The Proteolytic System of *Methylophilus Methylotrophus*

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Paul Watt BSc MSc

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Biological Science Department

University of Stirling

ABSTRACT

This thesis describes studies on the proteolytic system of Methylophilus methylotrophus. Proteinases have been partially purified and characterized from its cytoplasmic, periplasmic and membrane fractions. These enzymes are serine- and metalloproteinases maximally active in the pH range 7.3-8.2 at 37°C. They hydrolyze a broad range of substrates and several are dependent on metal-ions for maximum activity and/or stability.

The levels of proteolytic activity towards both endogenous and exogenous protein substrates are much lower in extracts from cells grown exponentially than in those from the stationary phase of growth.

Storage of cell extracts results in the breakdown of most intracellular proteins and an increased ability to degrade exogenous protein substrates. PMSF and EDTA reduce proteolysis in both cases.

A non-dialyzable heat-stable factor isolated from soluble extracts partly inactivates trypsin and chymotrypsin in addition to certain endogenous proteolytic enzymes. The inhibitory factor(s) responsible for proteinase inactivation have not been purified.

A sudden increase in temperature during exponential growth results in (1) a decrease in the synthesis of the majority of cellular proteins and (2) the induction of a number of unique proteins called 'heat-shock proteins' (hsps). These hsps have apparent molecular weights of 83, 78, 63, 60, 27, 20, 16 and 14 kD. A similar although not identical response is observed when cells are treated with methanol or ethanol. Hsps are induced that are unique to the type of 'stress-shock' treatment. Simultaneous exposure to

heat-shock and either methanol or ethanol results in the synthesis of both sets of hsps. Hsps are found in all three subcellular fractions.

Stress-shock of growing cells also results in the specific induction and repression of proteolytic enzymes with a net increase in cellular proteolysis. A comparable response is observed in Escherichia coli cells.

Overall this thesis provides information on the basic features of the proteolytic system of the obligate methylotroph, Methylophilus methylotrophus.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

Abbreviations

AAAPA	acetyl-alanyl-alanyl-prolyl-alanyl-nitroanilide
APNE	N-acetyl-D-L-phenylalanine-napthyl ester
Azc	azocasein
BTI	bovine trypsin inhibitor
Butyl-PBD	2-(4'-t-butyl phenyl)-5-(4''-biphenylyl)-1,3,4-oxadiazole
BzArgNA	Benzoyl-arginyl-nitroanilide
C	proteolytic enzymes from the cytoplasmic fraction
D	dalton
DCPIP	2,6-dichloroindophenol
DFP	diisopropylfluorophosphate
DMAM	Difco methionine assay medium
DMF	dimethylformamide
DOC	sodium deoxycholate
DTT	dithiothreitol
HPA	hide powder azure
hsp	heat shock protein
IAA	iodoacetic acid
Ie	inhibitor extract
IFN	interferon
LeuNa	leucyl-nitroanilide
MDH	methanol dehydrogenase
NG	N-methyl-N-nitro-N-nitrosoguanidine
P	proteolytic enzymes from the cytoplasmic fraction
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PMS	phenazine methosulphate

PMSF	phenylmethane sulphonyl fluoride
SAAPL	succinyl-alanyl-alanyl-prolyl-leucyl-nitroanilide
SAAPP	succinyl-alanyl-alanyl-prolyl-phenylalanyl-nitroanilide
Sp. Ac.	specific activity
ssp	stress-shock protein
STI	soybean trypsin inhibitor
TLCK	N-∝-tosyl-L-lysine chloromethyl ketone
TMPD	N,N,N',N'-tetra methyl-p-phenylenediamine
TPCK	L-tosyl-amido-2-phenylalanine chloromethyl ketone
W	proteolytic enzymes from the whole cell extract

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' The struggle itself towards the heights is enough to fill a man's heart. One must imagine Sisyphus happy '

Albert Camus

CHAPTER ONE

1:1 INTRODUCTION

Proteolytic degradation is a ubiquitous function of all living cells so far investigated. However compared to other aspects of cellular metabolism our present understanding of intracellular proteolysis and its regulation, particularly in bacterial cells, is limited. This thesis describes attempts to address this problem by an investigation of the proteolytic system of the gram-negative bacterium Methylophilus methylotrophus.

To introduce this topic Chapter 1 has been divided into three sections. The first section describes the nomenclature, taxonomy and the biochemistry and physiology of methylotrophic bacteria, as well as the application of these bacteria in biotechnological processes. The second section is a review of intracellular proteolysis in bacteria, the proteolytic enzymes already isolated and characterized and the functional roles attributed to some of these enzymes. The third section of Chapter 1 describes the objectives of this study.

PART 1

1:2a Methylotrophic Organisms

The existence of Bacillus methanicus, a bacteria capable of growth on methane and methanol has been known since 1906 (Sohngen, 1909), however the ubiquity, taxonomic diversity and physiology of these microorganisms was not described to any extent until 1970 (Haber et al., 1983).

Organisms capable of utilizing one or more reduced carbon atoms (but not carbon-carbon bonds) as their sole source of energy are

known as methylotrophs (Anthony, 1982; Large, 1983). Those organisms capable of utilizing only a few C_1 compounds are known as obligate methylotrophs while organisms capable of growing on reduced C_1 compounds and multicarbon compounds are called facultative methylotrophs. The term methylotroph may itself be misleading since some of the C_1 compounds supporting methylotrophic growth do not contain any methyl groups (e.g. CO and CO_2).

The methylotrophs have been classified as a distinct group as they occupy a border line position between autotrophic organisms, i.e. those that use CO_2 as their carbon source but require an additional energy source, and heterotrophic organisms, i.e. those that use organic compounds as a simultaneous source of carbon and energy. Methylotrophs are heterotrophic as far as energy metabolism is concerned but there are distinct biochemical differences; in methylotrophs the energy-yielding pathway of oxidation of substrate to CO_2 does not involve the tricarboxylic acid cycle and the assimilation of substrates to cellular materials is generally cyclic.

The subclassification of the methylotrophs has proved an almost impossible task due to the large differences in growth substrates, C_1 assimilatory pathways and intracellular structure between members of the same species. Anthony (1982) has simplified this problem by subdividing the methylotrophs according to their growth properties and structural features alone. These groups will not necessarily be taxonomic when genetic comparisons are made.

The methylotrophs have been subdivided into microorganisms capable of growing on methane, these are the methanotrophic bacteria, and those unable to grow on methane, these are the

methylotrophic bacteria and yeasts. The methylotrophic yeasts will not be discussed.

1:2b Methanotrophic bacteria

Most methane-utilizing bacteria are obligate methanotrophs in that they only grow on methane or methanol, but facultative methanotrophs have also been identified that grow poorly on methane but will utilize multicarbon compounds.

On entering the stationary phase of growth methanotrophic bacteria are capable of forming resting stages i.e. exospores and cysts. This ability to form resting stages is believed to be a consequence of the restrictions imposed in the environment due to growth on methane as the only carbon substrate. All the methanotrophs have complex internal membrane structures with a surface area of up to 8 times that of the cytoplasmic membrane. Methanotrophs have been further subdivided according to their particular type of membrane structure. Type I bacteria have bundles of disc-shaped vesicles formed by invagination of the cytoplasmic membrane while type II bacteria have a system of paired peripheral membranes (Anthony, 1982). The special oxidative properties in methanotrophs appear to be a consequence of the type of membrane structure and are comparable to those found in different bacteria with a specific electron transport requirement e.g. photosynthetic bacteria, ammonia and nitrite oxidizers and cyanobacteria. The absence of such membranes in methylotrophs that are unable to utilize methane also suggests a special function for these membranes. Methanotrophs are generally motile due to a single polar flagellum.

The majority of methanotrophs are characteristically pink or red on solid media. Type II methanotrophs form rosettes of bacteria held together and anchored by polysaccharide material at their non-flagellated poles.

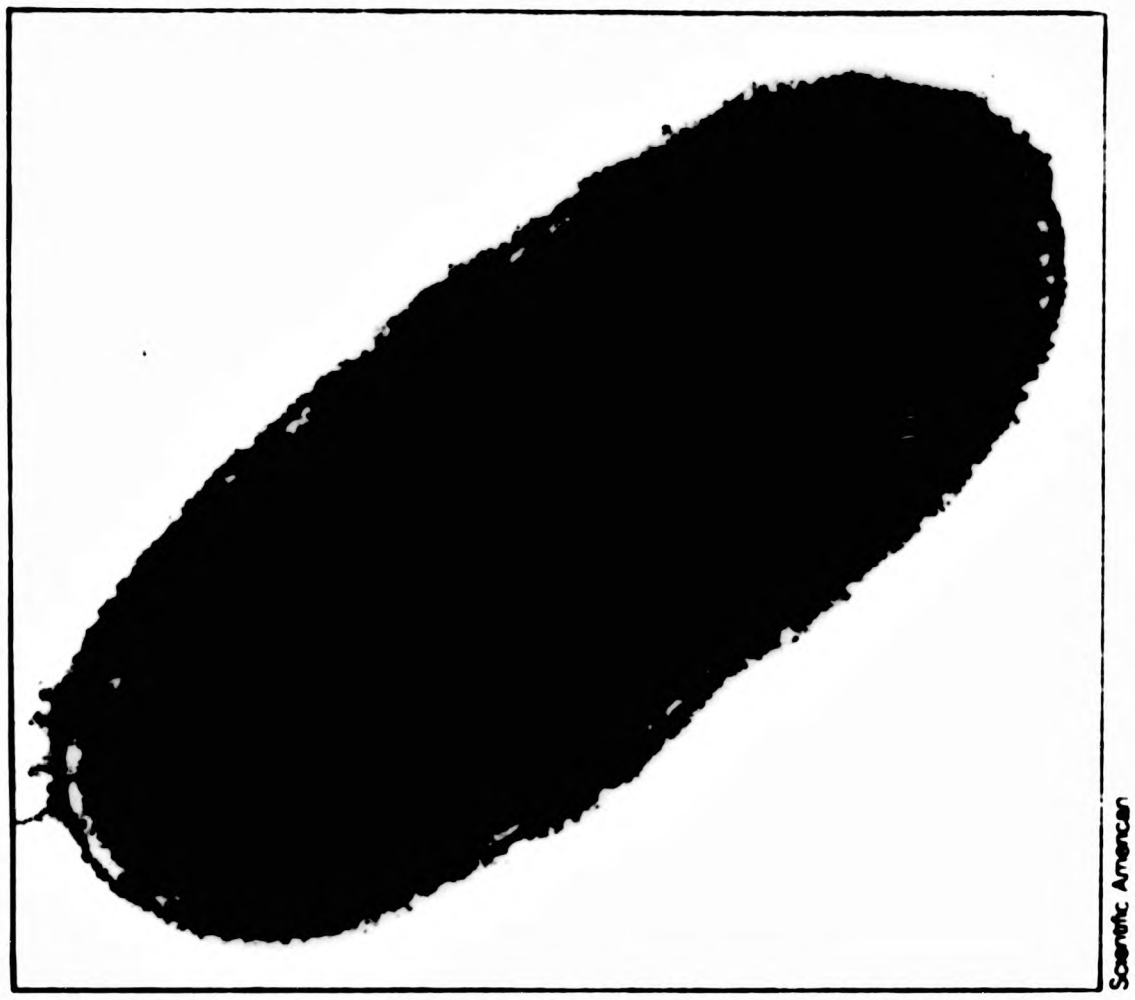
C_1 anabolism in methanotrophic bacteria follows different assimilatory pathways depending on the type of membrane structure. Type I bacteria assimilate C_1 through the ribulose monophosphate (RuMP) pathway, while type II bacteria use the serine pathway (see section 1:4a for details on these pathways). A list of most of the known obligate and facultative methanotrophs and some of their biochemical and physiological properties can be found in Anthony (1982) pages 6-8.

1:2c Methylotrophic bacteria

According to biochemical and physiological differences the methylotrophs have been subdivided into two distinct groups, (i) obligate methylotophs and (ii) facultative methylotrophs.

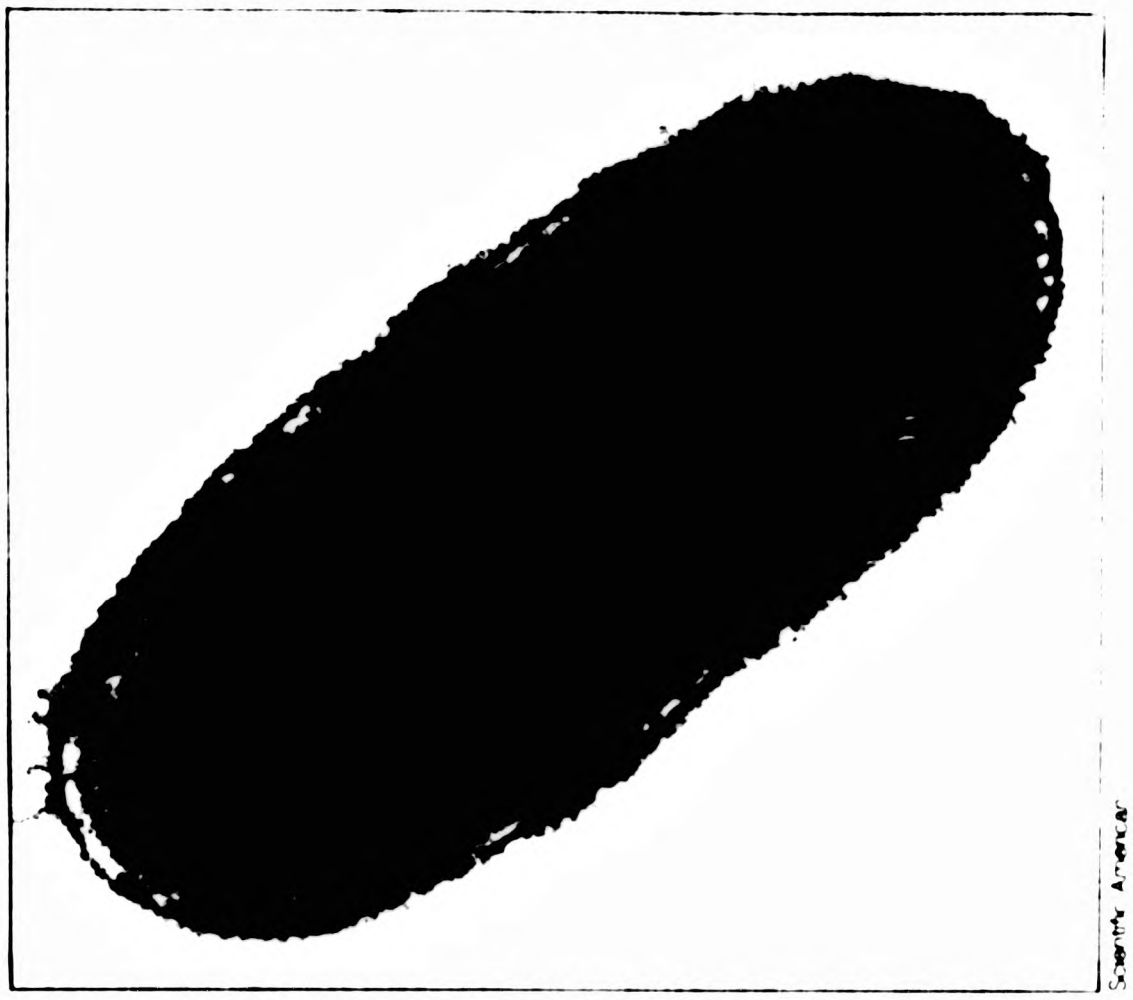
(i) The obligate methylotrophs have some similarity with the obligate methanotrophs in that they utilize the RuMP pathway for assimilation of C_1 compounds, contain predominantly 16C fatty acids and are rod-shaped and motile. However these bacteria do not utilize methane, do not have complex internal membranes, do not form spores or resting stages or fix nitrogen. The obligate methylotrophs all grow well on methanol.

A characteristic of certain obligate methylotrophs is a thick cell envelope with a wavy outline as observed by by electron-microscopy, plate 1:1 (Rokem et al., 1978; New Scientist,



The bacterium* Methylophilus methylotrophus, *a source of SCP

Plate 1:1 Electron micrograph of the obligate methylotroph, Methylophilus methylotrophus. Reprinted from New Scientist (1985) Jun 27th ed. 1462, p13.



The bacterium Methylophilus methylotrophus, *a source of SCP*

Plate 1:1 Electron micrograph of the obligate methylotroph, Methylophilus methylotrophus. Reprinted from New Scientist (1985) Jun 27th ed. 1462, p13.

1985). Such cell envelopes are not apparent with facultative methylotrophs. It is proposed that the thick cell envelopes are a result of high concentrations of lipid. A list of most of the known obligate methylotrophs and some of their biochemical and physical properties can be found in Anthony (1982) pages 25-26.

(ii) Facultative methylotrophs. Facultative methylotrophs utilize methanol and certain methylated amines as growth substrates but do not grow on methane. Although those growing on methanol may also use methylated amines, the converse is not true, with the notable exceptions of the pink facultative methylotophs and Hyphomicrobia. Examples of facultative methylotrophs unable to grow on methane are the pink facultative methylotrophs, non-pigmented "pseudomonads", the gram-negative (or variable), non-motile rods and coccal rods, gram-positve facultative methylotrophs, facultative autotrophs, hyphomicrobia and the marine bacteria.

A further class of facultative methylotrophs, the restricted facultative methylotrophs, has also been suggested to account for those methylotrophs that grow very slowly on one or two multicarbon substrates (e.g. glucose or fructose). The general biochemical and physiological properties of the facultative methylotrophs are described in Anthony (1982) pages 27-38.

1:3 SUBSTRATES THAT SUPPORT GROWTH OF METHYLOTROPHIC BACTERIA

One of the most important aspects of an organism used in large scale biotechnological processes is its ability to grow on relatively cheap and abundant substrates (Dijkhuizen et al., 1985). The next section summarizes the sources and availability of certain

naturally occuring and processed substrates that support growth of methylotrophic bacteria.

1:3a Methane

If a habitat is rich in biodegradable organic compounds, a nitrogen source and other essential elements, but lacks 0_2 then an anaerobic environment exists which allows the development of methanogenic (methane-producing) bacteria. Almost 50% of all organic carbon degraded anaerobically is converted to methane. The total amount of methane released into the atmosphere (10^{15} g/year) is distributed as follows: from aquatic sediments, swamps, tundra, decaying heartwood and trees and anaerobic sludge digesters (45%); from the gastro-intestinal regions of animals (20%); from paddy fields (25%) and the remainder from river and lake mud. Those figures are only partly representitive of the total methane produced as the greater part is probably oxidized by the methanotrophs before it reaches the atmosphere.

1:3b Methanol

Methanol may be found in small quantities in the atmosphere due to photo-oxidation of methane. Naturally occuring methanol arises from the hydrolysis of methyl esters and esters present in the pectin and lignin of plant material. The majority of methanol produced for industrial purposes is made from synthesis gas, a mixture of carbon monoxide and hydrogen. Most synthesis gas is currently manufactured by steam reforming natural gas although coal and residual oil are alternative resources (Dijkhuizen et al., 1985).

1:3c Formaldehyde, formate and formamide

All these substrates may be found in industrial waste from the tanning industry, chemical processing plants and in the case of formate as a product of mixed acid fermentation.

1:3d Cyanide and carbon monoxide

Cyanide is produced from electroplating and metal-extraction as industrial waste and naturally from cyanogenic plants, fungi and bacteria. Carbon monoxide is produced from vehicle exhaust and blast furnaces as well as from decaying organic matter.

1:3e Mono-, di- and tri-methylamines

These substrates are produced from decaying fish in the food industry and as industrial waste in the tanning industry.

1:3f Substrates not used as a source of carbon and energy

The oxidation of a large number of substrates, that are not used as a carbon and energy source for the bacteria, may have vast commercial potential, particularly in the transformation of hydrocarbons produced in the petroleum industry. The range of biotransformations performed by these organisms is extremely broad and includes reactions that produce potentially commercial products such as propylene oxide, a substrate for synthetic polymers. A comprehensive list of oxidations carried out by these bacteria is shown in Haber et al., (1983). Other reactions such as the dehalogenation of organic compounds may have important ecological significance.

1:4 THE BIOCHEMISTRY OF METHYLOTROPHIC BACTERIA

Other than the biochemical and physiological properties referred to in sections 1:2b and 1:2c, very few other aspects of the biochemistry of these microorganisms have received attention. The notable exceptions to these are discussed below.

1:4a The C_1 assimilatory pathways of methylotrophic organisms

Three assimilatory pathways are used by the various physiological groups of methylotrophs; (a) Ribulose bisphosphate (RuBP) pathway or Calvin cycle; (b) Ribulose monophosphate (RuMP) pathway and (c) Serine pathways (Anthony, 1982; Large, 1983; Dijkhuizen et al., 1985).

(i) Ribulose bisphosphate pathway : This pathway is used in cells where all the carbon is assimilated at the oxidation level of CO_2, some or all of which arises from oxidation of reduced C_1 compounds during methylotrophic growth. A summary of the RuBP cycle is shown in Fig. 1:1. Details of the enzymes involved in this cycle may be found in several comprehensive reviews (Anthony, 1982; Large, 1983; Dijkhuizen et al., 1985). The overall stoichiometry of the assimilatory reaction is shown below. It is obvious from this reaction that

$$3\ CO_2 + 6NAD(P)H + 6H^+ + 9ATP \longrightarrow \underset{\text{phosphate}}{\overset{\text{Glyceraldehyde}}{3}} + 6NAD(P^+) + 9ADP + 8P_i$$

there are heavy demands on ATP and reducing power. Examples of methylotrophic bacteria that use this pathway are shown in table 1:1.

