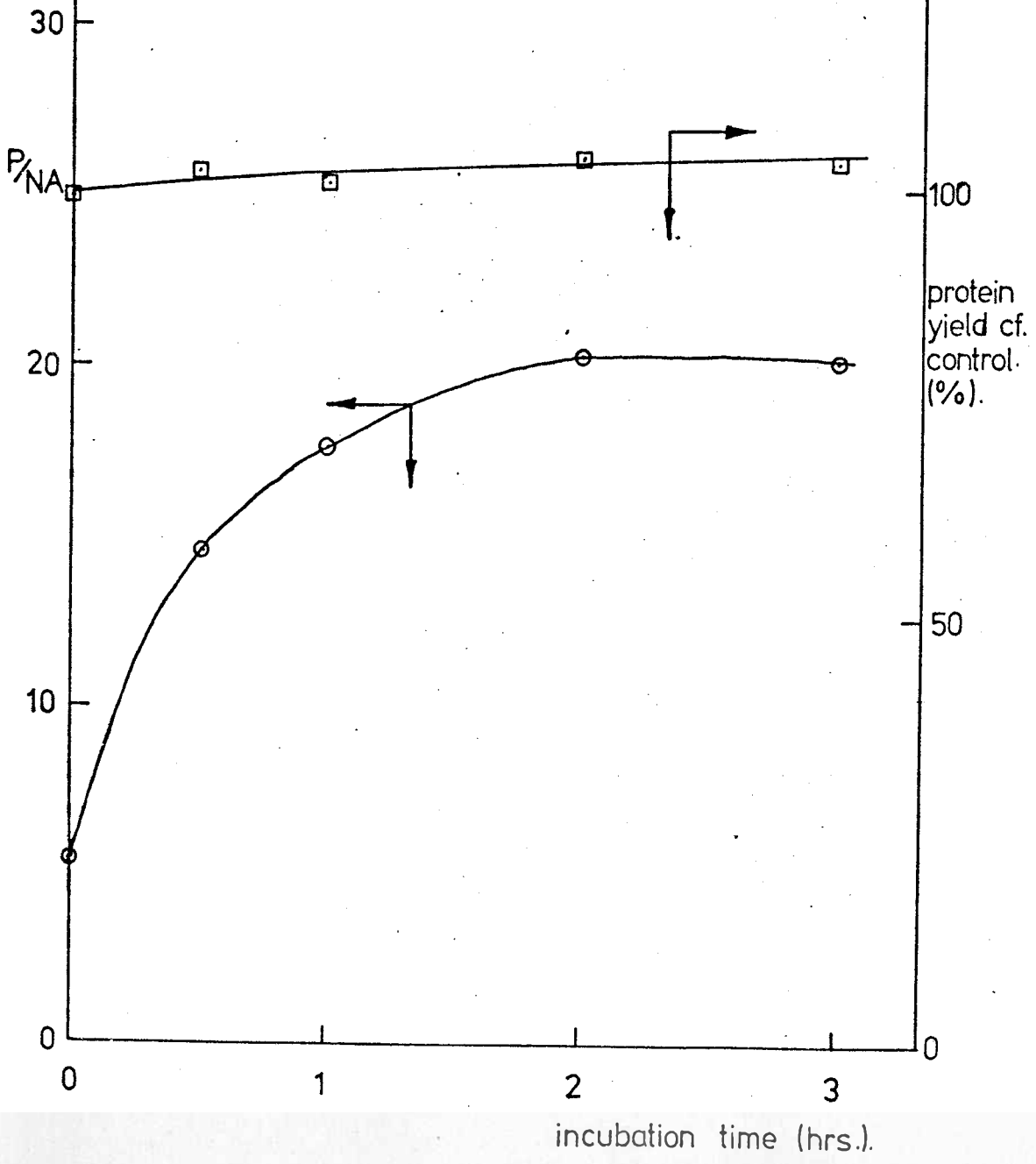
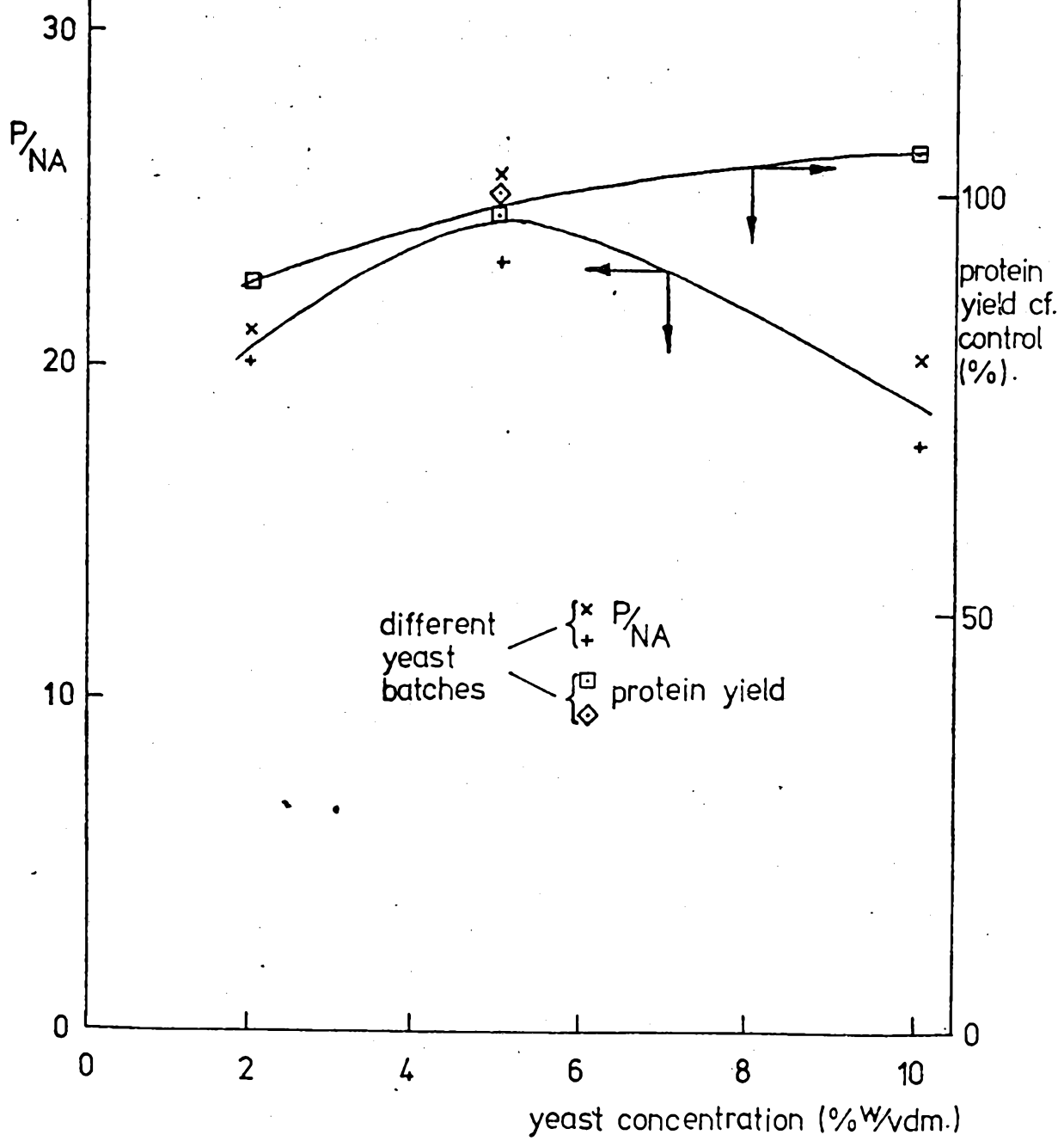


Fig. 5.4.16. Incubation Following Extraction - Yeast Concentration. (Extraction, pH 11; incubation 50°C, 2hrs., pH 6.)



visual observations of protein precipitation at this stage).

3. The nucleases were then free to work with little inhibition.
4. However, at lower concentrations the inhibitor was more soluble and not so easily precipitated following extraction.
5. The inhibition by nucleotides was still most evident at high concentrations so that a balance between these two effects yielded an optimum concentration, about 5%w/v in this case.

Protein yields were higher at high concentrations, again due to the decreased protein solubility at high pH.

Although 5%w/v yeast suspensions gave the high protein to nucleic acid ratio it was decided to continue using 10%w/v suspensions since this would be more economical and the protein yield was higher.

Extraction pH. It was expected that extraction at lower pH's would give higher protein to nucleic acid ratios, due to less nuclease denaturation, but lower yields (as in section 5.3.2.3). Yields were lower but not as low as was anticipated from Figure 5.3.1. This was probably due to increased protein precipitation caused by heat denaturation and aggregation during incubation.

Very surprisingly there was a dramatic fall in the P/NA ratios at extraction pH's less than pH 11. On reflection it seems that extraction at high pH denatures the inhibitor so that it is precipitated at pH 6 and 50°C. However extraction at pH 9 and 10 does not cause sufficient denaturation of the inhibitor to ensure

its precipitation and nuclease action is therefore much lower. This explanation seems more likely than that the nuclease is not extracted at lower pH's since the nuclease is highly active and therefore presumably soluble at much lower pH's (optimum enzyme activity being at pH 6.0) (Figure 5.4.17).

Incubation with NaCl. Incubation with 3%w/v NaCl increased the protein to nucleic acid ratio substantially, but not so dramatically as in earlier experiments. This was probably because the inhibitor had already been substantially inactivated by the alkaline extraction process (Figures 5.4.18. & 5.4.19).

Optimum conditions. The optimum conditions for nucleic acid removal using this method were considered to be:- extraction at pH 11, incubation at pH 6.0, 50°C with 3%NaCl for 2 hours giving a P/NA ratio of more than 25:1. Incubation without salt gave a P/NA ratio of about 20:1 and the protein content of the isolate was over 80%w/w of the dry matter (c/f 70%w/w without nucleic acid removal). With 3%w/v salt the protein content was about 70%w/.. presumably due to the higher salt (and therefore the higher ash) content in the isolate. On the whole these were a very encouraging set of results and further work on texturising properties were carried out on isolates prepared in this way. It will be appreciated that the spinning work and the nucleic acid removal work were conducted concurrently, even though, for convenience, they appear in different sections of this thesis.

5.4.4.3. Spinning.

It had been established (in section 5.5.) that the optimum conditions for spinning were to prepare a dope at pH10.0

Fig. 5.4.17. Incubation Following Extraction - Extraction pH.
(Yeast conc., 10% w/v; incubation, 50°C, 2hrs., pH 6.)

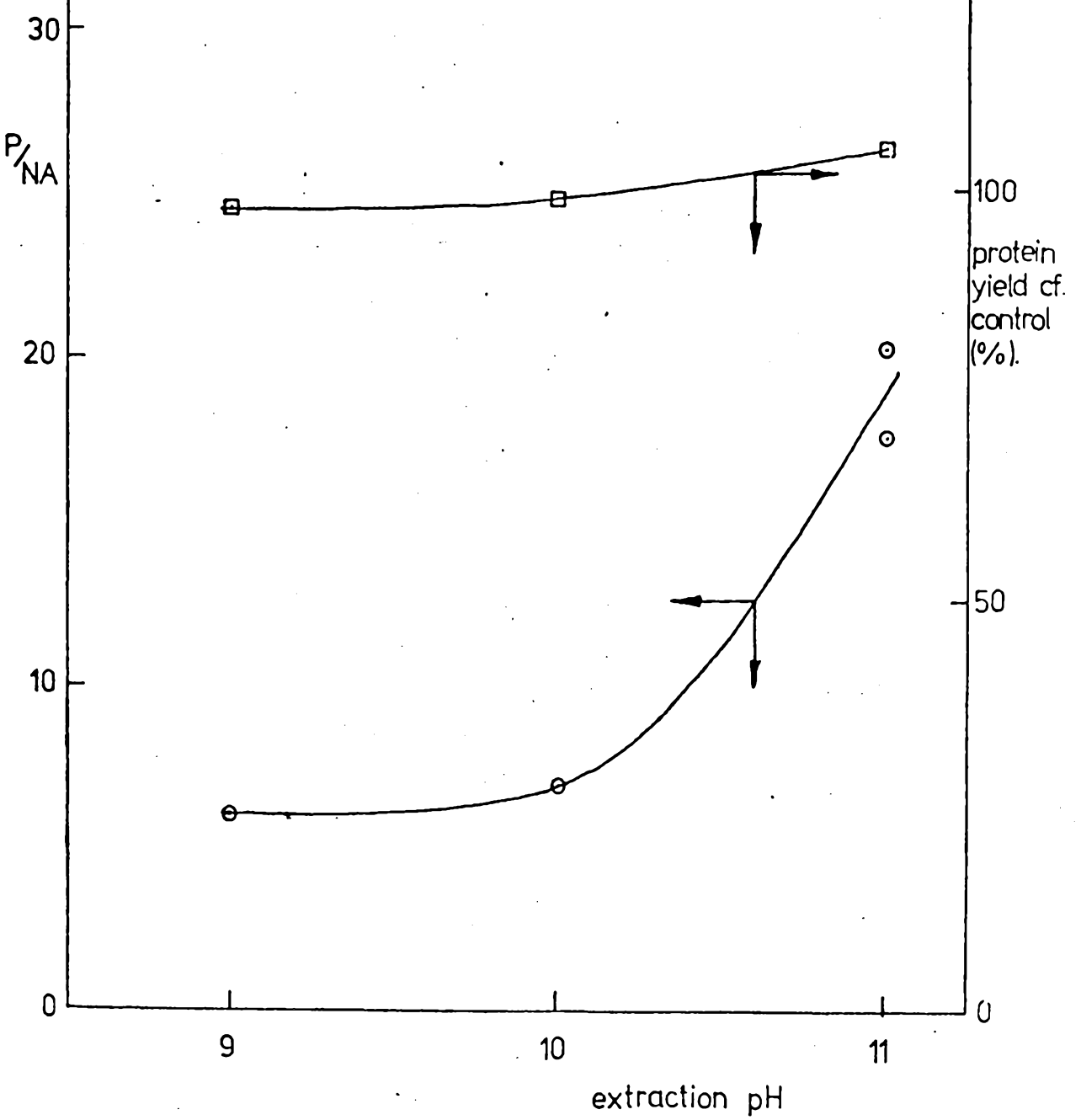


Fig. 5.4.18. Incubation Following Extraction - NaCl Concentration.
(Yeast conc.,10%$^{W}/_{V}$;extration, pH11, incubation pH6, 50°C, 2hrs.)

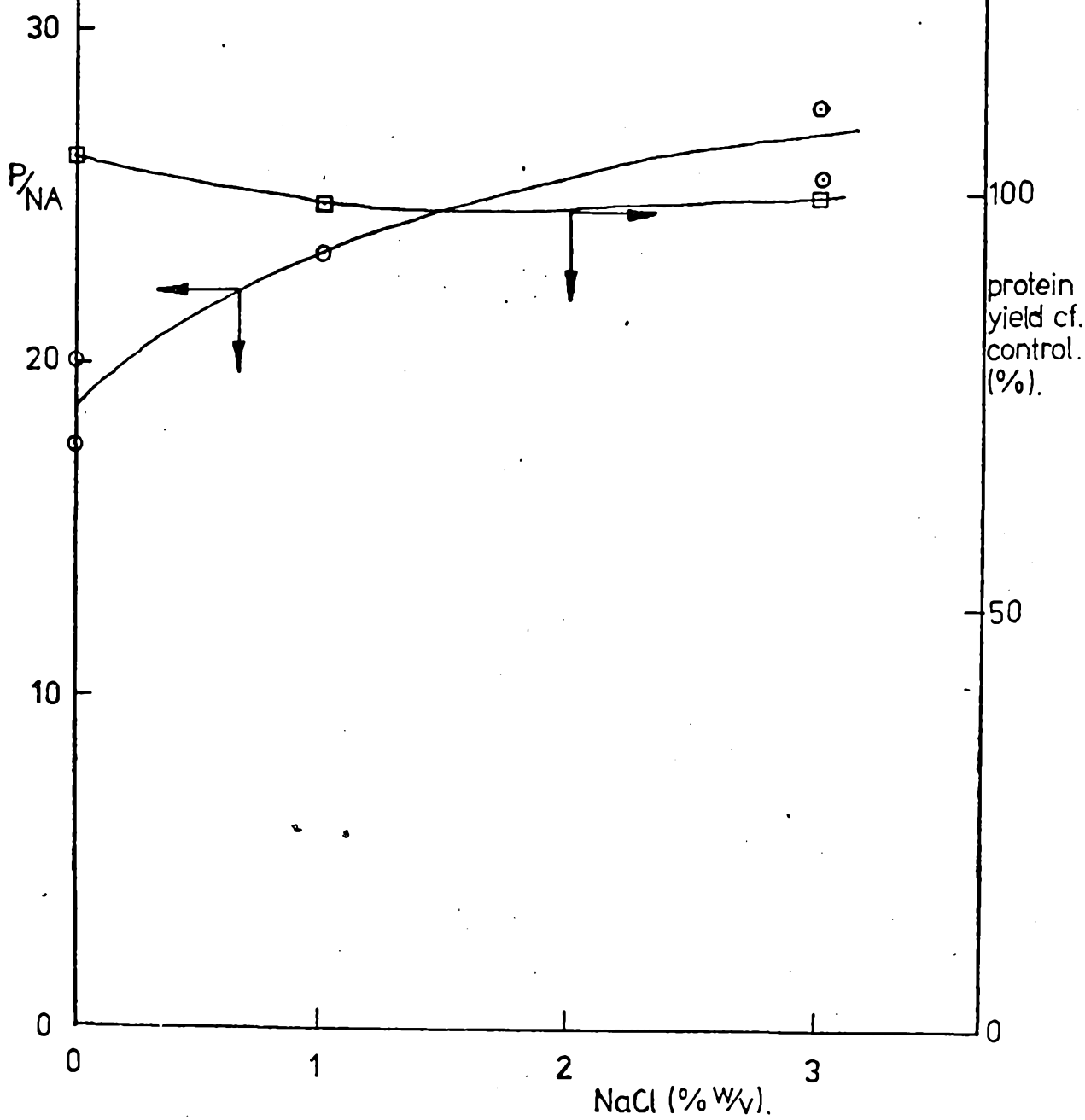
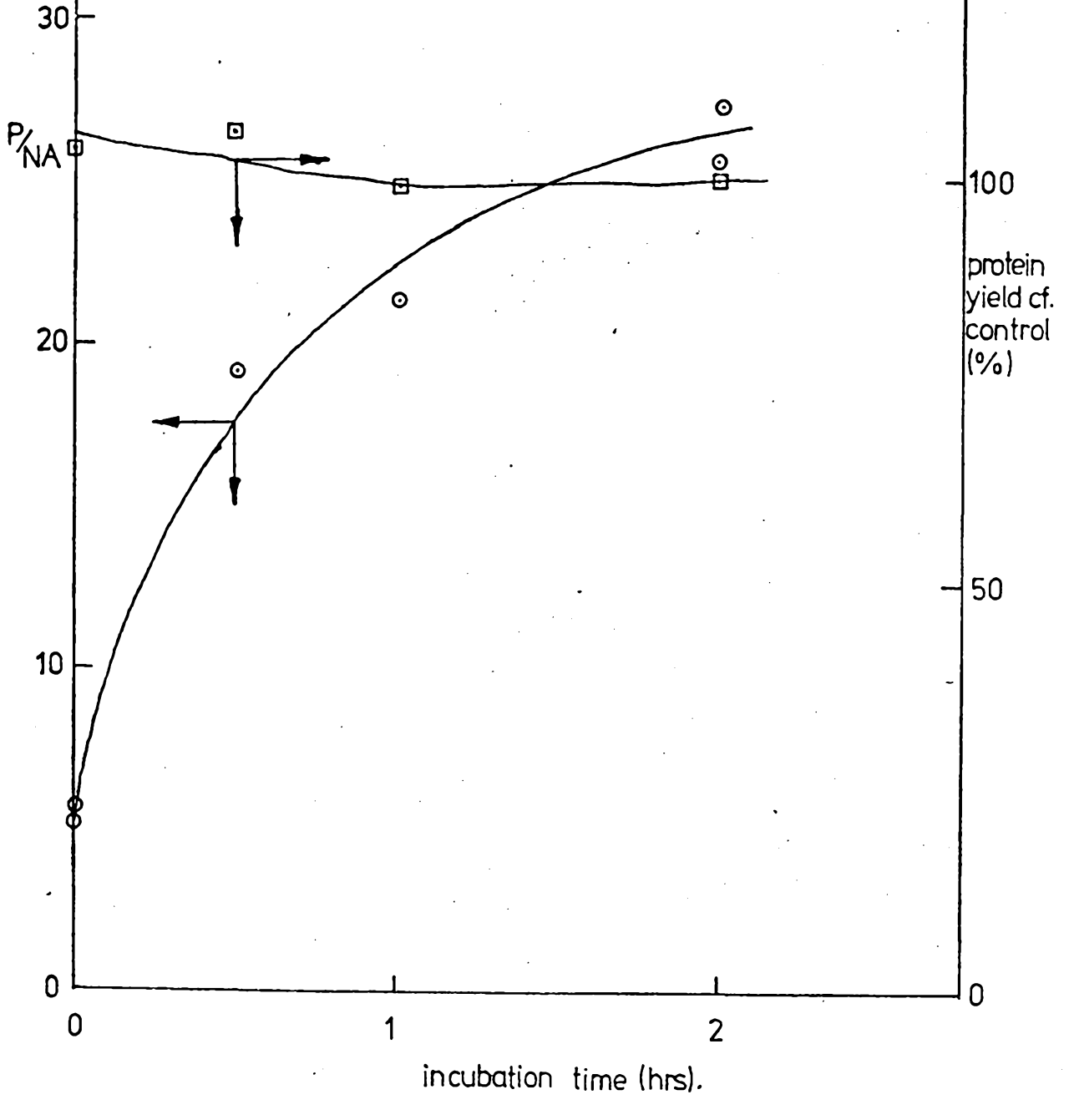


Fig. 5.4.19. Incubation with NaCl Following Extraction - Incubation Time. (Yeast conc.: 10% w/w dm.; extn.: pH 11; incubn.: pH 6, 50°C, 3% w/v NaCl.)



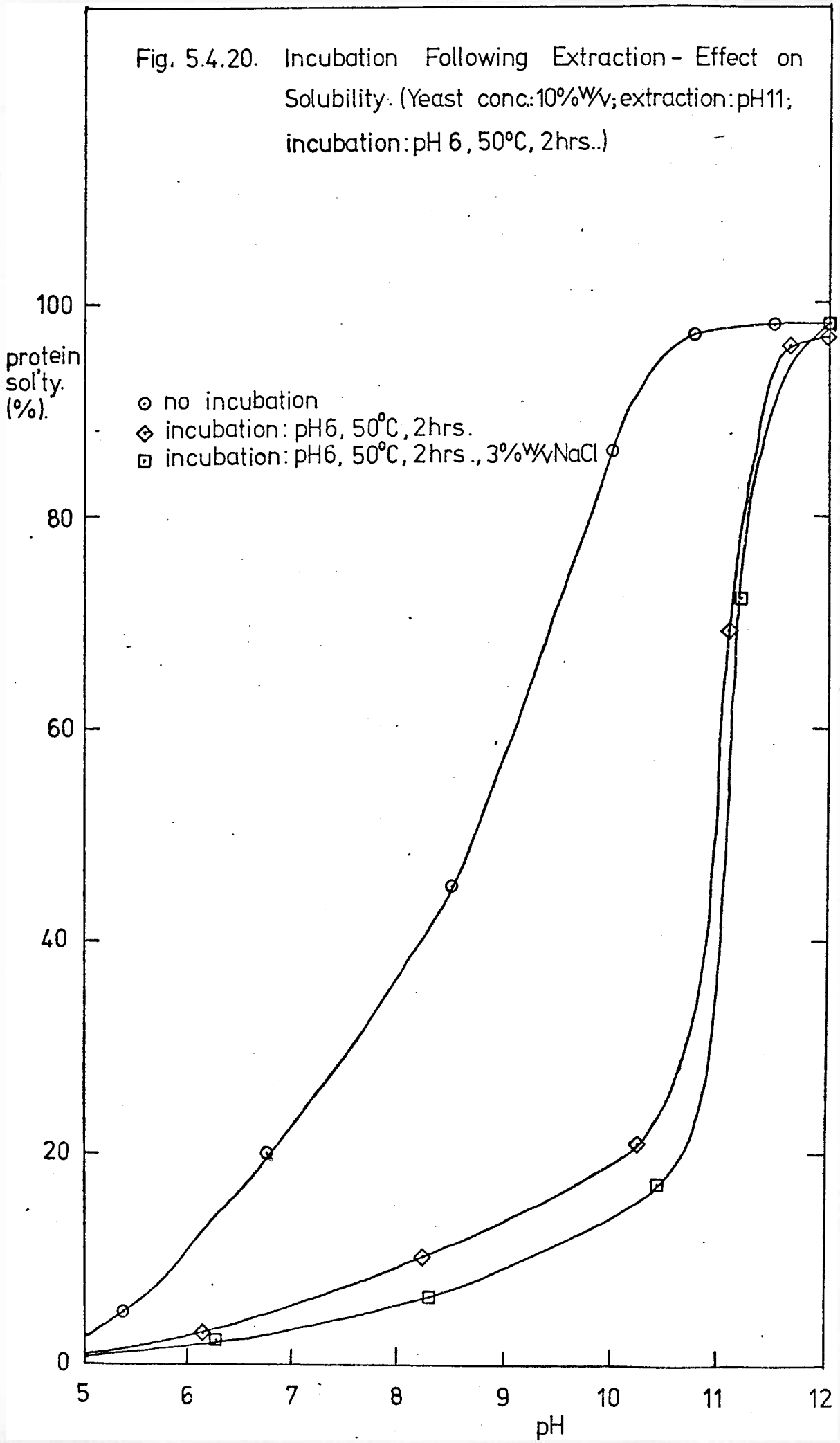
containing 20% dry matter and to spin this into an acid / salt bath. This procedure was carried out using isolates which had been subjected to incubation at pH 6.0, 50°C for 2 hours following extraction at pH 11 with no salt and with 3%w/v NaCl. The results were extremely disappointing, the fibre formation being very poor. The solubilities of the isolates were also checked and it was found that heat incubation led to a drastic reduction in solubility, especially incubation with NaCl. The results are summarised in Table 5.4.5. and Figure 5.4.20.

Table 5.4.4. Spinning Following Extraction and Incubation to Remove Nucleic Acid.

isolate preparation conditions.	solubility of protein in isolate at pH 10.0 (%)	length of wet fibre supported under its own weight in air (cm).
control - no incubation	86	250
incubation: 50°C, 2hrs., pH 6.0; extraction: pH 11.0.	19	15
incubation: 50°C, 2hrs., pH 6.0, 3% NaCl; extraction: pH 11.0.	13	5

Clearly the heat treatment resulted in reduced solubility which led to poor fibre spinning. Robbins et al (1975 (1)) reported that isolates prepared in a similar way could be textured. Examples were given of a simple extrusion process. Fibre formation was also claimed but it seems unlikely that this would have been very satisfactory in view of the above results. No detailed information or examples were given.

Fig. 5.4.20. Incubation Following Extraction - Effect on Solubility. (Yeast conc.: 10%$^{W}/_{V}$; extraction: pH 11; incubation: pH 6, 50°C, 2hrs..)



5.4.5. Incubation of Fibres after Spinning.

After much thought it was realised that since the endogenous nucleases have maximum activity at pH 6 and since the nucleases are of relatively low molecular weight (about 12,500 - Pharmacia (1)) they would be soluble at pH 6 and probably at pH's less than 6. The normal protein precipitation pH is 3.8 so it was likely that some nucleases at least would remain in the supernatant following protein precipitation. Indeed although 80% of the protein is precipitated at pH 3.8, 20% remains in solution - mostly relatively low molecular weight material. Furthermore, although pH 3.8 gives the highest protein precipitation, considerable precipitation occurs at pH 6.0 following extraction at pH 11.0. Hence it may be worth precipitating at pH 6 and sacrificing some protein yield in order to leave a large amount of nuclease in the supernatant.

All the effective and economical nucleic acid removal methods considered involved some heating which rendered the protein less soluble and therefore less suitable for texturising. However if the endogenous nucleases could be separated from the protein isolate, the isolate could be used for spinning and the fibres could then be incubated with the supernatant (containing the nucleases) at a later stage. After fibre spinning protein solubility is no longer important - indeed decreasing the protein solubility by incubation at high temperatures would prevent fibre dissolution on cooking. The idea was investigated as follows.