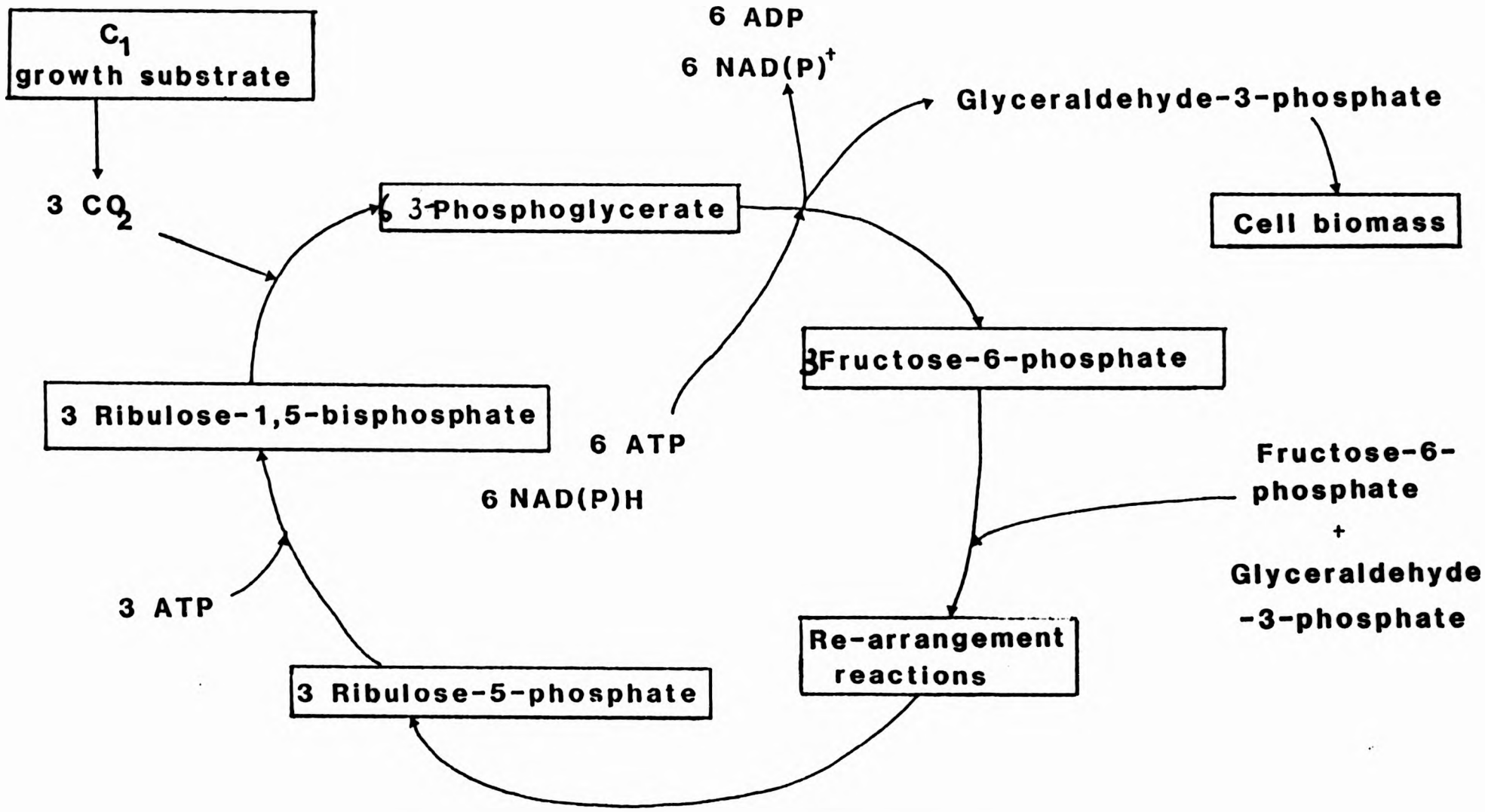


Fig. 1:1 Ribulose bisphosphate pathway.

(ii) Ribulose monophosphate pathway : In this pathway of carbon assimilation, carbon is fixed at the oxidation level of formaldehyde, produced by oxidation of methane, methanol or methylated amines. A summary of the two variants of the RuMP cycle is shown in Fig. 1:2a & b. It is apparent from the RuMP cycles that there are small demands on ATP and NAD(P)H compared to the RuBP cycle. The significance of these low levels of metabolic energy required to assimilate substrates will be discussed in a later section. The similarity between the RuBP and RuMP pathways has led to the suggestion that there is a close genetic relationship between bacteria using either of these pathways. Examples of methylotrophic organisms using the RuMP cycle are shown in table 1:1.

(iii)Serine Pathway : The serine pathway differs from other formaldehyde assimilation pathways in the nature of its intermediates which are carboxylic acids and amino acids rather than carbohydrates, and in the key formaldehyde-assimilating enzyme serine transhydroxymethylase which catalyzes the addition of formaldehyde to glycine producing serine. The serine pathway operates in bacteria using a variety of reduced C_1 compounds. A summary of the serine pathway is shown in Fig. 1:3. It is clear that cell carbon assimilated as CO_2, or in which part of the cell carbon arises from CO_2, require higher ATP requirements and are therefore metabolically less efficient than the RuMP pathway. Examples of bacteria using the serine pathway are shown in table 1:1.

1:4b The respiratory chain of methylotropic bacteria

There has been much work done on electron transport in

TABLE 1:1

Examples of bacteria using the various pathways of C_1 assimilation.

Ribulose biphosphate	Ribulose monophosphate	Serine
1) PHOTOSYNTHETIC BACTERIA	1) OBLIGATE METHANOTROPHS	1) OBLIGATE METHYLOTROPHS (able to use methanol)
Rhodopseudomonas sp.	Methylomonas methanica Methylobacteria capsulatus Methylobacter bovis	Methylocytis parvus Methylosinus sporium
2) NON-PHOTOSYNTHETIC BACTERIA	2) OBLIGATE METHYLOTROPHS (unable to use methanol)	2) FACULTATIVE METHYLOTROPHS (able to use methanol)
Pseudomonas oxalaticus Paracoccus denitrificans Pseudomonas carboxydovorans	Methylophilus methylotrophus	Methylobacterium organophilum XX Methylobacterium R6
	3) OBLIGATE METHYLOTROPHS (able to use methane)	3) FACULTATIVE METHYLOTROPHS (unable to use methane)
	Methylococcus capsulatus	Pseudomonas aminovorans Pseudomonas AMI
	4) FACULTATIVE METHYLOTROPHS (unable to use methane)	Pseudomonas extorquens
	Arthrobacter globiformis Pseudomonas olevorans	

a

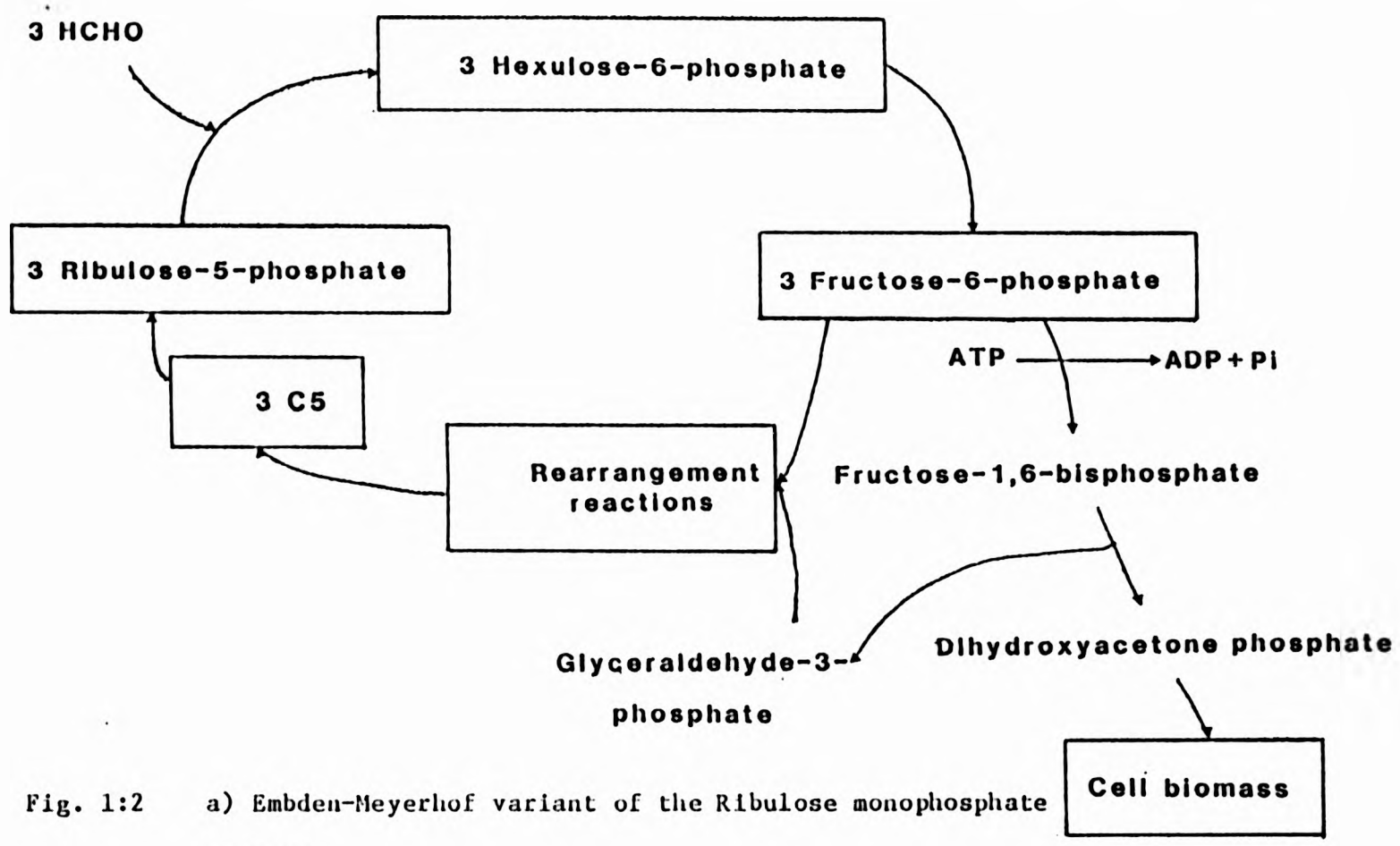
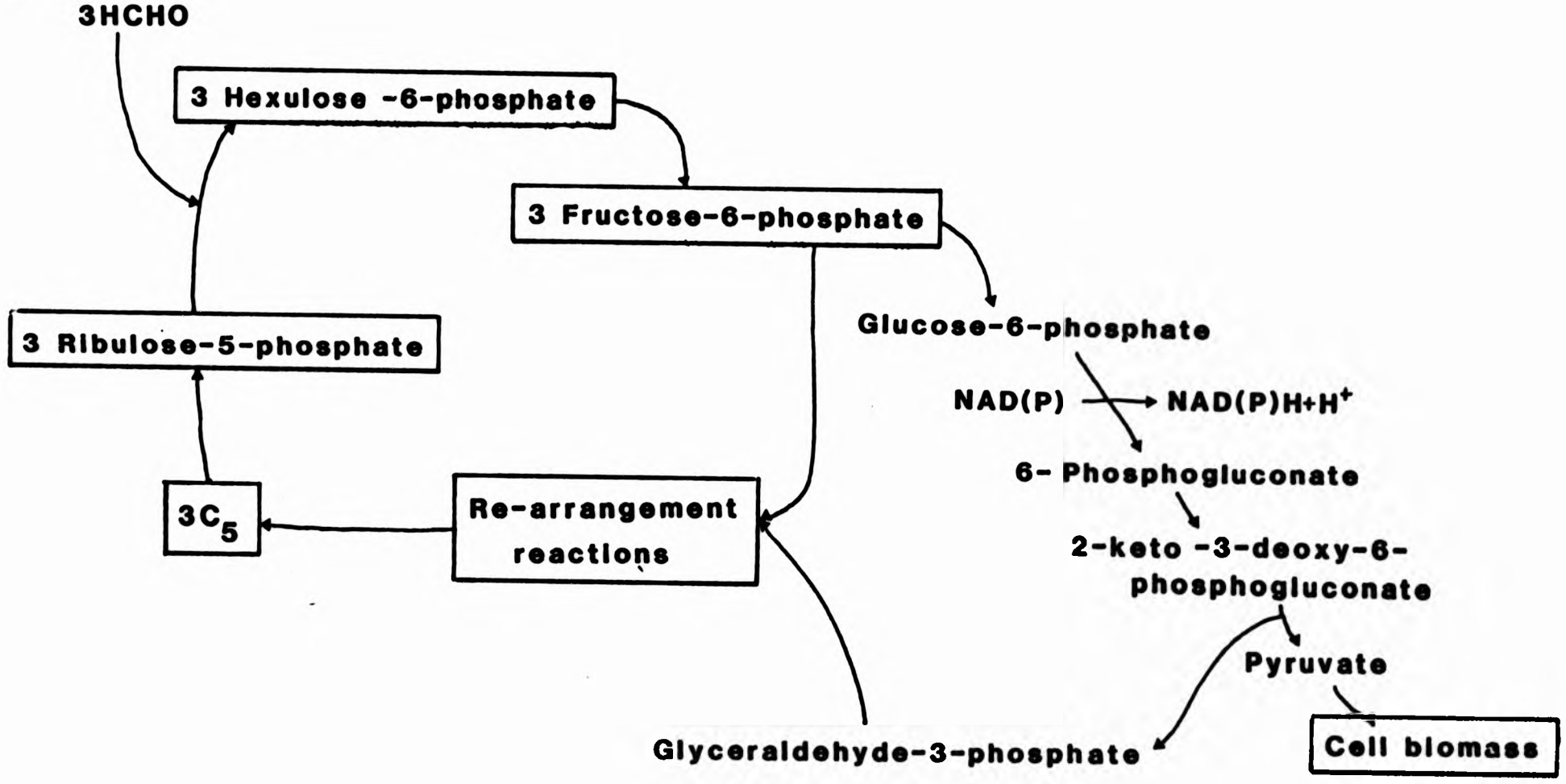


Fig. 1:2 a) Embden-Meyerhof variant of the Ribulose monophosphate pathway.

b) Entner-Doudoroff variant of the Ribulose monophosphate pathway.

b)


3HCHO
3 Hexulose -6-phosphate
3 Fructose-6-phosphate
Glucose-6-phosphate
NAD(P)
NAD(P)H+H+
6- Phosphogluconate
2-keto -3-deoxy-6-phosphogluconate
Pyruvate
Cell biomass
Glyceraldehyde-3-phosphate
Re-arrangement reactions
3C5
3 Ribulose-5-phosphate

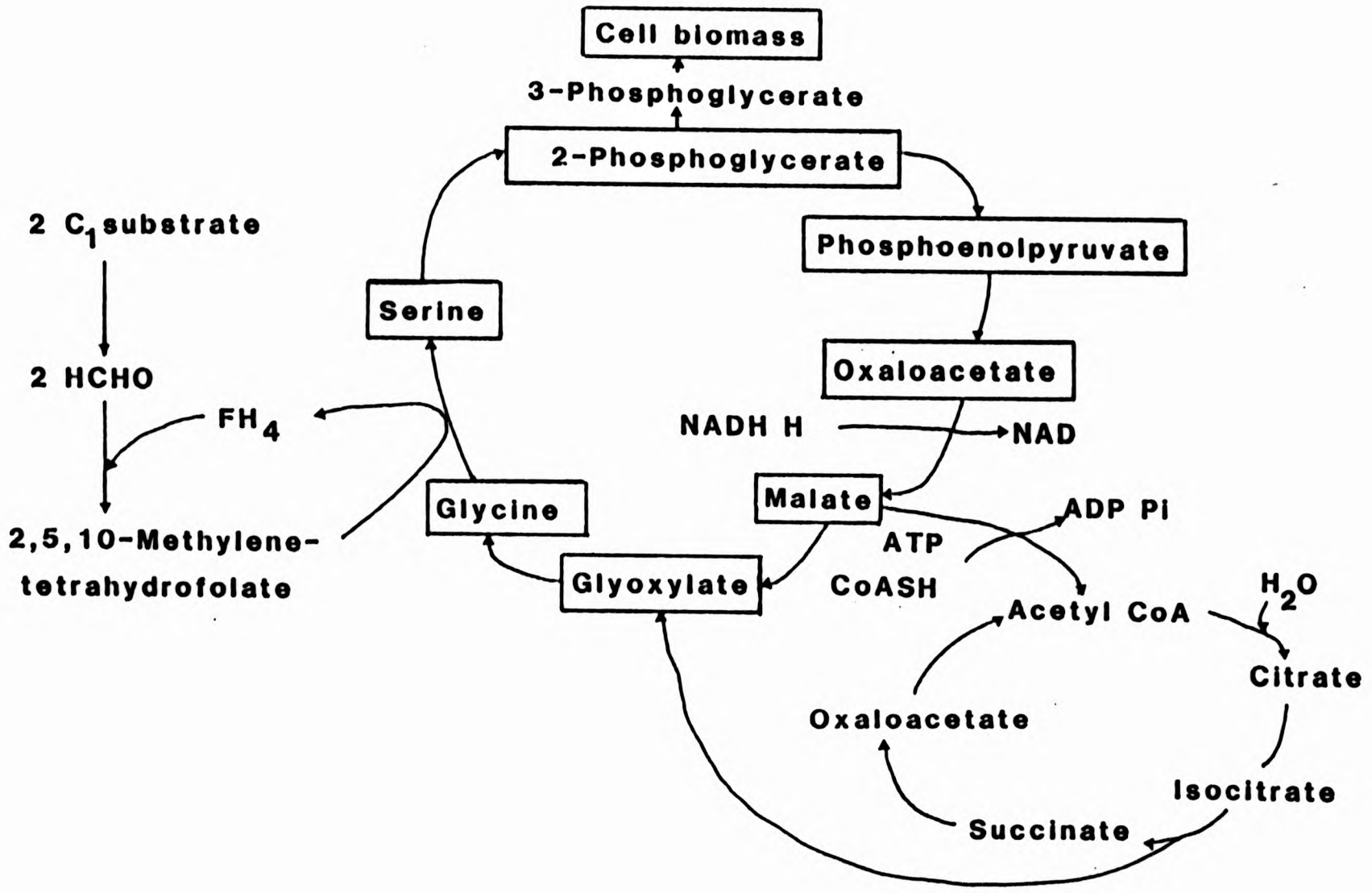


Fig. 1:3 Serine pathway.

methylotrophic bacteria and in particular their cytochromes (Dawson and Jones, 1981; Froud and Anthony; 1984; Carver et al., 1984). All methylotrophic bacteria growing on methanol, regardless of the type of methylotroph or growth conditions, contain the two soluble cytochromes c_H and c_L and a membrane-bound cytochrome c. In the case of Methylophilus methylotrophus, cytochrome c is composed of 8% cytochrome c_H, 37% cytochrome c_L and 55% the cytochrome c component of the oxidase, cytochrome c_o. Whole cells of M. methylotrophus oxidize methanol to formaldehyde via a pyrroloquinoline quinone-linked methanol dehydrogenase which is loosely attached to the periplasmic surface of the respiratory membrane. The subsequent transfer of electrons from methanol dehydrogenase to oxygen involves the cytochromes c_H, c_L and c_o. The remainder of the membrane-bound respiratory chain is composed of NADH dehydrogenase (containing flavin and multiple iron-sulphur centres), ubiquinone, a high redox potential iron-sulphur protein and two species of cytochrome b. Under these conditions the formaldehyde produced must pass through the membrane into the cytoplasm to combine with ribulose-5-phosphate in the first step of assimilation. This process is believed to occur by simple or facilitated diffusion. The overall methanol oxidase system in M. methylotrophus and all methylotrophic bacteria have been shown to catalyze effective proton translocation and hence to generate a proton motive force which can subsequently drive ATP synthesis. Recent work has shown that enzymes involved in the methanol oxidase system of M. methylotrophus are regulated by the concentrations of methanol for methanol dehydrogenase and dissolved oxygen for cytochrome oxidases c_o and aa_3 (Greenwood and Jones, 1986).

Methanol dehydrogenase and cytochrome oxidase aa_3 are repressed under methanol-sufficient growth conditions as is cytochrome oxidase c_o under excess oxygen. Conversely these systems are derepressed under conditions of insufficient methanol or oxygen. Such a system is believed to maintain *in situ* rates of respiration under substrate limiting conditions.

1:4c Genetic procedures for determining the biochemistry of methylotrophs

A lack of detailed knowledge of the biochemistry and physiology of methylotrophic bacteria has impeded the progress in obtaining nutritional mutants for genetic studies. One notable exception to this is the glutamate synthase mutants of *M. methylotrophus* isolated by Windmass et al., (1980). *M. methylotrophus* has a high carbon conversion efficiency but it was found that it had inefficient usage of methanol by its mode of ammonia assimilation. Instead of using glutamate dehydrogenase (GDH) to catalyze conversion of ammonia and 2-oxoglutarate into glutamate, as in *E. coli*, it has a two-stage pathway using glutamate synthetase (GS) and glutarate synthase (GOGAT) for the same function but requiring an extra molecule of ATP per molecule of glutamate formed. It is likely that the GS system which has a greater affinity for ammonia than GDH is a consequence of the environment in which *M. methylotrophus* lives. However since ammonia would not be growth limiting under laboratory conditions, the *E. coli* gene which encodes GDH was introduced into a mutant of *M. methylotrophus* lacking GOGAT. The result was an energetically more efficient ammonia assimilation pathway resulting in more methanol being converted into cellular carbon (an important factor

in the biotechnology processing of single-cell protein).

C_1 metabolism mutants have been successfully generated in the facultative methylotrophs Methylobacterium organophilum strain xx and the facultative methanol - and methylamine-utilizing Pseudomonas strain AMI, and Pseudomonas aminovorans (Haber et al., 1983). These mutants have demonstrated that several of the genes that encode proteins of the assimilatory pathway of C_1 are on the same chromosome and may therefore be under the same regulatory control.

DNA encoding oxidation and assimilatory enzymes of methane and methanol have been expressed in vitro using E. coli extracts and minicells capable of synthesizing proteins. Methane monooxygenase from Methylococcus capsulatus has been characterized using this method (Haber et al., 1983).

A naturally occuring plasmid in Methylobacterium organophilum strain xx has been shown to be associated with growth on methane. Other plasmids in methylotrophs have been discovered and may also have some role in producing proteins involved with metabolic pathways, however these plasmids are at present cryptic in that the functions they encode are not known (Haber et al., 1983).

It is apparent therefore that there is still a great deal to be learnt about the biochemistry and physiology of the methylotrophs, but continued attempts to isolate mutants may prove the most successful approach to this problem as has been the case in E. coli.

1:5 THE APPLICATION OF METHYLOTROPHS IN BIOTECHNOLOGY

Recent advances in our understanding of the physiology and biochemistry of methylotrophs have made it possible to evaluate the potential of these microorganisms in biotechnological processes.

The following section summarizes the variety of biotechnological applications for this group of bacteria and its enzymes.

1:5a Single-cell protein production

The increasing inability of agriculture to meet the protein demands of the world's population has necessitated alternative sources of protein for feeding live stock or as a direct nutrient for human consumption. Protein-rich foods such as soyabean meal and fish meal are at present the main protein alternative. Such protein sources however result in large demands on arable land and the limited supply of fish. Over the last 20 years, microbiologists and technologists have developed methods for the production of protein from microorganisms. Protein from microbial sources is known as 'single-cell protein' (Wiseman, 1983; Smith, 1985).

The two most important factors in the production of single-cell protein on an industrial scale are the choices of substrate and organism. When choosing a substrate the carbon source should not itself be a valuable food source but one that has little commercial or nutritional value. Initially these substrates were petroleum by-products such as long-chain hydrocarbons, natural gas and certain petrochemicals, however political and economic changes in the world market influence the supply and cost of these substrates and as such are not stable factors for commercial production. It was necessary therefore to find other substrates for single-cell protein production. The most promising alternative substrates examined so far are methane and methanol. There are problems however when methane is used as the source of carbon in that it is potentially explosive when mixed with air. Methanol offers many advantages over

alternative carbon sources: it is soluble in water and therefore avoids the phase-transfer problems encountered with hydrocarbons; there is no risk of explosion; it is readily available as a synthesis product of hydrocarbon sources e.g. methane, naphtha, synthesis gas and coal; it is easily purified; it requires less O_2 for metabolism and so requires less cooling; it is easy to handle, transport and store; it is available at a low cost and is available in the vast surplus in the world of a billion gallons/year (1985 value) (Dijkhuizen et al., 1985).

Another important factor in the choice of substrate when producing single-cell protein on an industrial scale is the "yield coefficient" (Anthony, 1982; Large, 1983; Wiseman, 1983; Smith, 1985). This is the mass of dry microbial cells (biomass) produced per unit mass of substrate utilized. A comparison of the carbon yield coefficient of many reduced carbon sources has shown that alcohols are more efficiently utilized for microbial growth than hydrocarbons.

The most important factor in the production of single-cell protein on an industrial scale is the choice of organism (Anthony, 1982; Large, 1983). The microorganism chosen must satisfy certain important criteria in order to be commercially viable: it must have a high growth rate, a high affinity for the substrate, a high optimum growth temperature and the ability to grow to a high cell density, it must be stable when growing in continuous culture, be resistant to contamination, have a low infectivity and should not require additional expensive growth-factors other than the carbon substrate.

Methylotrophic bacteria grown on methanol as the sole carbon

source satisfy the majority of the criteria descibed above and have been the microorganisms most commonly used by industrial companies in the production of single-cell protein (Anthony, 1982; Large, 1983; Wiseman, 1983). Examples of several industrial companies using methylotrophic bacteria for single-cell protein production are shown in table 1:2.

1:5b Overproduction of metabolites (Fermentation processes)

In addition to single-cell production, methylotrophs have a great potential in many other bioprocesses (Anthony, 1982; Dijkhuizen et al., 1985). Interest is now turning towards the production of metabolites and primary products, such as polysaccharides, amino acids, vitamins and coenzymes. The procedures used to produce the materials described depend on the selection of mutants that lead to metabolite over-production. Bacteria with the serine pathway of C_1 metabolism are generally preferred for these processes as they contain a complement of tricarboxylic acid cycle enzymes which themselves may be of considerable commercial value. Strains have been isolated that accumulate the following metabolites extracellularly, glutamate, valine, leucine, serine and vitamin B_{12}. Large-scale commercial production of any of these metabolites has not been reported. Certain intracellular metabolites have also been isolated in large amounts from methylotrophs and these include coenzymes Q_{10}, polysaccharide (glucan) and poly- β -hydroxybutyrate. The latter may provide a source of a non-petrochemical- based polymer for plastic manufacturing.

TABLE 1:2

Single-cell protein produced by various companies from methanol-grown bacteria.

COMPANY	Methylotrophic bacteria	Product name
ICI (UK)	Methylophilus methylotrophus	Pruteen
Uhde/Hoechst (W. Germany)	Methylomonas clara	Probion
Norprotein (Scandanavia)	Methylomonas methanolica	norprotein
Mitsubushi Gas Chemical Company (Japan)	Methylomonas methanolis BNK-84	-

1:5c Methylotrophs as biocatalysts

The methylotrophs and their enzymes may also have important roles as biocatalysts (Anthony, 1982; Dijkhuizen et al., 1985). Methanol-utilizing marine bacteria have been used in the bioassay of vitamin B_{12} in saline solutions. Primary amine dehydrogenases of Pseudomonas AMI have been used to estimate methylamine, ethylamine and n-propylamine. Methanol dehydrogenase and methane monooxygenase have been used to estimate primary and secondary alcohols respectively. The conversion by oxidation of hydrocarbons such as long-chain terminal alkenes to long-chain epoxides catalyzed by methane monooxygenase may prove to be an important biocatalytic method of producing epoxy homo- and copolymers.

1:5d Methylotrophs and their enzymes in electroenzymology and biofuel cells

If an enzyme-catalyzed reduction reaction uses electrons provided by a cathode, or if an oxidation reaction can donate its electron to the anode, then there is an electroenzymological process or enzymic reactor (Anthony, 1982; Dijkhuizen et al., 1985). An example of such a reactor utilizes methane monooxygenase to catalyze the oxidation of alkanes or alkenes by oxygen using electrons provided directly by the cathode. The electrons from the cathode replace those normally provided by NADH. If microorganisms are used instead of enzymes, driven by an electrical current, then it is a 'microbial reactor'.

A biofuel cell is one in which two molecules are kept physically separate, but by means of electrodes an electron transfer between the molecules occur. If the oxidizing and reducing

reactions are catalyzed by enzymes or whole microorganisms then the fuel cell is an enzymic or microbial fuel cell respectively. An example of an enzymic fuel cell uses methanol dehydrogenase and methanol (Anthony, 1982; Dijkhuizen et al., 1985).

1:5e Methylotrophs as hosts for eukaryotic-gene expression

The expression of eukaryotic DNA in microorganisms has a potential for producing biological products which would normally have to be extracted from human or animal tissues. For such gene-cloning procedures to be of commercial value the existing source of supply of the potential product must be very expensive or inadequate and the demand such that the cost of research and development can be recovered. There are several likely candidates for such commercial ventures, human growth hormone (Goeddel et al., 1979a), insulin (Goeddel et al., 1979b), interferon (Taniguchi et al., 1980), enzymes and vaccines (Wiseman, 1983). Although the cloning of foreign genes is now routinely performed with E. coli (Smith, 1985), other non-infectious organisms such as Bacillus subtilis (Reid et al., 1986) and Saccharomyces cerevisiae (Ingram, 1986) may be more suitable host systems for large scale production of gene-cloned products.

The many advantages described earlier for M. methylotrophus in commercial single-cell protein production in addition to the non-infectivity towards man also make this a potentially suitable organism for the expression of eukaryotic genes on an industrial scale. The fact that eukaryotic genes for chicken ovalbumin and mouse dihydrofolate reductase have already been expressed in this bacterium (Hennam et al., 1982) demonstrates the feasability of

using this organism.

Although the potential for using M. methylotrophus as a host for gene-cloning procedures is great, there are still certain practical problems, common to most other microorganisms, which must be first overcome.

The first problem is how to export the eukaryotic protein product using an organism that has no known export proteins. This problem may be partly solved by fusing the gene for the desired product with another foreign gene whose product is exported. A second practical problem involves the susceptibility of foreign protein to host proteinases (Taniguchi et al., 1980; Talmadge and Gilbert, 1982; Cheng et al., 1984). One method of solving this problem may be by using proteinase-deficient mutants (Fahnestock and Fisher, 1987). An alternative solution to this difficulty could involve selective overexpression of specific endogenous proteinase inhibitors in conjunction with the gene-cloned product (Cheng et al., 1981). A third problem is the absence of enzymes required for posttranslational modification of foreign products in prokaryotic host systems. This difficulty might be solved either by developing mutants that produce the enzymes responsible for posttranslational processing or alternatively these processes may be carried out under in vitro conditions.

In addition to the problems described above; lack of knowledge concerning the chromosome location of desired genes; unavailability of suitable vectors; processes requiring multiple gene involvement and the loss of plasmids through growth dilution are all factors that must first be considered before gene-cloning on an industrial scale can be undertaken (Caulcott and Rhodes, 1986).

PART 2

1:6 PROTEOLYSIS IN BACTERIAL CELLS

Intracellular protein degradation or proteolysis is a fundamental process in all organisms. The majority of studies on proteolysis however have been done using eukaryotic organisms (Matsubara and Feber, 1971). Since the results presented in this thesis are based on work in the gram-negative bacterium *M. methylotrophus* this review will deal in the main with bacterial proteolysis with particular emphasis on the work that has been done on *E. coli*.

Three aspects of intracellular proteolysis in bacteria will be reviewed : (1) proteolysis, (2) proteinases and (3) functional roles of bacterial proteinases.

1:7 PROTEOLYSIS

More than 40 years ago Schoenheimer (1942) in his concept of a "dynamic state of body constituents" suggested that cellular material, particularly proteins, was being continually degraded and replaced by newly synthesized constituents. The continuous degradation of proteins was, according to Schimke (Schimke and Doyle, 1970), necessary in order to regulate intracellular concentrations of enzymes controlling all metabolic functions. This regulation of intracellular levels of enzymes was believed to be the result of a delicate equilibrium between two equivalent processes, the synthesis and the degradation of proteins (Mandelstam, 1960; Goldberg and Dice, 1974).

The concept of a dynamic equilibrium for intracellular proteins in bacteria has since been brought into question with the knowledge

that bacterial proteins are relatively stable during normal exponential growth (Mandelstam, 1957; Coccuci and Davies, 1983) with only a few proteins being continuously turned over.

Under what conditions and for what reasons therefore are proteins degraded? In their earlier work Goldberg and his colleagues (Goldberg and Dice, 1974; Goldberg and St John, 1976) suggested that proteolysis was necessary for several critically important functions : (1) the removal of abnormal proteins produced by mutations, errors in gene expression, denaturation or chemical modification; (2) in the continuous turnover of proteins with short half-lives in order to allow delicate controls of crucial enzymes during changes in environmental conditions and (3) in the increase of proteolysis during starvation in order to provide essential amino acids required to synthesize important enzymes. It was proposed that metabolic energy was necessary for proteolytic degradation in all these cases.

In addition to the general concept described above Goldberg and Dice (1974) further proposed a relationship between protein degradation rates and protein structure in an attempt to explain the differential rates of degradation observed for proteins. It was suggested that larger, more acidic or more hydrophobic proteins were degraded faster than smaller proteins, the reason suggested being : (1) that larger proteins had more proteinase - sensitive sites; (2) certain conformations of proteins were more susceptible to cleavage, and larger proteins had more of these sites; (3) the native conformation of larger proteins might be more susceptible to denaturation or once denatured might be less likely to refold and (4) larger proteins were more likely to have errors of transcription

or translation.

Although the basis of selectivity and the mechanism of intracellular proteolysis in vivo are still not clear, much of the earlier evidence for energy-dependent degradation of abnormal proteins and normal proteins during starvation has been substantiated (Mandelstam, 1960; Olden and Goldberg, 1978; Murakami et al., 1979; Chung and Goldberg, 1981; Etlinger et al., 1985; Goldberg et al., 1985; Goldberg and Waxman, 1985). A likely role for proteolysis in the rapid turnover of crucial enzymes has also been further indicated with the discovery of several regulatory enzymes with short- half-lives (Mayer and Doherty, 1986).

The major point of contention concerning the earlier work is largely the result of new evidence on protein structure and protein degradation rates (Kempstead and Hipkiss, 1976; Coccuci and Davies, 1983; Mayer and Doherty, 1986). The relationship between protein structure and protein degradation as indicated earlier would involve a specific proteinase for each individually degraded protein and an entire regulatory system would be necessary to activate and deactivate these proteinases. It has since been demonstrated that neither protein subunit size, thermal stability, protein charge or amino acid composition are related to the rate of protein degradation (Coccuci and Davies, 1983). An alternative explanation for selective degradation of proteins was therefore required.

It is likely that selectivity of protein degradation is in fact determined not by one, but by a number of physical properties and only in special cases will a single factor dominate.

Other factors that are proposed to act as signals for protein degradation and hence control the overall extent of proteolysis

include : (1) the proportion of reduced sulphydryl groups in proteins which are more susceptible to degradation than proteins containing S-S groups (Coccuci and Davies, 1983); (2) modification of proteins by ligands or other proteins (Goldberg and Dice, 1974; Mayer and Doherty, 1986), (3) modification of proteolytic enzymes by substrate binding and association with high-energy compounds (Goldberg and St John, 1976; Waxman and Goldberg, 1986) and (4) the N-terminal amino acid of individual proteins (Bachmair et al., 1986; Kolata, 1986).

One other important factor that cannot be ignored is the role that endogenous inhibitors might play in the overall control of intracellular proteolysis (Prouty and Goldberg, 1972; Millet, 1976; Chung et al., 1983), however the mechanism regulating the interactions of these proteins with proteinases remains to be determined.

Although many of the factors described above may effect selective degradation, the possibility that a specific proteolytic system in which a protein is specifically degraded by a proteinase or proteinases cannot be discounted. The identification of proteinases that catalyze limited proteolysis (see section 1:9 on proteinase function) indicates that such a mechanism must play an integral role in intracellular degradation. It is clear that the elucidation of the mechanisms of proteolysis will require characterization of the enzymes and substrates involved, the inhibitors and cofactors regulating it, and the determination of the specific functions of the proteolytic enzymes involved in these processes.

1:8 PROTEINASES

1:8a Nomenclature

The terms 'protease' and 'proteinase' have both been used extensively in the literature to describe proteolytic enzymes. It has now been recommended by the International Union of Biochemistry that 'peptidase' be used as the general term for 'peptide (bond) hydrolase'. This therefore means that the previous nomenclature of 'proteases' and 'proteinases' has been replaced by 'peptidases' and 'endopeptidases' respectively. The term 'exopeptidase' remains unchanged. To avoid confusion the term 'proteinase' has been selected for use throughout this thesis.

1:8b Classification

Exopeptidases : enzymes that degrade polypeptides by cleaving the terminal amino acids are called exopeptidases and have been classified according to their specificities, table 1:3a.

Endopeptidases : enzymes that cleave internal peptide bonds (although not exclusively) are called endopeptidases. The complex specificities of these proteolytic enzymes has led to several methods of classification: (1) their similarity to well characterized proteinases such as pepsin, trypsin or chymotrypsin; (2) their ability to cleave specific proteins. Proteinases that degrade keratin, collagen and elastin are called keratinase, collagenase and elastinase respectively; (3) the pH range in which the enzyme is active. Proteinases most active in the following pH ranges, 2-5, 7-9 and 10-12 are classified as, acidic, neutral and alkaline respectively; (4) on the basis of their catalytic mechanism. Classification according to catalytic mechanism forms

TABLE 1:3

Enzyme classification

a) Exopeptidases (peptide hydrolases)

α-aminoacylpeptide hydrolyases
peptidylamino-acid or acylamino-acid hydrolases
dipeptide hydrolases
dipeptidylpeptide hydrolases
peptidyldipeptide hydrolases

b) Endopeptidases

Class	Specific inhibitors	Other inhibitors	approximate pH range
Serine (E.C.3.4.21)	DFP, PMSF soybean trypsin inhibitor	TPCK, TLCK leupeptin, antipain chymostatin	7-9
Cysteine (thiol) (E.C.3.4.22)	iodoacetate, iodoacetamide, N-Ethylmaleimide, heavy metals	TPCK, TLCK p-chloromercuri-benzoate, antipain, leupeptin, chymostatin	4-7
Metallo (Neutral) (E.C.3.4.24)	EDTA, EGTA o-phenanthroline,		7-9
Aspartate (acid) (E.C.3.4.23)	Diazoacetyl norleucine methylester (Cu^{2+}) Pepstatin, epoxy (p-nitrophenoxy) propane		2-5

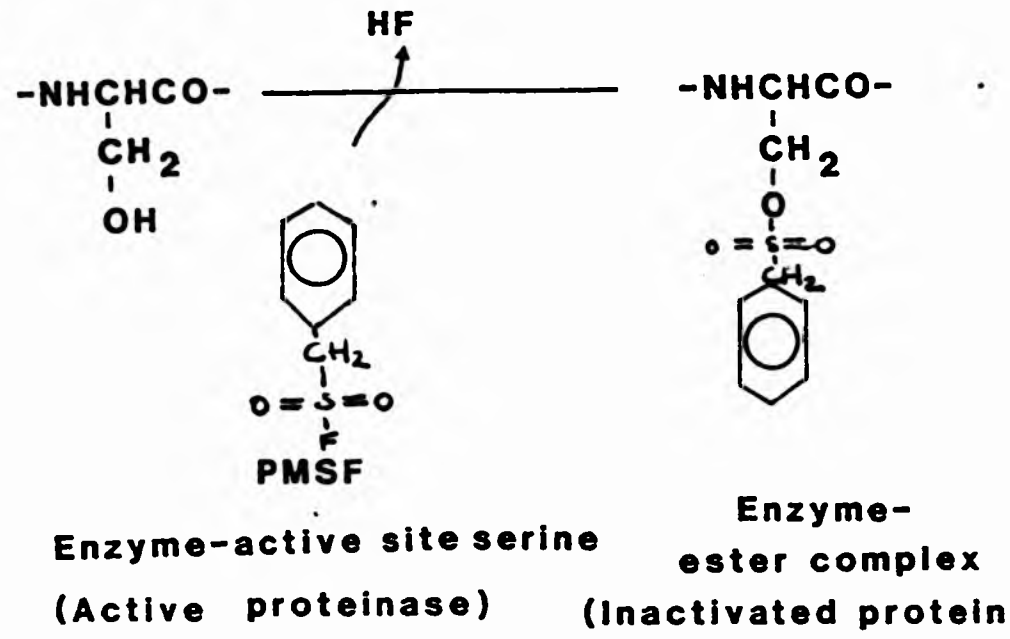
the basis for the Enzyme Commission classification shown in table 1:3b.

The identification of the catalytic mechanism of a particular proteinase relies on the chemical modification of essential amino acids at or near the active site of the enzyme. If the modified proteinase intermediate is then stabilized and sequenced, the amino acids at the active site of the enzyme can be determined. The most convenient method of chemically modifying these amino acid residues is by using specific inhibitors of the enzymes activity. Four classes of proteinases have been identified by this procedure.

1) Serine proteinases : these enzymes have been identified using diisopropyl fluorophosphate (DFP) and phenylmethanesulphonylfluoride (PMSF). These inhibitors inactivate serine proteinase by forming complexes with the hydroxyl group of an essential serine residue at the active site of the enzyme, fig. 1:4a.