5.4.5.1. Procedure.

10%w/v yeast suspensions were sonicated and extracted at pH 11.0

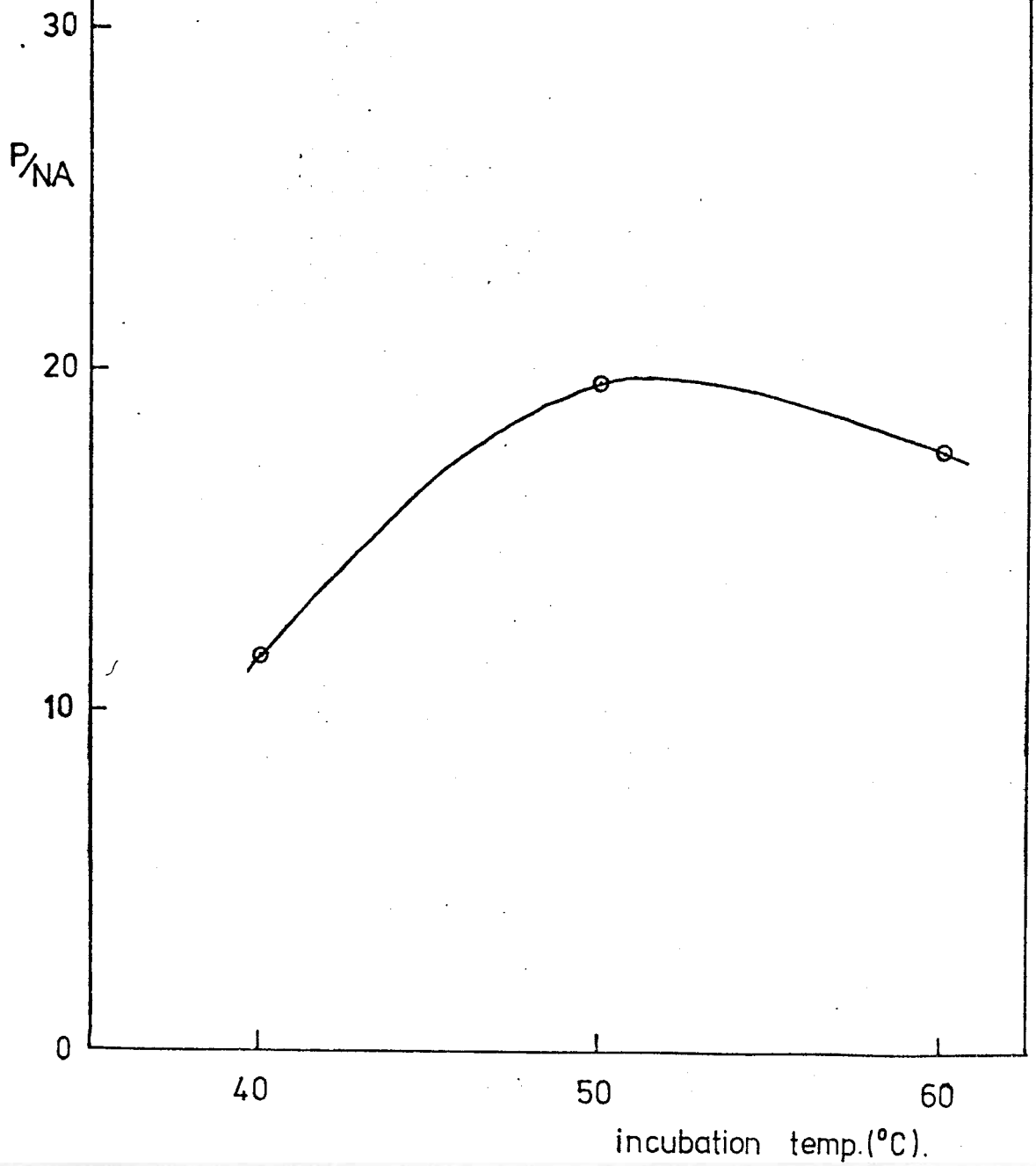
in the usual way and the cell debris removed. 3 N HCl was added to the alkali extract to give the desired pH (referred to as the precipitation pH) and the suspension was separated by centrifuging. The protein content of a sample of the supernatant was determined and the rest was stored in the refrigerator at 4°C until required (usually used within two days). The protein isolate was washed, recentrifuged and used for spinning. The dopes contained 20%w/w dry matter at pH 10.0 (see section 5.5.). Fibres were spun through a needle or a spinnerette into an acid / salt bath. They were washed in distilled water and weighed chunks of fibre were put into centrifuge tubes. Samples of the fibres were taken for protein, nucleic acid and dry matter analysis. To the fibres was added 10ml of the supernatant solution. The pH was adjusted to 6.0 since it had already been established that this was optimal for endogenous nucleases. NaCl was added in some cases. The tubes were incubated in a water shaker bath and then boiled for five minutes to pasteurise the fibres. The samples were cooled, centrifuged, washed in distilled water and recentrifuged. The fibrous precipitate was homogenised with distilled water to a fine suspension which was analysed for its protein, nucleic acid and dry matter contents.

In some experiments bovine pancreatic ribonuclease solutions were used instead of the supernatants. In this case the incubation step was at pH 7.0, the optimum for this enzyme.

5.4.5.2. Results.

Incubation temperature. The optimum incubation temperature was again 50°C (Figure 5.4.21).

Fig. 5.4.21. Incubation of Fibres - Incubation Temperature.
(Extn.: pH 11; pptn.: pH 6·1; incubn.: pH 6, 2hrs.;
fibre/sn. protein: 1·5; capillary ø: 75μm.)



Incubation time. P/NA levels increased with increasing incubation period as shown in Figure 5.4.22. Fibre spinning alone did not affect the P/NA ratio.

NaCl concentration. Incubation with 3%w/v NaCl had a very marked effect upon nucleic acid removal (Figure 5.4.22). A linear relationship was found to exist between NaCl concentration and the P/NA ratio (Fig.5.4.23 cf. Figs.5.4.8.& 5.4.18.). It seems likely that the NaCl was not simply inactivating the inhibitor in this case but may have been effective in, for example, increasing diffusion rates into the fibres. This is not well understood and merits further investigation using higher NaCl concentrations.

Precipitation pH. The optimum precipitation pH from the point of view of P/NA ratio of the product is pH 5 (Figure 5.4.24). This is probably representing a balance between extraction of the nuclease enzyme and precipitation of the inhibitor. However, the amount of protein precipitated was lower at pH 6.1 and 5.0 than at pH 3.8, being 68%, 75% and 80% of the protein in the alkali extract respectively. When the supernatants were incubated with the fibres, however, the excess unprecipitated protein at pH 6.1 and 5.0 was recovered since it was heat precipitated. However this formed as a sludge on the fibres and spoiled their appearance. For this reason precipitation at pH 3.8 was chosen for further work since this still gave reasonably high P/NA ratios.

There was no loss in protein from the fibres following incubation with the supernatant following precipitation at pH 3.8. (Table 5.4.5. overleaf).

Fig. 5.4.22. Incubation of Fibres - Incubation Time. (Extn.: pH 11, pptn.: pH 6·1; incubn.: pH 6, 50°C; fibre/sn. protein: 1·5; capillary ϕ: 75 μm.)

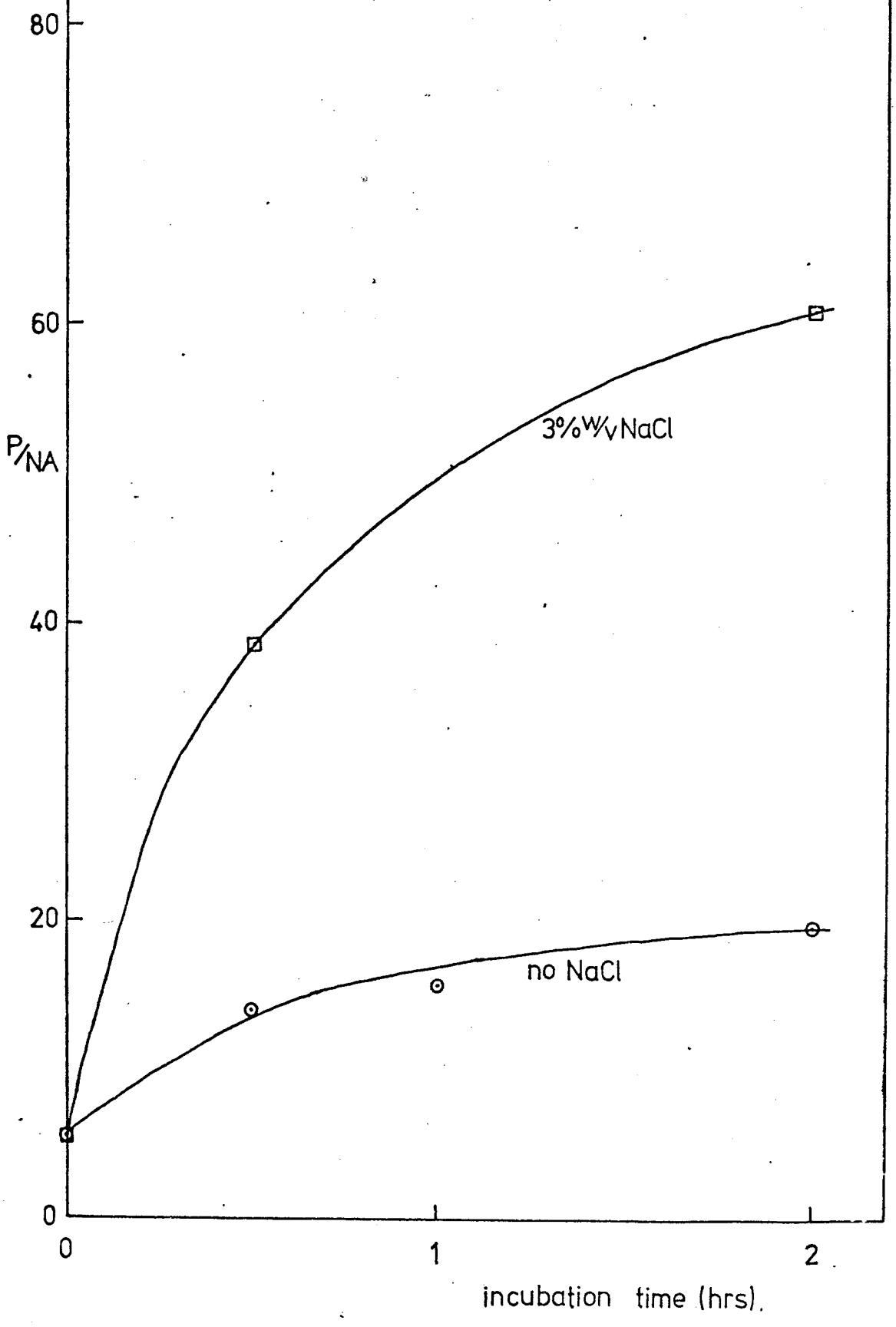


Fig. 5.4.23. Incubation of Fibres - NaCl Concentration.
(Extn.: pH 11; pptn.: pH 6.1, incubn.: pH 6, 50°C, 2hrs.;
fibre/sn. protein : 1·5; capillary ø: 75 μm.)

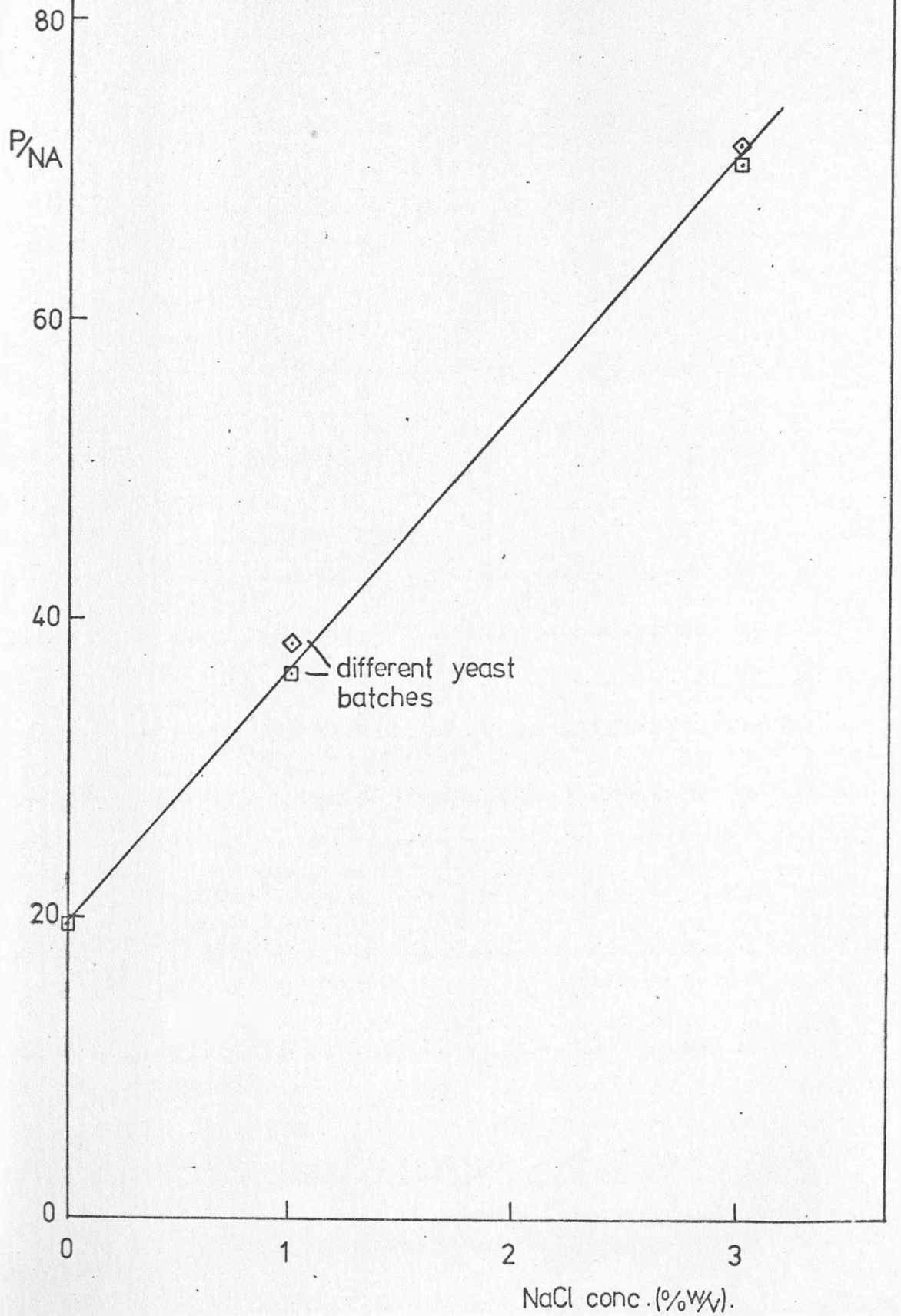


Fig. 5.4.24. Incubation of Fibres - Precipitation pH. (Extn.: pH 11; incubn.: pH 6, 50°C, 2hrs.; 85mg. protein in fibre incubated with 10ml. sn.; capillary ø: 75μ.)

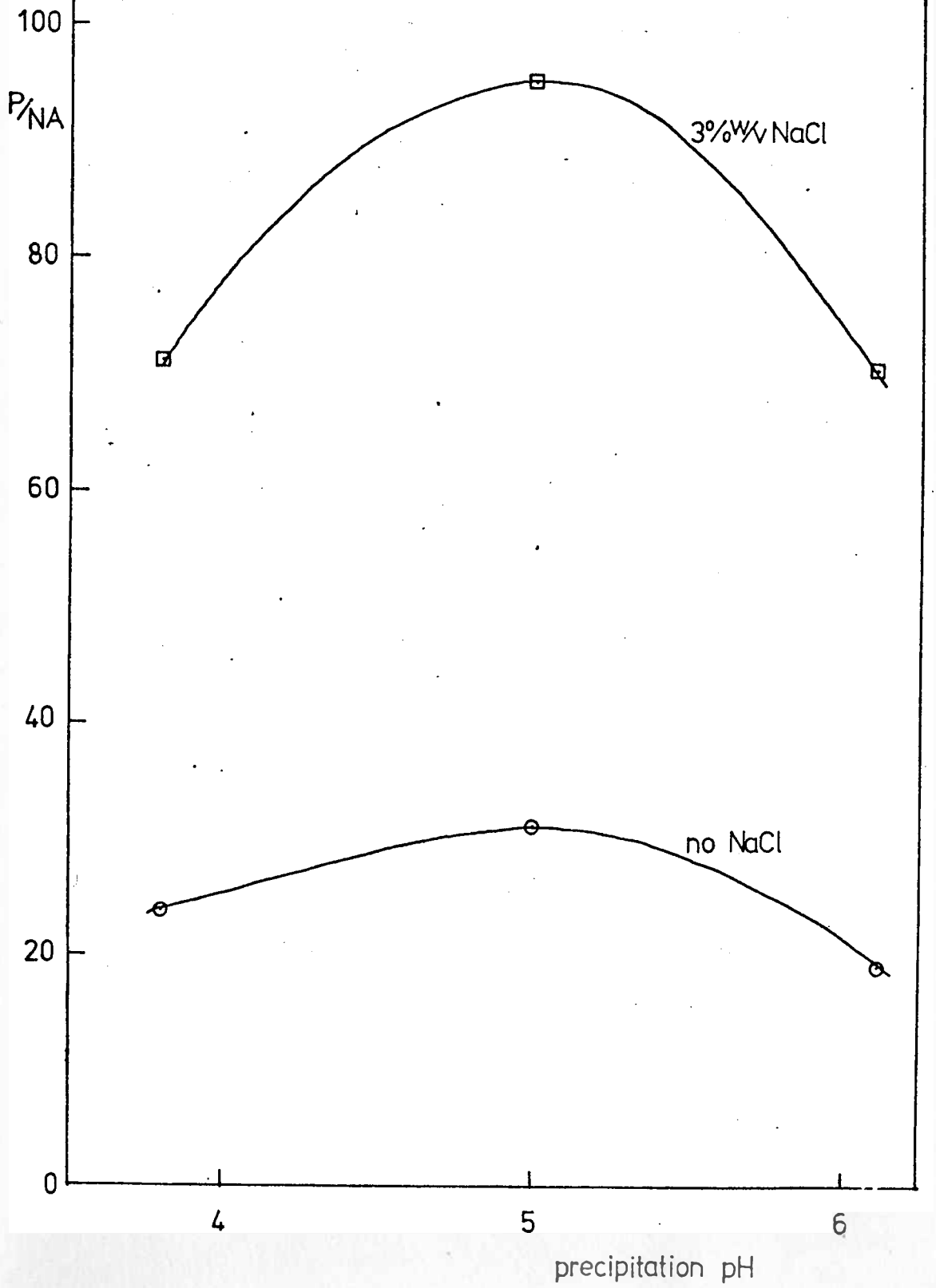


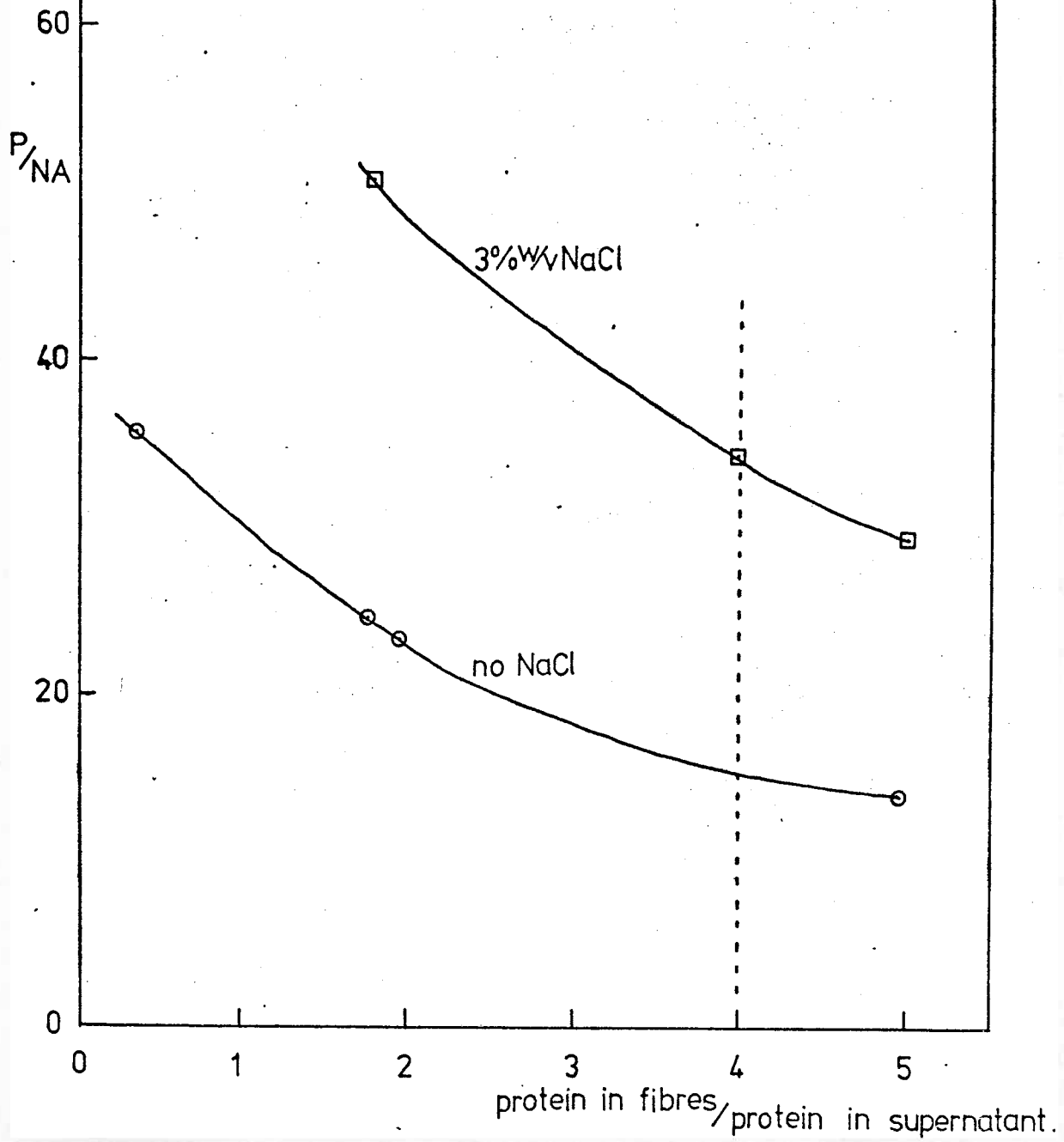
Table 5.4.5. Protein Yield in Fibres Following Incubation with Endogenous Enzymes.(Protein pptd. at pH3.8).

incubation conditions				protein yield (%) cf. control
temperature (°C)	time (hrs)	NaCl conc. (%W/v)	fibre protein : sn. protein	
control - no incubation				100
40	2·0	0	1·8	97
50	2·0	0	1·8	96
60	2·0	0	1·8	100
50	0·5	0	1·8	104
50	0·5	1·0	1·8	97
50	0·5	3·0	1·8	100
50	2·0	0	1·8	96
50	2·0	1·0	1·8	99
50	2·0	3·0	1·8	101
50	2·0	0	0·4	103
50	2·0	0	1·8	96
50	2·0	0	5·0	103

Enzyme concentration. The P/NA ratio increased as the fibre protein / supernatant protein ratio (an indication of protein / enzyme ratio) decreased. In the limiting case, if all the protein isolate were used for spinning the fibre protein / supernatant protein ratio would be 4.0 (since 80% of the protein is precipitated at pH 3.8). Figure 5.4.25. shows that in this case the P/NA ratios obtainable on incubation at 50°C, pH 6.0 for 2 hours would be 15:1 without salt and 34:1 with 3%w/v NaCl.

Boiling after incubation. This was found to increase the P/NA ratio, probably due to enhanced diffusion of nucleotides out of the fibres and into the supernatant fluid, at high temperatures. It is unlikely that nucleic acid hydrolysis would occur on boiling at pH 6.

Fig. 5.4.25. Incubation of Fibres - Fibre/Supernant Protein Ratio. (Extn.: pH 11; pptn.: pH 3.8; incubn.: pH 6, 50°C, 2hrs.: capillary ø: 75µm..)



Effect of fibre diameter. Fibres spun through a spinnerette (75μm diameter holes) had much higher P/NA ratios (70:1 and 23:1) than fibres spun through a capillary of 320μm diameter (27:1 and 13:1) following incubation at 50°C, pH 6 for 2 hours with and without 3%w/v NaCl respectively, using a fibre protein to supernatant ratio of 1.5. It would appear that diffusion of the enzyme into the fibres and diffusion of nucleotides out of the fibres is an important rate controlling step and warrants further investigation. In fact the conditions for mass transfer were poor in these experiments since the fibres sunk in a heap to the bottom of the test tube and remained there despite agitation. This is an area which may be of considerable interest in the future.

Effect of incubation on fibre properties. A larger sample of fibres was incubated at 50°C, pH 6 for 2 hours, boiled, cooled, decanted from the supernatant fluid and washed. The fibres did not appear to have suffered in any way as a result of incubation. They had not broken up nor changed in colour. There was no apparent difference in 'chewiness' or flavour.

Exogenous nucleases. Excellent results were obtained using bovine pancreatic ribonuclease (Figures 5.4.26.,5.4.27.& 5.4.28.). NaCl improved the nucleic acid removal rate although not to such a great extent as with endogenous nucleases. Again the relationship between NaCl concentration and the P/NA ratio was almost linear and quite dissimilar to that observed in earlier work (e.g. Figures 5.4.8.& 5.4.18). There was no loss of protein from the fibres in any of these experiments.

Protein content of the dry fibres. For P/NA ratios greater than

Fig. 5.4.26. Incubation of Fibres with Exogenous Nucleases – Incubation Time. (Extn.: pH11; pptn.: pH 3.8; incubn.: pH 7, 50°C; enzyme/protein: 0.001; capillary ø: 75μm.)

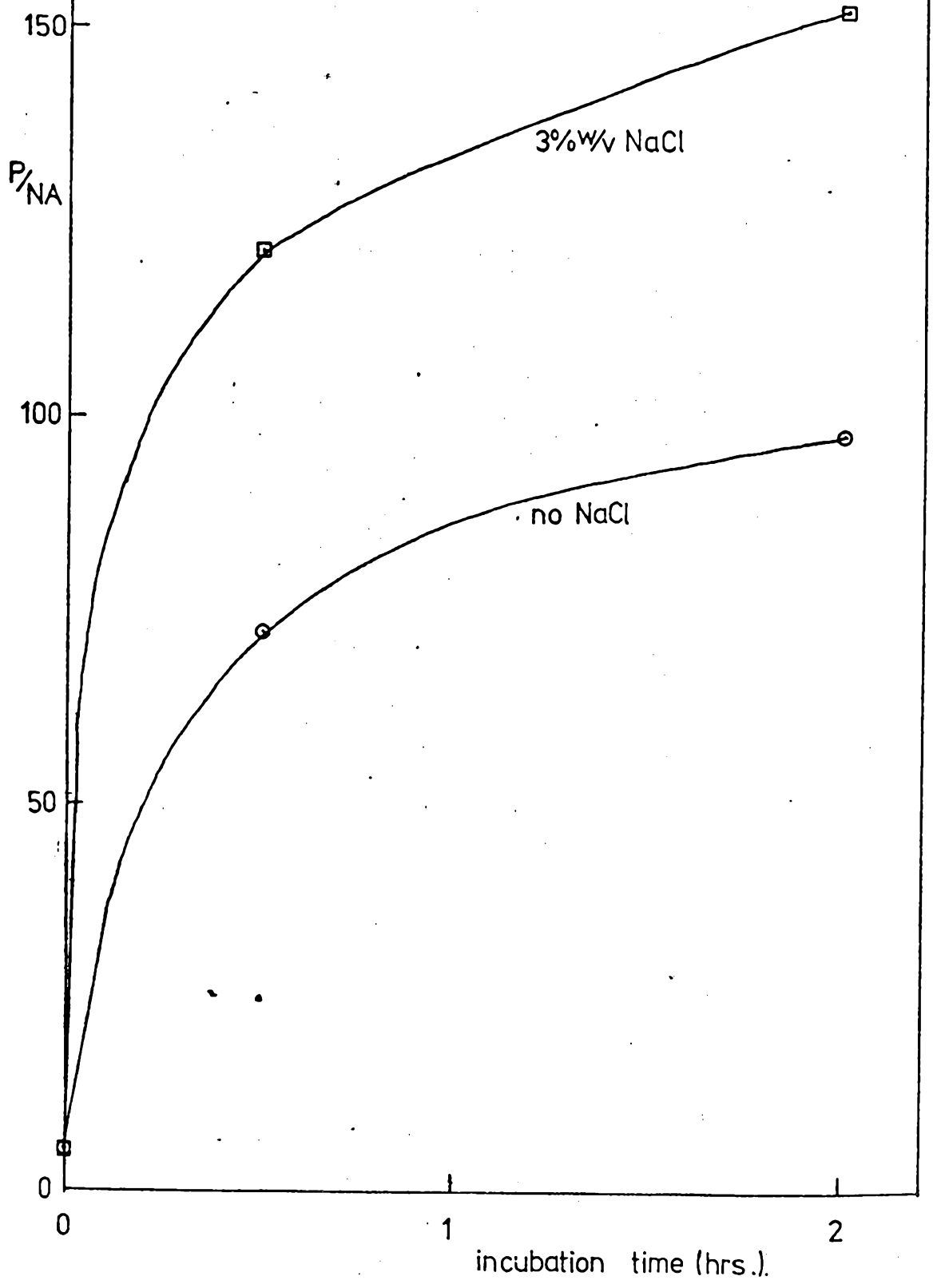


Fig. 5.4.27. Incubation of Fibres with Exogenous Nucleases -Enzyme / Fibre Protein Ratio. (Extn.: pH 11; pptn.: pH 3·8; incubn.: pH 7, 50°C, 2hrs.; capillary ø: 75μ.)