2) Cysteine proteinases : identified by inhibitors such as iodoacetate and iodoacetamide which inactivate cysteine proteinases by binding to the thiol group of the cysteine residue at the active site of the enzyme, fig. 1:4b. Since iodoacetic acid and iodoacetamide will also bind to thiol groups unassociated with the active site of the enzyme, these compounds may also result in some inactivation of other proteinases due to conformational changes. Therefore, in order to be certain that the enzyme is a cysteine proteinase, in addition to inhibition by iodocetate or iodoacetamide, the enzyme must also be shown to be insensitive to DFP and EDTA and to be stimulated by reducing agents such as

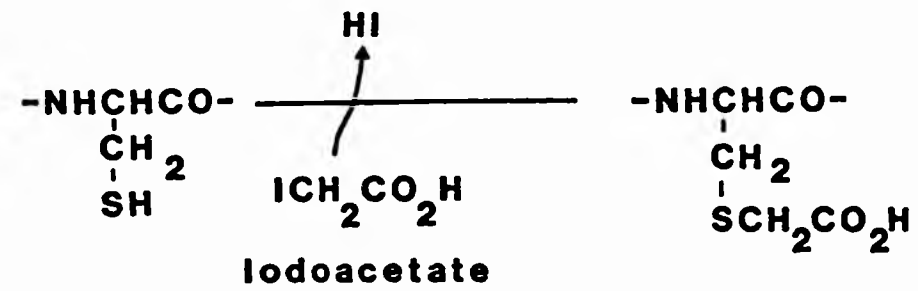
a



Enzyme-active site serine (Active proteinase)

Enzyme-ester complex (Inactivated proteinase)

b



Enzyme-active site cysteine (Active proteinase)

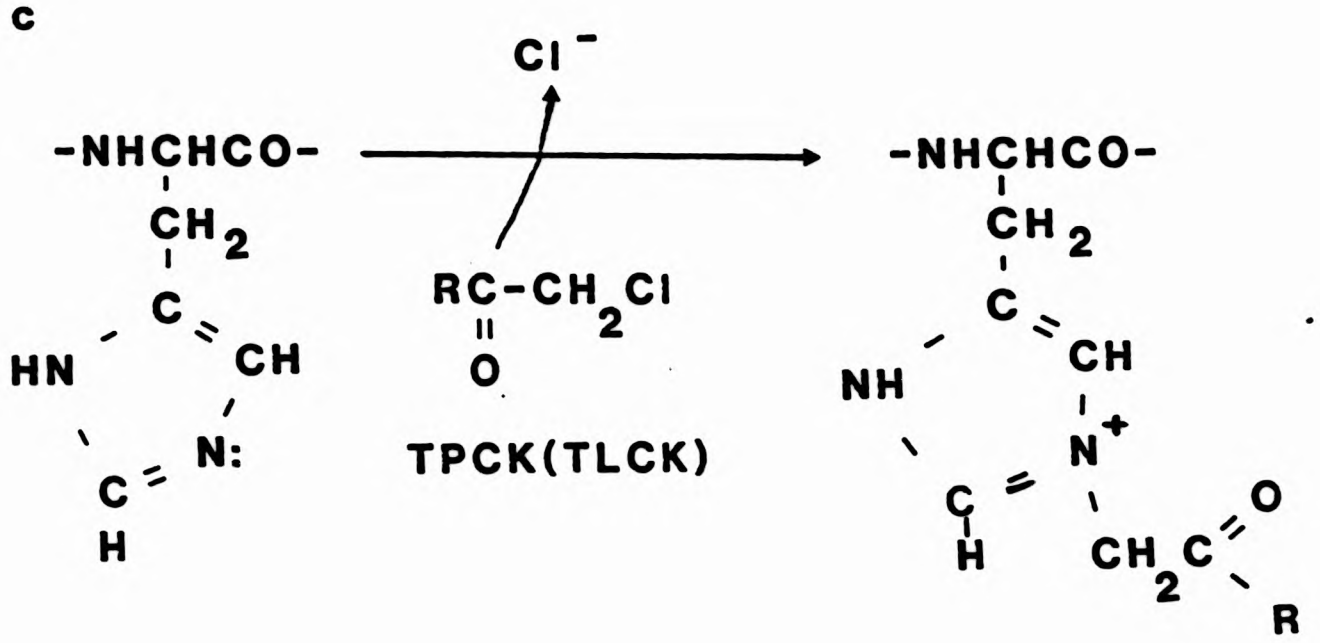
s-carboxymethyl derivative of proteinase (Inactivated proteinase)

Fig. 1:4 a) Inactivation of serine proteinases by PMSF.

b) Inactivation of cysteine proteinases by iodoacetic acid.

c) Inactivation of proteinases by TPCK and TLCK.

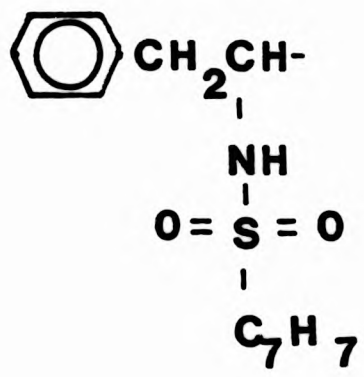
c



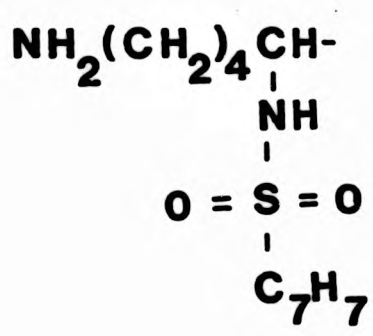
Enzyme-active centre histidine (Active proteinase)

alkylated histidine group (Inactivated proteinase

TPCK (R)



TLCK (R)



dithiothreitol (DTT) and KCN.

3) Metalloproteinases : identified by metal-chelating compounds such as EDTA and o-phenanthroline. These proteinase inhibitors are believed to remove an essential metal ion from the active site of the enzyme. However since metal-chelators will also bind metal ions required for enzyme stability not associated with the active site, proteinases inhibited by these agents must also be insensitive to DFP, PMSF and thiol-binding reagents.

4) Aspartic proteinases : these enzymes are inhibited by diazoketone compounds, such as diazoacetyl norleucine methylester (with copper) and are insensitive to DFP, PMSF, EDTA and thiol-binding agents. Diazoketone compounds inactivate aspartic proteinases by binding to an essential aspartate residue at the active site of the enzyme. Aspartic proteinases are also specifically inhibited by pepstatin which has little or no effect on other proteolytic enzymes.

5) Unclassified proteinases : this fifth group of proteinases is proposed to account for those proteolytic enzymes that do not exhibit any of the catalytic mechanisms described above.

In addition to the inhibitors described above that specifically bind to residues at the active site of the enzyme, there are a number of other inhibitors that inactivate more than one type of proteinase. L-tosyl-amido-2-phenylalanine chloromethyl ketone (TPCK) was designed as an active centre probe for chymotrypsin.

This inhibitor contains a phenylalanine since chymotrypsin acts hydrolytically at the carbonyl of phenylalanine in esters, amides and proteins. In addition TPCK contains a chloromethyl ketone function designed to achieve covalent binding with some functonal group on the enzyme. TPCK inactivates chymotrypsin by alkylation of a histidine residue at the active centre of the enzyme, fig. 1:4c. Inactivation of a bacterial proteinase with TPCK indicates some similarity between the active centre of this enzyme and that of chymotrypsin. N-∝-tosyl-L-lysine chloromethyl ketone (TLCK) was designed containing a lysine group to specifically inactivate trypsin. In addition this inhibitor contains a chloromethyl ketone group which alkylates a histidine residue at the active centre, fig. 1:4c. Proteinases that are inactivated by this inhibitor are said to be trypsin-like proteinases. Other inhibitors that are known to inactivate more than one proteinase are the microbial inhibitors, antipain, leupeptin and chymostatin. Interpretation of results using TPCK, TLCK or the microbial inhibitors must therefore be made in conjunction with results from other more specific inhibitors.

1:8c Intracellular proteinases

Serine and metalloproteinases are the proteolytic enzymes most commonly observed in bacteria. Although several cysteine proteinases have been isolated from extracellular extracts of certain bacteria (Gilles et al., 1983; Lo et al., 1984), there are no well characterized bacterial intracellular cysteine proteinases. In addition there have been no reports of aspartic proteinases existing in bacteria (Tang, 1979).

Intracellular proteinases have been reported to be found in a

broad range of microorganisms including thermophiles (Bromme and Kleine, 1984), gram-positive (Hiroishi and Kadota, 1976, Cheng and Aronson, 1977, Dignam and Setlow, 1980; Muro et al., 1985) and gram-negative bacteria (Orlowski and White, 1974; Miller at el., 1976; Car and Woods, 1984; Kohayashi et al., 1985), although by far the best studied of these have been isolated from the gram-negative bacteria E. coli (Goldberg et al., 1981).

1:8d Intracellular proteinases of E. coli

The first intracellular proteinase isolated and characterized from E. coli was protease I (Kowit et al., 1976). This proteinase has a molecular weight of 43000 D as determined by SDS-PAGE and sucrose density gradients. It is active in the pH range 6-8 and shows typical serine proteinase properties, i.e. it is inhibited by DFP, but not by metal chelators, sulphydryl inhibitors or metal ions. A second proteinase, Protease II, has also been isolated and characterized from E. coli (Pacaud, 1976). This proteinase has a molecular weight of 58000 D as determined by SDS-polyacrylamide gel electrophoresis and gel-filtration. It has a pH optimum of 8.0 and is also inhibited by DFP, the serine-proteinase inhibitor.

Since then Goldberg and his co-workers (Kowit et al., 1976; Pacaud, 1976; Goldberg et al., 1981) have identified eight intracellular proteolytic enzymes in E. coli. Six of these proteinases have been named according to the Sol-fa scale.

Protease Do was located in the cytoplasm with activity in the pH range 6-8.5. It has an apparent molecular weight of 320000-520000 D depending on the method of isolation. It is a serine proteinase which is not sensitive to metal chelators or

sulphydryl inhibitors.

Protease Re was found in both periplasm and cytoplasm and has a molecular weight of 82000 D as determined by SDS-polyacrylamide gel electrophoresis and gel-filtration. It is active in the pH range 7-8.5 with an optimum pH 8.0. It appears to be a serine proteinase but is also inhibited by EDTA, o-phenanthroline and TPCK.

Protease Mi was located in the periplasm. It has a molecular weight of 110000 D as determined by gel-filtration. It is a serine proteinase that is also inhibited by metal chelators but not by TPCK or TLCK.

Protease Fa was located in the cytoplasm and has a molecular weight of 110000 D. It is a serine proteinase that is also inhibited by EDTA and TPCK.

Protease So is a cytoplasmic enzyme of molecular weight 140000 D as determined by gel-filtration and 70000 D by SDS-polyacrylamide gel electrophoresis, indicating that this proteinase is a dimer of identical subunits. It is active over the pH range 6-8, but is maximal at 6.5. It is a serine proteinase that is also inhibited by TPCK but not metal chelators, sulphydryl inhibitors or TLCK.

Protease La is by far the best characterized bacterial intracellular proteolytic enzyme. It is a cytoplasmic enzyme (although some evidence suggests it is membrane-associated (Voellmy and Goldberg, 1981)) of molecular weight 450000 D as determined by gel-filtration and 94000 D by SDS-polyacrylamide gel electrophoresis. This enzyme is therefore a tetramer of equal subunits. It is a serine proteinase that is not sensitive to metal chelators or sulphydryl inhibitors. It is a proteinase that

requires ATP and Mg^{2+} for maximum proteolytic activity. The role of ATP in Protease La activity has received much attention. One way this has been studied is by observing the degradation of specific fluorogenic peptides in the presence of this nucleotide. The results of this work have eliminated previous models for the role of ATP in proteolysis by Protease La (Goldberg et al., 1985; Waxman and Goldberg, 1986). These peptides lacked amino acids that could be phosphorylated, they lacked amino acids that could be modified as in the analagous fashion to the covalent binding of ubiquitin in eukaryotic systems and they also lacked any secondary and tertiary structure and therefore do not require energy for unfolding. It was concluded that the ATP and Mg^{2+} altered the proteinase conformation and not the substrate. Furthermore, protein substrate (not any other proteins) appeared to stimulate ATP hydrolysis and activate Protease La activity. It was thought that these proteins activate by binding to the proteinase, without being degraded themselves. ATP and protein substrate then have additive effects in enhancing proteinase activity. These findings suggested two recognition sites on Protease La, (1) active site, specific for certain amino acids and (2) regulatory site, responds to unfolded proteins which activate the proteinase. Such a system may be important for proteolytic regulation and may explain why proteinases do not destroy critical cell components. A scheme for the mechanism of ATP-dependent proteolysis by Protease La has been proposed by Goldberg and his colleagues (Goldberg et al., 1985), fig. 1:5. The scheme implies that degradation of large proteins to peptides involves multiple rounds of the reaction cycle. This model is based on the following observations; (1) Proteins and ATP activate peptide

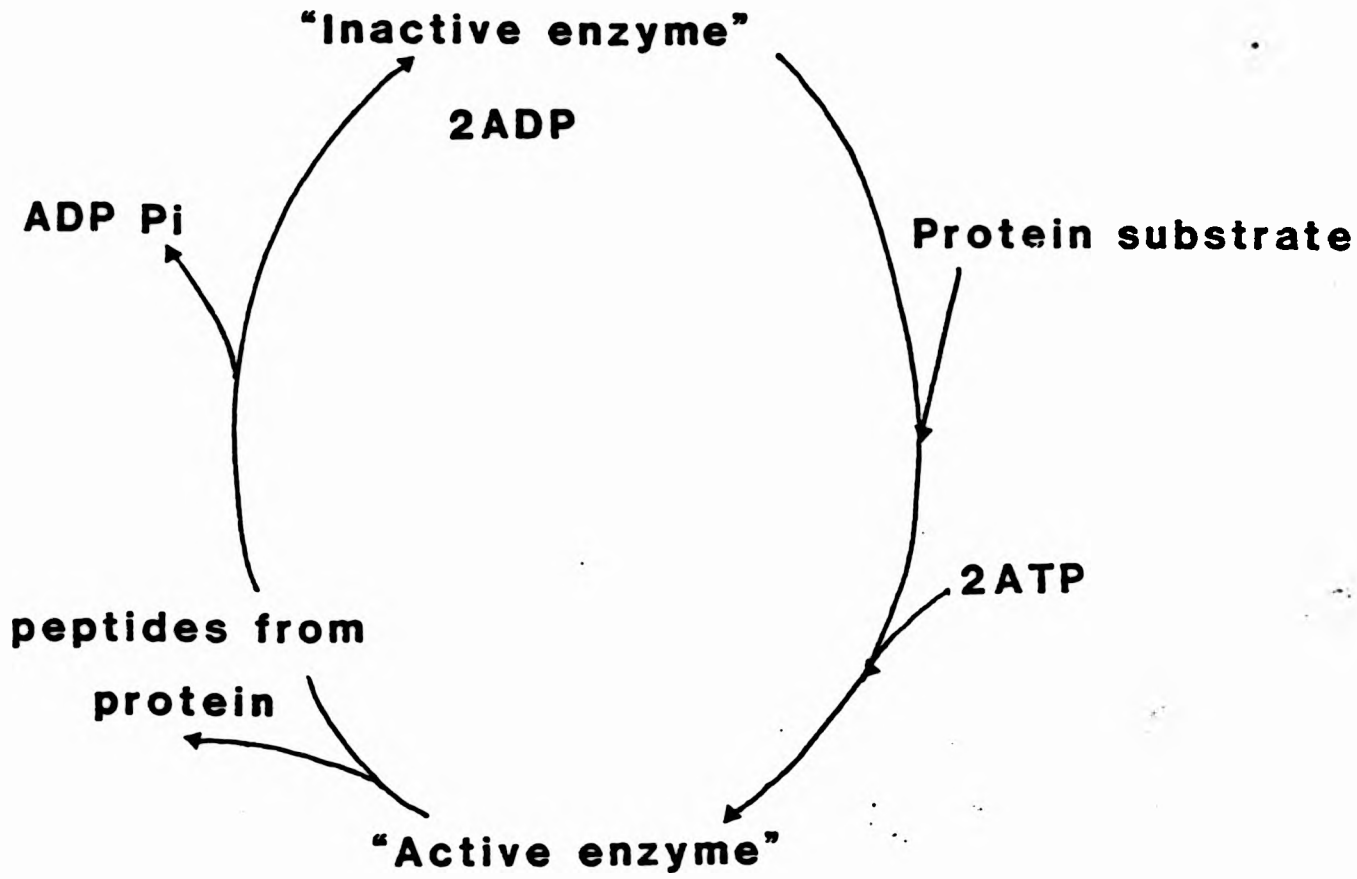


Fig. 1:5 A scheme for the mechanism of ATP-dependent proteolysis by Protease La. (From Goldberg et al., 1985)

hydrolysis allosterically; (2) ATP hydrolysis is not required for peptide bond cleavage; (3) ATP hydrolysis inhibits peptide bond cleavage and (4) 2 molecules of ATP are used for each peptide bond hydrolysed.

Two metalloproteinases have also been identified in E. coli (Goldberg et al., 1981). Protease Pi was located in the periplasm and has an optimum pH of 7.5 and a molecular weight of 110000 D as determined by gel-filtration. This proteinase is inhibited by EDTA, o-phenanthroline and DTT but not by serine proteinase inhibitors or sulphydryl inhibitors. This proteinase is identical to Protease III isolated independently by Cheng and Zipser (1979). It is also one of the proteinases in the complex of proteinases previously known as Protease A (Régnier and Thang, 1975).

The second metalloproteinase, Protease Ci, was located in the cytoplasm and has a pH optimum of 7.5. This proteinase has a molecular weight of 125000 D as determined by gel-filtration and is inhibited by o-phenanthroline but not EDTA. This proteolytic activity is stimulated 2-4 fold by Mn^{2+} and Co^{2+} and to a lesser extent by Mg^{2+} and Ca^{2+}.

1:8e Membrane-bound (associated) proteinases of E. coli

In addition to the increasing number of intracellular proteinases being isolated from E. coli, a small number of membrane-associated proteinases have also been described. Régnier and Thang (1973) first reported the existance of membrane associated proteinases after treatment of membrane fragments by sonication. Since this time two novel proteolytic enzymes known as Protease IV and V have been isolated and partially characterized (Pacaud,

1982a). Protease IV has a maximum activity at pH 7-7.2, while Protease V is maximally active at pH 7.9-8.1. Both are inhibited by serine hydrolase inhibitors, but only Protease V was inhibited by TLCK and TPCK. Another membrane bound proteinase, also known as Protease IV, has been isolated from E. coli (Régnier, 1981a & Régnier 1981b). This proteinase is inhibited by EDTA but not by DFP.

In addition to the endoproteolytic enzymes described above, two membrane-bound peptidases have also been isolated from E. coli (Pacaud, 1982b). Both these peptidases cleave leader (signal) peptides, are maximally active at pH 8.0 and are insensitive to serine proteinase inhibitors.

Table 1:4 summarizes characteristics of proteinases isolated from E. coli.

1:9 FUNCTIONAL ROLES OF BACTERIAL PROTEINASES

For most of the proteinases described thus far, clear physiological functions have not been established. The difficulties in establishing functions for many of these proteinases is most likely due to the inability to isolate mutants deficient in any one specific proteolytic enzyme. This is compounded by the ability of certain proteinase deficient mutants to use other proteolytic enzymes to replace the lost function (Miller et al., 1976). Another possible reason for lack of knowledge about proteinase functions is the fact that protein catabolism involves multiple mechanisms and as such imposes many problems when studying in vitro proteolysis (Mayer and Doherty, 1986).

In spite of the problems described, functions for several proteolytic enzymes from a variety of microorganisms (Reysset and

TABLE 1:4

Proteinases from E. coli

Proteinases	Stimulation	Inhibitors	Mol. weight	location
Protease I	-	DFP	43000	Soluble fraction
Protease II	-	DFP	58000	Soluble fraction
Protease Do	-	DFP	520000	Cytoplasm
Protease Re	-	DFP, EDTA, O-phen-anthroline	82000	Cytoplasm/ periplasm
Protease Mi	-	DFP, EDTA, o-phen-anthroline	110000	Periplasm
Protease Fa	-	DFP, EDTA, o-phen-anthroline, TPCK	110000	Cytoplasm
Protease So	-	DFP, TPCK	140000	Cytoplasm
Protease La	+	DFP, N-ethyl-maleimide, EDTA	450000	Cytoplasm/ membrane
Protease Pi (III)	-	EDTA, o-phen-throline	110000	Periplasm
Protease Ci	-	o-phenanthroline, p-hydroxymercuri-benzoate	125000	Cytoplasm
Protease IV*	-	DFP	-	Membrane (inner and outer)
Protease V	-	DFP, TLCK, TPCK	34000	Membrane (inner)
Protease IV#	-	DFP, EDTA	-	Membrane

*, # See text for references.

Millet, 1972; Cheng and Aronson, 1977; Switzer et al., 1975; Yoshimoto et al., 1980; Muro et al., 1985; Nara and Morioka, 1986) have been described. These functions include many important cellular processes, such as maturational processing of secretory and membrane proteins (Wickner, 1979), phage morphogenesis (Cavard and Lazdunski, 1979), breakdown of colicins (Miller, 1975) inactivation of regulatory proteins (Roberts et al., 1978), processing of spore coat precursors (Reysset and Millet, 1972; Cheng and Aronson, 1977), germination (Jenkinson and Lord, 1983), and inactivation of specific enzymes (Switzer et al., 1979; Nara and Morioka, 1986).