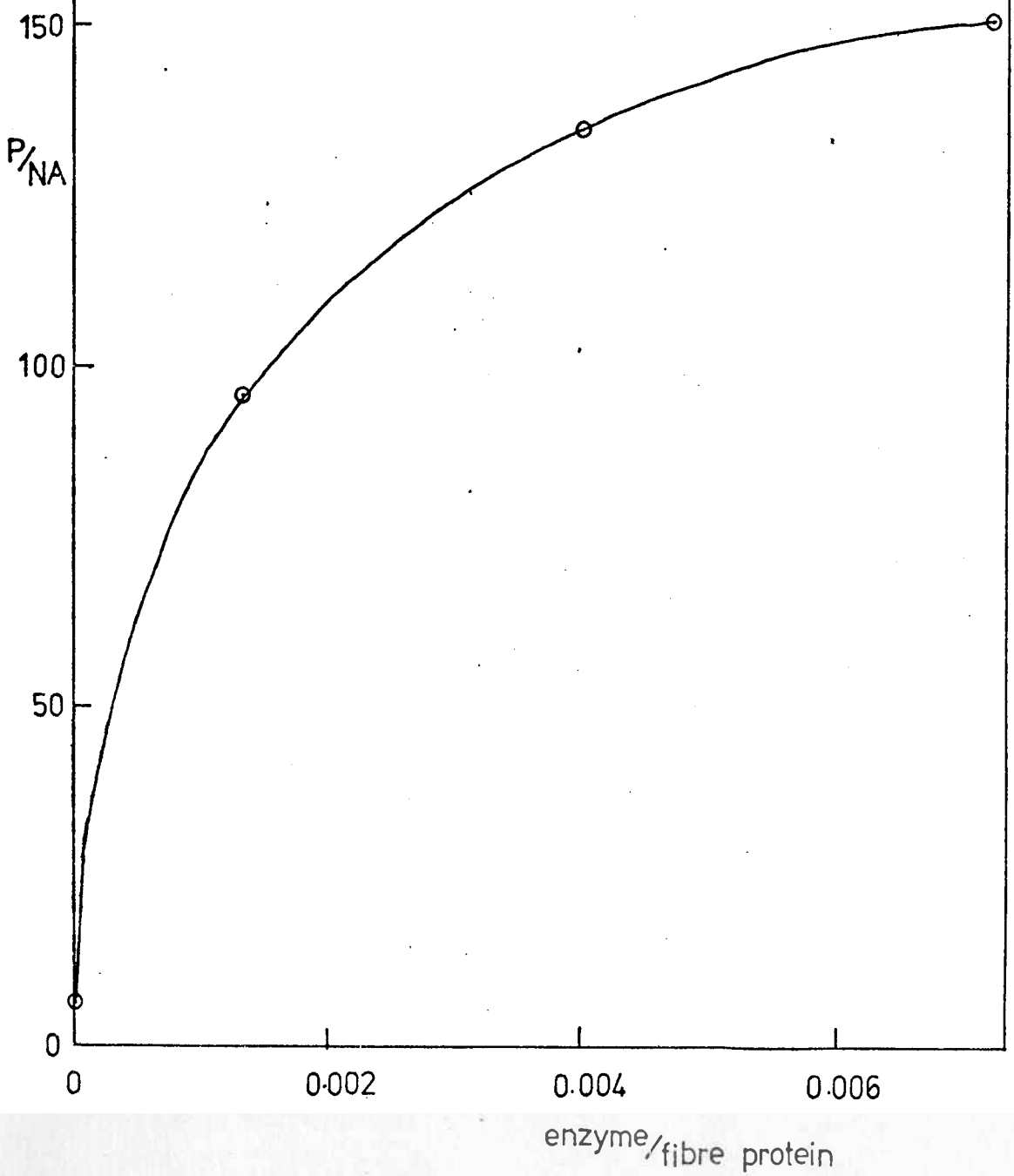
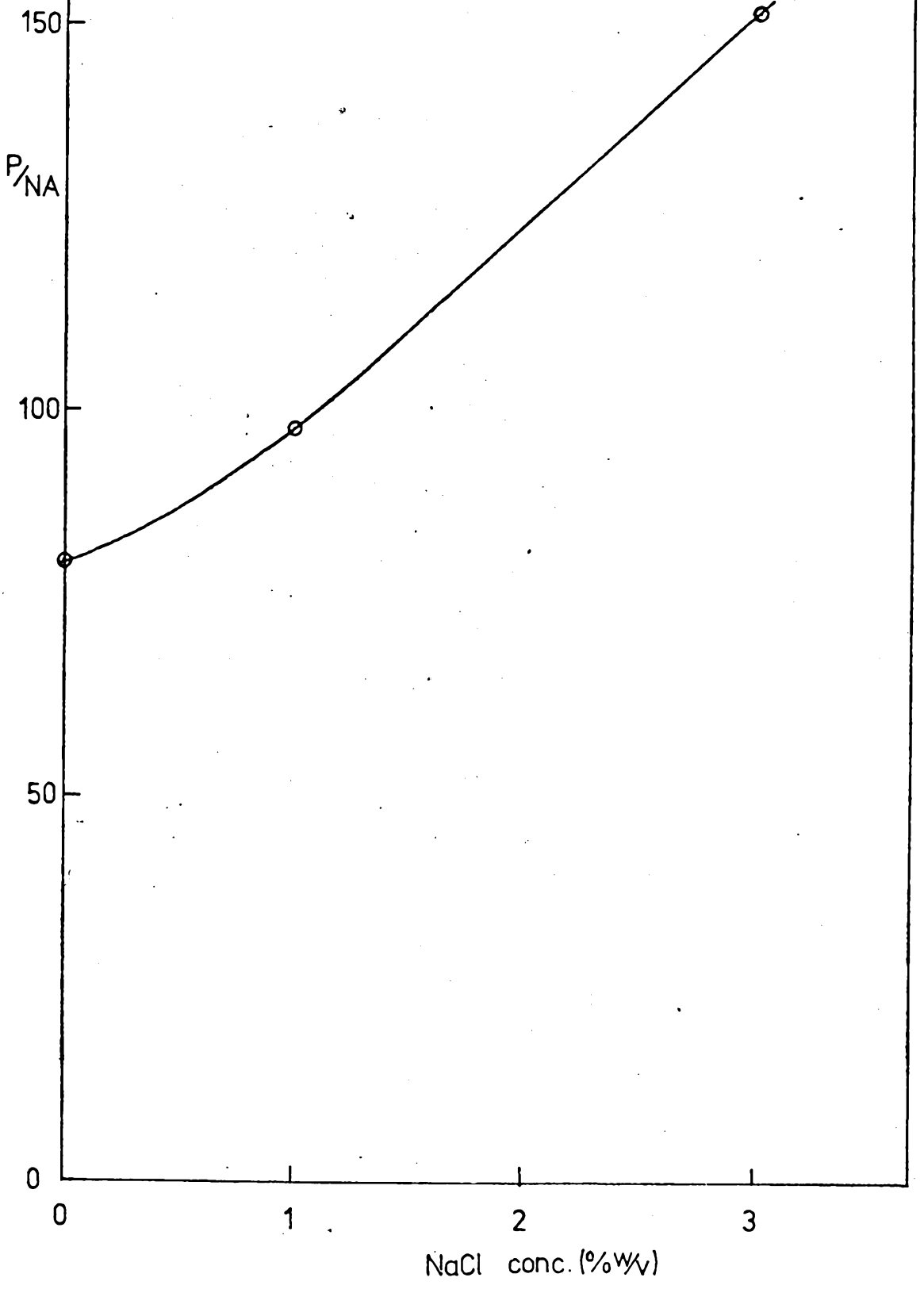


Fig. 5.4.28. Incubation of Fibres with Exogenous Enzymes- NaCl Concentration. (Extn.: pH11; pptn.: pH3.8; incubn.: pH7, 50°C, 2hrs.; enzyme/protein: 0.001; capillary ø: 75µm.)



20:1 the protein content in the dry matter exceeded 80%w/w except when using 3%w/v NaCl, when the protein contents were about 75%w/w. The protein content of the original fibres was about 70%w/w.

5.4.5.3. Conclusions.

This was in fact the final series of experiments performed for this thesis and represents the culmination of the work. It appears to be a most successful method of incorporating nucleic acid removal and fibre spinning.

5.5. Fibre Spinning.

5.5.1. Introduction.

It has already been observed that it is possible to produce single cell protein materials with nutritional properties similar to some meat products (section2.2.). Assuming that the toxicological problems involved in feeding SCP to human subjects can be overcome, SCP could be used as a replacement for at least some of the meat in the diet. The production of animal protein is an expensive and rather wasteful process when compared with SCP production (Senez, 1972). However, to gain wide acceptance as a foodstuff SCP will probably have to be textured and processed into an attractive food (Shelef & Morton, 1976), thus following a similar pattern to the development of textured vegetable protein foods.

Many different techniques have been reported (section 2.4.1.) for texturing vegetable proteins. Of these the most sophisticated is the spinning process. Using this method fibres are produced

which can be made into meat analogues. Furthermore the technology of spinning is well documented. However there are two major reasons for choosing this technique which are:-

1. The rheological properties of the dopes and the protein fibre properties (particularly wet strength) could be quantified. It was hoped that it would be possible to relate previous protein processing conditions to these properties.
2. A higher quality isolate (in terms of protein content and degree of denaturation) was required for spinning than for other techniques (Ashton et al, 1970; Burke, 1971). Hence the 'spinability' of the isolate was a more sensitive test of protein properties than for example extrusion.

This is not to say that spinning would be the choice of texturing technique for industrial SCP production, since the cost of processing is relatively high. It was however thought to be the most useful technique for the present work.

There are a large number of variables involved in the spinning process. For this reason it was decided to perform a series of rather qualitative, preliminary experiments designed to reveal the most important variables. The aim of these experiments was to use the isolates obtained with the different processing methods outlined in section 5.4. to find the spinning conditions which produced the best fibres, hence integrating the extraction and texturisation processes.

It was found at an early stage in the work that the fibres produced were very weak and therefore difficult to handle. Hence, in this

case fibre strength, as measured by the simple test described in section 4.2.7., was used throughout the experiments as the main criterion for assessing fibre quality. (It is worth noting that in the field of TVP it is possible to produce fibres which are too strong to be used to simulate meat. In that case other parameters are used to assess the fibres and the analysis of fibre quality becomes much more complex (Society of Chemical Industry, 1960)).

5.5.2. Preliminary Experiments.

The results in this section are essentially qualitative, largely based on visual observation rather than precise measurement. All the isolates used for this set of experiments were prepared by the standard method as outlined in section 5.3.3.

5.5.2.1. Dope preparation.

For the preliminary trials dopes were prepared in the following way. Duplicate samples of isolate, prepared by sonication, extraction at pH 11 and precipitation at pH 3.8, were analysed for protein content (usually 17 - 20%w/w of the wet isolate cake). 30g of isolate were weighed accurately into a 250ml beaker and the amount of fluid needed to give the required protein concentration was calculated. 3 N NaOH was added to the isolate in 0.5ml or 0.1ml portions and the isolate and NaOH were mixed using a thick glass rod. The dope pH was measured by taking a small sample of the dope and diluting it with sufficient distilled water to allow pH measurement with a standard pH probe. Further additions of NaOH were made until the pH reached the required level. Distilled water was added to the dope and mixed in thoroughly to give the required

final concentration allowing for the volume of NaOH solution which had already been added.

The dopes produced were generally light brown, rubbery gels. Raising the dope pH led to darker colouration and, up to pH 11, firmer gels. Higher protein concentrations also led to firmer gels.

Unfortunately the method was rather slow and tedious and did not yield a homogeneous dope. The dope contained lumps and air bubbles which interfered with the spinning process by blocking the syringe needle. A more successful method of dope mixing is described later in the text but the above method was used for all the experiments in which hand syringes were used for spinning.

5.5.2.2. Hand spinning.

Rather crude spinning trials were performed by transferring the dope to the barrel of a 20ml syringe and extruding it through a capillary tube, 0.5cm long and approximately 0.03cm internal diameter, into a coagulating bath containing (unless otherwise stated) 1%w/w acetic acid solution and 3%w/v NaCl. The bath was generally a 1 litre glass beaker.

Using the simple techniques described above it was possible, under the best conditions, to produce wet fibres which could support their own weight in air in lengths up to about 30cm when removed from the coagulating bath (see section 4.2.7.).

5.5.2.3. Bath residence time.

Visual observation showed that the fibres rapidly lost their brown colour following extrusion and after 1 - 2 minutes in the

bath were a light cream-white colour. Furthermore their strength increased to a maximum during this period of coagulation. Immediately after leaving the capillary the fibres were very weak and often could not be removed from the bath without breaking up. For this reason fibres were always left for 2 minutes after spinning before handling to test the fibre strength (section 4.2.7.).

5.5.2.4. Dope pH.

The strongest fibres were obtained using a dope pH in the range pH 9 - 10.5. As alkali was added to the thick isolate paste a more fluid material was produced at first. However as the pH rose above pH 8 the dope became firmer, having the appearance of a rubbery gel at pH 9 - 10. The colour of the material changed from the light cream / grey of the isolate to a translucent light brown at pH 10. The brown colour became darker as the pH increased.

5.5.2.5. Protein concentration.

Dopes containing about 15%w/w protein gave the strongest fibres. At concentrations above 15%w/w protein dopes were very thick and difficult to mix.

The above two parameters, pH and concentration, proved to be the most important in influencing fibre formation.

5.5.2.6. Coagulating bath composition.

The composition of the spinning bath was not reported to be critical in the literature reviewed (Ashton et al, 1970; Burke, 1971). Baths contained an acid / salt solution (usually NaCl). Acetic acid or hydrochloric acid were most frequently used. For this work acetic acid was preferred since, being a weak acid, it maintained a pH

of about 2.5 even after a considerable quantity of dope had been spun into the bath. No difference in fibre strength was observed using concentrations from 0.5 to 5%w/v acetic acid. The addition of 1%w/v sodium chloride to the bath slightly improved fibre strength but no further improvement was noticed when salt concentrations in the range 1 - 10%w/v were used. Salt is reported to cause hardening of the fibres and is used to help prevent redissolution of the fibres after spinning (Ashton et al, 1970; Burke, 1971; Gutcho, 1973).

It was decided to use a bath composition of 1%w/v acetic acid and 3%w/v sodium chloride in further experiments.

5.5.2.7. Coagulating bath temperature.

Spinning trials were conducted using various coagulating bath temperatures in the range 20°C - 100°C. From 20 - 40°C there was no noticeable difference in fibre strength. Fibre strength increased with increasing temperature in the bath, particularly above 60°C providing the capillary was held above the surface of the acid / salt solution. When the needle was immersed in the solution the needle itself rapidly heated up causing heat coagulation of the protein inside the capillary. When this occurred the material extruded was no longer in a fibrous form but emerged as a spray of white particles. Furthermore even when the needle was held above the surface of the solution, air bubbles in the fibre rapidly expanded at the higher temperatures (particularly above 80°C) causing the fibres to break where a sufficiently large bubble occurred. It was not therefore, possible to produce a continuous fibre by spinning into a bath at more than 60°C.

Temperatures less than 60°C gave little improvement in fibre strength compared with spinning at room temperature. For this reason it was decided to operate at room temperature.

5.5.2.8. 'Mesophase' type dopes.

Tombs (1972) developed a method of spinning using a high concentration of protein dissolved in salt solutions at an ionic strength of about 0.5. This method had the advantages of operating at low pH (preventing protein damage by alkali) and of giving dopes whose rheological properties did not vary with time (see section 2.4.1.5.). For the present work dopes were prepared by adding sodium chloride or calcium chloride to the isolates at various concentrations. However the protein in the isolate was only sparingly soluble in 0.2 - 1.0 M NaCl or $CaCl_2$ at pH 3.8. Using 0.5 M NaCl and adjusting the pH to higher levels did not lead to any improvement over fibres produced from dopes without NaCl. In the patent Tombs points out that an undenatured protein material is required. It seems probable that extraction at pH 11 renders the protein in the isolate sufficiently denatured to prevent its dissolution in salt solutions.

5.5.2.9. Sodium sulphite.

Sodium sulphite has been used widely in spinning of vegetable proteins to prevent disulphide bonding and hence to limit gelation. Under the preferred conditions for fibre spinning (approximately pH 10 and 15%w/w protein) the dope was a very viscous gel which was extremely difficult to extrude by hand. Sodium sulphite was therefore added, either directly to the isolate prior to dope formation or after sonication and prior to alkaline extraction, in the hope of limiting protein aggregation at that stage. However

the use of sodium sulphite at concentrations from 0.1 - 1.0%w/v did not significantly affect the gel-like structure of the dopes or the fibre strength. One advantage of adding Na_2SO_3 during extraction was that the isolate could be kept in the refrigerator in the wet state for over three weeks without microbial degradation. Without Na_2SO_3 an unpleasant smell developed in the isolate after about ten days storage. However care must be taken in the use of sodium sulphite and similar materials if it is required to use enzyme breakdown of nucleic acids at a later stage in the protein processing since it is well known that sulphites inhibit enzyme activity.

The use of sodium sulphite was not continued.

5.5.2.10. Isolate storage.

There was no noticeable difference in fibre strength when dopes were prepared from samples of the same isolate immediately after preparation and following storage in a refrigerator for five days. In general, however, isolates were used for spinning within 24 hours of preparation to minimise the risk of microbial attack.

5.5.2.11. Conclusions.

Following the preliminary experiments it was clear that dope pH and concentration were the major factors affecting the fibre strength and it was decided to investigate these parameters in more detail. The hand spinning method was unsatisfactory as it was impossible to produce identical conditions of shear rate during spinning. The dope preparation technique also left much to be desired, particularly due to the problems of lumps in the dope, which often blocked the capillaries and air bubbles which caused

breaks in the fibre. A spinning apparatus was therefore devised capable of giving controlled shear conditions and dope mixing techniques were investigated in order to produce deaerated, homogeneous dopes.

5.5.3. Development of the Spinning Apparatus.

5.5.3.1. Objectives and limitations.

The major objectives were to design an apparatus capable of filtering the dope, giving controlled and reproducible shear rates through capillaries and incorporating measurement of the pressure before the capillaries in order to study the rheological properties of the dopes. Provision was also made for the use of spinnerettes.

It became apparent that the use of sonication for cell disruption placed a constraint on the size of the spinning apparatus that could be built. On average 40% of the protein in the original yeast suspension appeared in the isolate. The isolate normally contained about 20%w/w protein so that, using a flowrate of 10ml/min with a yeast suspension of 10%w/v dry solids, only about 50g of isolate were produced per hour of sonication. It was not possible to run the sonicator for periods of more than three hours due both to overheating of the transducer and pipe blockage caused by yeast sedimentation at the low flowrates employed. Furthermore it was considered desirable to carry out the extraction process as rapidly as possible to avoid denaturation of the sonicated suspension. For these reasons sonication for two hours was generally employed, giving approximately 100g of wet isolate material.

5.5.3.2. Design of the dope extruder.

Because of the limited amount of isolate available the industrially preferred method of screw extrusion (Gutcho, 1973) which would have required a much larger quantity of isolate was ruled out. Quantities were also too small for use with a metering pump such as a gear pump. Furthermore, following the preliminary trials it was felt that there would be considerable problems in feeding the gel-like material to the pump. Pushing the dope using compressed air was another possibility but problems of flowrate measurement were foreseen. It was therefore decided to use a piston in a barrel device to push dope through a filter to the capillary. The main features of this device were:- (see also Figures 5.5.1. & 5.5.2).

1. The device was of a robust construction to withstand the considerable horizontal forces between the static and moving parts (barrel and piston respectively).
2. The device was designed to allow easy assembly and dismantling of all the component parts.
3. The pushing mechanism consisted of a fine pitch, screw threaded syringe pusher powered by a geared down, ¼hp, variable speed motor.
4. All the parts of the apparatus which came into contact with the dope were of stainless steel, with the exceptions of the 'O'ring piston seals and the chrome plated 'Leur' type syringe fittings.
5. The barrel was designed to handle 20ml batches of dope.
6. The dope flowrates obtainable using this device were from 0.1 to 0.33ml/min. The precise flowrate for a particular run was calculated by noting the distance moved by the piston, marked at 1mm intervals, in a given time.

Figure 5.5.1. Spinning Apparatus - breakdown of major components.

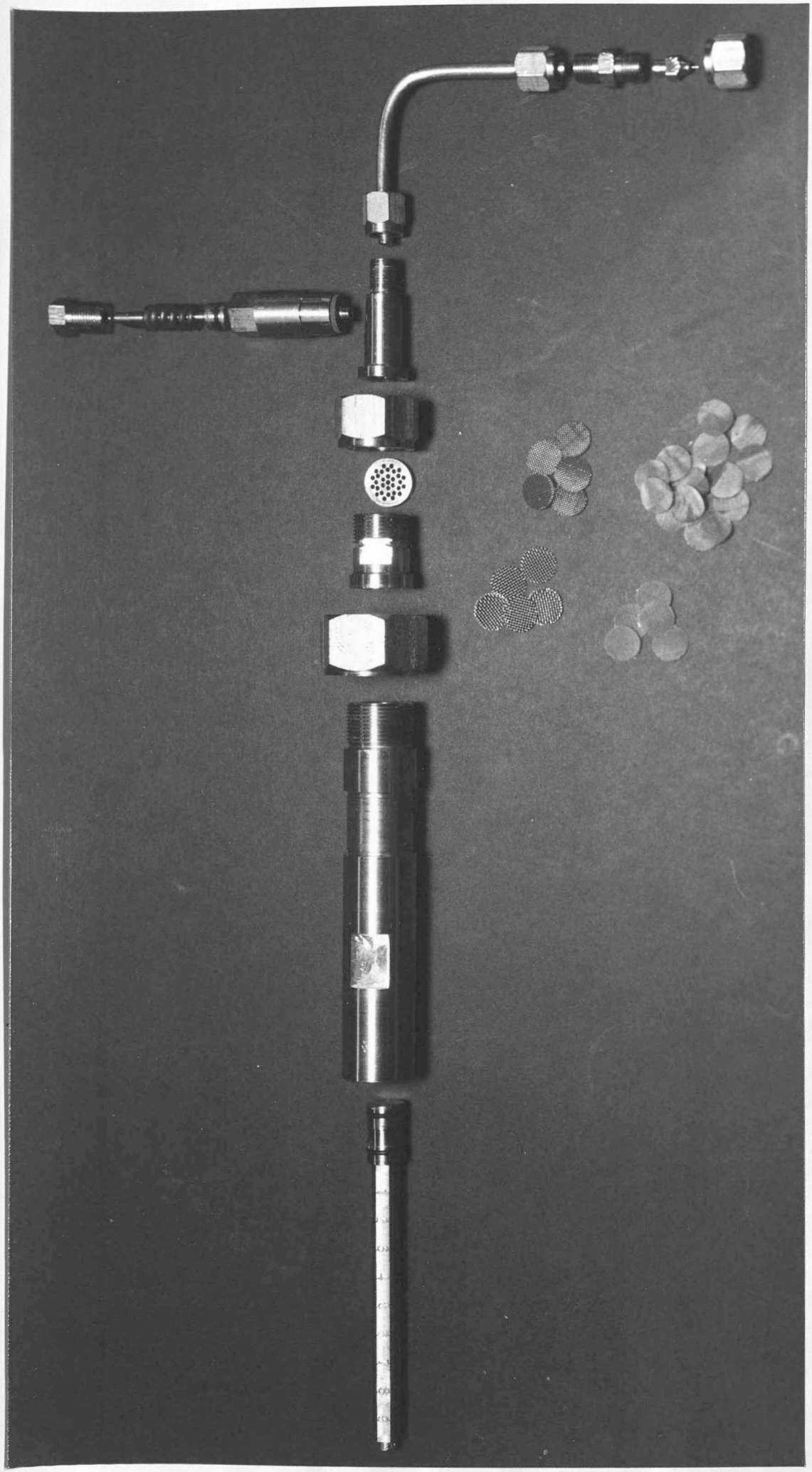
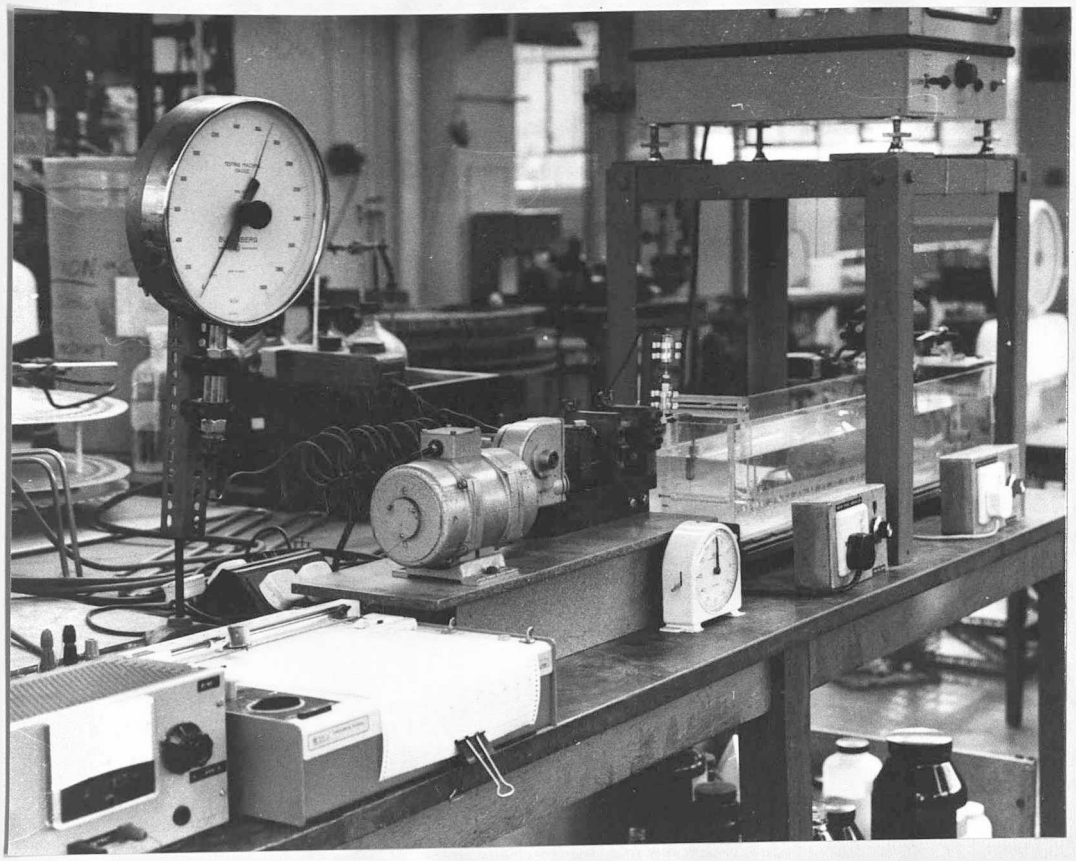
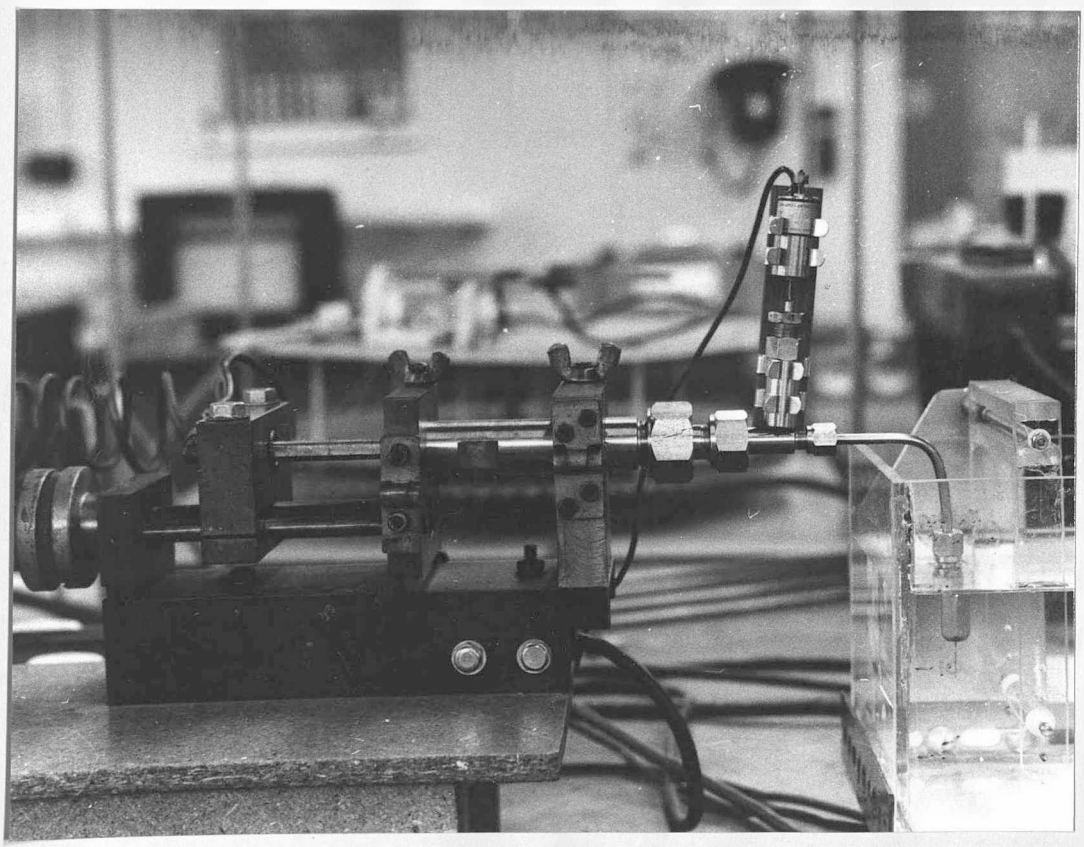


Figure 5.5.2. Spinning Apparatus - overall view.