By far the best recognised and detailed roles for intracellular proteinases are those of the rec A gene product (Roberts et al., 1978) and lon gene product of E. coli (Etlinger et al., 1985; Chung and Goldberg, 1981; Goff and Goldberg, 1985; Waxman and Goldberg, 1986).

rec A gene product : the rec A gene product is a 40000 D monomeric protein that has been shown to have multiple enzyme activities. This protein is essential for genetic recombination (Walker, 1985) and for catalyzing the endoproteolytic inactivation of lamba phage-repressor during the lysogenic induction of the prophage (Roberts et al., 1978). The rec A protein is thought to cleave the repressor into two fragments, in the presence of ATP and a polynucleotide, preventing transcription from the critical early phage promoters (Roberts et al., 1978). The rec A protein also inactivates the lex A gene product in a similar reaction during the SOS response (Little et al., 1980). In contrast to many other endoproteinases this enzyme does not digest protein substrates commonly used to detect proteolysis such as [^{14}C]-globin (Goldberg

et al., 1981), .

lon gene product : the lon gene product is a soluble, ATP-dependent, proteolytic enzyme called Protease La (Chung and Goldberg, 1981). It was discovered that mutants in the lon gene (also cap R and deg) lead to a reduced capacity of the cells to degrade abnormal proteins in vivo (Chung and Goldberg, 1981). In addition cells containing multicopy plasmids carrying the genes for eukaryotic proteins, or cells treated with amino acid analogues, or antibiotics, all showed an increase in lon transcription (Goff and Goldberg, 1985). It appears therefore that Protease La is involved in the rapid degradation of abnormal and foreign proteins (Waxman and Goldberg, 1986).

The lon gene product has also been implicated in the response of E. coli to various stress conditions, including heat, antibiotic and alcohol shock of growing cells (Phillips et al., 1984; Neidhardt et al., 1984; Carr et al., 1985). It is now known that Protease La is infact one of 17 heat-shock proteins induced in response to stress-shock (Neidhardt et al., 1984).

Protease La is also thought to be involved in the response of E. coli to ultraviolet irradiation (Mayer and Doherty, 1986). Lon mutants showed a decreased ability to recover from ultraviolet irradiation as cells failed to divide. Cell septation is thought to be inhibited by the sul A gene product which is induced after ultraviolet irradiation. The fusion of the sul A gene in a lon mutant resulted in increasing the half-life of the sul A gene product, thus implicating Protease La in regulation of this cell process. So in addition to the breakdown of abnormal proteins Protease La is involved in the regulation of other important cell

processes by controlling the concentration of critical proteins (Maurizi, 1986; Maurizi, 1987; Torres-Cabassa and Gottesman, 1987).

PART 3

1:10 AIMS OF THE STUDY

The interference of protein-degradation systems with the expression of eukaryotic proteins in bacteria may present a major problem in developing the production of commercially important proteins using recombinant DNA technology. The instability of proteins produced from cloned genes in E. coli has been observed by a number of workers (Itakura et al., 1977; Goeddel et al, 1979a&b; Taniguchi et al., 1980; Talmadge and Gilbert, 1982). This project was designed to gain information on the proteolytic system of another bacterium which might be suitable for the production of eukaryotic proteins and peptides.

The obligate methylotroph M. methylotrophus, as discussed in Part 1 of the introduction, is an organism which grows well on cheap substrates and it has been demonstrated that eukaryotic cDNAs encoding chick ovalbumin, mouse dihydrofolate reductase and human interferon can be expressed in the bacteria (Hennam et al., 1982; De Maeyer et al., 1982). However little is known about the biochemistry of this organism and this project represented the first attempt to characterize its proteinases. It was anticipated that the results of this study would provide a useful backgound which may allow problems with protein degradation to be minimized. Since M. methylotrophus occupies a different environmental niche from other bacteria such as E. coli, whose proteolytic systems have been studied, the project may also shed light on the role of proteinases in the growth and metabolism of the bacterium.

Initially the project involved the screening of cell extracts for proteolytic enzyme activity using a range of substrates

(proteins, chromogenic peptide and amino acid derivatives) and assay conditions. Particular attention was paid to the localization of the activities within the cell (cytoplasmic, periplasmic and membrane bound) and to the possible presence of an ATP-dependent protein degradation system involving ATP-dependent proteinases, as has been demonstrated in E. coli (Sreedhara Swamy and Goldberg, 1982). Proteinases were fractionated and characterized using methods adapted from those used with other bacteria, particularly E. coli. Although many of the physical properties of the enzymes were determined, the most important aspects of the characterization were substrate specificity (including the in vitro activity on potential commercial products e.g. interferon) and the sensitivity of the enzymes to proteinase inhibitors. This also included attempts to isolate endogenous inhibitors. These inhibitors may provide a means of controlling protein degradation during commercial processes. The investigation also considered the effects of culture conditions (age of culture and changes in growth affected by temperature, alcohols and antibiotics) on the level of proteolytic enzymes since this could influence the conditions selected for commercial production. Attempts were made to isolate mutant strains, with altered levels of proteolytic enzymes, within which eukaryotic proteins might be more stable.

' Basic research is when I'm doing

what I don't know I'm doing '

Wernher Von Braun

CHAPTER TWO

2:1 MATERIALS AND METHODS

2:1a ORGANISMS AND CULTURE CONDITIONS

(i) Methylophilus methylotrophus.

Methylophilus methylotrophus AS1 (supplied by Dr.L.Hugh, I.C.I. Agricultural Division, Billingham) cells were grown in MM media, table 2:1, at 37°C with shaking at 120 rpm in an orbital incubator (Gallenkamp) (Windmass et al., 1980). A typical growth curve is shown in fig. 2:1. Cells were harvested (unless otherwise stated) at mid-log phase by centrifugation at 5000 g for 20 min at 4°C. Changes to growth conditions are detailed in the appropriate sections. Solid media were prepared by adding 7.5 g agar (1.5% (w/v)) to 500 ml distilled H_2O prior to autoclaving and addition of MM media.

(ii) Escherichia coli

Unless indicated Escherichia coli K12 cells were used. E. coli K12 cells (supplied by Dr.M.J.North, Department of Biological Science, Stirling) were grown in nutrient broth (Oxoid) (13 g/l) at 30°C with shaking at 120 rpm in a orbital incubator. A typical growth curve is shown in fig. 2:2. Cells were harvested at mid-log phase by centrifugation at 5000 g for 20 min at 4°C. Changes to growth conditions are detailed in the appropriate sections. Solid media were prepared by autoclaving nutrient agar (Oxoid) at 1.2% (w/v) in distilled H_2O.

TABLE 2:1

MM media constituents and preparation

Constituents

1. Phosphates (100 ml) : NaH_2PO_4 $2H_2O$ 7 g
   K_2HPO_4 9.5 g
   adjusted to pH 6.85 with K_2HPO_4 and autoclaved.

2. Sulphates (100 ml) : $(NH_4)_2SO_4$ 9 g
   $MgSO_4$ $7H_2O$ 1 g
   autoclaved

3. Ferric chloride (100 ml) : $FeCl_3$ 98mg
   $FeCl_3$ filter sterilized using a 0.45 µm filter unit (Millipore)

4. Trace elements (100 ml) : $CuSO_4$ $5H_2O$ 2 mg
   $MnSO_4$ $4H_2O$ 10 mg
   $Zn\ SO_4$ $7H_2O$ 10 mg
   $CaCO_3$ 180 mg
   each constituent was disolved separately in dH_2O with the exception of $CaCO_3$ which was dissolved with HCl (3.5 ml). Trace elements were filter sterilized using a 0.45 µm filter unit.

5. dH_2O : autoclaved.

Preparation

MM media (500 ml) was prepared as follows : phosphates (10 ml); sulphates (10 ml); $FeCl_3$ (0.5 ml); Trace elements (0.5 ml) and dH_2O (475 ml).

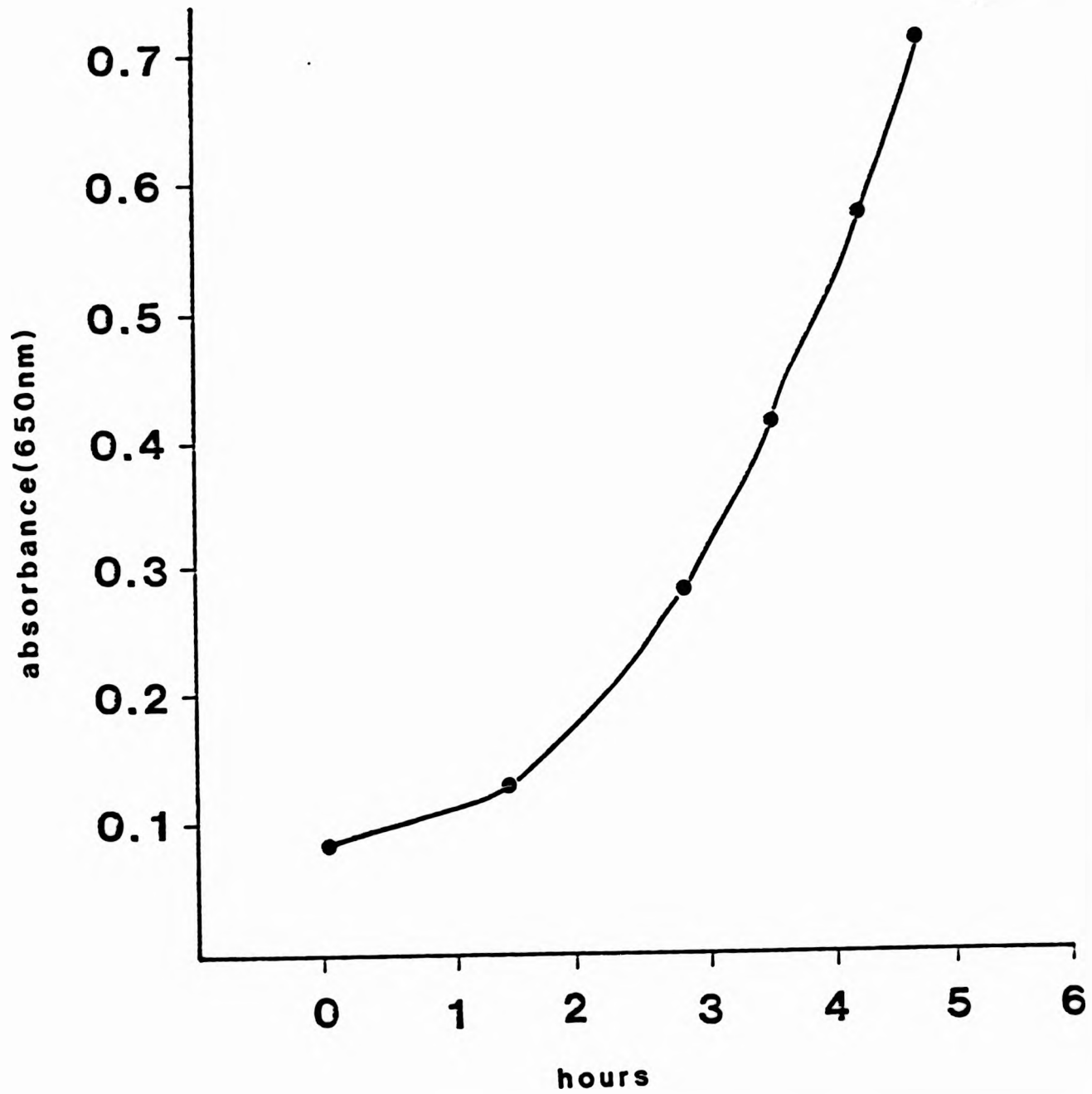


Fig. 2:1 Typical growth curve for M. methylotrophus AS1 cells.

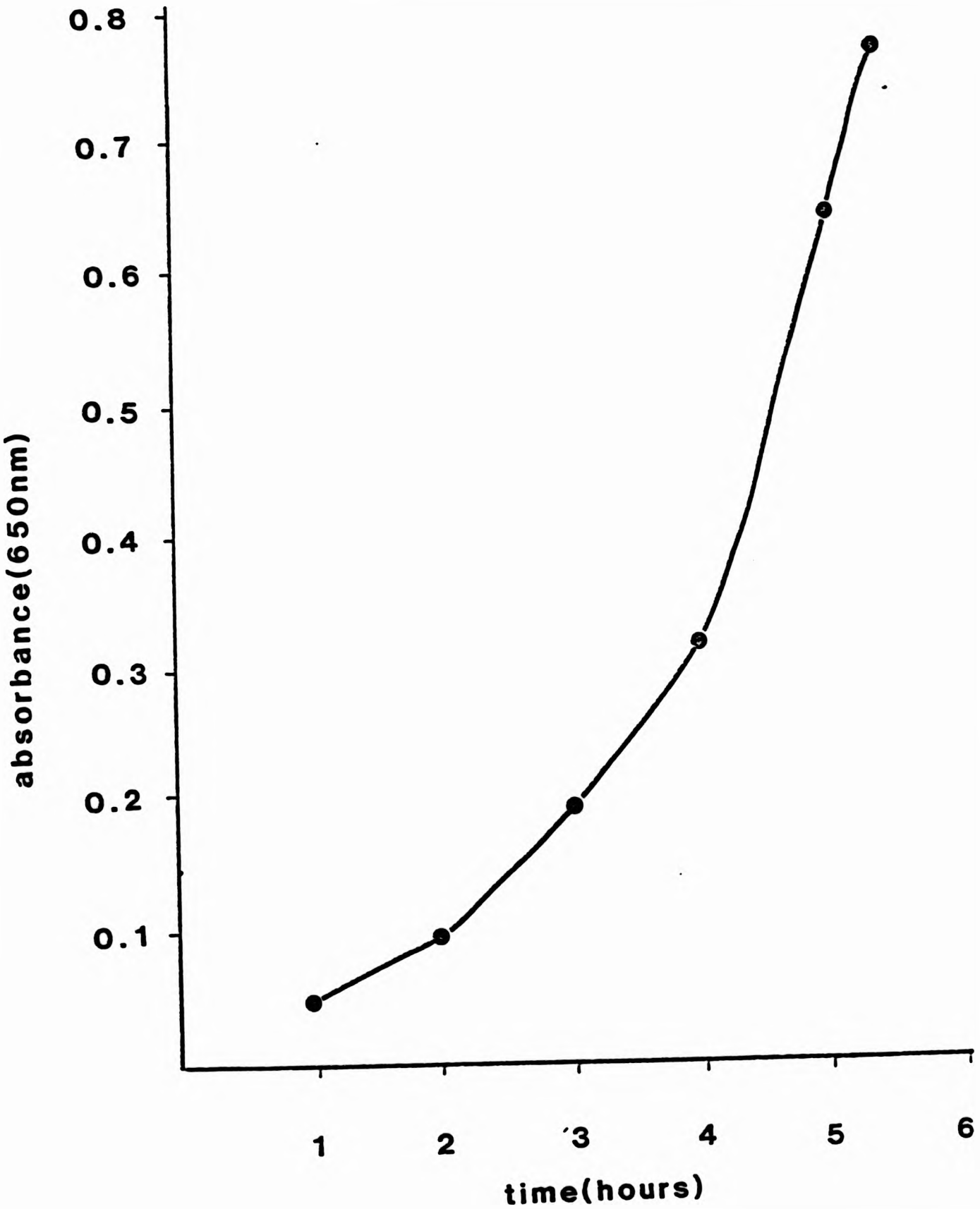


Fig. 2:2 Typical growth curve for E. coli K12 cells.

2:2 PREPARATION OF CELLULAR EXTRACTS

2:2a Whole cell extracts

(i) *M. methylotrophus* AS1 cells were washed several times by resuspension in ice-cold washing buffer (22 mM KH_2PO_4; 50 mM Na_2HPO_4; 85 mM NaCl and 0.1 mM $MgSO_4$) and centrifugation at 5000 g for 20 min at 4°C (Windmass et al., 1980). Cells were resuspended in washing buffer and sonicated (4 x 10s) on ice. Unbroken cells were removed by centrifugation at 11000 g for 20 min at 4°C. The whole cell extract was prepared by pelleting cell debris at 80000 g for 60 min at 4°C. Whole cell extracts were stored at -20°C when not being assayed.

(ii) *E. coli* K12 cells were washed by resuspension in ice-cold washing buffer (50 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 200 mM KCl) and centrifugation at 5000 g for 20 min at 4°C. Cells were again resuspended in washing buffer and sonicated (20 x 30s) on ice. Unbroken cells were removed by centrifugation at 11000 g for 20 min. The whole cell extract was prepared by pelleting cell debris at 130000 g for 60 min at 4°C. Whole cell extracts were stored at -20°C when not being used.

2:2b Subcellular fractions

Subcellular fractions of *M. methylotrophus* AS1 cells were prepared according to the method of Jones et al. (1982). Cells grown to mid-log phase were pelleted at 5000 g for 20 min at 4°C. Cells were then resuspended (5 mg dry weight/ml) in 20 mM Tricine, pH 7.5, 0.75M D-mannitol. Lysozyme (1 mg) was added to the whole cell suspension (8.5 ml) and agitated at 30°C for 12 min. 100 mM

EDTA was added in 10 x 0.1 ml aliquots at 1 min intervals and incubated for a further 5 min. 1M $MgCl_2$ (0.5 ml) and 1 mg of both Ribonuclease A and Deoxyribonuclease I (Sigma) were added and incubated for 2 min before cooling on ice. The suspension was then centrifuged at 15000 g for 15 min. The supernatant was pink-orange (periplasm) while the pellet of sphaeroplasts was paler than the original whole cell. The sphaeroplasts were resuspended in 20 mM Tricine, pH 7.5 (8.5 ml) and centrifuged at 150000 g for 60 min. This yielded a straw coloured supernatant (cytoplasm) and a red-brown pellet (membrane). The membranes were resuspended in 20 mM Tricine, pH 7.5, 0.75M D-mannitol. All subcellular fractions were stored at -20°C when not being assayed.

2:3 ASSAYS FOR PROTEOLYTIC ACTIVITY

2:3a Detection of proteolytic activity using hide powder azure (HPA)

HPA (10 mg/ml) was suspended in distilled H_2O and sonicated (20 x 15s) to produce a fine suspension. Assay mixtures routinely contained, HPA (0.5 ml), (20 mM Tris HCl pH 7.8, 10 mM $MgCl_2$) (0.5 ml) and sample (0.1 ml) (North, 1978). Incubations were carried out at 37°C (unless otherwise indicated) and reactions stopped by addition of ice-cold 50% (w/v) trichloroacetic acid (200 µl). Mixtures were left at 4°C for 30 min. Supernatants containing trichloroacetic acid-soluble products were obtained by centrifugation at 5000 g in a bench centrifuge (MSE microcentaur). The absorbance at 595 nm of the supernatants were then measured in an Ultraspec 4050 (LKB). Under these conditions an increase in absorbance of 1.0 was equivalent to the hydrolysis of 3.4 mg HPA (North, 1978). Specific activities were expressed as mg HPA

hydrolyzed/min/mg protein.

2:3b Detection of proteolytic activity using azocasein

Azocasein was prepared in distilled H_2O (10 mg/ml). Assay mixtures and conditions were as described for HPA. The absorbance of the trichloroacetic acid-soluble products were measured at 366 nm (Coombs, 1982). Specific activities were expressed as absorbance units/min/mg protein.

2:3c Detection of peptidase and proteinase activities using peptide nitroanilides

1 mM solutions of peptide nitroanilides were prepared in distilled water or dimethylformamide (DMF) depending on their solubilities. Assay mixtures routinely contained, 10 mM Tris-HCl pH 7.8, 5 mM $MgCl_2$ (0.85 ml), peptide nitroanilide (0.05 ml) and sample (0.1 ml) (North and Whyte, 1984). Incubations were carried out at 37°C and reactions stopped by cooling on ice. Absorbances were read at 405 nm and converted to nmol 4-nitroaniline released using a standard curve of 4-nitroaniline concentrations versus absorbance at 405 nm (fig. 2:3). Specific activities were expressed as nmol 4-nitroaniline released/min/mg protein. When necessary aminopeptidase M (4 μg) was added to assay mixtures (North and Whyte, 1984).

2:3d Detection of aminopeptidase activities

Aminopeptidase activity was detected by measuring the hydrolysis of Leu NA (North and Whyte, 1984). The assay mixtures contained, 10 mM Tris-HCl pH 7.8, 5 mM $MgCl_2$ (0.85 ml), 1 mM Leu

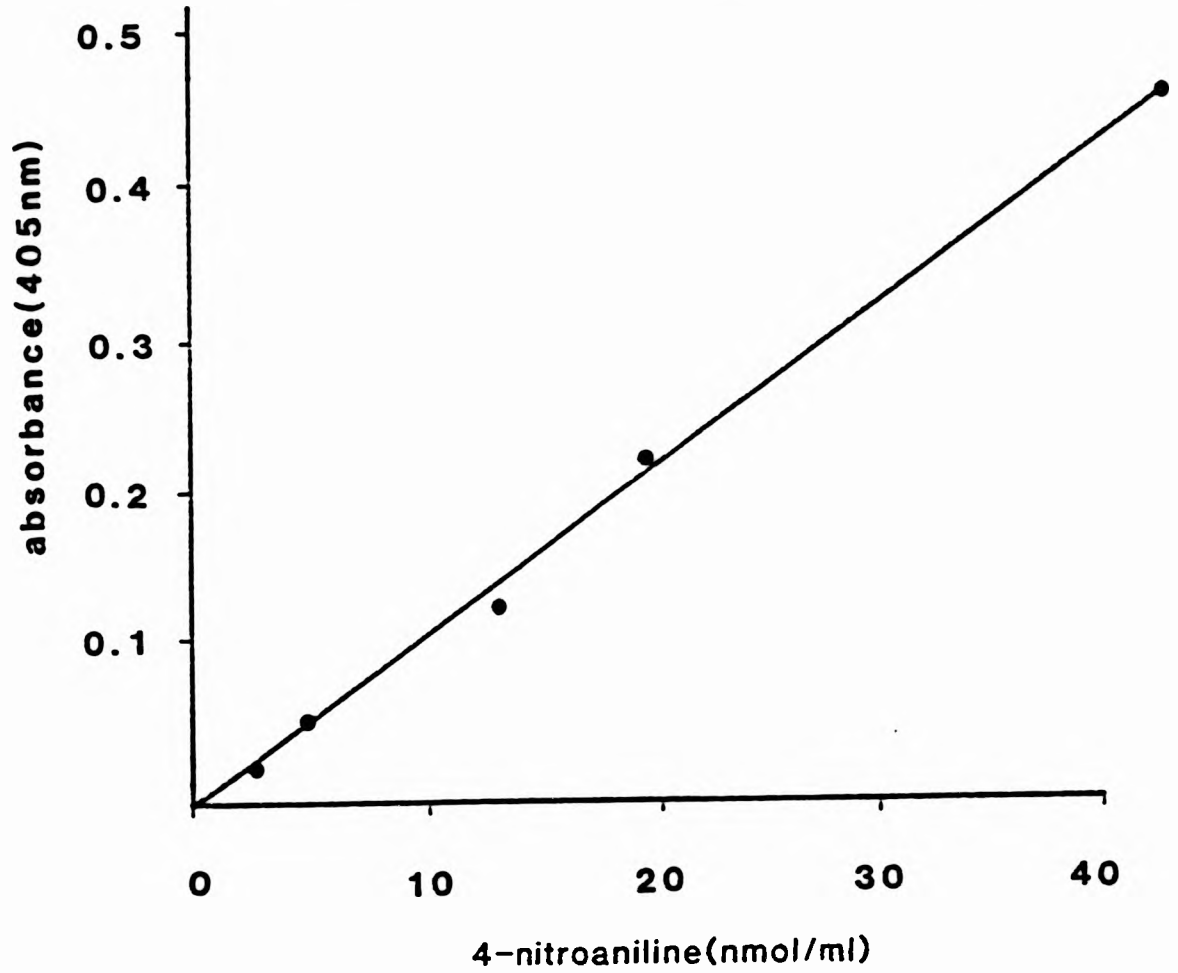


Fig. 2:3 Standard curve for conversion of A_{405} values to nmoles of 4-nitroaniline released/ml.

NA (0.05 ml) and sample (0.1 ml). Incubations were at 37°C and reactions stopped by cooling on ice. The release of 4-nitroaniline was measured spectrophotometrically at 405 nm.

2:3e Microtitre assay procedure for detecting peptide-nitroanilide hydrolysis

For the routine assay of large numbers of samples, assays were performed in the wells of a microtitre plate. Assay mixtures contained, 50 mM Tris-HCl pH 7.8, 25 mM $MgCl_2$ (0.1 ml), 1 mM peptide nitroanilide (0.03 ml) and sample (0.1 ml). Plates were then sealed with a transparent cover and incubated at 37°C. Hydrolysis of peptide nitroanilides was detected by observation of yellow colour.

2:3f Detection of proteolytic activity using radiolabelled substrates

Proteolytic activity was measured by following the degradation of [^{14}C]-methyl haemoglobin (10^6 cpm/ml), [^{14}C]-α_1-casein (10^6 cpm/ml) and [^{35}S]-α_2-interferon (9×10^5 cpm/ml) to products soluble in 5 % w/v trichloroacetic acid (Goldberg et al., 1981). Incubation mixtures contained, 10 mM Tris-HCl pH 7.8, 5 mM $MgCl_2$ (0.4 ml), sample (0.1 ml) and [^{14}C]-methyl hemoglobin (0.005 ml), [^{14}C]-α_1-casein (0.005 ml) or [^{35}S]-α_2-interferon (0.02 ml). Assays were performed at 37°C and reactions stopped by the addition of 50% w/v trichloroacetic acid (0.06 ml) and 1.2 mg bovine serum albumin. The assay mixtures were kept on ice for 30 min. Samples were centrifuged at 5000 g for 5 min and 0.4 ml of the acid-soluble products were removed and counted in 10 ml of scintillation cocktail (Methanol (100 ml), Toluene (600 ml), Triton

X-100 (300 ml) and Butyl-BPD (6.3 g)).

2:4 PREPARATION OF RADIOLABELLED SUBSTRATES

2:4a Conversion of oxyhaemoglobin to [^{14}C]-globin

(i) Production of methaemoglobin by oxidation of oxyhaemoglobin using $K_3Fe(CN)_6$

Globin has previously been observed to be a much better substrate for proteolysis than haemoglobin (Goldberg et al., 1981). In order to extract the haem moiety from haemoglobin the iron must first be converted to the ferric state with ferricyanide (Austin and Drabkin, 1935). $K_3Fe(CN)_6$ was reacted with oxyhaemoglobin (bovine) in a molar ratio of 8.16:1. The reaction conditions were as follows: $K_3Fe(CN)_6$ (8 mg) was incubated with haemoglobin (0.2 g) in H_2O (10 ml) for 5 min and then dialyzed against 3 x 2 litre changes of distilled water to remove salts.

(ii) Extraction of the haem moiety from methaemoglobin

The haem and protein components of methaemoglobin were separated by an acid-methylethylketone two phase procedure (Teale, 1959). Methaemoglobin was adjusted to pH 2.0 with 0.1M HCl before adding ice-cold methylethylketone (10 ml). The immiscible solution was then shaken in a separating flask and allowed to separate in the cold. Haem was isolated in the upper ketonic layer while the globin was removed in the lower aqueous phase. The globin solution was extensively dialyzed against distilled water to remove dissolved ketone. Powdered globin was prepared by shell-freeze drying the dialyzed protein solution at -40°C.

(iii) Labelling globin with [^{14}C]-HCHO

Globin (1 mg) was dissolved in sodium borate buffer pH 9.0 (1 ml) (Goldberg et al., 1981). After dialysis against the same buffer the globin solution (1 mg/ml) was cooled on ice and 100 µl of [^{14}C]-HCHO (85 mCi/mmol) added. After 30s, four 200 µl aliquots of sodium borohydride (5 mg/ml) were added followed by a further 100 µl of sodium borohydride after 1 min. Low molecular weight components were removed by extensive dialysis against dH_2O. Dialysis was continued until the dialyzate recorded background level counts. The dialyzed suspension was then shell freeze-dried at -40°C. The solid [^{14}C]-globin was then dissolved in sodium borate buffer pH 9.0 ready to be used as a substrate for proteolysis.

2:4b Preparation of [^{14}C]-α_1-casein

α_1-casein was kindly supplied by Phillipe Régnier (Institut de Biologie Physico Chimique, Paris). The labelling procedure for α_1-casein with [^{14}C]-HCHO was as described for globin.

2:4c Preparation of [^{35}S]-α_2-Interferon (α_2-IFN)

(i) Intrinsic labelling of p-encoded α_2-IFN with [^{35}S]-methionine

E. coli JA221 (pl205) cells containing the α_2-IFN plasmid (pl205) (kindly supplied by Dr. R Hockney, ICI Pharmaceuticals Division, Alderley Park) were subcultured in M9 minimal media (per litre : Na_2HPO_4, 6 g, K_2HPO_4, 3 g, NaCl, 0.5 g, NH_4Cl, 1 g pH 7.0) supplemented with thiamine (4 mg/ml), tryptophan (100 mg/ml), leucine (20 mg/ml) and ampicillin (0.1 mg/ml) and grown

overnight at 37°C. The overnight culture (0.75 ml) was then used to subculture the supplemented M9 medium (75 ml) and grown at 37°C, with shaking at 3000 rpm, up to an A550 of 1.0. Chloramphenicol was then added (0.478 ml : 34 mg/ml in ethanol) and incubated at 37°C overnight with shaking at 3000 rpm. The addition of chloramphenicol to the cell culture inhibits protein synthesis preventing the initiation of new rounds of chromosome replication. However replication of the plasmid coding for α_2-interferon is not affected resulting in a considerable degree of amplification of the plasmid relative to the chromosome.

A 25 ml aliquot of the overnight culture was centifuged at 5000 g for 5 min at 4°C. The cells were then washed with 2 x 25 ml of labelling medium (M9 media, thiamine (4 mg/ml) and 10% DMAM). This medium supplies all nutrients except methionine which is added in the form of [^{35}S]-methionine. The washed pellet was then resuspended in 5 ml of prewarmed (22°C) labelling medium and 2 mCi [^{35}S]-methionine added. It was also important that the [^{35}S]-methionine was also prewarmed in order to facilitate fast incorporation into the cells. Incubation was continued at 22°C with gentle agitation until [^{35}S]-methionine incorporation was complete. [^{35}S]-methionine corporation was monitored as follows: Aliquots (25 μl) of culture media were removed at 10 min intervals. The cells were centrifuged on a bench centrifuge and 10 μl aliquots of supernatant removed. The aliquots were diluted 1000 fold with dH_2O and 10 μl removed and counted in 5 ml of scintillation cocktail. As [^{35}S]-methionine is incorporated into the cells it is expected that the counts in the supernatant will decrease and maintain a minimal count as shown in the theoretical curve Fig.

2:4. The actual incorporation curve was found to be different Fig. 2:4, although the amount of incorporation was still the same (personal communication from Dr R. Hockney).

After of [^{35}S]-methionine incorporation the cells were spun down at 10000 rpm in an SS34 Sorval rotor for 5 min at 4^{o}C and then stored at -20^{o}C for several hours.

The cells were then resuspended in 62.5 mM Tris-HCl, pH 6.8, 1% SDS (1.25 ml) and left at RT for 1 hour with gentle agitation. The cell suspension was then diluted 20-fold with PBS and centrifuged at 40000 rpm in a Beckman 50.2 Ti rotor for 30 min at 4^{o}C. The supernatant was then diluted 2-fold prior to loading onto an NK2-sepharose column.

(ii) Purification of [^{35}S]- α_2-IFN on NK2-Sepharose

A Gilson tip (1000 μl) was treated with blocking solution (PBS, 0.5% w/v BSA, 0.1% w/v NaN_3) overnight at 22^{o}C. The tip was then washed with dH_2O and plugged with glass wool before adding NK2-sepharose (supplied by Dr.R.Hockney). NK2-sepharose has the monoclonal antibody to α_2-IFN bound to the sepharose. The column was blocked with blocking solution (500 μl) followed by the elution of the blocking solution with PBS (5 ml) at 15 ml/hr. The column was then washed with 2 x 1 ml aliquots of citrate buffer (per 100 ml : 0.5M citric acid, pH 2.0, 1.5M NaCl) followed by PBS (5 ml). At this point the column was ready for use.

The supernatant (50 ml) was loaded at 15 ml/hr and then the column washed with 10 ml PBS in order to elute contaminating protein. The PBS was then washed through with citrate buffer (200 μl) and 100 μl fractions collected as the eluent turned acidic

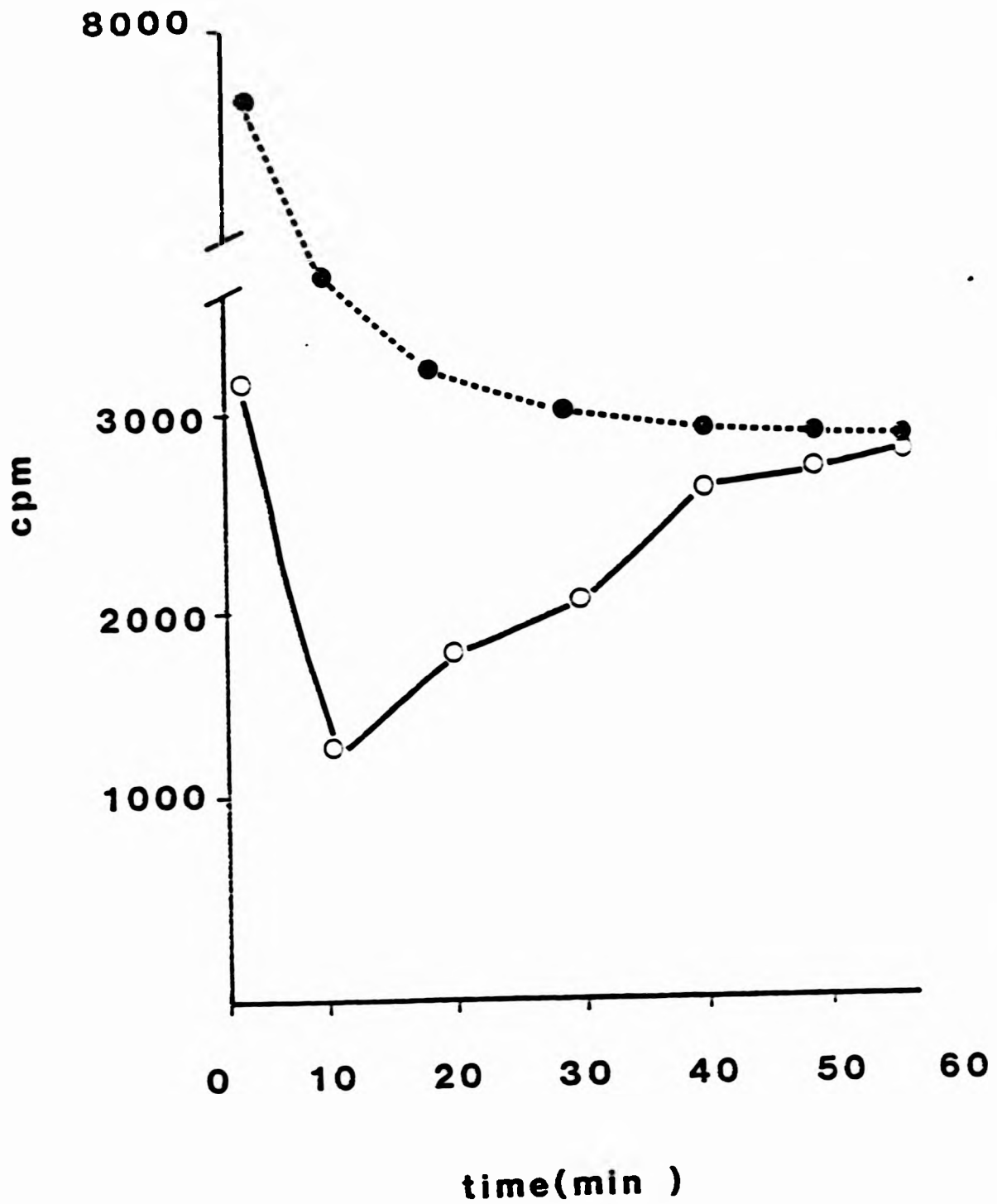


Fig. 2:4 [^{35}S]-methionine incorporation into E. coli JA221 (P 1205) cells. Theoretical curve (●---●), actual curve (○—○).

(indicated with litmus paper). The eluted fractions were immediately neutralized with 100 µl of neutralizing solution (0.25M NaH_2PO_4 (16 ml), 0.25M Na_2HPO_4 (84 ml), pH 7.5). Neutralized samples (10 µl dil 1:1000) were counted in 5 ml of scintillation cocktail. Lysates and all wash throughs were also measured in this way. Results of the purification procedures are shown in table 2:2.

The eluted fractions were run on SDS-polyacrylamide gels and autoradiographs produced (Plate 2:1). The autoradiographs indicated that [^{35}S]-α_2-IFN was eluted in the first 3 citrate wash fractions. The eluted fractions were pooled and used for proteolysis assays.

2:5a Molecular exclusion (gel filtration)

Chromatography was carried out using the following columns; Sephadex G75 (430 x 26 mm), Sephadex G200 (150 x 26 mm) and Sephacryl S300 (100 x 26 mm). Columns were equilibrated with 10 mM Tris-HCl, pH 7.8, 5 mM $MgCl_2$. Void volumes were determined using 0.2 ml blue dextran 2000 (mol wt ~ 2 x 10^6) at a concentration of 4 mg/ml. Calibration of gel filtration columns was achieved using the appropriate molecular weight standards; cytochrome C (12,500 D); bovine serum albumin (68,000 D); ovalbumin (45,000 D), aldolase (158,000 D), catalase (240,000 D) and ferritin (450,000 D).

2:5b Ion-exchange chromatography

(i) DEAE-cellulose (DE52) columns (115 x 52 mm) equilibrated with 10 mM Tris-HCl, pH 7.8, 5 mM $MgCl_2$. Proteins were eluted with a 0-0.2M NaCl linear gradient in running buffer (see

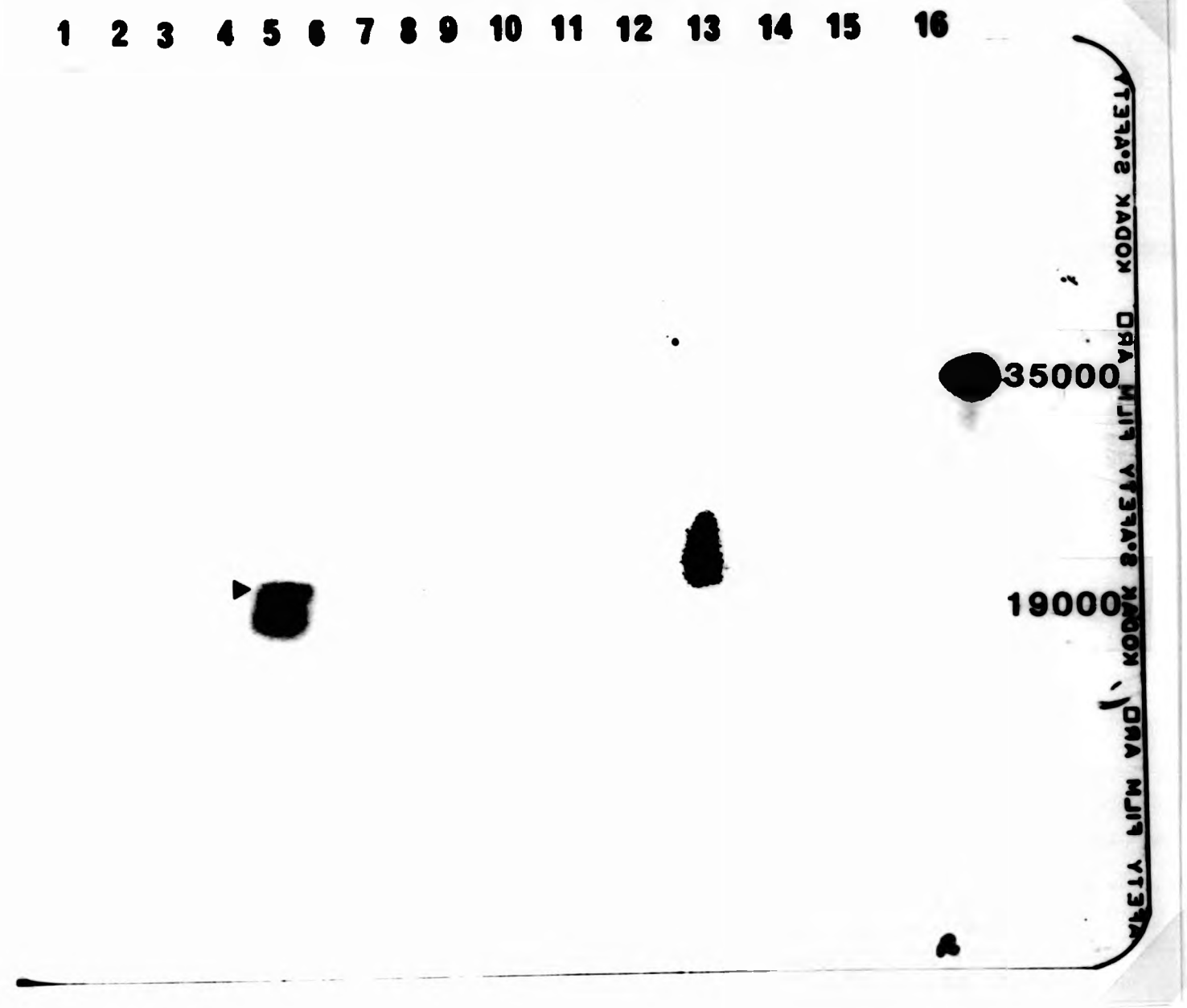


Plate 2:1 Autoradiogram showing the elution of [^{35}S]-IFN from an NK2-Sepharose column. Lane 2, PBS wash; lane 3, citrate wash; lanes 4-15, fractions eluted with citrate; lane 16, [^{14}C]-α_1-casein. Lanes 4, 5 and 6 show eluted [^{35}S]-IFN indicated with an arrow.