With hindsight an obvious improvement to the design would be to ensure that bending moments between the pushing system and piston were eliminated by ensuring that they were exactly co-axial.

Where a particular variable was being studied (e.g. the effect of dope pH on fibre strength) the same batch of isolate was used to prepare dopes at different levels of that variable.

5.5.3.3. Dope filters.

Several different types of filter packing were considered. Sand and similar materials were rejected due to the possibility of sand particles passing through into the product. Glass wool and stainless steel wool were tried but these easily compacted causing very high pressure drops across the filter. It was finally decided, following advice (Auchard, 1974), to use a series of stainless steel mesh discs of different grades. The combination detailed in Table 5.5.1. yielded a filtered dope which did not block even the smallest capillaries used (0.0108cm internal diameter) or the spinnerette (0.0075cm diameter holes). This combination was used for all subsequent experiments without further investigation.

Table 5.5.1. Filter Packing.

mesh size (number of holes per inch)	wire grade (SWG)	number of discs used
28	28	5
50	28	5
100	41	5
200	47	20

Several filter holders were constructed such that they could be rapidly interchanged when transferring from one dope to another.

The filters were the most difficult part of the apparatus to clean so that much time was saved between runs by having several similar filter units available. The filter discs were cleaned by placing them in a small glass beaker of detergent solution and subjecting them to sonication for 15 minutes.

5.5.3.4. Pressure measurement.

Accurate measurement of the pressure of the dope prior to extrusion was a severe problem. The major constraint was again the limited amount of dope available. Furthermore, because of the nature of the dope, the measuring device needed to be easy to dismantle for cleaning. A simple spring device was designed such that dope acted on a small piston, restrained in its movement by a powerful compression spring (Figures 5.5.2. & 5.5.3.). The movement of the piston was measured using a Sangamo linear displacement transducer (type ND1) which gave a DC output proportional to the movement of the piston.

The pressure tapping was made immediately after the filter section. The barrel diameter was reduced at this point from 1.2cm to 0.25cm to reduce dead volume. It was considered that the pressure drop in the tube between the pressure gauge and the capillary would be negligible compared with that across the capillary itself. However this proved not to be the case.

One of two springs could be used in the barrel of the pressure measuring device. These gave a total range of measurement from 20 - 800 psig. They were calibrated by filling the barrel with oil attaching a bourdon gauge to the pipe which normally lead to the capillary and using the pusher slowly to increase the pressure.

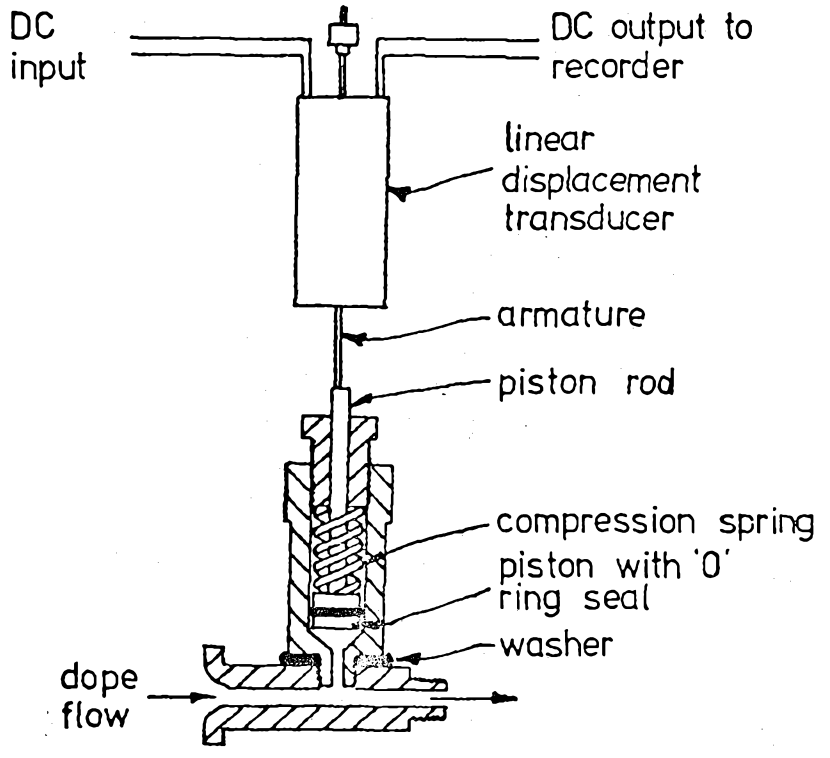


Fig. 5.5.3. Pressure Measuring Device.

Finally when the springs become fully compressed, no further movement of the transducer occurred as the pressure increased. The pressure in the system was then slowly reduced to zero. The calibration graphs are shown in Figures 5.5.4. and 5.5.5. It appeared that there was some sticking of the pressure gauge piston in the barrel since the lines for increasing and decreasing pressures were not coincident. It was difficult, particularly with the weaker spring (no.2) to locate exactly the top of the spring with the lock nut. For this reason the lines were sometimes displaced but as they were always linear their slope was always constant within the working limits. To ensure reproducibility pressure readings were always taken with the pressure increasing (i.e. increasing shear rates) and pressure drop across the capillary was calculated by taking the difference in pressure between the pressure drop with the capillary attached and that without it, at the same shear rate. In addition pressure readings were only taken after recorder output had been steady for two minutes (i.e. steady state had been achieved).

An indication of the pressure before the filters was gained by attaching a bourdon gauge to a small piston of the same diameter as that of the extruder barrel to the pusher device (Figure 5.5.2.). This did not yield very reproducible results, perhaps because of high friction between the pistons and cylinders both of the pressure gauge and of the extruder. Nevertheless it did give a useful indication of the pressure drop across the filters.

5.5.3.5. Connecting tubing.

A stainless steel tube 10cm long and 0.25cm in diameter was used to join the reducing section to the capillary holder. Assuming

Fig. 5.5.4. Pressure Calibration Curve - Spring no.1
(Transducer input, 20v D.C.)

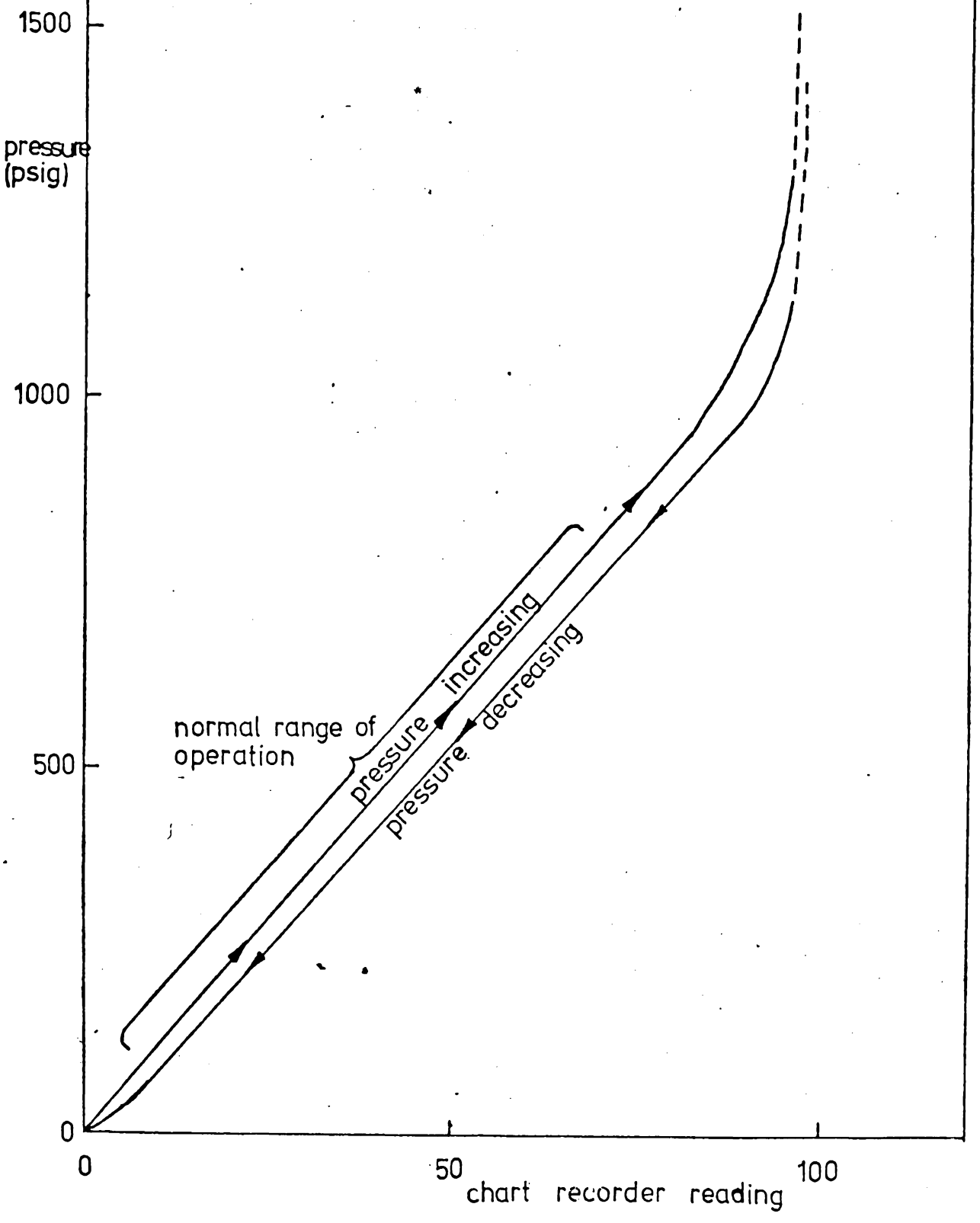
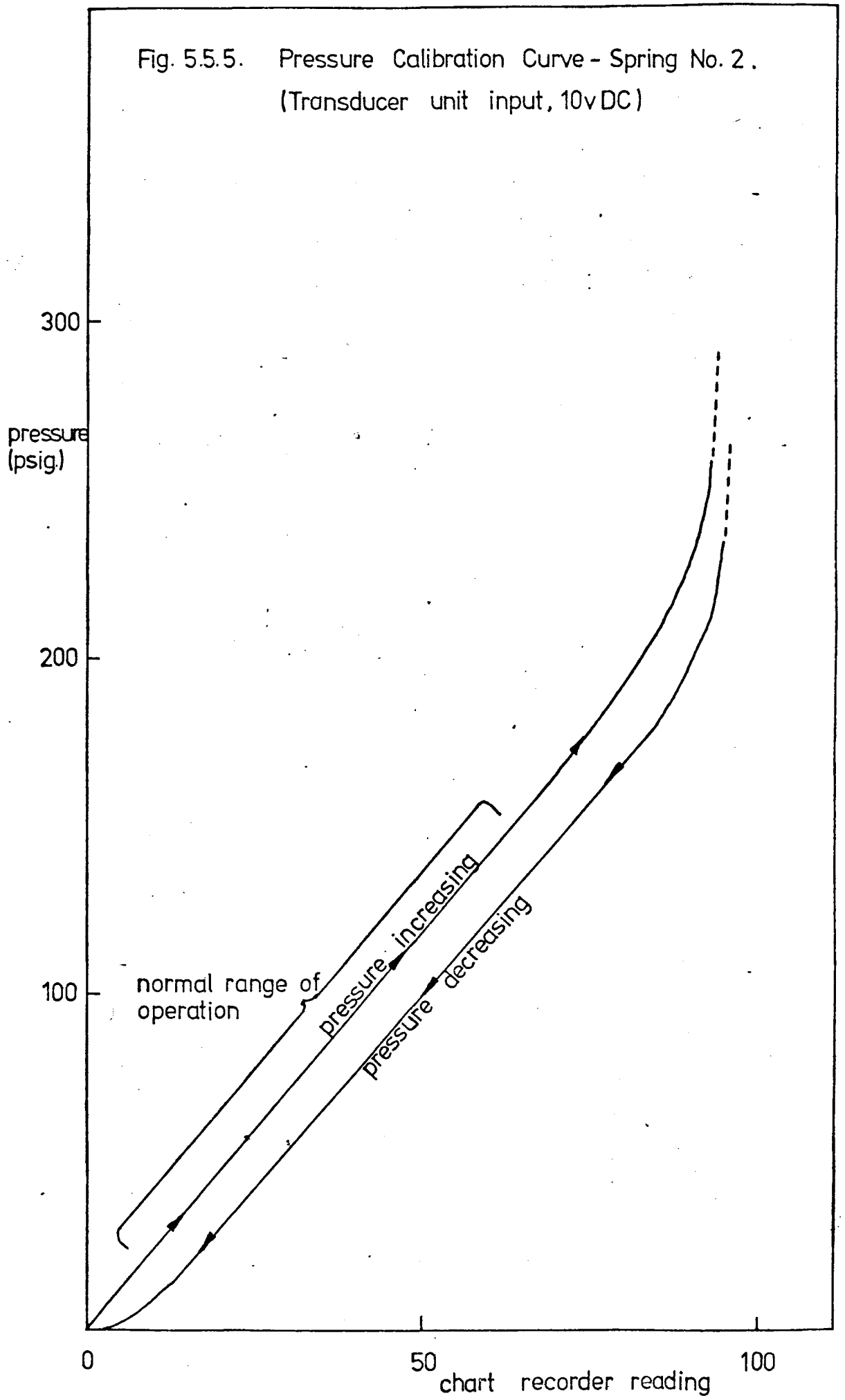


Fig. 5.5.5. Pressure Calibration Curve - Spring No. 2.
(Transducer unit input, 10v DC)



Newtonian flow (since the rheological characteristics of the dopes were not known at this time) it was estimated that the pressure drop in this connecting tube would be less than 1% of that in the capillary, even in the limiting case using the largest diameter capillary.

When the equipment was tested it was found that this assumption was invalid, due mainly to the very high yield stress of the dopes. For example, with a dope of 20%w/w dry matter, pH 10.0, the pressure drops were 108 and 191psi in the connecting tube and the capillary respectively at a flowrate of 0.1ml/min using a 1cm long 0.02cm diameter capillary. For this reason the pressure drops through the connecting tubes were always measured at all the appropriate flowrates prior to attaching the capillary.

5.5.3.6. Capillary and spinnerette attachment.

The capillary holder (Figure 5.5.6.) was designed to fit into standard 'Leur' type syringe needle fittings. A lock nut was provided to prevent the needles blowing off at high pressure. The capillaries themselves were stainless steel tubing cut to length and countersunk at the ends to remove burrs. These were then soldered into Leur fittings. The capillary diameters were calculated by weighing known lengths of tubing, measuring the outside diameter using a micrometer screw gauge and hence calculating the internal diameter, based on a knowledge of the density of the steel. Hence:-

$$d = \sqrt{D^2 - \frac{4M}{\pi \rho l}}$$

Where d = internal diameter of tube

D = external diameter of tube

M = mass of tube

l = tube length

ρ = density of stainless steel type 304 ($8.03 g/cm^3$)

Figure 5.5.6. Spinning using Capillaries or Spinnerette.

a) Capillary and spinnerette holders.

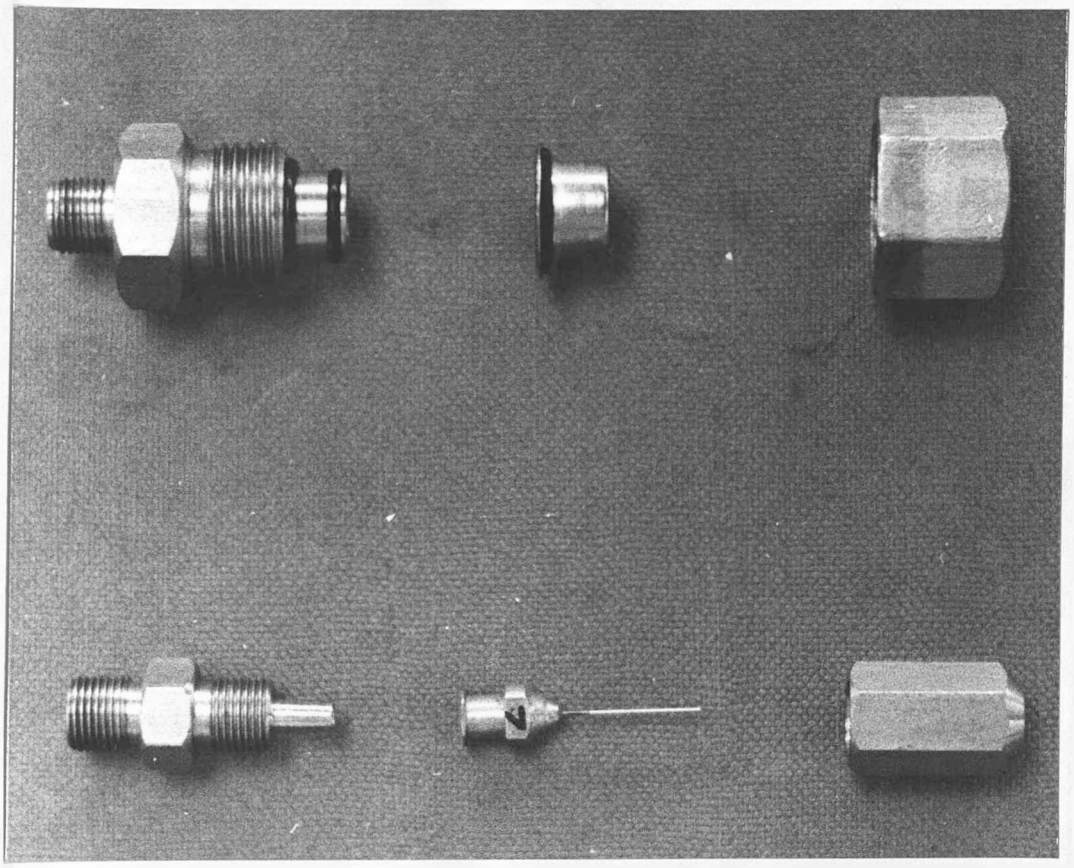
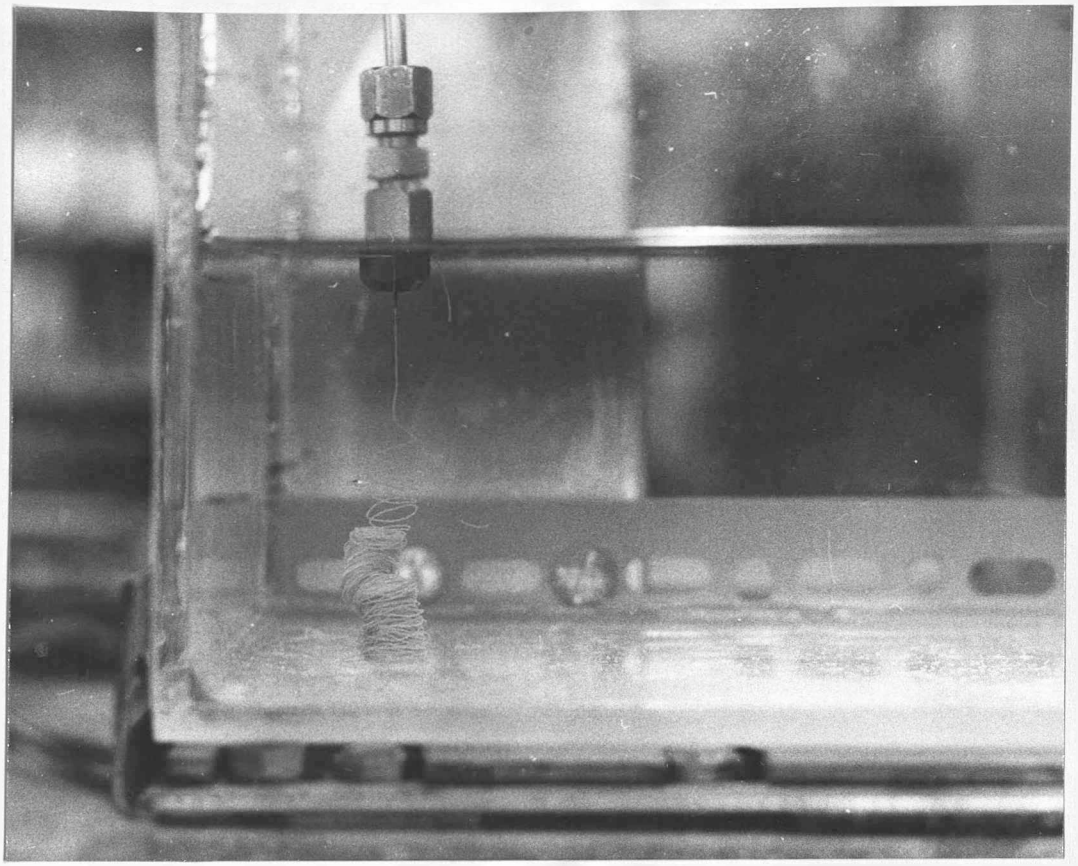
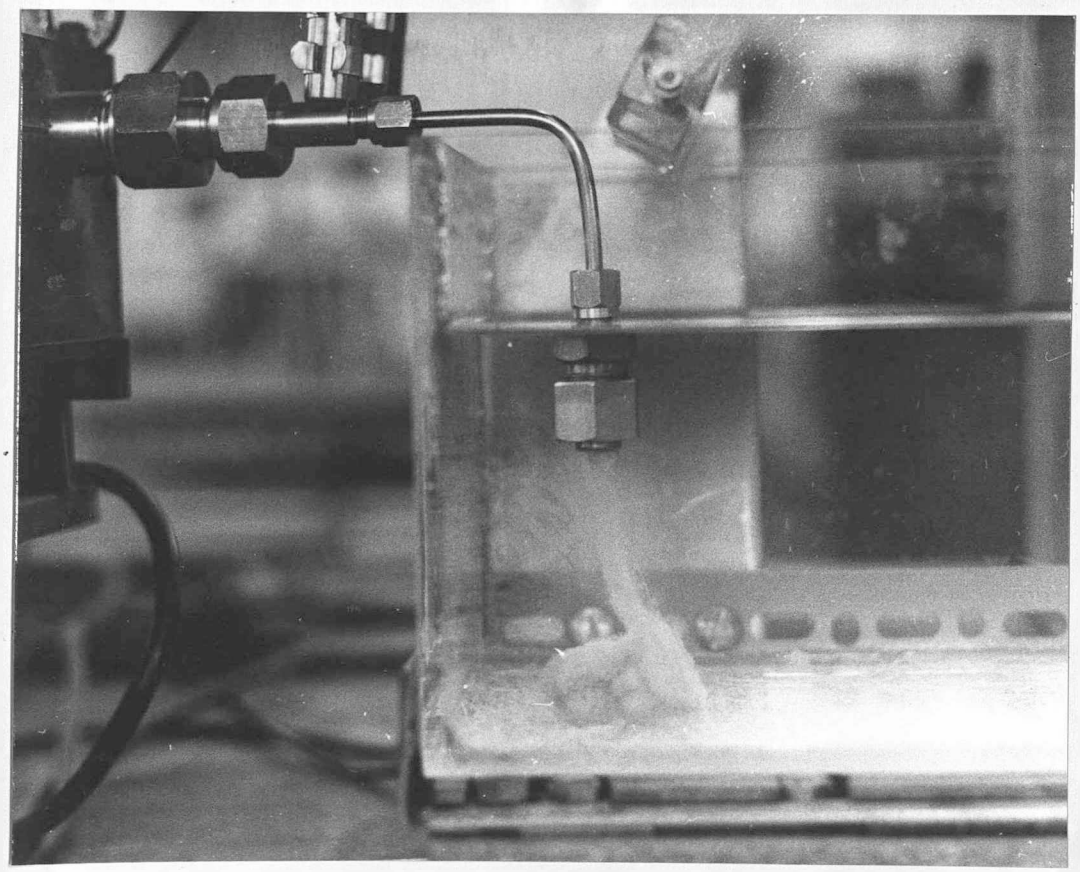


Figure 5.5.6. b) Spinning through a capillary.



c) spinning through a spinnerette.



The half inch diameter spinnerette which had 1,000 holes of 0.0075cm diameter was made from gold / platinum alloy by Courtaulds Engineering Ltd. This slotted over the holder and was held in place and sealed by rubber 'O' rings (Figure 5.5.6.). Again effort was made to reduce the dead volume by allowing only a 3mm gap between the holder and the spinnerette face. Unfortunately however there was considerable lateral pressure drop across the inside face leading to a parabolic profile of fibre velocities leaving the spinnerette. In some cases most of the holes were blocked off by inserting a small teflon washer inside the spinnerette. This led to more uniform fibre velocity except at the edges of the tow where, in many cases, the washer only half occluded the holes. Washers with different hole sizes were used to alter the fibre velocity through the spinnerette since if all the holes were used even at the maximum flowrate of 0.33ml/min, the mean fibre velocity was only about 0.1cm/sec.

5.5.3.7. Sealing.

Rubber 'O' ring seals were used to seal pistons and the spinnerette holder. Because of the viscous nature of the dopes it was found that metal to metal surfaces provided an adequate seal where barrel sections were joined together. Rubber gaskets were used when pressure calibrations were performed using oil.

5.5.3.8. Coagulating bath design.

A coagulating bath was constructed so that fibres could be drawn and wound up on a reel. From preliminary experiments it appeared that full fibre srength, and therefore presumably complete coagulation, took about 1 - 2 minutes to achieve. Even at spinning velocities as low as 10cm/sec a 6 metre long bath would

have been required. This was impractical and it was decided to use a much shorter bath, one metre in length, and to wind up the fibre in the coagulating solution. This restricted the possibility of stretching after fibre coagulation but allowed for investigation of fibre drawing.

In industry much longer coagulating baths are used and inaddition, small spinnerette holes (less than 100μm diameter) allow for more rapid diffusion and hence more rapid coagulation of the fibres (Booth, 1967). In this work it was desired to use a wide range of capillary diameters. Therefore it was unlikely that complete coagulation would occur before wind up in most of the experimental runs.

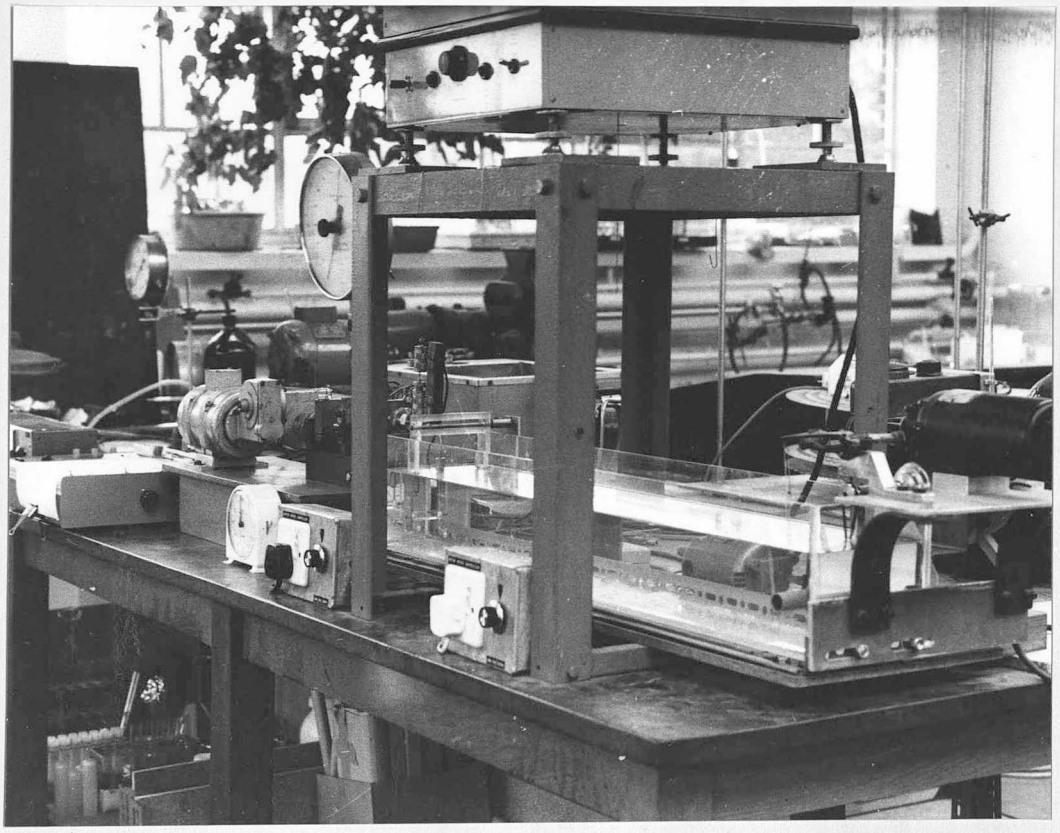
A perspex bath (Figure 5.5.7. a), one metre long and 15cm in width and depth was constructed. Perspex was resistant to dilute acids at ambient temperatures and allowed for visual observation of the spinning process.

A winding device, capable of working at speeds from 10 - 600 revs/min drove a 2cm diameter, removable plastic spool. A fibre guide system consisting of a modified sewing machine bobbin winder was employed to give even fibre wind up onto the spool (Figure 5.5.7. b).

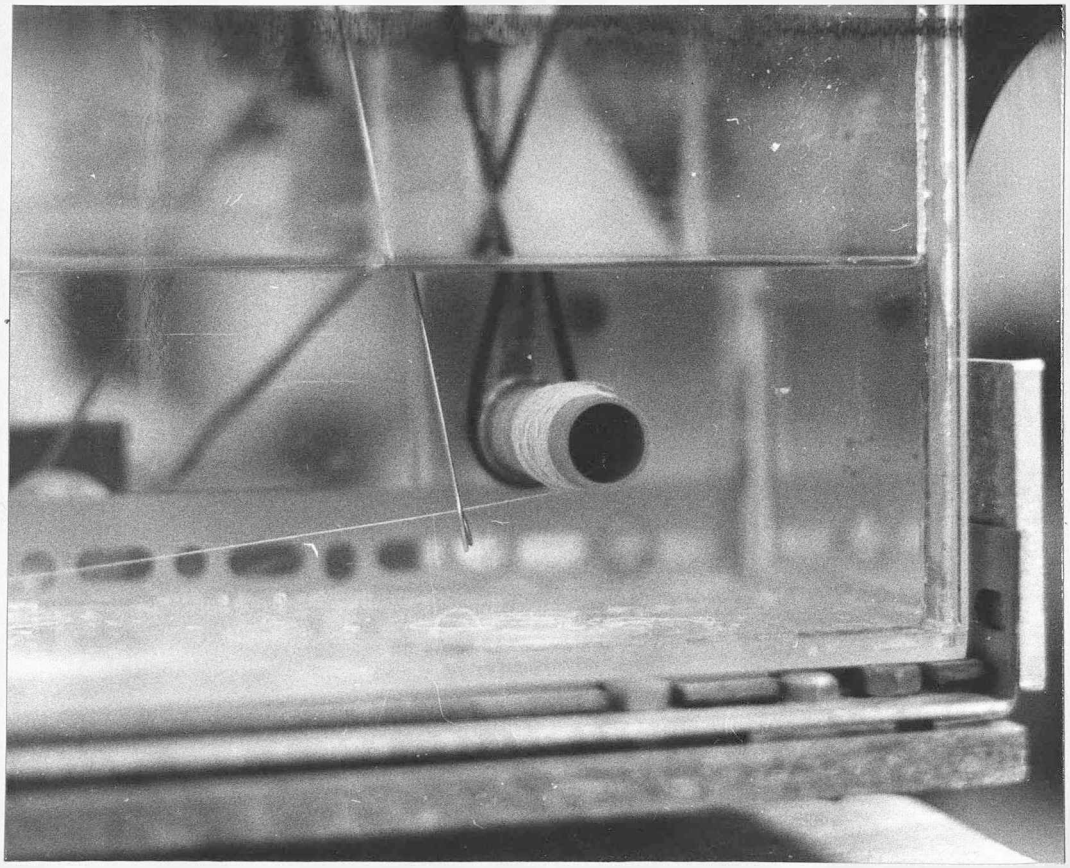
Fibres were spun vertically downwards onto the bath, passed under a pin bearing, perspex roller and wound up as described above. A tachometer attached to the winder made it possible to calculate the winding velocity.

Figure 5.5.7. Fibre Winding.

a) Overall view.



b) Close up of winding spool and fibre guide.



5.5.3.9. Measurement of dope rheological properties.

It was decided to investigate shear stress to shear rate relationships for the dopes by using the spinning needle as a capillary rheometer. Several workers have investigated the rheological properties of protein spinning dopes, as mentioned previously, but most used cylindrical spindle or similar viscometers (e.g. Huang & Rha, 1971) which operated at low shear rates (less than 100 $secs^{-1}$). Some workers simply measured the apparent dope viscocity based on shear stress measurement at a single shear rate value (e.g. Circle et al, 1964). The technique used in this work not only allowed measurement of shear stress over a range of shear rate values but also involved measurement of rheological properties under actual spinning conditions.

For laminar flow with no slip at the tube wall the shear stress $(\frac{\Delta PD}{4L})$ can be related to the shear rate $(\frac{8V}{D})$ by:-

$$\frac{\Delta PD}{4L} = K \left(\frac{8V}{D}\right)^n \qquad \text{(Skelland, 1967)}$$

Where ΔP = pressure drop across the capillary.

D = tube diameter

L = tube length

V = mean fluid velocity

K = consistancy index

n = flow behaviour index.

The apparent viscosity is defined by:-

$$\mu app = \frac{\Delta PD}{4L} \Big/ \frac{8V}{D}$$

For a Newtonian fluid $n = 1.0$ and

$$\mu app = K$$

In many cases n and K are found to vary with shear rate.

Errors of interpretation of the results are reported to occur if account is not taken of kinetic energy effects, entrance and exit effects, if flow is not laminar or if slip flow occurs at the tube walls (Bowen, 1961; Skelland, 1967).

In this case kinetic energy was negligible due to the low velocities used. Furthermore it was calculated that, even under the worst conditions, flow would be well within the laminar region. In fact the highest Reynolds number recorded was for a dope at pH 10.0 containing 10%w/w dry matter passing through a 0.0202cm diameter capillary at 20cm/sec. Under these conditions Re = 2.3.

Experiments were carried out to determine the importance of entrance and exit effects and of slip flow. These are reported in section 5.5.4.

Graphs of $\frac{\Delta PD}{4L}$ vs. $\frac{8V}{D}$ gave a useful indication of dope properties.

5.5.3.10 Dope preparation.

The disadvantages of the original method of dope mixing have already been pointed out. When this technique was used in conjunction with the new apparatus it was found that the reproducibility of data was extremely poor (Figures 5.5.8. & 5.5.9). Several attempts were made to improve on this.

A screw mixer with a 0.5 inch pitch was made from teflon (Figure 5.5.10 a). A close fitting stainless steel jacket was made with

Fig. 5.5.8. Rheology of Dopes Prepared on Protein Content Basis from Three Different Yeast Batches. (15% w/w protein, pH10; capillary: 2·54 cm × 0·0259 cm. ø.)

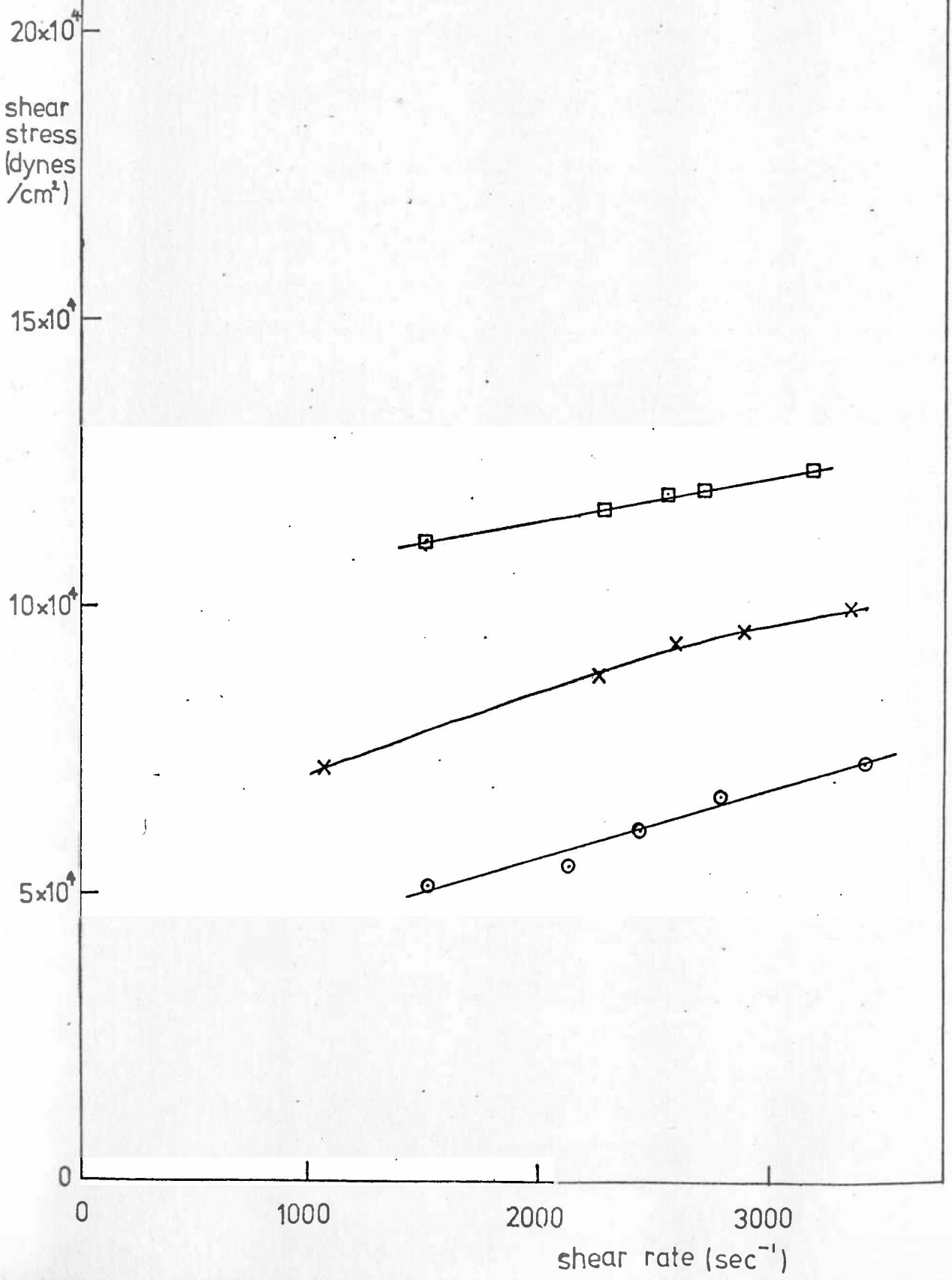


Fig. 5.5.9. Rheology of Dopes Prepared on Protein Content Basis from 3 Different Yeast Batches. (15% w/w protein, pH 10; capillary: 0.560cm × 0.0108cm .ø.)

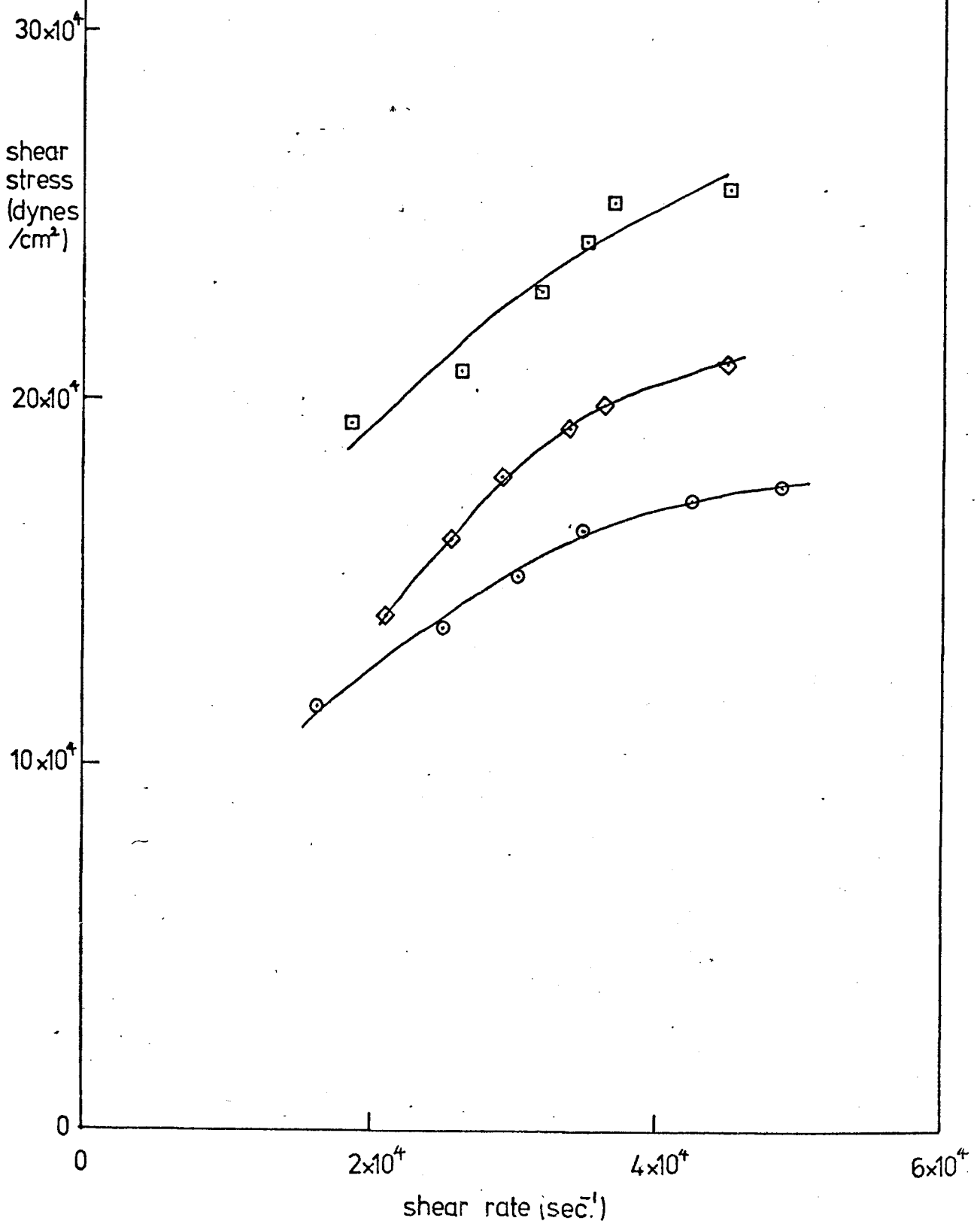
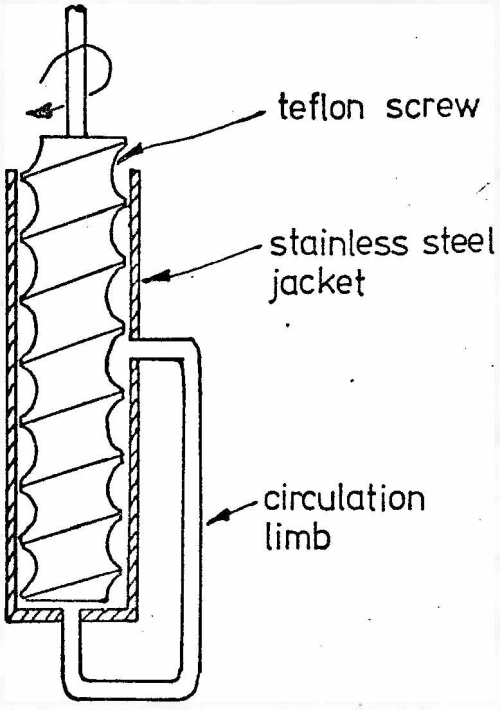
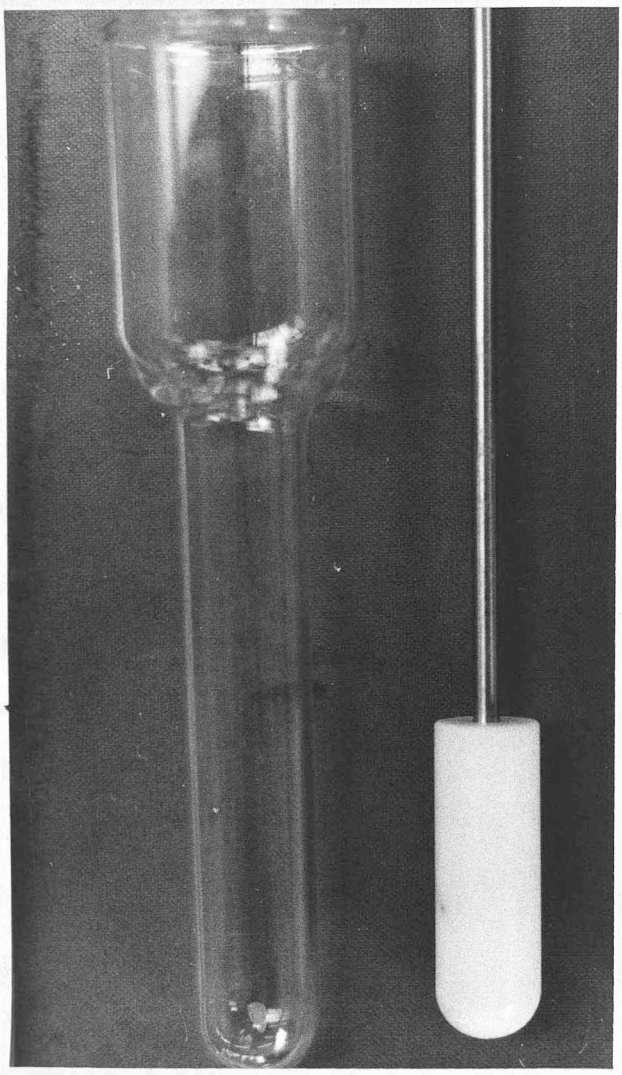


Fig. 5.5.10.
(a) Screw Mixing Device.



(b) Laboratory Homogenizer.



a circulation pipe leading from the bottom of the jacket to a side arm on the jacket. Isolate and NaOH were fed into the jacket and it was hoped that, on turning the screw, dope would be forced to circulate through the side arm and back into the screw. However, in practice, the dope clung to the teflon screw and yielded at the tube walls, so that no circulation took place.

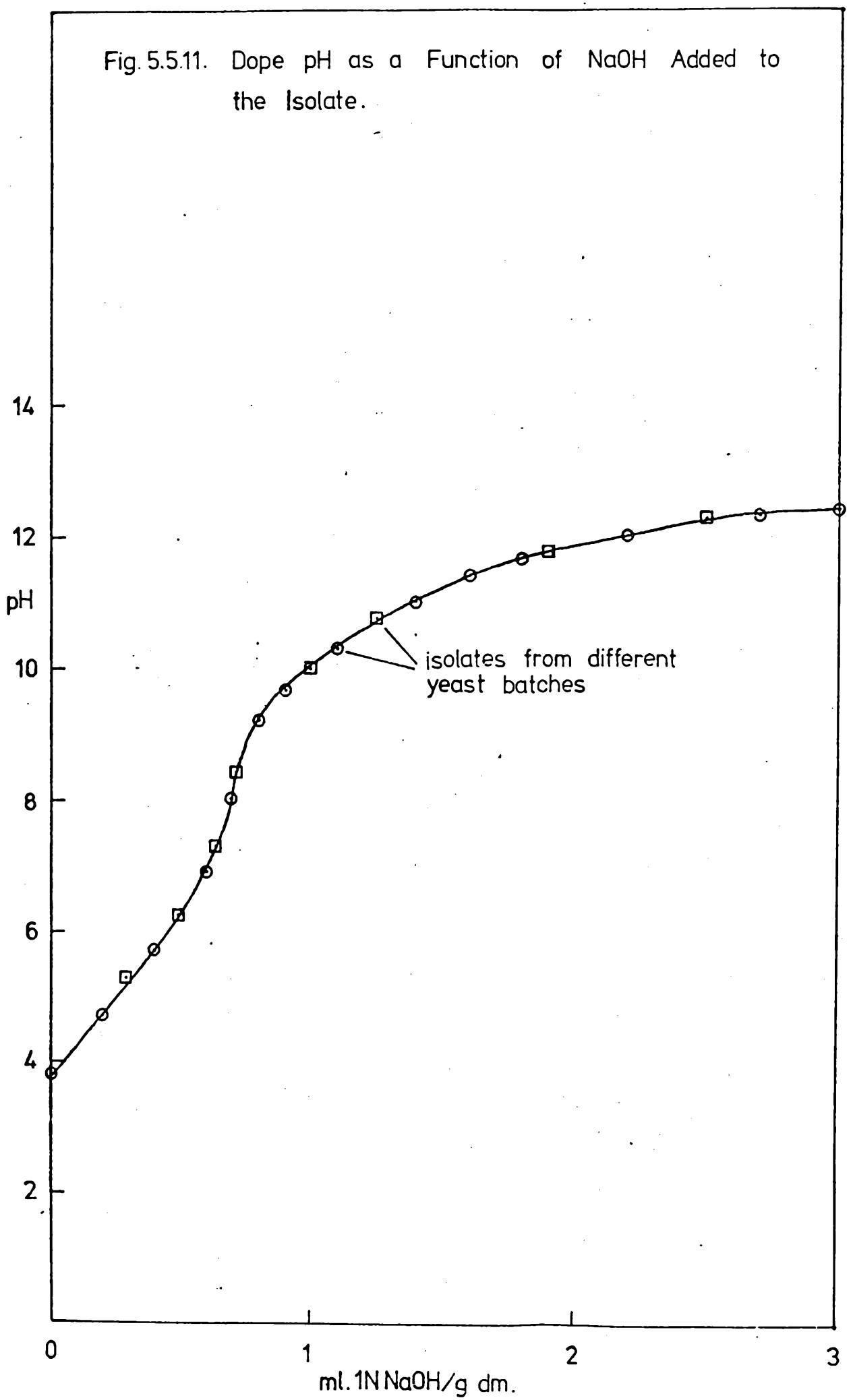
Attempts were made to mix the dope in a laboratory homogeniser (Figure 5.5.10 b) which was used in analysis for suspending precipitates in water. However the force required to push the dope past the teflon core through the narrow annulus was too great to be achieved by hand.

The main limitation was again the small amount of isolate available. Eventually a modified hand mixing technique was developed.

The isolate had a very high buffering capacity and the pH was dependant almost entirely on the ratio of dry matter to NaOH and not on the concentration of the isolate suspension. The protein content of the isolates varied from batch to batch between 65 and 75%w/w of the dry matter, but this did not appear to have a significant effect on the relationship between the NaOH content and pH for a given quantity of isolate dry matter. Figure 5.5.11. shows pH as a function of volume of 1 N NaOH/g of dry matter in the isolate.

It was also found that it was far easier to mix homogeneous dopes if the isolate had previously been diluted and thoroughly

Fig. 5.5.11. Dope pH as a Function of NaOH Added to the Isolate.



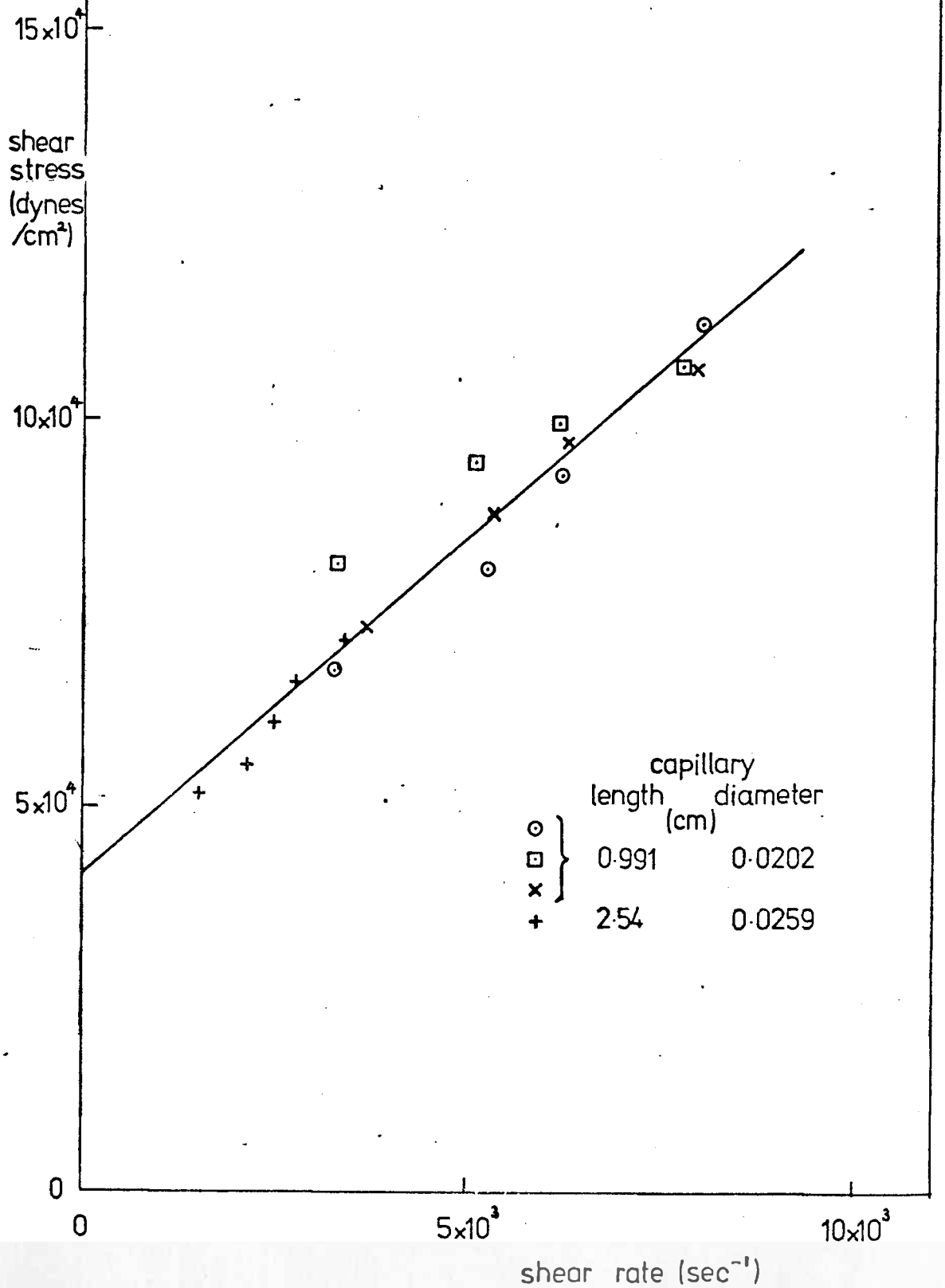
mixed with water. The following technique was therefore adopted.

1. The isolate was analysed for its dry matter content.
2. The required amount of isolate was weighed into a 250ml beaker.
3. The required dry matter content and pH were decided upon and the amount of 3 N NaOH required to achieve this pH was calculated from Figure 5.5.11.
4. The amount of distilled water required to adjust the dry matter content to the desired level was calculated and, after subtracting the water to be added from the NaOH solution, it was added to the isolate and mixed with it to as uniform a suspension (or paste) as possible.
5. All the required NaOH was added to the isolate and the dope was mixed as rapidly as possible with a thick glass rod to a uniform consistency.

By using this method far fewer lumps occurred. Air was removed by centrifuging the dope at 2,000g for five minutes in a tube just large enough to accommodate the extruder barrel. The barrel was then pushed into the centrifuge tube and deaerated dope passed into the barrel. The plunger was pushed into the barrel which was then fitted into the pusher. Dope passed through the filter pack to the capillary and was spun into the coagulating bath.

Although the reproducibility of the results still left much to be desired a considerable improvement over the earlier method of dope preparation was achieved (Figure 5.5.12.). The revised method was used in all further spinning experiments.

Fig. 5.5.12. Rheology of Dopes Prepared using the Improved Mixing Technique for Different Yeast Batches. (20%$^{W}/_{w}$ dm, pH 10.0)



5.5.3.11. Conclusion.

Generally speaking the apparatus gave satisfactory performance. However rheological data were not entirely reproducible partly due to differences in different batches of isolate, partly due to the difficulty of mixing the dopes and partly due to the problems of piston friction in the pressure gauge. It is felt that these problems could be solved if it were possible to produce larger batches of isolate and hence work on a bigger scale.

Because of the problems of reproducibility of data from different isolates where experiments were performed to investigate a particular parameter the same batch of isolate was used.

As mentioned previously (section 5.5.3.2.) the apparatus could be improved by using a pushing system which was co-axial with the piston, thus preventing the large bending stresses caused by pressures of up to 1,000psi.

5.5.4. Fibre Formation.

5.5.4.1. Introduction.

The experiments in this section were aimed at finding the optimum conditions of dope pH, concentration and shear rate which gave the strongest fibres using capillary tubes. The effect of dope age on its spinning characteristics was also investigated since it was known that in Boyer type spinning processes, using vegetable proteins, viscosity changed considerably with time following dope preparation (Boyer, 1953). Fibres were analysed to see what effect spinning had on the protein and nucleic acid

contents of the material. Tests were also aimed at seeing to what extent the fibres could be drawn out on spinning. The use of a multihole spinnerette was also investigated. The above tests were carried out using isolates prepared by the standard procedure of extraction at pH 11.0 and precipitation at pH 3.8.