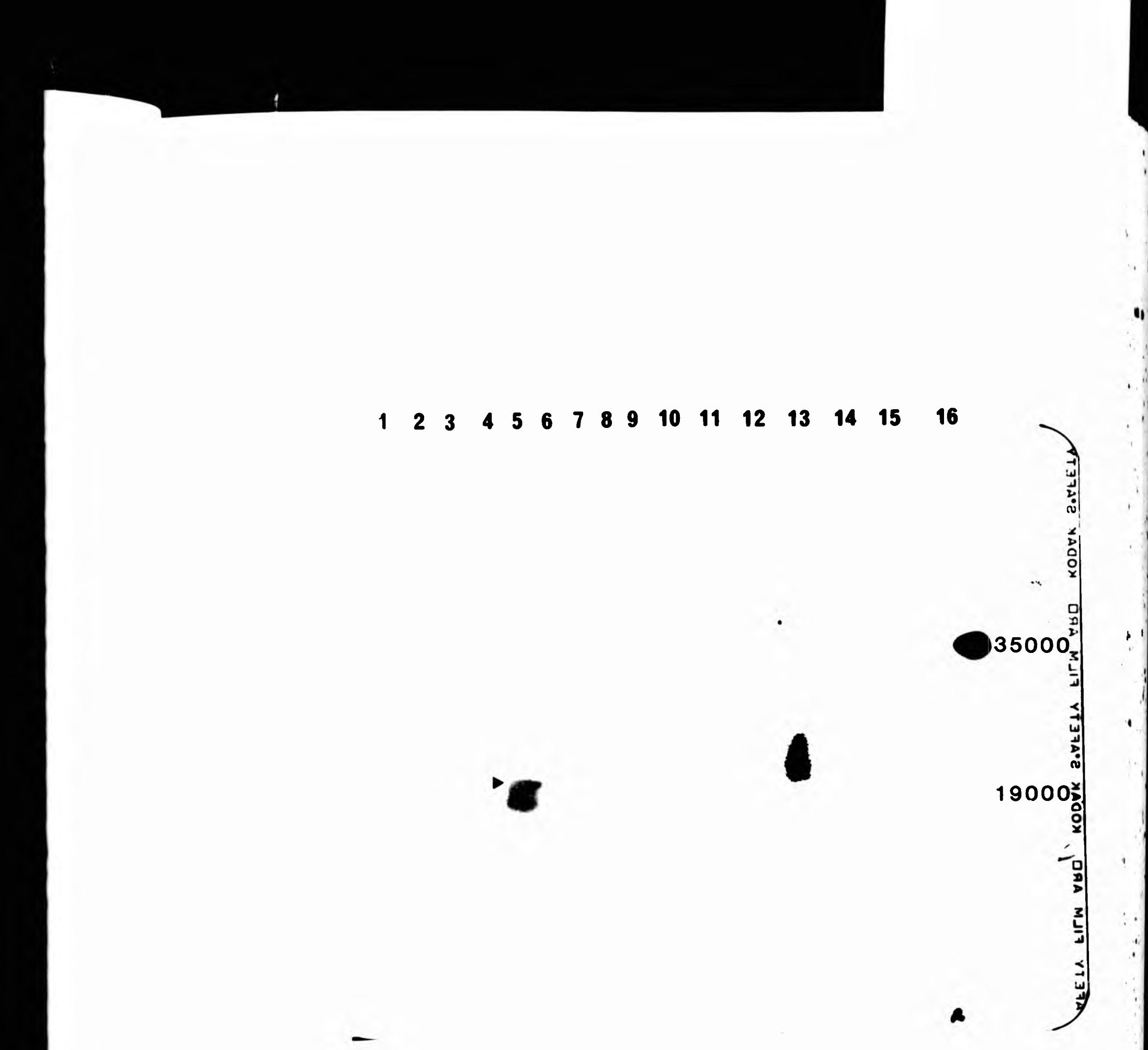


Plate 2:1 Autoradiogram showing the elution of [^{35}S]-IFN from an NK2-Sepharose column. Lane 2, PBS wash; lane 3, citrate wash; lanes 4-15, fractions eluted with citrate; lane 16, [^{14}C]-α_1-casein. Lanes 4, 5 and 6 show eluted [^{35}S]-IFN indicated with an arrow.

TABLE 2:2

Purification of [^{35}S] -α_2-Interferon on an NK2-Sepharose column

Sample	cpm	Total cpm
25 ml lysate	8950	2.2×10^9
50 ml lysate	4118	2.1×10^9
straight through	4232	2.0×10^9
PBS wash	484	4.8×10^7
Citrate wash	31	3×10^4
Eluent 1	90	9×10^4
2	78	7.8×10^4
3	48	4.8×10^4

see text for an explanation of samples.

equilibration buffer).

(ii) CM-cellulose (CM52) columns (30 x 25 mm) equilibrated with 10 mM Na-acetate, pH 5.5. Proteins were eluted with a 0-0.2M NaCl linear gradient in running buffer (see equilibration buffer).

(iii) DEAE-sephacel column (115 x 52 mm) equilibrated with 10 mM Tris-HCl, pH 7.8, 5 mM $MgCl_2$. Proteins were eluted with a 0-0.2M NaCl linear gradient in running buffer (see equilibration buffer).

2:5c Affinity chromatography

A trypsin-Sepharose affinity column (3.5 ml) was prepared as directed in the Pharmacia Fine Chemicals catalogue (1986). CNBr-activated Sepharose (1 g) was swollen in 1 mM HCl (200 ml) for 15 min and then loaded onto a sintered glass filter and washed with the acid. Trypsin (35 mg) was dissolved in 7 ml coupling buffer (0.1M $NaHCO_3$, pH 8.3, 0.5M NaCl) and added to Sepharose prewashed with 5 ml coupling buffer. The trypsin/Sepharose mixture was stirred overnight at 4°C. The trypsin-Sepharose suspension was then transferred to a solution containing a blocking agent (0.2M glycine, pH 8.0) for 2 hours at 4°C. The column was then washed 5 times alternatively with 10 ml of coupling buffer and 10 ml of acetate buffer (0.1M Na-acetate, pH 4.0, 0.5M NaCl). The trypsin-Sepharose affinity column was stored in coupling buffer at 4°C. Before use the column was equilibrated with 10 vols of 10 mM Tris-HCl, pH 7.8. The protein inhibitor solution was then loaded onto the column and inhibitors eluted with a 0-1.0M NaCl stepwise gradient in running buffer.

2:6 SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

2:6a SDS-polyacrylamide gels

Solutions used in the preparation of polyacrylamide gels are shown in table 2:3 (Neville, 1971). Samples [~ 1 µg protein per band] were prepared for electrophoresis by mixing with an equal volume of solubilization-reduction mix. Electrophoresis was carried out at 6 mA/slab (120 x 120 x 1.5 mm) through the stacking gel, as determined by the position of the bromophenol blue tracking dye, and 32-40 mA/slab through the separating gel until the bromophenol blue was 1 cm from the bottom of the gel. Electrophoresis was toward the anode. Polyacrylamide gels were stained as follows: (1) 2 hours in stain A, (2) 1 hour in stain B and (3) left in stain C until all the background was removed (Table 2:4).

2:6b Linear gradient SDS-polyacrylamide gels

Solutions used in the preparation of linear gradient SDS-polyacrylamide gels are shown in table 2:5 (Hames, 1981). Separating gels were prepared by the gradient mixing of 8% and 16% polyacrylamide mixtures. The stacking gel was prepared as in section 2:6a. Electrophoresis was carried out at 6 mA/slab through the stacking gel and 32 mA/slab through the separating gel.

2:6c SDS-polyacrylamide gels containing gelatin

This method was adapted from that of Heussen and Dowdle (1980). Gelatin gels were prepared as described for SDS-polyacrylamide gels (section 2:6a) with the following differences: the separating gel contained 8.9 ml dH_2O with 3 ml gelatin solution (1 g%). Electrophoresis was as described

TABLE 2:3

Solutions for SDS-polyacrylamide gels

1. 4 x Lower gel buffer (pH 9.18)

0.123M HCl
0.698M Tris
0.28M NaCl

2. 4 x Upper gel buffer (pH 6.1)

0.1068M H_2SO_4
0.216M Tris

3. 10 x Reservoir buffer (pH 8.64)

0.40M Boric Acid
0.41M Tris
1.0% (w/v) SDS

4. Stock acrylamide-stacking gel

30% acrylamide : 2% bisacrylamide

5. Stock acrylamide-separating gel

42% acrylamide : 0.36% bisacrylamide

6. Solubilization Reduction Mix

	1:1
SDS (w/v)	4%
β-mercaptoethanol (v/v)	10%
Upper gel buffer	2x
glycerol (v/v)	20%

7. Separating gel 14%

lower gel buffer (4x)	7.5 ml
42% acrylamide : 0.36% bisacrylamide	10.0 ml
water	11.9 ml
TEMED	42 µl
1% ammonium persulphate	0.6 ml

8. Stacking gel 3%

Upper gel buffer (4x)	2.5 ml
30% acrylamide : 2% bisacrylamide	1.0 ml
water	6.25 ml
TEMED	20 µl
1% ammonium persulphate	0.24 ml

TABLE 2:4

Protein staining and destaining solutions

Stain	Isopropanol (%)	Glacial Acetic Acid (%)	Coomassie Blue (%)
A	25	10	0.03
B	10	10	0.003
C	-	10	-

TABLE 2:5

Solutions for Linear gradient SDS-polyacrylamide gels

Stock solutions

1. 30/0.8 acrylamide : 30 g acrylamide, 0.8 g bisacrylamide/100 ml.
2. Tris-HCl pH 8.9 : 36.3 g Tris in 100 ml, adjusted to pH 8.9 with c. HCl.
3. 20% SDS : 2 g SDS in 10 ml.
4. 2.5M sucrose : 8.56 g sucrose in 10 ml.
5. 5 x electrophoresis buffer : 30 g Tris, 144 g glycine and 5 g SDS in 1 litre.

Lower gel preparation

	8%	16%
30/0.8 acrylamide	4 ml	8 ml
Tris-HCl pH 8.9	1.92 ml	1.92 ml
20% SDS	76 µl	76 µl
TEMED	8 µl	8 µl
2.5M sucrose	-	1 ml
H_2O	9 ml	4 ml

Polymerize each percentage gel with 40 µl ammonium persulphate (10% w/v).

previously. After the gels were run, SDS was removed by dialysis for 1 hr against 2.5% Triton X-100 (1 litre). Dialysis was followed by incubation of the gels in 0.1M Tris-HCl, pH 7.8, 20 mM $MgCl_2$ at 37°C. Gels were than stained and destained as described in section 2:6a. Proteinase activities towards gelatin were visualised as clear bands against a blue background.

Linear gradient gels containing gelatin were prepared as described in section 2:6b, except for the following differences: the 8% polyacrylamide mixture contained 7.5 ml distilled H_2O and 1.5 ml gelatin (1 g%) and the 16% polyacylamide mixture contained 2.5 ml distilled H_2O and 1.5 ml gelatin (1 g%). Proteinases were visualised as described above.

2:6d Detection of inhibitors using SDS-polyacrylamide gels incorporating gelatin

Gelatin gels were prepared and run as described in section 2:6c. After dialysis with Triton X-100 they were incubated at 37°C in the presence of various proteinase solutions according to the method of Pellegrini et al. (1984). Gels were stained and destained as described in section 2:6a. Inhibitors would have been visualised as dark blue bands against a light background.

2:6e Preparative SDS-polyacrylamide gel electrophoresis

A 1.5 cm stacking gel (3%) minus well former, was loaded onto an SDS-polyacrylamide gel prepared as described in section 2:6a or 2:6b. Extracts (1.2 ml) were prepared in 1-2 ml of solubilization mix and run as described in section 2:6a. Gels were then dialyzed for 1 hour against Triton X-100 and sliced into 2-5 mm horizontal

slices.

The horizontal slices were mashed and then incubated overnight in 10 ml of 50 mM Tris-HCl, pH 7.8, 25 mM $MgCl_2$ at 25°C. The supernatants from the gel slices were then used in assaying for proteolysis.

2:7 FLUOROGRAPHY

Autoradiograms of SDS-polyacrylamide gels containing radiolabelled proteins were prepared as follows (New England Nuclear Autoradiography brochure, 1986).

2:7a Impregnation with EN^{3}HANCE

10% (v/v) glacial acetic acid was removed from destained gels and replaced with EN^{3}HANCE. Gels were then gently agitated for 1 hour at room temperature.

2:7b Precipitation

Following impregnation the EN^{3}HANCE was discarded and an excess of cold water added to precipitate the fluorescent material inside the gel. After 30 min the opaque gel was ready for drying.

2:7c Drying and exposing

After precipitation was complete the gels were placed on 2 layers of 3 mm Whatman filter paper and dried under heat (60-70°C) and vacuum on a gel drying apparatus (Biorad) for 3 hours. Dry gels were then placed against a high speed blue-sensitive X-ray film (XOMAT, Kodak) in a cassette (Okamoto 18 x 24 cm) and then left to expose for several days (dependent on the isotope) at -70°C.

2:7d Development of films

Exposed film was developed for 3-5 min at 18°C in developer (Technol) diluted 1:18. The film was then rinsed with water and fixed for 5 mins with fixer (Kodak FX-40) diluted 1:4. Fixed films were then thoroughly washed with water and left to dry at room temperature.

2:8 PROTEIN ASSAYS

Protein concentrations were estimated by measurement of the change in absorption spectra when proteins bind Coomassie Brilliant Blue G-250 according to the method of Sedmak & Grossberg (1977). Bovine serum albumin was used to produce a standard curve (Fig. 2:5). The limitations of this procedure are as follows: (1) Coomassie Brilliant Blue G-250 only interacts with polypeptides and proteins greater than 3000D; (2) the measurement of protein depends on the interaction of the dye-anion with the NH_3^+ groups of proteins and since the proportion of these groups on proteins varies this method must be classed as a protein estimation. This problem is enhanced due to the fact that G-250 also reacts differently to NH_3^+ from different amino acids; (3) the dose response curve is not linear when plotted arithmetically, however it is rectilinear from 0.5-50 μg protein when the absorbance ratio at 620 nm and 465 nm is plotted.

The advantages of this procedure are as follows: (1) it is simple and reproducible; (2) the dye has a long shelf life; (3) it has good sensitivity, detecting as little as 1 μg of protein (cf. 5-10 μg by the Lowry method of protein detection); (4) this procedure can be used for small and large volumes; (5) it does not

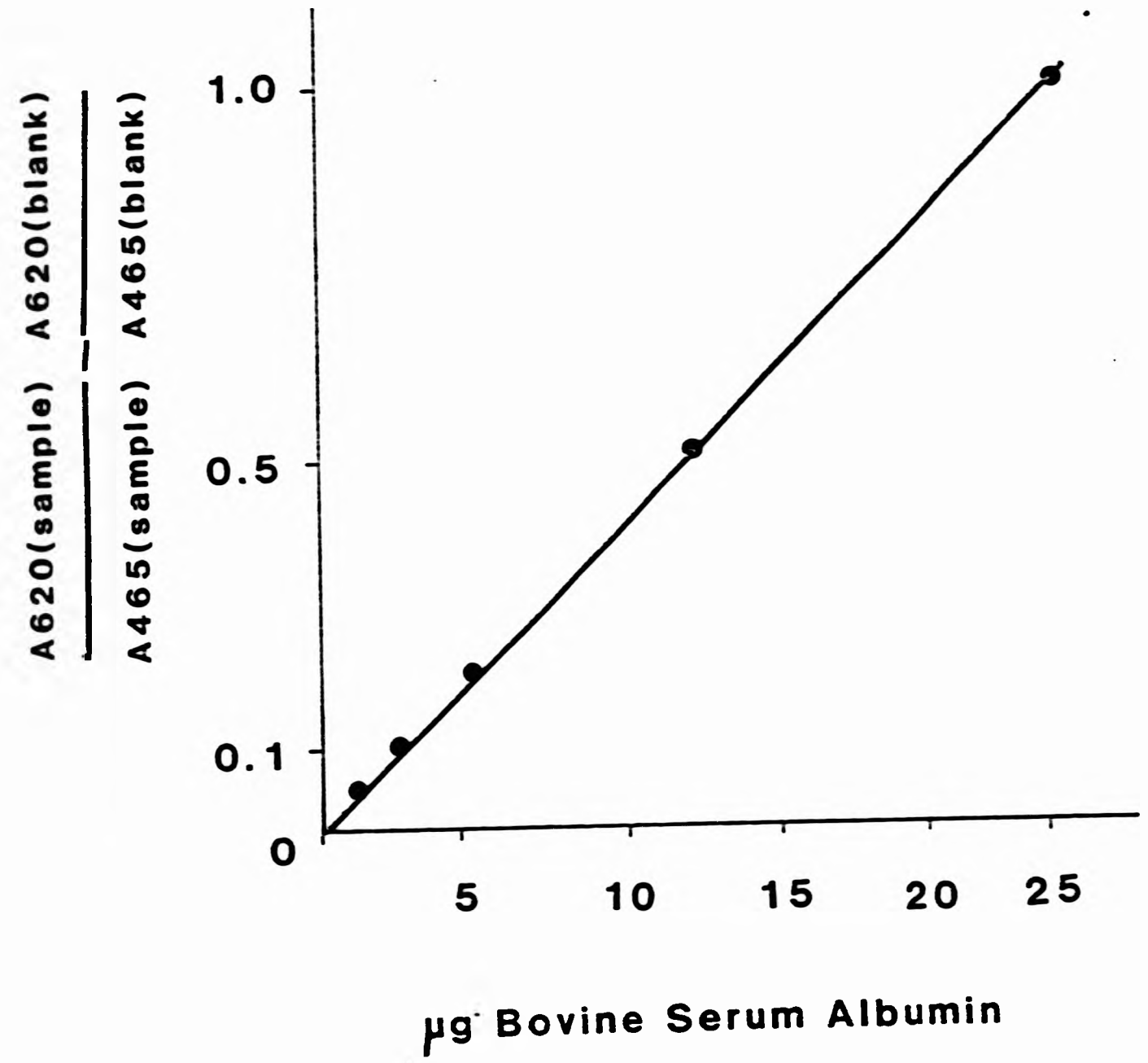


Fig. 2:5 Standard protein curve according to the method of Sedmak and Grossberg (1977) using method exactly as described in text.

interact with free amino acids and several chemicals that interfere with the Lowry protein assay and (6) the absorbances of the solutions after addition of protein are stable for 60-90 minutes at room temperature.

2:9 GLUCOSE-6-PHOSPHATE DEHYDROGENASE ASSAY

This assay procedure (Bergmeyer, 1974) is based on the conversion of glucose-6-phosphate to gluconate-6-phosphate and the reduction of the co-enzyme $NADP^+$ in the presence of glucose-6-phosphate dehydrogenase measured at 340 nm.

$$\text{Glucose-6-phosphate} + NADP^+ \xrightarrow[\text{dehydrogenase}]{\text{glucose-6-phosphate}} \text{gluconate-6-phosphate} + NADPH{+}H^+$$

The concentrations of components in the assay (3 ml) were as follows: 86.3 mM Tricine, pH 7.6; 6.7 mM $MgCl_2$; 1.2 mM glucose-6-phosphate, 0.37 mM $NADP^+$ solution and 5 mM glucose-6-phosphate dehydrogenase. Reactions were started by the addition of 0.01 ml subcellular extract.

2:10 METHANOL DEHYDROGENASE ASSAY

Methanol dehydrogenase activity was assayed (Anthony and Zatman, 1967) by measuring the rate of reduction of 2,6-dichlorophenol indophenol (DCPIP) at 40°C in the presence of 20 mM Tricine, pH 8.5, 0.43 mM phenazine methosulphate (PMS), 15 mM NH_4Cl and 1 mM KCN. The subcellular fraction (0.1 ml) was blown by pipette into 3 ml of assay mixture and the change in A600 measured between 15s and 45s. 1 unit of enzyme activity is the

amount of enzyme producing a change in A600 of 0.01/min. [A_{600} DCPIP = 1.91×10^7 1/mol/cm].

2:11 CYTOCHROME C (ASCORBATE-TMPD) OXIDASE ASSAY

An oxygen electrode was calibrated with 20 mM Tris-HCl pH 7.4, 0.75M D-mannitol (4 ml). Activities (Jones et al., 1982) were set up by the addition of subcellular extract (0.1 ml) and after 2 min the suspension was made 0.8 mM with ascorbate-TMPD (an artificial electron donor: used to replace cytochrome c). Oxygen levels were monitored for a further 5 min. The solubility of oxygen in air-saturated buffer at 40°C is 0.38 µg-atom 0/ml.

2:12 DETERMINATION OF THE OPTIMUM pH FOR PROTEOLYSIS

(Preference for buffers)

The optimum pH for proteolysis was determined using McIlvain's buffer in the pH range 2.9-7.6 and Tris-HCl pH 7.4-10.0. Having determined the optimum pH range a selection of buffers were investigated to determine the best buffering conditions (Table 2:6). A variety of substrates were also used to determine the optimum pH range including: Hide powder azure (HPA), azocasein (Azc), peptide nitroanilides and radiolabelled substrates. The proteolytic activity of extracts was determined as described in section 2:3.