As previously described (section 5.4.) various techniques were investigated for the removal of nucleic acid and one such technique resulted in the production of low nucleic acid isolates. The spinning properties of these isolates were therefore investigated since the main aim of this project was to integrate, as far as possible, the extraction and texturisation processes. Unless otherwise stated however, isolates were prepared by the standard method outlined in section 5.3.3.

Before these tests were conducted preliminary tests were performed to check for slip flow and end effects in order to validate the rheological data. It was not possible in the time available, to investigate all the dopes used, and it was therefore decided only to use dope conditions in the region of the optimum pH and concentration.

5.5.4.2. End effects.

The significance of pressure losses on entrance to and exit from the capillaries was checked by using a series of 0.0202cm diameter capillaries of different lengths (i.e. different aspect ratios). The same dope (20%w/w DM at pH 9.5) was used in each case and the pusher ran at the same speed so as to give the same shear rate. A plot of shear stress at the tube walls versus aspect ratio is

shown in Figure 5.5.13. Clearly for $\frac{L}{D}$ values greater than 50 the end effects are insignificant. Capillaries with aspect ratios of 50 or more were used in all subsequent tests. Another conclusion which can be drawn from this graph is that the absolute pressure does not affect the rheological properties, since the shear stress at the wall was constant above $\frac{L}{D} \geqslant 50$ even though the pressure required to push the dope through longer capillaries was much greater. Pressure dependancy has been observed for other polymers (Penwall & Porter, 1971).

There was no noticeable difference in fibre strength as a result of using different lengths of capillary tubing.

5.5.4.3. Slip flow.

A procedure for estimating slip flow has been outlined by Skelland (1967). Using this technique dope was passed through a series of tubes of different diameters but constant length. Where no slip occurs at the walls plots of shear stress versus shear rate should yield concurrent or coincident lines. If the lines were not concurrent it would be possible to estimate the slip flow coefficient and hence to correct the rheological data. The results of such an experiment are shown in Figure 5.5.14. It can be seen from the graph that the lines are approximately concurrent showing that slip flow did not occur to any great extent when dopes approximately 20%w/w DM and pH 10 were used. These dope conditions led to the production of the strongest fibres.

Changes in capillary diameter did not affect the fibre strengths.

Fig. 5.5.13. Effect of Aspect Ratio on Measured Shear Stress. (Dope: 20%$^{W}/_{W}$ dm., pH 9·5; shear rate: $7{\cdot}3\times10^{3}$ sec.$^{-1}$; capillary ø: 0·0202 cm.)

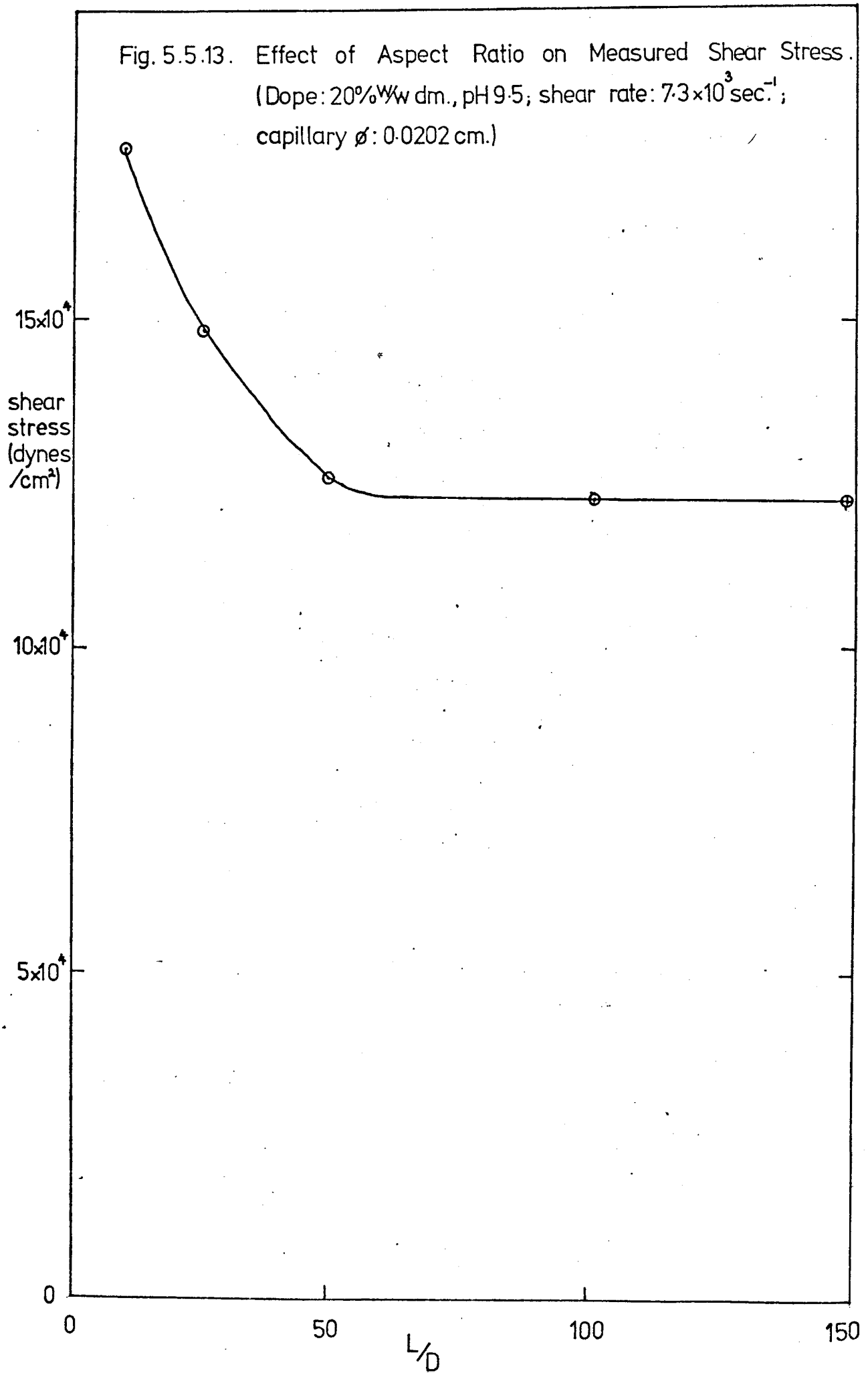
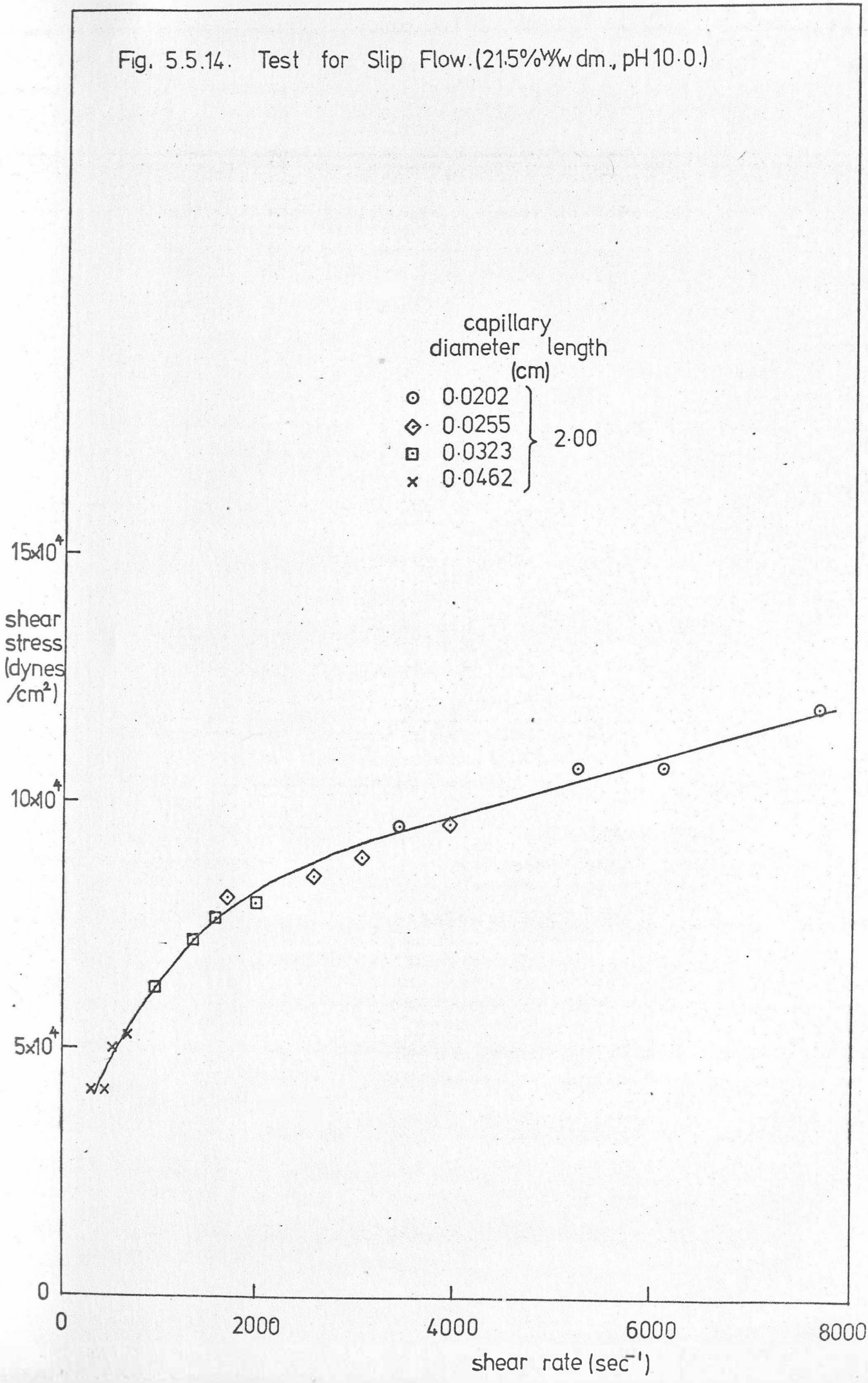


Fig. 5.5.14. Test for Slip Flow. (21.5% w/w dm., pH 10·0.)



5.5.4.4. Shear rate.

Generally speaking over the range of shear rate values used in the experiments (from 200 - 8,000 $secs^{-1}$) using needles of different lengths and diameters, fibre strength (measured as outlined in section 4.2.7.) increased only very slightly with increasing shear rate. For example, the length of fibre which could be drawn from the bath increased from 200 - 220 cm when the shear rate was increased from 3,000 - 8,000 $secs^{-1}$ for a 20%w/w DM, pH 10.0 dope. It had been thought that higher shear rates would lead to a greater degree of molecular orientation and hence much stronger fibres. However Ferguson and Ibrahim (1969), using cellulose acetate and Tombs (1972), using soya protein, reported that the increasing shear rate during spinning had no effect on the tensile properties of the fibres.

5.5.4.5. Dope ageing.

The phenomenon of dope ageing has already been mentioned (section 2.4.1.5.). In order to test for changes in rheological properties a dope containing 20%w/w DM at pH 10.0 was spun at the same shear rate for a period of 45 minutes (achieved by using a low flowrate). No change in the pressure drop across the capillary was noted during this time (although the pressure drop across the filters increased steadily as they became increasingly screened with lumps from the dope). It was concluded that no dope aging effect was present.

It is probable that, when the dope is mixed, gel formation occurs rapidly and as soon as a stable gel is formed the molecules are held within a rigid lattice after which no further cross linking occurs. This would not have been the case for the fluid

dopes in which the characteristic was observed (Ashton et al, 1970; Lundgren, 1949).

5.5.4.6. Dope pH.

The effect of using dopes with pH's in the range 7.3 - 11.75 is shown in Figure 5.5.15. The dry matter content of the dopes was 20%w/w. In the range of shear rates used the dopes conformed quite well to 'Bingham plastic behaviour' model. Based on this the yield stresses (i.e. the shear stress value when the shear rate is zero) and apparent viscosities (i.e. the gradient of the shear stress / shear rate lines) were estimated using linear regression analysis. Yield stress, apparent viscosity and fibre strength were plotted as functions of dope pH and these are shown in Figure 5.5.16.

Clearly the optimum pH, from the point of view of fibre strength is in the region 9.5 - 10.2. Above and below this range there is a rapid fall off in fibre strength. The highest viscosity, 8 - 9 poises, also occurred in this region, whereas the yield stress had a maximum in the region pH 10 - 11. Huang and Rha (1971 & 1972) reported highest viscosity and best fibre formation at pH 9 using protein from C. utilis.

It is interesting to note that the viscosity increased again between pH 10.75 - 11.75. Visual observation of the dopes gave an indication as to why this was so. The dopes varied from a smooth paste, light cream in colour, easy to mix and deaerate at pH 7.3 to a light brown, firm, rubbery gel at pH 10.75 which was difficult to mix and deaerate. Gel formation occurred due to

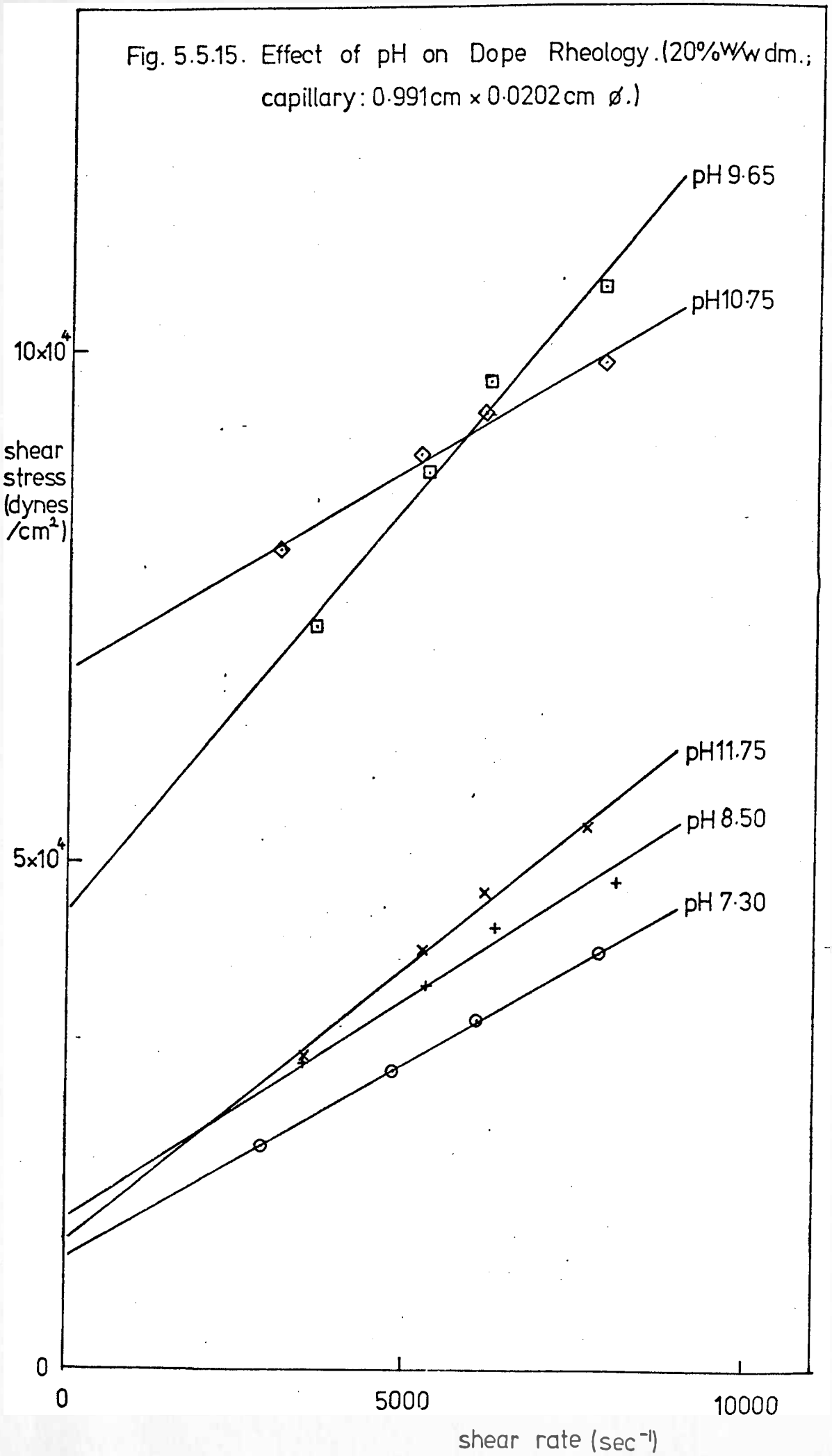


Fig. 5.5.15. Effect of pH on Dope Rheology. (20%w/w dm.; capillary: 0·991cm × 0·0202cm ø.)

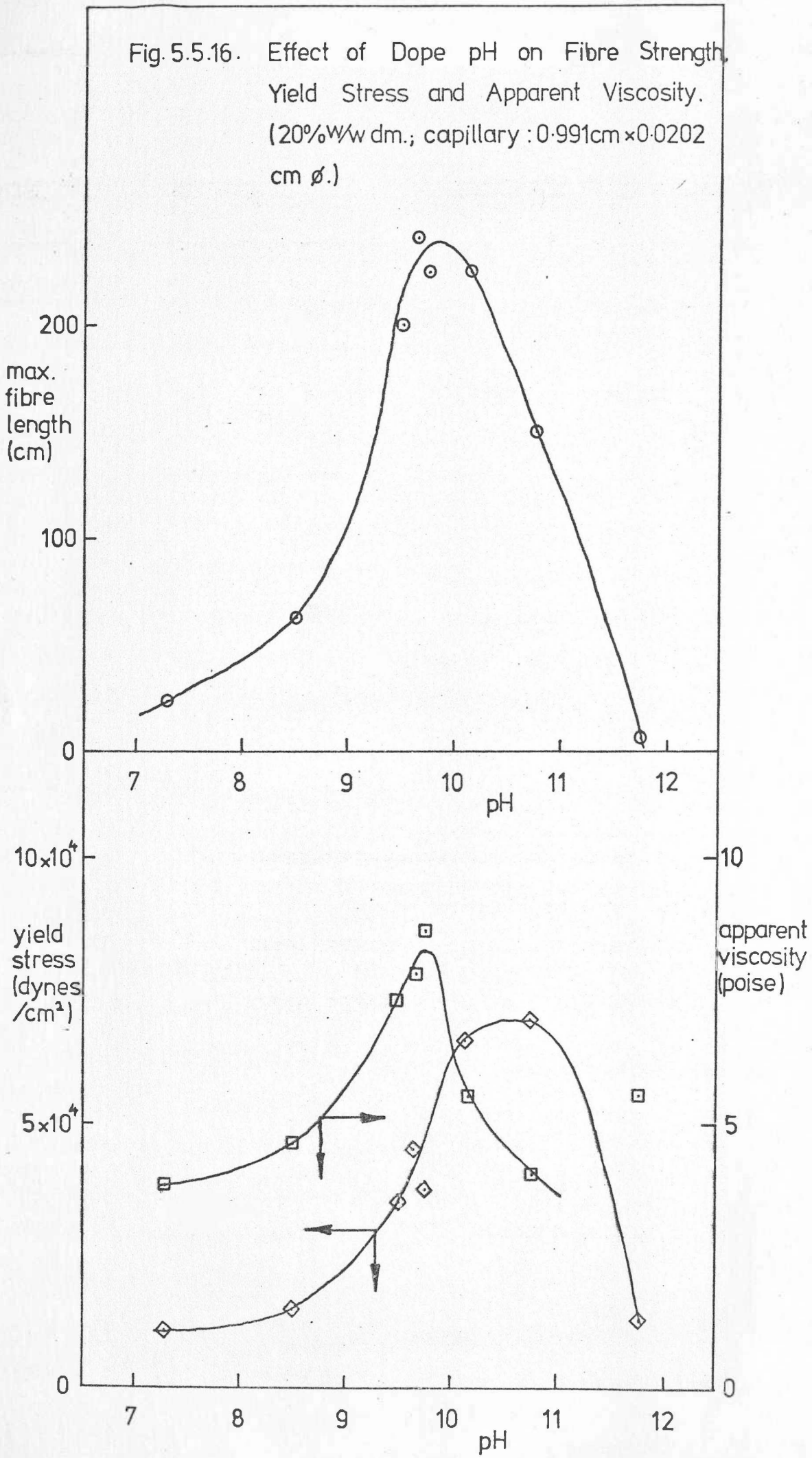


Fig. 5.5.16. Effect of Dope pH on Fibre Strength, Yield Stress and Apparent Viscosity. (20%w/w dm.; capillary : 0·991cm × 0·0202 cm ø.)

increased molecular unfolding and interaction at high pH, as discussed in section 2.4.1.3. However, at pH 11.75, after initial mixing to a firm, light brown, rubbery gel, the dope began to separate into two distinct phases - a thin, glistening, free flowing liquid and a matrix of small, hard gel granules. Upon spinning it was found that the pressure drop across the filter was very large (initially 300psi and increasing to 600psi) compared with a filter pressure drop of about 100psi for dopes at pH 10.0. After passing through the filter a homogeneous material was observed, but this rapidly reverted to the two phases system described previously. On emergence from the needle into the air the fibres glistened as though coated with liquid and appeared to 'slip' through the tube in a pulsating fashion. It was quite likely therefore that slip flow did occur in this case and that the rheological properties recorded were not the true values for the material. A possible explanation for these observations is that a rigid gel formed at first but, due to the high pH, the protein and the nucleic acid were hydrolysed and the gel began to break down. Because of the firmness of the original gel a high pressure drop was required across the filter to break up the gel granules. Build up of granules on the filter surface would account for the increase in pressure drop over the spinning period - about 30 minutes. The apparent re-mixing caused by filtering may be simply a re-suspension of the now much smaller gel particles in the suspending fluid, which then rapidly separated out again. Fibres were extremely weak and became partially dispersed in the acid bath. Unlike fibres spun at lower pH's which were white to light cream in colour and had a smooth surface these fibres were light brown and had an uneven

and grannular texture when observed under a microscope.

Maximum solubility of the isolates occurred at pH 10.75 as already shown (Figure 5.4.20.). Solubility alone does not, therefore, account for the production of the strongest fibres at pH 10. Clearly most of the protein must be solublised to produce good fibres. As pH increases the gel structure becomes firmer (shown by increasing yield stress in Figure 5.5.16) and this would tend to give poorer fibres since breakdown of the gel during extrusion through the capillary would be more difficult and may even lead to a non-homogeneous product composed of small gel granules instead of a smooth, continuous, partially orientated fibre. In spinning groundnut protein Thompson and Johnston (1947) found that the highest viscosity short of gel formation gave the strongest fibres. In their case however the protein was completely solublised at lower pH's before the onset of gelation which occurred at higher pH's. In this case, however, solublisation and gel formation occurred over the same range of pH's, maximum solubility (Figure 5.4.20.) and firmest gels (as indicated by yield stress in Figure 5.5.16.) both occurring at pH 10.75.

It is recommended that investigations into agents capable of preventing gelation are undertaken, since this may lead to stronger fibres being produced. It may also be possible to produce isolates more soluble at lower pH's. This would probably involve extraction at lower pH's following cell disruption. It may prove beneficial to accept some loss of protein yield in order to gain higher solubility and possibly stronger fibres. It is also therefore recommended that the effect of protein

extraction pH on spinning be investigated.

For further work dopes were used at pH 10.0.

5.5.4.7. Dope concentration.

Dopes of 10 - 25%w/w DM were prepared at pH 10.0. Again the Bingham plastic model was found to apply in defining the yield stress and viscosity. The results are plotted in Figures 5.5.17. and 5.5.18.

Maximum fibre strength occurred when dopes of 20%w/w DM or more were used. The dopes varied from a jelly like fluid (similar in texture to custard) at 10%w/w to a thick, rubbery elastic solid at 25%w/w. The 25%w/w dope proved extremely difficult to mix. All the dopes were light brown in colour.

There was an exponential rise in yield stress with increasing dope concentration (Figure 5.5.18.) whereas the viscosity increased rapidly to a maximum at 20%w/w and then levelled off at a value of about 10 poise. Fibre strength followed a similar pattern to that of dope viscosity and it is therefore concluded that it is the viscosity of the dope rather than the yield stress which has the greatest influence on fibre strength.

Yield stress depends upon gel formation (Circle et al, 1964) so again it is seen that dope gelation does not improve fibre strength.

5.5.4.8. Dope rheology.

It has already been observed that dopes in general behaved according to the Bingham plastic model, having a high yield stress

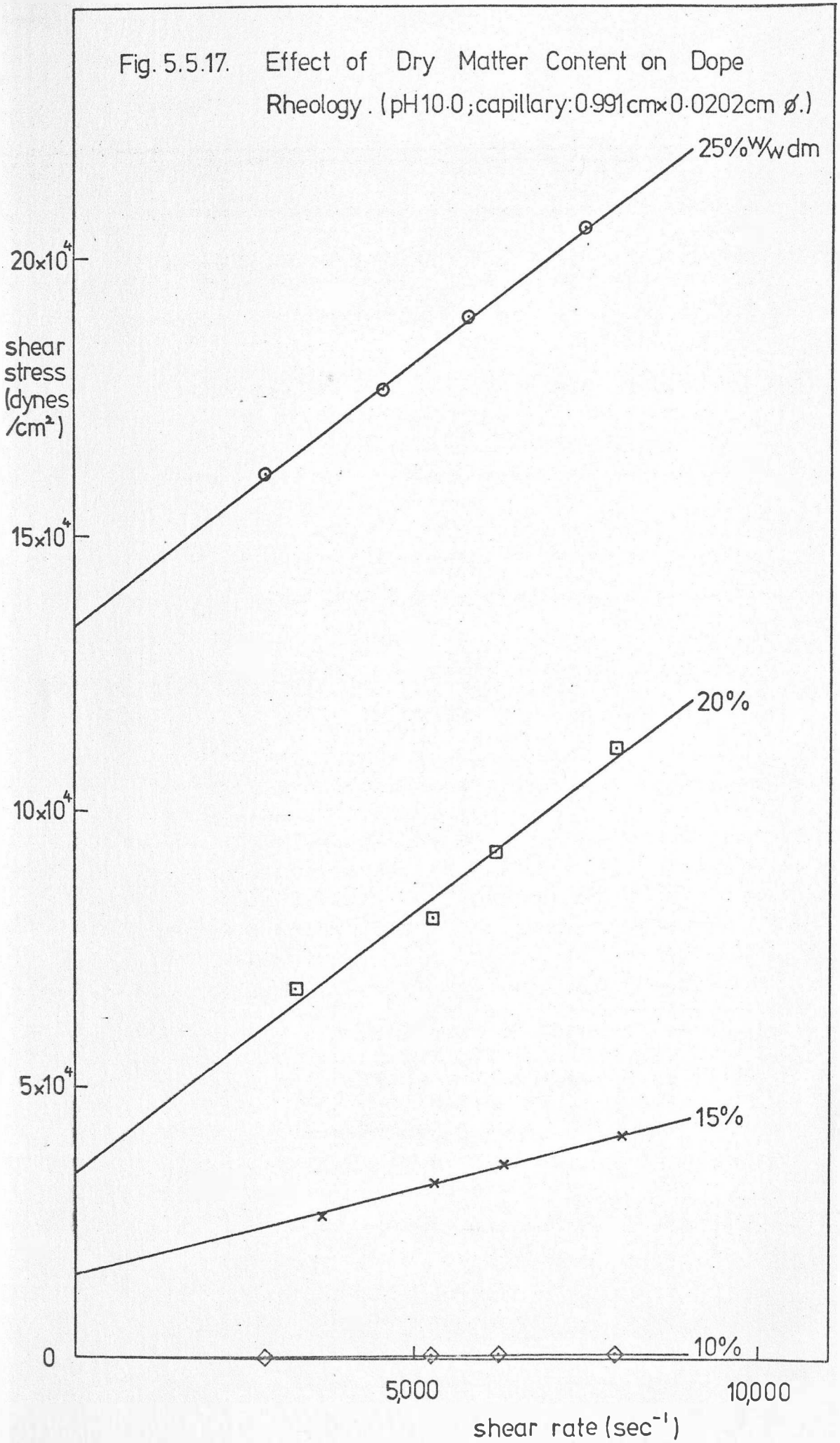
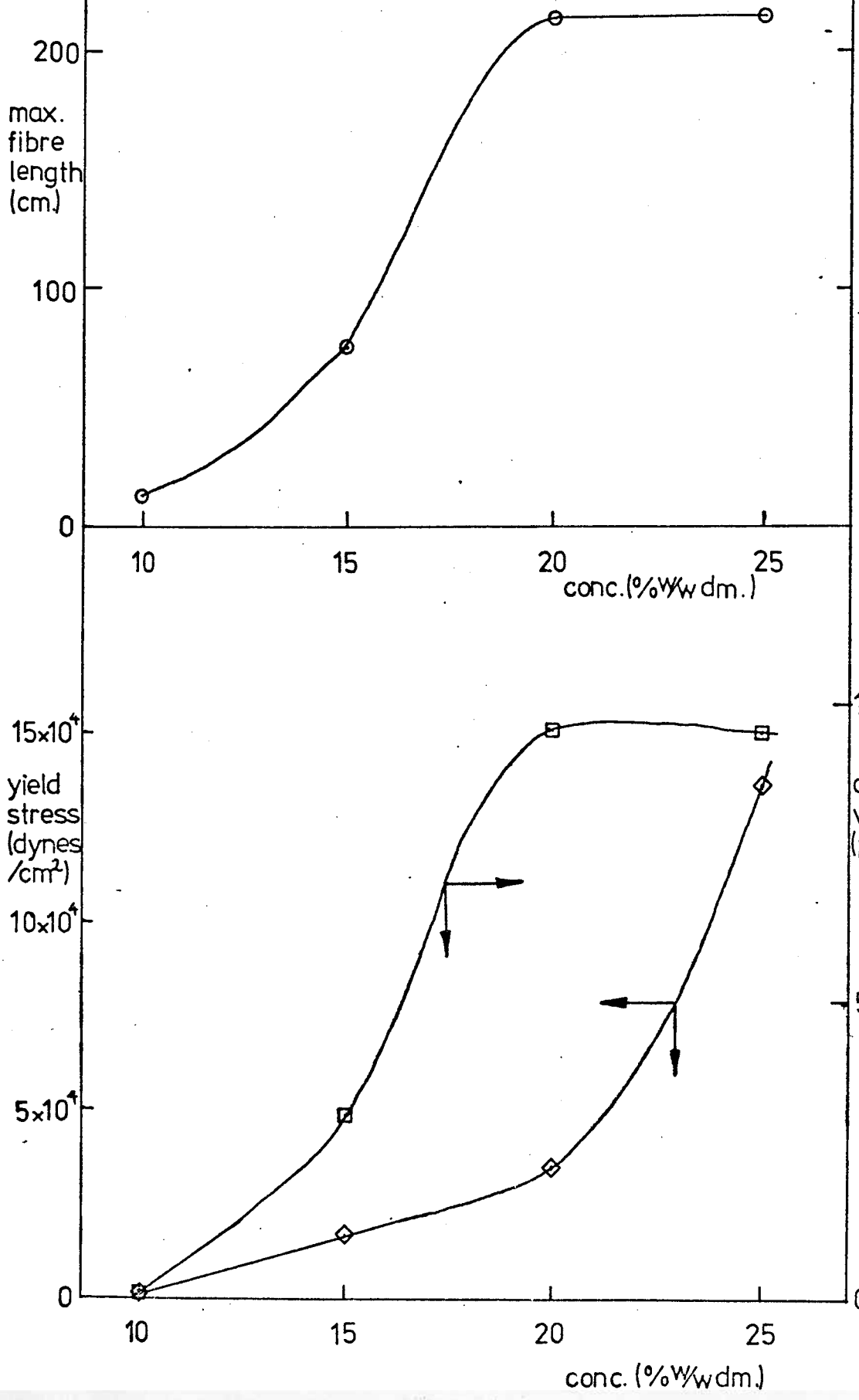


Fig. 5.5.17. Effect of Dry Matter Content on Dope Rheology. (pH 10·0; capillary: 0·991 cm × 0·0202 cm ø.)
25% w/w dm
20%
15%
10%
20×10⁴
15×10⁴
10×10⁴
5×10⁴
0
shear stress (dynes /cm²)
5,000
10,000
shear rate (sec⁻¹)

Fig. 5.5.18. Effect of Dry Matter Content on Fibre Strength, Yield Stress and Apparent Viscosity. (pH 10·0, capillary: 0·0991cm × 0·0202cm ø.)



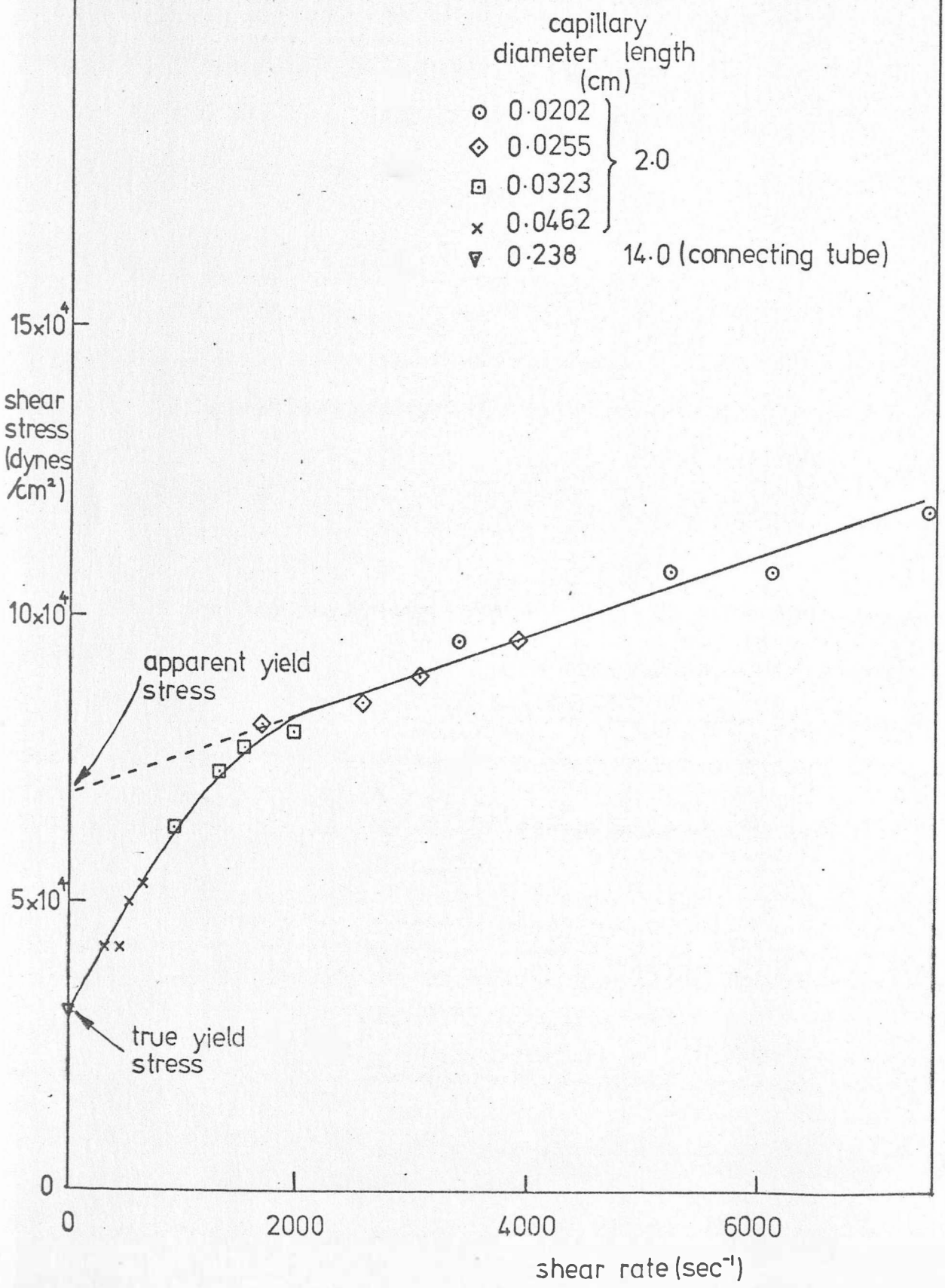
and a linear relationship between the shear stress and shear rate (e.g. Figures 5.5.15. & 5.5.17.). However when a very wide range of shear rates was used, as shown in Figure 5.5.19., it was seen that at shear rates less than 2,000 sec^{-1} the dope demonstrated pseudoplastic behaviour. Hence, over the range of shear rates studied,(0 - 8,000 sec^{-1}) the dope exhibited a combination of Bingham plastic and pseudoplastic properties.

Bingham plastic materials are typically suspensions of fine particles at high concentrations. Pseudoplastic fluids are typically polymer solutions (Van Wezer, 1963). A possible explanation of the behaviour observed in Figure 5.5.19 is that a high yield stress is required to break up the gel structure of the dope, giving large molecular aggregates suspended in a viscous polymer (largely protein) solution. The gels reformed when stress was removed. The gelation of the dope would therefore explain the Bingham plastic effect and the dissolved protein would explain the pseudoplastic effect.

It is clear from Figure 5.5.19. that when only high shear rates were used (as in Figures 5.5.15. & 5.5.17.) the inferred yield stresses were well above the true yield stress. For a dope of 21.5%w/w DM at pH 10.0 the true and apparent yield stresses were 31,000 and 69,000 $dynes/cm^2$ respectively. All the yield stresses referred to in sections 5.5.4.6. and 5.5.4.7. and in Figures 5.5.16. and 5.5.18. are, therefore, apparent rather than true yield stresses.

It is interesting to note that Naismith and Thompson (1955), having

Fig. 5.5.19. Dope Rheology over a Wide Range of Shear Rates. (21·5%$^{W}/_{w}$ dm, pH10·0).



investigated the rheological properties of groundnut protein dopes over a wide range of concentrations and pH's concluded that about one half of the dopes fitted the Bingham plastic model. The highest yield stress value reported by Huang and Rha (1971) was only 73 dynes/cm^2 for a 25%w/w Torula yeast protein dope at pH 9. This was very much less than the yield stress values observed in the present work.

The apparent viscosities shown in Figures 5.5.16. and 5.5.18. were calculated by measuring the slope of the shear stress versus shear rate lines (which were approximately linear) (section 5.5.3.9.). Apparent viscosities did not exceed 10 poise. The viscosities recorded are low compared with those reported to give good fibre formation for vegetable protein dopes (25 - 300 poise) (Ashton et al, 1971; Burke, 1972). However, comparison with other results is difficult since the apparent viscosities reported were calculated either by measurement of a single value of shear rate at an arbitary (and generally rather low) shear rate, assuming Newtonian behaviour (e.g. Circle et al, 1964) or else they were calculated as shown in section 5.5.3.9. but at very low shear rates (of the order of 10 sec^{-1}) (e.g. Huang and Rha, 1971). In many cases, particularly in the patent literature, the methods of assessing apparent viscosity were not reported at all.

The method of calculating μ_{app} is obviously very important. For example, from the results shown in Figure 5.5.19. the apparent viscosities were calculated on the basis of single shear rate measurements with the assumption of Newtonian behaviour. The values were 340 and 3,200 poise at shear rates of 100 and

10 sec^{-1} respectively. Obviously the use of the Newtonian model is inappropriate.

Using the method described in section 5.5.3.9., again using data from Figure 5.5.19., the apparent viscosity at a shear rate of 10 sec^{-1} was 33 poise. This is, in fact, very similar to the highest viscosity reported by Huang and Rha (1971) which was 30 poise (for a 25%w/w protein dope at pH 9.0 prepared from dried Torula yeast).

With the exception of the present work no data are available either for vegetable or SCP protein dopes in which the rheological properties of the dopes were measured at shear rates similar to those occurring during spinning. Clearly μ_{app} is very dependant upon shear rate.

As already stated in sections 5.5.4.6. and 5.5.4.7. the fibre strength (section 4.2.7.) was found to be dependant on the apparent viscosity of the dopes rather than on their yield stress. The strongest fibres produced, in the wet state, would support their own weight in air up to a height of 250cm. It was not possible to compare fibre strength with other workers since those involved in spinning fibres from microbial protein used only qualitative terms to describe fibre strength (e.g. poor, fair, good, excellent) (Heden et al, 1971; Huang & Rha, 1972).

5.5.4.9. Use of the spinnerette.

The available spinnerette had 1,000 holes each being 75 microns in diameter. This made it too large for direct use with the

spinning apparatus if reasonable fibre velocities (greater than 5cm/sec) were to be achieved. As mentioned in section 5.5.3.6. teflon discs were used to cut off all but a few holes. Thus fibre velocities up to 8cm/sec were attained using a disc with a 0.15cm diameter hole. This compares with the 0.0202cm diameter capillaries which were most frequently used, which gave velocities of 7 - 10cm/sec.

Using 20%w/w dopes at pH 10.0 fibres appeared to be considerably weaker than those achieved using capillaries. For example, with a spinning velocity of 7cm/sec 75cm of the fibre tow could be removed from the bath under its own weight compared with 200cm of fibre using a 0.0202cm diameter, 0.991cm long capillary at the same velocity. However a considerable amount of water was observed to be retained in the tow between the fibres, thus increasing the load on the fibres. For this reason the comparison was not valid. Heden et al (1971) and Huang and Rha (1972) and Daly and Ruiz (1974) all stated that capillaries of various sizes gave better fibres, using microbial protein dopes, than spinnerettes, although no quantitative data were reported.

The problem of uneven flow of dope through the spinnerette holes was discussed in section 5.5.3.6. This made tows difficult to handle since all the fibres were not moving at the same speed.

The importance of end effects in determining pressure drop through capillaries was demonstrated in section 5.5.4.2., an aspect ratio of 50 being the minimum required to generate useful rheological data. The aspect ratio of the spinnerette was only

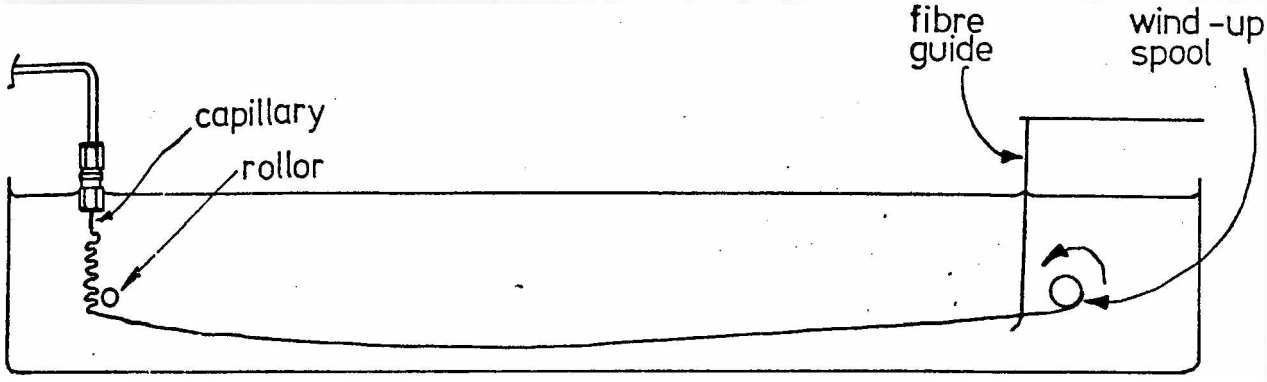
about 2, making it impossible to gain meaningful flow data.

As expected the pressure drop across the spinnerette was very low compared with that across capillaries. At the highest fibre velocity, 8cm/sec, a pressure drop of only 30psi was recorded.

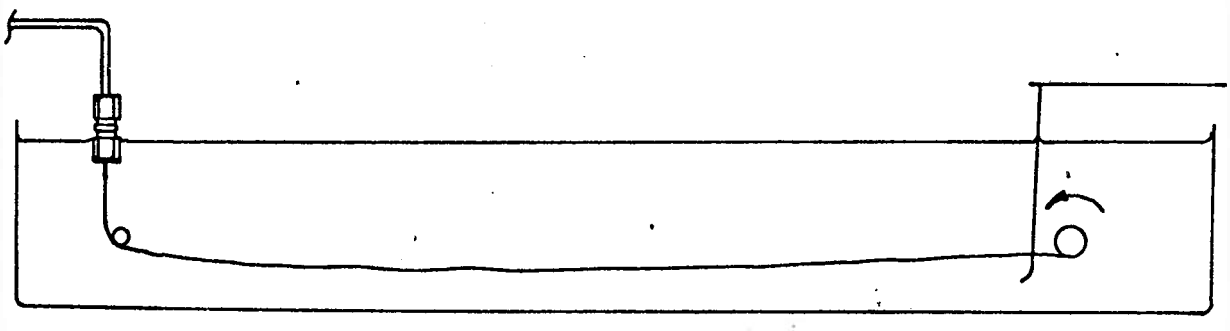
Although it has already been stated that shear rate had little effect on fibre strength (section 5.5.4.4.) it may be that some minimum residence time at a particular shear rate is necessary to allow a degree of molecular rearrangement sufficient to give good fibre formation. This was hinted at in the 'mesophase' spinning patent (Tombs, 1972) where spinnerettes with 0.01cm diameter holes, 1cm long were used, although it was said that the shear rate alone had no effect on fibre formation. Interestingly enough Tombs did not draw the fibres from the spinnerette to any extent as is customary in other processes, but caught the tow on a conveyor belt in the bath and allowed it to coagulate before stretching it at a later stage. It may be, therefore, that drawing of the fibres from the spinnerette serves the same purpose as using a longer hole size; i.e. to allow some degree of orientation before coagulation. This warrants further investigation.

5.5.4.10. Fibre winding and drawing.

When estimates of fibre strength were made, fibres were simply allowed to fall into the bottom of the bath and were left to coagulate for two minutes before their strength was assessed (section 4.2.7.). However, it was possible to wind up fibres onto the two cm diameter spool (Figures 5.5.7. & 5.5.20) and to draw out the fibres at a more rapid rate than they were being



(a) minimum wind-up speed



(b) just before break

Fig. 5.5.20. Fibre Winding.

produced, thus stretching them to some extent in the coagulation bath.

Fibres were transported to the roller from the capillary outlet using a spatula, passed through the fibre traversing guide and attached to the spool. Use of the fibre guide prevented a build up of fibres at one point on the spool. The rotation speed of the spool was increased to take up slack and then adjusted so that it was just sufficient to prevent fibres building up in a pile on the bottom of the bath. The speed of the spool was then increased gradually until the fibre broke. By measuring the speed of rotation (see section 5.5.3.8.) at break and that which was just sufficient to maintain steady fibre wind up, it was possible to calculate the degree of stretch imparted. Tests were repeated several times and the mean values were recorded. 20%w/w dopes were used at pH 10.0 with a 0.0202 cm diameter, 1cm long capillary.

It was found that, at minimum wind up speed, the fibre emerged from the capillary in a coiled configuration which remained stable as shown in Figure 5.5.20. a). It was not until just before the maximum wind up speed that the fibres travelled in a straight path (Figure 5.5.20. b)).

It was found that the fibres swelled on leaving the capillary so that the minimum uptake velocity was less than the velocity of the fibres through the capillary. Assuming that there was no swell due to absorption of water from the bath, the fibre diameter with no stretching could be calculated as overleaf.

$$df = dc\sqrt{\frac{Vc}{Vf}}$$

Where:- df,c = diameter of the fibre & capillary respectively.

Vf,c = velocity of the fibre at minimum wind up speed and of the dope in the capillary respectively.

The actual diameter was measured using a travelling microscope with a vernier scale. This was repeated several times and mean values were recorded. The results are shown in Table 5.5.2.

Table 5.5.2. Fibre Drawing.

shear rate (sec^{-1})	velocity (cm/sec)			measured fibre ø (cm)	calculated fibre ø (cm)	capillary ø, d_c (cm)
	in the capillary	during wind up				
		minimum	maximum	-no stretch	-no stretch	
3300	8·3	4·9	9·0	0·0270	0·0272	0·0202
5200	13·2	7·5	15·0	0·0265	0·0266	
8000	20·1	12·0	16·5	0·0255	0·0260	

The measured values for fibre diameter without stretch were very similar to the calculated values. Hence we can conclude that swell is not due to absorption of liquid from the coagulation bath. The swell is probably due to stress relaxation, a characteristic of viscoelastic bahaviour.

Generally speaking the fibres could be drawn sufficiently for them to regain their original capillary velocity but no further stretching could then be imparted. Greater stretch could be achieved at lower shear rates, probably as a result of the

greater fibre swell at lower shear rates. However, the reason for this is not clear.

It was not possible to assess the fibre strength after drawing since the fibres stuck to each other on the spool and could not easily be individually removed. The sticking was probably because the fibres were not completely coagulated when they reached the wind up spool.

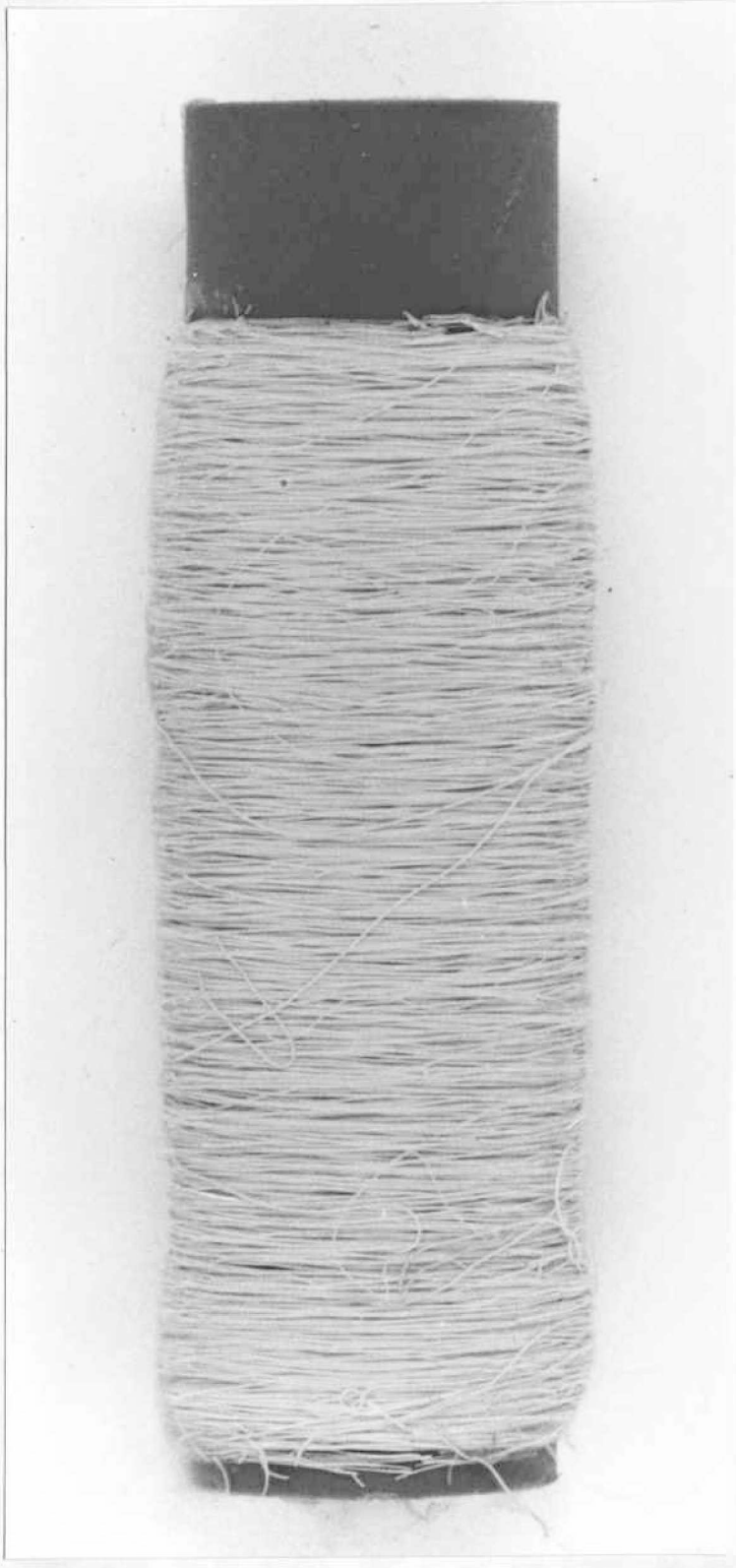
A spool of spun fibres is shown in Figure 5.5.21. The loose ends are due to breaking of fibres during testing of the maximum wind up rates.

It was also possible to wind up fibres spun from the spinnerette under conditions described in section 5.5.4.9.

5.5.4.11. Fibre composition.

The composition of the fibres produced, from the point of view of percentage protein in the dried fibres and protein to nucleic acid ratios, was not significantly different from the composition of the original protein isolates. In particular no loss of nucleic acid occurred during spinning. For example, fibres spun through the spinnerette (dope conditions 20%w/w DM, pH 10.0) into a 1%w/v acetic acid and 3%w/v sodium chloride solution were wound up and removed from the bath after 15 minutes. They contained 73%w/w protein, on a dry basis, with a P/NA ratio of 5.4. The original isolate contained 72%w/w protein and had a P/NA ratio of 5.2.

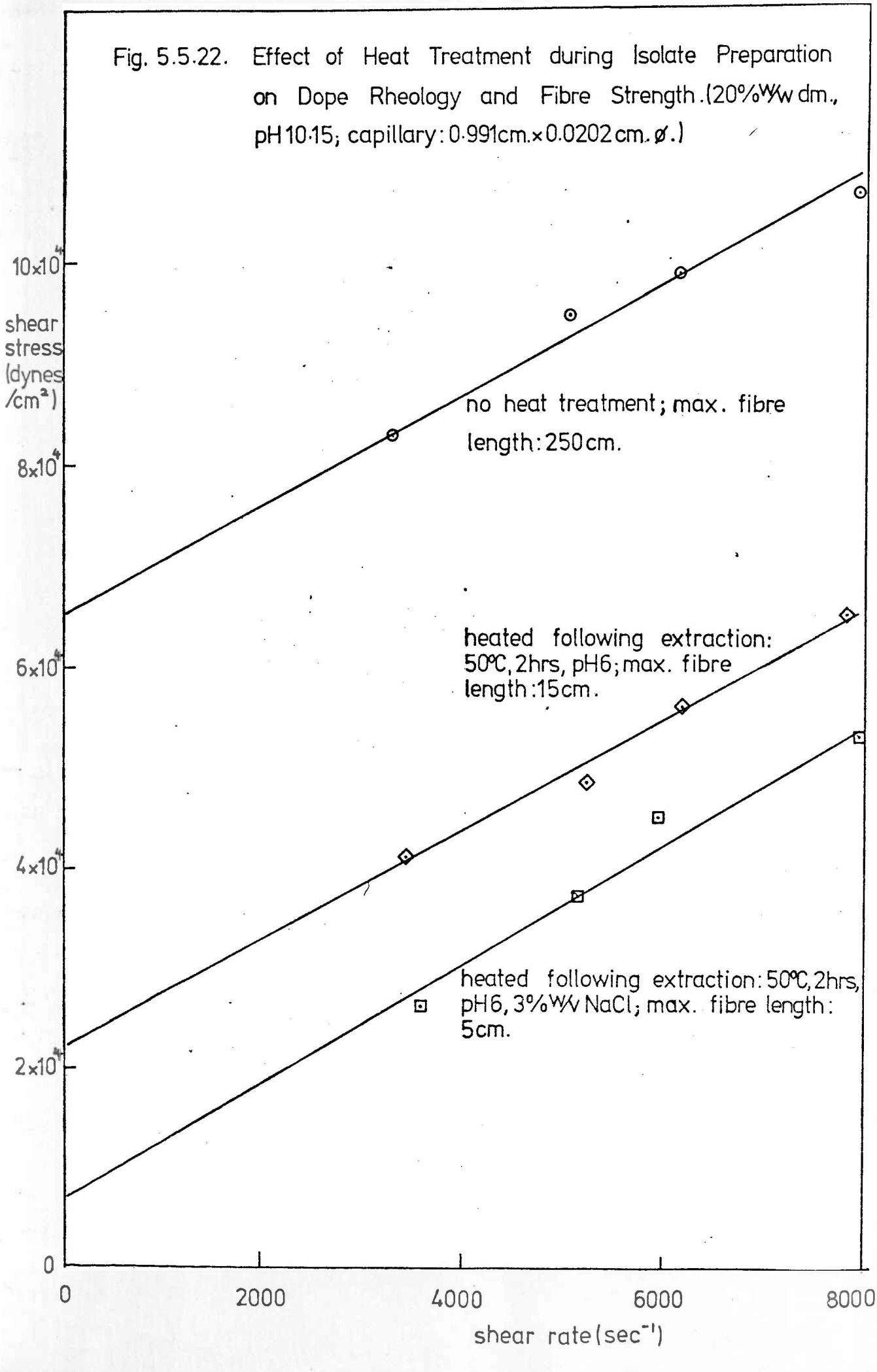
Figure 5.5.21. Yeast Protein Fibres.



5.5.4.12. The effect of heat treatment during isolate preparation. Up to this point all the spinning had been performed using isolates prepared by the standard method of extraction at pH 11 and precipitation at pH 3.8. As already discussed in section 5.4.4. successful nucleic acid removal could be accomplished by extraction at pH 11, followed by incubation at pH 6.0 and 50°C to allow the endogenous nucleases to hydrolyse the nucleic acid and finally precipitation at pH 3.8 to separate the nucleic acid fragments which were then too small to be precipitated. Two isolates were prepared in this way, one which had undergone heat treatment as described and the other in which 3%w/v NaCl had been added prior to incubation. There was no loss in protein yield following heat treatment. Dopes were prepared from these and from a standard isolate at 20%w/w and pH 10.15. These were spun in the usual way using a 0.0202 diameter, 1cm long capillary. The results are presented in Figure 5.5.22.

Heat treatment resulted in a dramatic fall in fibre strength. The dopes of the heated isolates were lighter in colour than the standard dope and much less viscous, particularly when NaCl had been used during heating. Having previously discovered that fibre strength was more a function of viscosity than yield stress it was interesting to note here that the apparent viscosities were very similar (5.0, 5.7 and 5.9 poise for the standard, heat treated and heat and salt treated dopes respectively) even though the fibre strengths dropped dramatically from 250cm without heating to only 5cm with heat and salt incubation during extraction. However apparent yield stress fell from 65,000 dynes/cm^2 using the standard isolate to 6,700 dynes/cm^2 with the heat /salt treated isolate. Hence there appeared to be a correlation between

Fig. 5.5.22. Effect of Heat Treatment during Isolate Preparation on Dope Rheology and Fibre Strength. (20%w/w dm., pH 10·15; capillary: 0·991cm.×0.0202cm.ø.)



Fibre strength and apparent yield stress, rather than viscosity in this case.

As already shown in Figure 5.4.20 (section 5.4.4.3.) it is clear that the isolates, following heat treatment, were only slightly soluble at the dope pH used (pH 10.15). A pH of 11.5 would have been required to achieve a solubility similar to that of the standard isolate at pH 10 (i.e. 85 - 90%) but this would probably have resulted in alkaline hydrolysis similar to that which occurred using the standard isolates at pH 11.65 (section 5.5.4.6.).

Clearly there is a considerable change in the isolates following heat treatment reflected both in their solubility and in the rheological characteristics of the dopes prepared from them. The dopes probably resemble an emulsion of fine particles in a solution containing a low concentration of the more soluble and lower molecular weight material of the isolates rather than the firm gels which are formed when alkali is added to the isolates prepared in the usual way.

It was concluded that such heat treated material was unsuitable for spinning, although it may find application where functional properties are of little importance.

5.5.4.13. Overall conclusions with regard to spinning.
As already stated it is difficult to compare the results with those of other workers, due to ill defined or incompatible rheological data and lack of information on the wet strength of fibres.

However there is good reason to believe that the fibres produced were superior to those previously spun using yeast protein (Rha, 1976). The fibres were still relatively difficult to handle and it is suggested that additives may be used in future to try to increase fibre strength. Such materials could include carageenan, carboxymethyl cellulose and wheat flour, all of which are known to improve fibre strength (Circle et al, 1964; Rha, 1975). As already mentioned it may also be beneficial to produce isolates using milder extraction conditions to give more soluble and less denatured protein. The use of other additives for reducing gelation may also prove beneficial.

The use of spinnerettes should be investigated further as industrial application of this technique would involve spinnerettes rather than capillaries. A short hole is preferable since this gives the smallest pressure drop and thus the lowest energy input.

The method of assessing fibre strength outlined in section 4.2.7. proved unsuitable for fibre tows from spinnerettes due to the weight of the water held between fibres (section 5.5.4.9.). This method could be modified by removing a tow of fibres from the bath, weighing it, laying it on a filter paper to remove excess water, and reweighing it. Allowance could then be made for the increased weight by estimating the length of tow equivalent in weight to the entrained water. By adding this length to the actual length of tow removed an indication of the true length of tow which could be removed without excess water could be gained. Fibre strengths thus measured would be comparable to strengths of fibres spun from capillaries.

The use of spinnerettes matched in size to dope pumping capacity would be advantageous.

Dopes containing 20%w/w DM at pH 10.0, made from isolates prepared by the standard method described in section 5.5.3. gave the strongest fibres. Fibre strength was almost entirely dependant on the conditions of isolate and dope preparation rather than on the physical spinning conditions or the coagulating bath composition.

5.5.5. Post Treatment of Fibres.

5.5.5.1. Introduction.

It was of obvious importance to see whether or not the fibres produced as described in section 5.5.4. had characteristics similar to meat. The tests described below are largely qualitative but they do provide an interesting conclusion to this work.

Unless otherwise stated fibres were spun through 0.0202cm diameter, 0.991cm long capillaries from dopes containing 20%w/w DM at pH 10.0 prepared from isolates extracted at pH 11.0 and precipitated at pH 3.8 without heat treatment.

5.5.5.2. Texture of wet fibres.

Following wind up onto the spool fibres were removed from the coagulation bath on the spool and washed twice in distilled water. They were then cut from the spool giving a flat sheet of fibres about 10cm square. This was rolled up so that the fibres formed a bundle 10cm long and about 2cm in diameter. Excess water was squeezed from the fibres and they were cut into chunks

about 1.5cm in length.

These chunks were found to have quite a bland flavour, being free of the bitter taste of yeast and of the characteristic taste of yeast extract. They were white / cream in colour and did not have any significant aroma. Such characteristics would be desirable in a product for food use since flavours and colours could be added to simulate any desired product.

However the chunks were not resistant to mastication, being easily broken up in the mouth. Furthermore they could be rubbed between the fingers and broken up into a paste. Clearly further treatment was required before the chunks had the required chewiness and resistance to shear forces.

Storage of fibres in 1%w/v acetic acid solution and in water for two days at 40°C did not improve the texture of the fibres which had turned light brown in colour. Boiling in 1%w/v acetic acid for one minute resulted in the break up of the fibres into small fragments. Boiling for one minute in distilled water resulted in an improvement in texture. The fibres became springy and were less easily broken up when rubbed in the fingers. However they were still lacking in chewiness.

Boiling in acetic acid caused a reduction in the protein content of the fibres from 70%w/w DM to 59%w/w DM. This was probably due to protein hydrolysis. There was no reduction in nucleic acid content. Other treatments did not significantly affect the fibre composition.

The improvement in fibres on boiling in water was probably a result of heat denaturation causing loss of bound water and hence fibre shrinkage. Similar effects are seen in an initial cooking of meat. The shrinkage is due to the dehydration as more protein - protein and protein - carbohydrate interaction occur and the number of sites available for hydrogen bonding of water are diminished (Bender, 1972; Meyer, 1960).

5.5.5.3. Drying.

Chunks were prepared as described above and dried overnight at 105°C and 50°C and by dessication at room temperature. Drying by dessication resulted in a final water content of about 3%w/w compared with fibres dried at 50°C and 105°C. Chunks dried at 105°C are shown in Figure 5.5.23. These were a red brown colour and crisp to handle. Although no binding agent had been added they did not break up into discrete fibres but retained their shape. Fibres dried at 50°C were similar except that they were fawn in colour and not quite so brittle as those dried at 105°C. Dessicated chunks were light fawn / grey in colour and less brittle than either of the samples dried at the higher temperature.

The drying process was also carried out on chunks which had been boiled in distilled water for one minute and then drained. All the dried samples were noticeably lighter in colour than those without boiling prior to drying. The samples dried by dessication were very light grey in colour, showing no sign of the brown colour previously seen.

All the samples were similar in composition to the original protein isolates, containing about 70%w/w protein with a P/NA

Figure 5.5.23. Dried Fibre Chunks (dried at 105°C).

ratio of about 5.0.

5.5.5.4. Rehydration.

Samples were rehydrated by boiling in distilled water for five minutes.

Fibres dried at 105°C were tough and did not rehydrate well. The chunks had retained their shape but were considerably smaller than they had been prior to drying. They were light brown in colour and proved difficult to chew, resembling liquorice root in texture. The fibres had an unpleasant, bitter flavour with a hint of yeast extract. Fibres dried at 105°C which had previously been boiled in water for one minute were similar in texture but the colour was lighter and the bitter flavour was somewhat less distinct.

Fibres dried at 50°C were well hydrated but still rather tough. They were light brown in colour. The texture was superior to that of the chunks dried at 105°C in the sense that the fibres were more juicy and chewy rather than stringy. There was still a hint of bitterness and of the yeast extract flavour but this was not present to any great extent in fibres which had been boiled prior to drying.

Fibres dried by dessication were well hydrated to form springy white / grey chunks. There was a slight bitterness in fibres which had not been boiled prior to drying but this was not present in fibres which had undergone boiling. Fibres were chewy with a bland flavour, rather dry, characteristic of chicken or more particularly turkey which they also resembled in colour

and appearance.

Boiling of the fibres prior to drying probably serves to remove traces of low molecular weight components such as nucleotides and other agents which develop the characteristic yeast extract flavour after prolonged heating. This would explain the improvement in colour and flavour following boiling before drying.

It is worth noting at this stage that fibres both prior to drying and after rehydration did not break up on boiling. Compared with vegetable protein isolate, for example soya protein isolates, the yeast protein isolates were considerably less soluble. Chemical hardening agents such as aluminium sulphate and sodium chloride are commonly added to the coagulating baths used for spun TVP manufature to help prevent the fibres from redissolving on cooking (Gutcho, 1973). Lack of solubility of the yeast protein was seen as a disadvantage in extraction, nucleic acid removal and dope formation. However it was an advantage following spinning since the fibres, even when spun into baths containing no sodium chloride, were stable on boiling. Furthermore drying reduced water holding capacity, giving stronger fibres. Hence although the wet fibres following spinning were weak and unsuitable for food use, texture was improved following drying and rehydration. It is foreseen therefore that for food use, the product could be conveniently marketed as dry chunks.

5.5.5.5. Nucleic acid removal following fibre formation.
Removal of nucleic acid from the fibres as described in section 5.4.5. did not affect the fibre characteristics. From the point

of view of texture and flavour the fibres, both before drying and after rehydration, were indistinguishable from those which had not undergone nucleic acid removal.

Hence the overall process, which produced fibres high in protein and low in nucleic acid with a bland flavour and with a texture and colour similar to that of chicken or turkey, involved the following steps:-

1. A 10% w/v (on a dry basis) suspension of fresh Bakers' yeast was disrupted using sonication.
2. Protein was extracted at pH 11.0 using caustic soda and the cell wall debris were removed by centrifugation.
3. The supernatant was acidified to pH 3.8 using hydrochloric acid and the precipitate was recovered by centrifugation and washing with distilled water. The precipitate, referred to as the protein isolate contained about 70%w/w protein and about 13%w/w nucleic acid on a dry solids basis. The supernatant following precipitation was retained for later use.
4. Dopes were prepared by hand mixing the isolate with water and 3 N NaOH solution to give a 20%w/w dry matter concentration at pH 10.
5. The dope was spun through a 0.02cm diameter capillary into a bath containing 1%w/v acetic acid and 5%w/v sodium chloride. Fibres were wound onto a spool, left for 15 minutes, to ensure complete protein coagulation and removed from the bath.
6. The fibre tow was washed in distilled water and put in a shaker flask with the supernatant remaining after acid precipitation at pH 3.8. The pH was adjusted to 6.0 and 3%w/v NaCl was added. Incubation with agitation was carried out for 30 minutes at 50°C.

7. The fibre tow was carefully drained and boiled in distilled water for one minute. The water was drained off and the tow cut into chunks and dried by dessication at room temperature.
8. The chunks were rehydrated by boiling in distilled water for 5 minutes. The resulting material contained 70 - 75%w/w protein and had a protein to nucleic acid ratio in excess of 30:1. Assuming 100% disruption about 70% of the original protein in the yeast was recovered in the fibres.

5.5.5.6. Conclusions.

Clearly drying is essential in producing a texture similar to meat. The increased denaturation in this step reduces the water absorption capacity of the fibres which become firmer and more resilient on rehydration.

Drying at low temperatures is preferred as this gives the best texture on rehydration as well as the best colour and flavour.

Boiling prior to drying serves to reduce the presence of undesirable flavours and is also useful as a pasteuration step to reduce microbial contamination.

The nucleic acid removal step following fibre spinning does not appear to have any adverse effect on the fibre quality and does not cause loss of protein.

The strongest fibres produced were, in the wet state, much weaker than fibres made with soya or other vegetable proteins. However, on drying and rehydration the fibre chunks were similar in character to those produced by TVP manufacturers.

6. DISCUSSION.

6.1. Introduction.

Individual results have already been discussed to a large extent in section 5. In this section the work is discussed as a whole in the light of the objectives set in section 3. Briefly, these were to develop an integrated process for the production of low nucleic acid, high protein (both in terms of content and yield), cell wall free fibres suitable for food use. This was to be done using simple techniques and inexpensive chemicals. It was considered important to keep protein denaturation to a minimum prior to texturing. Finally it was hoped that a deeper understanding of the processes involved would be gained.

6.2. Process Assessment.

A process which fulfills many of the objectives was developed and is outlined in detail in section 5.5.5.5.

Considerable efforts were made to remove nucleic acid prior to texturing but these failed largely because successful nucleic acid removal involved heating to temperatures which caused loss of protein solubility and hence either loss of protein yield or fibre forming ability.

It is considered that all the techniques were simple and the only chemicals required were sodium hydroxide, hydrochloric and acetic acids and sodium chloride.

It is interesting to compare the process with the well developed

techniques used for spinning vegetable and in particular soya protein. In price fresh SCP material can now compete with soya beans. (section 2.1.7.). Protein isolation and texturisation techniques are similar and would therefore incur similar costs. For soya two other major processes remain - dehulling and oil extraction. For yeast the remaining processes are cell disruption and nucleic acid removal. It is not possible, at present, to compare the economics of these remaining processes but it would appear that the overall costs of textured soya and textured SCP would be similar.

The yeast protein fibres have been produced from a 'wet' protein isolate and from 'wet' fresh yeast. Drying has been found to result in substantially reduced solubility (Labuza, 1975; Labuza & Jones, 1973). If this system were to be used at a commercial level it would be an advantage if the yeast fermentation, protein isolation and fibre spinning were all carried out on the same site, to minimise problems in transporting and storing the wet materials. This is not the case with TVP manufacture where protein isolates are generally dried and transported elsewhere for texturing.

It is worth noting here that there are two main bi-products of the process - the cell wall debris and the supernatant liquor following nucleic acid removal. It is envisaged that the cell debris could be used for animal feed or as a fat substitute in salad cream, ice cream or dietary products (Sucher et al, 1975). The supernatant fluid contains low molecular weight nucleic acid and protein fragments and could be dehydrated to give yeast

extract type products as described by Robbins et al (1975 (1)) (see section 2.5.).

6.3. Process Development.

No work has as yet been undertaken on the nutritional properties of the fibres. For such work and for more thorough testing of the fibres' texture characteristics the scale of operation will need to be considerably increased. It has already been pointed out that increased scale of operation (currently limited by the rate of cell disruption using sonication) would make dope mixing easier and would allow the use of more sensitive pressure measuring equipment for the generation of rheological data (section 5.5.3.4.).

For commercial operation spinnerettes would obviously be preferred over single capillary tubes.

The fibre strength measuring technique needs to be revised (section 5.5.4.13) to allow comparison of single fibres produced from capillaries with tows from spinnerettes.

It is interesting to note that the nucleic acid removal from the fibres was greatly influenced by the diameter of the fibres and presumably, therefore, by diffusion of material into and/or out of the fibres (section 5.4.5.2.). Diffusion is also reported to be the rate limiting step in fibre coagulation on spinning (Booth, 1967; Paul, 1968; Watson & Hadley, 1973). Here again the importance of using small hole spinnerettes is seen. Some

information is already available on the rates of diffusion during coagulation of Torula yeast protein dopes (Balmaceda & Rha, 1973). It would be of great interest to study the fibre incubation - nucleic acid removal process from the point of view of diffusion of the enzymes and the hydrolysis products into and out of the fibres.

The so called functional properties of the isolates produced prior to spinning (in particular solubility) were found to be less desirable than those of soya isolates. Protein was less soluble, more easily denatured by heating and extremes of pH and formed fibres which were far less easy to handle than those from soya (Leslie, 1975). This is confirmed by the results of other workers who have spun yeast protein dopes and obtained only weak fibres (Daly & Ruiz, 1974; Huang & Rha, 1972). Furthermore dried Torula yeast gave poor results when used in breadmaking (Labuza, 1975). Much effort is required in this area to produce yeast protein which is more soluble and less susceptible to gelation than isolates produced to date.

Finally it would be of interest to investigate other texturisation methods and in particular extrusion, which are reported to be effective with lower quality protein material (Ashton et al ,1971).

6.4. Protein Denaturation and Aggregation.

Part of the aim of this work was to gain a deeper understanding of the fundamental nature of the processes involved in protein extraction and texturisation. The processes of protein denaturation were found to be important in many areas of the work. Protein

aggregation was seen to occur as a result of heating and pH changes (section 5.3.2.6.). The observations on protein aggregation were in agreement with those of Lindblom (1974).

The secondary and tertiary structures of native proteins are stablised by hydrogen and other weak bonds. On heating at extremes of pH or at high salt concentrations etc. these bonds are broken. The protein molecules unfold and their reactive groups are exposed. Interaction with other protein molecules or with carbohydrates or nucleic acids occurs and large complexes or aggregates form. The degree of stability of the aggregates is dependant upon the nature of the bonds formed (Bate-Smith & Morris, 1952; Meyer, 1960; Taylor, 1964). Often disulphide bonds are formed in protein systems. These are strong bonds and lead to a firm structure (Fox & Foster, 1957). However these are not so likely to be of great importance in microbial protein systems as the content of sulphur containing amino acids is small (section 2.2.2.). Denaturation always results in loss of solubility since fewer sites are available for hydrogen bonding of water. The changes caused by denaturation are, in general, irreversible (Bate-Smith & Morris, 1952; Taylor, 1953).

Loss of nutritional value may result from the formation of bonds which are resistant to hydrolysis in the stomach or due to damage to individual amino acids (Bender, 1972; Meyer, 1960)

Moore and Carter (1974) studied protein / carbohydrate interactions occurring as a result of heating soya bean protein and starch hydrolysates. Using gel filtration, chemical modification of particular groups and selective precipitation techniques, they

found that non-covalently bonded aggregates between protein and carbohydrate were formed. The present work could be usefully extended in a similar manner to look at interactions of yeast protein with carbohydrate and more particularly with nucleic acids. Gel filtration studies have already shown that the molecular weight spectra of nucleic acids and protein are surprisingly similar following alkali extraction (compare Figure 5.3.11. with Figure 5.3.12. and Figure 5.3.13. with 5.3.16.). Furthermore protein and nucleic acid seem to have similar extraction and precipitation properties. It seems likely that protein / nucleic acid interactions are present. These may be formed as a result of extraction at high pH since it has been noted (Lindblom, 1974) that prior to alkali treatment the molecular weight spectra for protein and nucleic acid are significantly different. If the precise nature of these interactions could be determined and hence prevented, it may become feasible to remove nucleic acid with some simple, physical separation procedure. For example, it is suggested that ion exchange methods used before alkali extraction may be effective.

From the point of view of improving the functional properties of the protein isolates, it may be advantageous to sacrifice some protein yield by using less severe conditions of extraction and precipitation, thus reducing the degree of denaturation. The highest quality isolates from soya are prepared by extraction at pH 7 and precipitation at pH 5, which results in very little protein denaturation (Ashton et al, 1971; Tombs, 1972).

The denaturation of protein is also of importance in fibre spinning. Dopes gelled easily, this being a disadvantage from

the point of view of dope handling. Gelation appeared to add nothing to the fibre quality (section 5.5.4.8.). Even the strongest fibres produced were weak and subject to damage on handling. Strong fibres are reported to result from molecular alignment and the formation of strong bonds between adjacent molecules (Lundgren, 1949; Moncrieff, 1975). Interaction causing yeast protein gels was probably due to weak non-covalent bonds of a similar nature to those causing aggregation during protein extraction. It is suggested that the formation of a rigid gel lattice served to prevent molecular alignment and hence reduced fibre strength. Chemicals capable of preventing gelation may be useful in improving fibre strength, although there is some restriction on choice of methods as these must not inhibit nuclease enzyme action.

It is recommended that gel filtration studies are continued using chemical modification or disruption of particular groups (e.g. with urea or SDS) and selective precipitation to find the nature of the protein / carbohydrate / nucleic acid interactions which occur as a result of different pH's, temperatures, salt concentrations and protein concentrations. The results of such experiments would be of value in explaining reactions at all stages of the process and perhaps in suggesting ways of improving the process.

6.5. Accuracy and Reproducibility of the Results.

In general trends seen in all the experiments could be reproduced well in subsequent experiments, although the absolute values of various parameters showed some variation. An indication of the

degree of variation for several series of experiments is shown as follows:-
Protein extraction and precipitation - Figures 5.3.1. and 5.3.2.
Nucleic acid removal - Figures 5.4.3., 5.4.13., 5.4.16. and 5.4.23.
Solubility - Figure 5.3.4.

Molecular weight spectra were very reproducible.

Rheological data were more difficult to reproduce (Figure 5.5.12.) even after the dope mixing techniques had been improved (c/f Figures 5.5.8. and 5.5.9. with 5.5.12.). Spinning experiments were performed at room temperature which varied considerably. Closer control of dope temperature during spinning is recommended. The limitations of the pressure measuring device have been mentioned (section 5.5.3.4.) and it has been suggested that increasing the capacity of the spinning apparatus should facilitate more accurate measurement of pressure.

The limitations of the various analytical methods have been discussed in section 4.2. These were found to be satisfactory with the exception of fibre strength measurement.

6.6. Overall Assessment.

The present work outlines a method for the production of spun yeast protein fibres, low in nucleic acid and suitable for food use. Details of such a process are not currently available elsewhere in the literature. Robbins et al (1975 (1)) described a process in which a yeast protein isolate, low in nucleic acid and with fibre forming properties is formed. However no details

on the fibre forming process were given in their report.

The removal of nucleic acid from fibres by an endogenous enzyme process as described in the present work, is novel.

Improvements to existing nucleic acid removal processes were developed, particularly with reference to the use of sodium chloride during the incubation periods of the methods described by Maul et al (1970) and Robbins et al (1975 (1)).

Finally it is felt that the understanding of the processes involved in yeast protein extraction and texturisation has been enhanced by this work.

7. CONCLUSIONS AND RECOMMENDATIONS.

7.1. Conclusions.

The major conclusions of the work are outlined below.

7.1.1. Disruption.

1. Fresh Bakers' yeast suspensions could be disrupted by sonication using a Dawes 7530-1A soniprobe with a ½" titanium tip. The addition of glass beads to the cell suspension aided disruption.

2. For continuous sonication a Rosett cell gave a higher protein release than a Hughes cell at the same yeast concentration and flowrate. The preferred operating conditions for the Rosett cell were:- yeast concentration 10%w/v DM; yeast suspension flowrate 10ml/min; glass beads 10ml of 0.7mm ø beads.

3. Nuclease enzymes were inactivated by sonication, prolonged batch sonication led to a considerable loss of enzyme activity.

4. The slow rate of disruption using sonication imposed a restriction on the size of the spinning apparatus which could be used.

7.1.2. Protein Extraction and Precipitation.

7.1.2.1. Precipitation with ammonium sulphate and acetone.

Following alkaline extraction of disrupted yeast suspensions and fractional precipitation using acetone or $(NH_4)_2SO_4$ nigh

purity protein isolates were obtained (greater than 98%w/w protein) but with unacceptably low protein yields.

7.1.2.2. Precipitation with HCl.

1. Extraction at pH 11 and precipitation at pH 3.8 led to an isolate containing 70%w/w protein and a protein yield of 70% (based on 100% disruption). Considerable protein aggregation occurred as a result of non-covalent bonding during alkaline extraction. This led to increased protein yield on precipitation but reduced solubility.

2. The protein isolate contained an unacceptable amount of nucleic acid (about 13%w/w).

7.1.3. Nucleic Acid Removal.

7.1.3.1. Chemical extraction.

1. Precipitation of nucleic acid with manganous chloride led to isolates low in nucleic acid but with poor yields of protein.

2. Precipitation of protein with acetic acid rather than HCl did not give an increased protein to nucleic acid ratio (P/NA) in the product.

3. Precipitation of protein with perchloric acid at pH 3.8 and ambient temperature led to a P/NA ratio of 20 in the isolate with no loss of protein yield.

7.1.3.2. Alkali hydrolysis.

Incubation of alkaline extracted yeast at 37°C for 24 hours led

to considerable nucleic acid hydrolysis as shown by gel filtration studies. However this was insufficient to prevent nucleic acid being precipitated in the isolate at pH 3.8.

7.1.3.3. Heat shock and incubation.

1. When whole yeast suspensions were subjected to heat shock at 70°C followed by incubation at 55°C a large proportion of the nucleic acid was released from the cells in the form of low molecular weight fragments. This was due to the action of endogenous nucleases following inactivation of a macromolecular inhibitor.

2. Nucleic acid removal increased as yeast concentration decreased.

3. Nucleic acid removal was substantially increased when NaCl was added to the yeast suspensions.

4. When heat shock and incubation were followed by disintegration alkaline extraction and acid precipitation the protein yields were much lower than those obtained without heat treatment.

7.1.3.4. Incubation of disrupted yeast suspensions.

1. Incubation of disrupted yeast suspensions at 50°C prior to alkaline extraction and acid precipitation did not lead to reduced nucleic acid content in the isolate.

2. The addition of NaCl to the yeast suspensions gave isolates with a P/NA ratio of 14, but with protein yields considerably less than those obtained without incubation.

3. The use of applied enzymes did not result in significant reductions of nucleic acid in the isolates.

4. The nuclease inhibitor was still effective following cell disruption, although its action was somewhat reduced by the addition of NaCl to the yeast suspensions.

7.1.3.5. Incubation following alkaline extraction.

1. Incubation of yeast suspensions at pH 6 and 50°C, following alkaline extraction and prior to acid precipitation, led to isolates with considerably reduced nucleic acid contents without loss of protein yield.

2. The addition of NaCl to the yeast suspensions led to substantial increases in the rates of nucleic acid removal.

3. Isolates obtained by this process were much less soluble than those obtained without incubation, particularly if NaCl were added to the suspensions.

4. Fibres obtained on spinning the low nucleic acid isolates were much weaker than those from isolates prepared without incubation.

7.1.4. Fibre Spinning.

1. Isolates prepared by alkaline extraction and acid precipitation were mixed with NaOH to give dopes which, when filtered, could be spun through capillaries or spinnerettes into acid / salt coagulating baths to yield white fibres. The critical parameters

were dope concentration and pH. The strongest fibres were formed from pH 10 and 20%w/w DM dopes.

2. Fibres swelled on leaving the capillary and could be drawn, by increasing the winder speed, to the point where the winding velocities were equal to the mean velocity of the dope in the capillary.

3. Even the strongest fibres produced were rather weak, lacking resistance to mastication.

4. Dopes conformed , to a first approximation, to the 'Bingham Plastic Model'. Rheological data were obtained over a wider range of shear rates than had been reported previously.

5. The protein and nucleic acid contents of the isolates and the fibres were not significantly different.

7.1.5. Nucleic Acid Removal from Fibres.

1. Nucleic acid removal could be achieved by incubating the fibres at 50°C and pH 6 with the supernatant fluid remaining after protein precipitation.

2. Protein to nucleic acid ratio increased linearly with increasing NaCl concentration in the range 0 - 3%w/v NaCl.

3. Greater nucleic acid removal rates could be achieved by spinning fibres through narrower orifices. It is suggested that this was due to increased resistance to diffusion in the

thicker fibres.

4. P/NA ratio increased when the ratio of supernatant fluid to fibre protein was increased, the active nucleases being contained almost entirely in the supernatant rather than the fibres.

5. Very high nucleic acid removal rates could be obtained by incubating the fibres with exogenous nucleases.

6. There was no loss of protein from the fibres in any of the above processes.

7. No deterioration in fibre strength was observed following nucleic acid removal by the above method.

7.1.6. Post Treatment of Fibres.

An improvement in fibre texture could be obtained by washing the fibres in water, drying at ambient temperature and rehydration in boiling water. The resultant fibres were resistant to mastication, white in colour and had a bland flavour.

7.2. Recommendations for Further Work.

1. Increased scale of work would make dope mixing easier and allow the use of more sensitive pressure measuring equipment for the generation of rheological data. Furthermore, this should facilitate the assessment of fibre texture and nutritional characteristics.

2. For commercial operation spinnerettes would obviously be preferred over single capillary tubes and their use merits further investigation. Fibre strength measuring techniques which allow comparison of fibres from spinnerettes and capillaries need to be developed.

3. It would be of great interest to study the fibre incubation / nucleic acid removal process from the point of view of assessing the importance of diffusion of enzymes and hydrolysis products into and out of the fibres. The role of NaCl in the diffusion process may be of importance.

4. Efforts should be made to improve the functional properties of the yeast protein isolates. It may prove necessary to use less severe extraction and precipitation conditions to reduce the degree of protein denaturation. This may result in the loss of protein yield but the production of higher quality isolates. Chemical modification of the protein may also prove worthwhile.

5. It would be interesting to study other texturising methods and, in particular, extrusion, which are reported to be effective with lower quality protein material.

6. Finally it is recommended that gel filtration (or gel electrophoresis) studies be continued using chemical modification or disruption of particular groups and selective precipitation to find the nature of the protein / carbohydrate / nucleic acid interactions. The effects on these interactions of different pH's, temperatures, salt concentrations, process times and protein

concentrations are of particular interest. The results of such experiments would be of value in explaining reactions at all stages of processing and may suggest ways of improving the system.

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