2:13 TEST FOR THE EFFECTS OF INHIBITORS ON PROTEOLYTIC ACTIVITY

The effect of proteinase inhibitors on proteinase activity was tested by preincubating inhibitor (10 µl), at concentrations shown in table 2:7, with the enzyme extract (0.1 ml) for 30 min at room

TABLE 2:6

Buffers used to determine optimum pH for proteolysis

Buffer	pH range
McIlvains	2.9-7.6
Tris-HCl	7.0-9.0
MOPS	6.5-7.9
HEPES	6.8-8.2
TRICINE	7.4-8.8
CAPS	9.7-11.1
glycine	8.8-9.0
TAPS	7.7-9.1

TABLE 2:7

Inhibitors used to determine the types of proteinases in cells of M. methylotrophus

Inhibitor	Concentration of Stock Solution	Solvent
PMSF	10 mM	DMSO
EDTA	10 mM	dH_2O
TPCK	10 mM	DMSO
TLCK	10 mM	dH_2O
Chymostatin	10 µg/ml	dH_2O
Leupeptin	10 µg/ml	dH_2O
Phenanthroline	10 mM	dH_2O
Iodoacetic Acid	10 mM	dH_2O
α-1-antitrypsin	1 mg/ml	dH_2O
Aproptinin	1 mg/ml	dH_2O
Soybean Trypsin Inhibitor	1 mg/ml	dH_2O
Ovomucoid	1 mg/ml	dH_2O
Ovoininhibitor	1 mg/ml	dH_2O
Endogenous inhibitor extract	1.6 mg/ml	dH_2O

temperature. The inhibitor-proteinase mixture was then assayed for proteinase activity as described in section 2:3. When endogenous inhibitors were tested, the enzyme extract referred to the commercial proteinases, trypsin and chymotrypsin.

2:14 TEST FOR THE EFFECTS OF NUCLEOTIDES/NUCLEOSIDES ON PROTEOLYTIC ACTIVITY

The effect of nucleotides and nucleosides on proteinase activity were tested by preincubation of 10 µl nucleotide or nucleoside (30 mM) with enzyme extract (0.1 ml) in the presence or absence of 5 mM $MgCl_2$ for 60 minutes at room temperature. The nucleotide/nucleoside-proteinase mixtures were then assayed for proteinase activity as described in section 2:3.

2:15 THE EXTRACTION OF MEMBRANE-ASSOCIATED PROTEINASES

Membranes prepared as described in section 2:2b were washed 3-4 times with 20 mM Tris-HCl, pH 7.8. Membranes (2 mg protein/ml) were then solubilized using the following procedures: resuspension in 50 mM Tris-HCl, pH 7.5 and either; (1) 0.5% SDS (Regnier, 1981a) (2) 0.5% Deoxycholate, 500 mM NaCl (Regnier, 1981b) or (3) 2.25% Triton X-100, 100 mM $MgCl_2$ (Froud and Anthony, 1984). Suspensions were then incubated for 1 hour at room temperature before centrifugation at 130000 g. Both detergent-treated membrane pellets and supernatants were extensively dialyzed against 20 mM Tris-HCl pH 7.8 and then assayed for proteinase activities as described in section 2:3.

2:16 PROCEDURE FOR THE ISOLATION OF ENDOGENOUS INHIBITORS

The isolation of endogenous inhibitors from M. methylotrophus was based on the procedure used for isolating inhibitors from the periplasm of E. coli (Chung et al., 1983). Whole cells or subcellular fractions (50 ml) were adjusted to pH 3.5 with 1M HCl and incubated for 10 min at 4°C. The suspension was centrifuged at 14000 rpm for 30 min in an 8 x 50 ml Sorval SS34 rotor. The supernatant was adjusted to pH 7.4 with 1M Tris base. NaCl (solid) was then added to make a final concentration of 0.3M and the supernatant heated for 10 minutes in a boiling bath. The supernatant was then centrifuged as above and then made 65% with $(NH_4)_2SO_4$ and stirred at 4°C for 1 hour. The suspension was centrifuged as above and the pellet (inhibitor extract) resuspended in 2 ml 10 mM Tris-HCl pH 7.8, 5 mM $MgCl_2$. The resuspended pellet was then dialyzed overnight against the same buffer.

The endogenous inhibitors were purified further by running the endogenous extract through Sehadex G75 and trypsin-Sepharose columns (see section 2:5a/c for details).

2:17 PROCEDURE USED TO DETERMINE THE EFFECT OF STRESS-SHOCK ON GROWING CELLS OF M. METHYLOTROPHUS

A 1 ml inoculum was taken from an overnight culture of M. methylotrophus cells grown at 30°C and subcultured into 50 ml of minimal media. Cells were then grown to mid-log phase also at 30°C. At this point [^{35}S]-methionine (0.5 µl/ml) and the stress-shocking agent were added (alternatively there was a temperature shift). Stress-shocking treatments used are shown in table 2:8. Aliquots (0.5 ml) and (5 ml) were removed after 0, 5, 15

TABLE 2:8

Stress-shock treatments

1. Heat-shock, 30-40°C temperature shift (carried out in a water bath)
2. Methanol shock, cells made 5% (v/v) with methanol.
3. Ethanol shock, cells made 5% (v/v) with ethanol.
4. Puromycin shock, cells made 100 μg/ml with puromycin.

and 30 minutes stress-shock treatment from which total protein and subcellular fractions were prepared respectively. Total protein was isolated by the following procedure: 0.5 ml of ice-cold 10% w/v trichloroacetic acid was added to 0.5 cell culture. The sample was centrifuged in a bench centrifuge and resuspended in 0.5 ml of ice-cold 5% (w/v) trichloroacetic acid. The sample was centrifuged and the pellet resuspended in ice-cold acetone. The sample was again centrifuged and the pellet prepared for SDS-gel electrophoresis by resuspension in solubilization-reduction mix. In order to determine the effects of stress-shock treatment on the total number of proteinases in whole cell extracts of M. methylotrophus, larger batches (500 ml) of cells were prepared. The whole cell extracts were then run on DEAE-Sephacel columns as described in section 2:5b (iii) and eluted fractions assayed for proteinase activity as described in section 2:3.

The procedure used to determine the effects of stress-shock treatment on E. coli cells were as described above for M. methylotrophus with the exception that stress-shock treatment was only for 30 min and heat-shock treatment involved a temperature shift from 30-42^{o}C (Yamamori et al., 1978 ; Lindquist, 1986).

2:18 PROCEDURE FOR THE ISOLATION OF Ts MUTANTS OF M. METHYLOTROPHUS

An overnight culture of M. methylotrophus cells was subcultured in MMc media (minimal media with Na-citrate (100 µg/ml)) and grown to an A550 of 0.34 at 30^{o}C. The culture (5 ml) was centrifuged in

an MSE bench centrifuge and resuspended in 10 mM citrate buffer pH 5.5 (0.1M citric acid, 0.1M Na-citrate) containing 50 µg/ml fresh N-Methyl-N-Nitro-N-Nitrosoguanidine (NG) (solid NG was dissolved in citrate buffer and filter sterilized). Cells were incubated at 30°C for 20 min then spun down and washed several times with warm (30°C) MMc buffer. The culture (2 ml) was then subcultured in fresh MMc (18 ml) and incubated overnight at 30°C in shaking water bath at 120 rpm. At this point the culture would normally be enriched for Ts mutants using penicillin G and cycloserine. However since it is actually Ts lysis mutants that are selected for this procedure would in fact be counter selective. The absence of enrichment effectively reduces the number of Ts mutants by 100 fold.

The overnight culture was diluted with 10 mM citrate buffer to give a final dilution of 2.5×10^{-6}, 6.25×10^{-7} and 1.56×10^{-8}. The diluted cells (0.1 ml) were spread on agar plates. Cells were then grown at 30°C for 2 days and then replica plated and the replica incubated at 40°C for 2 days. Cells that did not grow at 40°C (Ts mutants) were picked from the original plate and streaked on fresh plates to isolate single colonies. The Ts mutants were cleaned and checked by replica plating and then shifting the plate to the permissive temperature.

2:19 DETECTION OF Ts LYSIS MUTANTS

Ts mutants that lyse on shifting to the non-permissive temperature should release their intracellular components. Since *M. methylotrophus* cells have intracellular proteinases, it should be possible to detect such Ts lysis mutants by replica plating colonies onto agar plates containing protein substrates (Wolf and Ehmann,

1978). Release of proteinases is apparent as clear zones surrounding the lysed colony.

Three protein substrates were incorporated into agar plates, (1) HPA (10 mg/ml) sonicated 10 x 15s, (2) Azc (10 mg/ml) and (3) skimmed milk (2 mg/ml), (5 mg/ml) and (10 mg/ml). Substrate agar plates were prepared by adding the protein in distilled water autoclaving and then adding to the normal agar plates. Attempts to isolate Ts lysis mutants were also made by using the napthyl ester N-acetyl-DL-phenylalanine-naphthyl ester (APNE), a known proteinase substrate (Jones, 1977). Replicas of mutagenized cells were made using Whatman No 1 filter paper. The replica was then layered into 20 ml of 0.05M Tris-HCl pH 7.5, 40 mg fast blue salt B and 6 ml of dioxan containing 20 mg APNE (2 mg APNE made up in dioxan prior to addition). Release of hydrolyzing activities (dark blue colonies) was followed at 40^{o}C.

2:20 GRAM-POSITIVE (AND NEGATIVE) STAIN FOR BACTERIA

A slurry of the bacteria was placed on a microscope slide and heated to dryness over a bunsen flame. The slide was soaked in aqueous crystal violet (0.5%) for 30s and then flushed with Lugols Iodine (1 g I_2, 2 g KI in 100 ml distilled H_2O) for 2 min. The slide was then washed with distilled H_2O. Ethanol was added dropwise to the bacteria for 10s (removes purple stain if bacteria not gram positive). The slide was washed again with water and counterstained with Carbol Fuschia 1/10 for 10s. The slide was washed again with distilled H_2O and prepared for microscopic observation by addition of a drop of immersion oil. Gram negative bacteria such as M. methylotrophus take up counterstain and show

characteristic pink rods.

This procedure was used over a 3 year period to monitor the purity of M. methylotrophus cells.

' Scientific discovery is a private
event, and the delight that
accompanies it, or the
despair of finding it
illusory does not travel '

Sir Peter Medawar

CHAPTER 3

RESULTS

3:1 OPTIMUM CONDITIONS FOR DETECTING PROTEINASE ACTIVITY OF WHOLE CELL EXTRACTS FROM M. METHYLOTROPHUS

The initial phase in the analysis of proteolytic enzymes involved determining the appropriate methods for detection and the best assay conditions.

There is a whole spectrum of substrates available to detect proteolytic enzymes, ranging from those likely to be substrates for a limited number of enzymes to those capable of being degraded by many enzymes. Protein substrates such as HPA, azocasein, [^{14}C]-α_1-casein and [^{14}C]-globin are typical substrates used to detect a broad range of proteolytic enzymes. Individual proteolytic enzymes preferentially hydrolyze peptide bonds involving a limited number of amino acids or amino acids of a certain type. It is also apparent that the activity of many proteinases can be influenced by the nature of just a few amino acids surrounding the hydrolysed bond (Achstetter et al., 1985). Consequently the use of peptide substrates of defined sequence can offer a means of differentiating between individual enzymes in cell extracts containing a mixture of proteinases and peptidases. Therefore in addition to protein substrates a group of synthetic peptide nitroanilide compounds were used to detect optimum conditions for protein/peptide hydrolysis. The types of peptide-nitroanilide substrates used can be divided into three distinct groups, table 3:1. Group 1 have the general structure RXYZArgNA, where X, Y and Z are different amino acids; group 2 have the general structure RAlaAlaProXNA, where X is either

TABLE 3:1

Classification of synthetic nitroanilide substrates

Group type	Substrate*
1	Bz-Ile-Glu-Gly-Arg-
	Tos-Gly-Pro-Arg-
	Bz-Val-Gly-Arg-
	Bz-Pro-Phe-Arg-
	Bz-Phe-Val-Arg-
2	Suc-Ala-Ala-Pro-Phe-
	Suc-Ala-Ala-Pro-Leu-
	Ac-Ala-Ala-Pro-Ala-
3	Bz-Tyr-
	Bz-Lys-
	Bz-Arg-

* All substrates had 4-Nitroaniline at the C-terminus

Ala, Leu or Phe and group 3 have the general structure $R\begin{pmatrix}X\\Y\\Z\end{pmatrix}NA$, where X, Y, and Z may be the same or different amino acids. In all cases R is the amino-terminal blocking group and NA is the nitroaniline carboxyl-terminal blocking group. Hydrolysis of the carboxyl-terminal arylamide bond of these synthetic peptide substrates results in the release of 4-nitroaniline, fig. 3:1. 4-nitroaniline has an absorption coefficient of 9500 litre mol^{-1} cm at 405 nm and can be measured spectrophotometrically (North et al., 1983).

3:1a <u>Hydrolyzing activity using protein substrates</u>

<u>pH and buffer dependence</u>

Hydrolysis of HPA by whole cell extract releases a blue chromophore attached to soluble peptides. After trichloroacetic acid precipitation of the undigested material the degree of hydrolysis was measured spectrophotometrically (section 2:3a).

The pH dependence for whole cell extract hydrolysis of HPA in a variety of buffers was measured, fig. 3:2a. The results suggested that there were two pH optima for the hydrolysis of HPA, at pH 6.1-7.0 and 7.1-8.2. The highest rate of substrate hydrolysis was at pH 7.3 in citrate/phosphate buffer. The rates of hydrolysis at pH 7.6 in either HEPES or Tris-HCl buffer however were not significantly different.

Hydrolysis of azocasein by whole cell extract releases an orange chromophore attached to soluble peptides. After trichloroacetic acid precipitation of the undigested material, the degree of substrate hydrolysis was measured spectrophotometrically (section 2:3b).

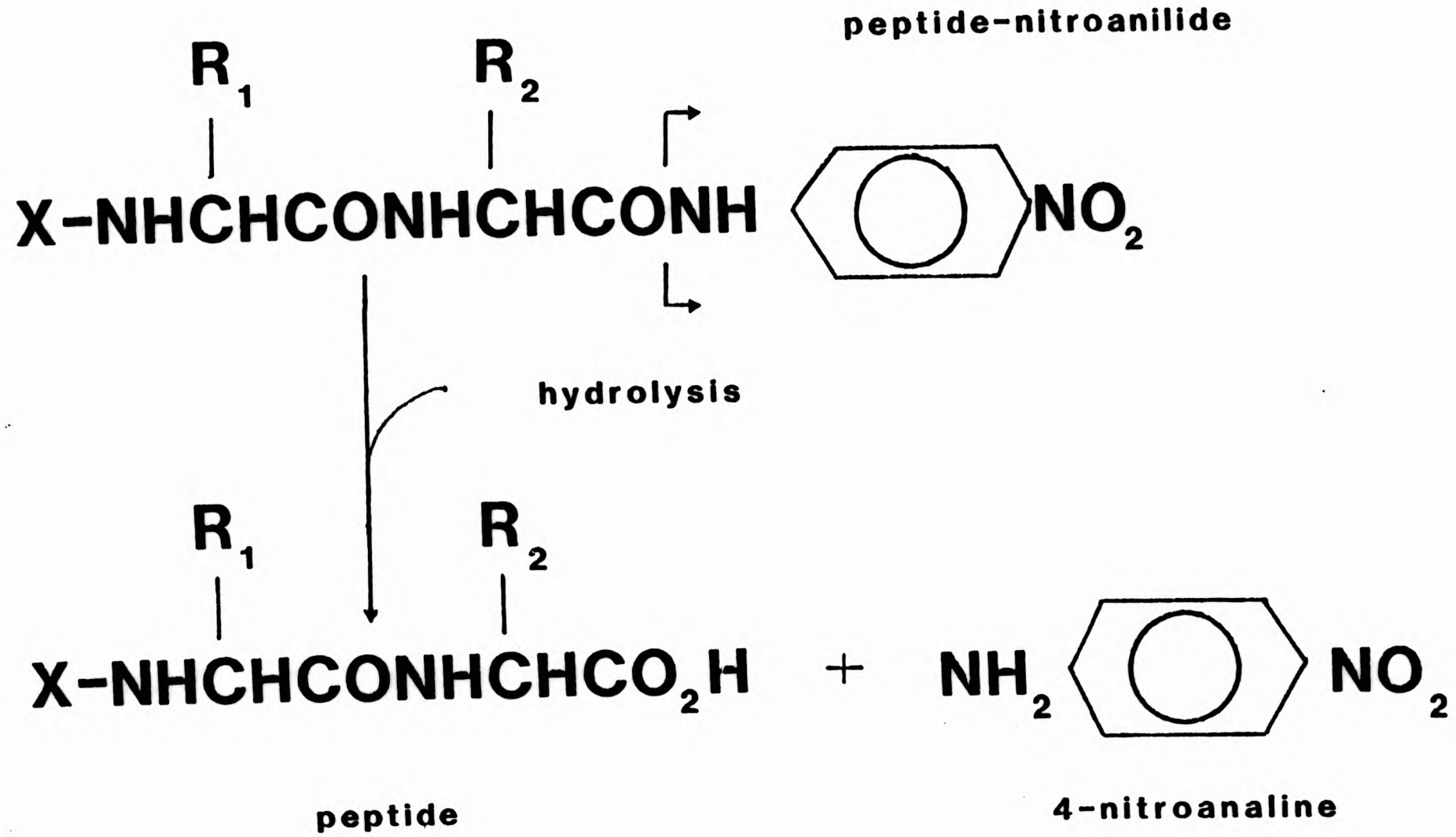
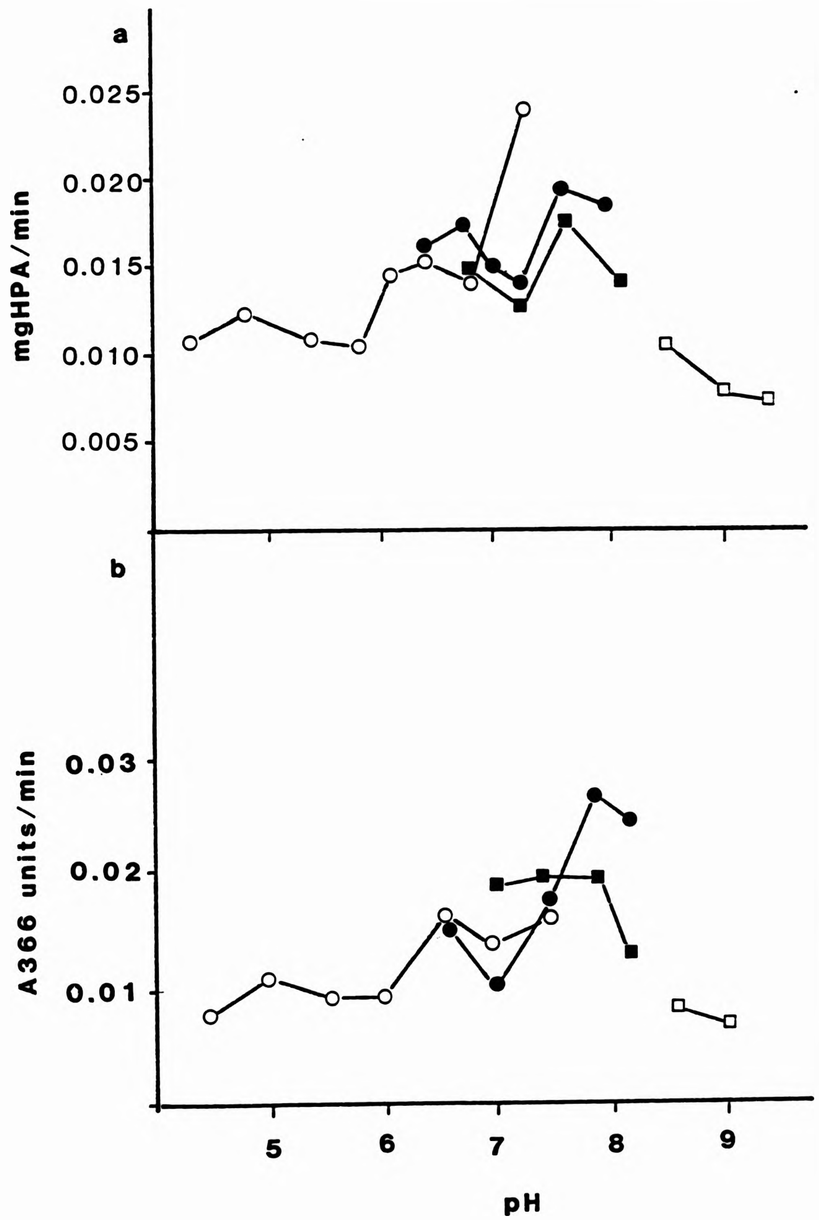


Fig. 3:1 Hydrolysis of peptide-nitroanilides.

Fig. 3:2 pH dependence and effects of various buffers on the hydrolysis of protein substrates. Activities were measured with (a) HPA and (b) azocasein. Buffers were: 20 mM McIlvaine's buffer (citrate/phosphate) (O—O); 20 mM HEPES (●—●); 20 mM Tris/HCl (■—■); 20 mM glycine (□—□) Samples used contained 70 µg protein per assay.



The pH dependence of whole cell extract hydrolysis of azocasein in a variety of buffers was measured, fig. 3:2b. The results suggested that there were two pH optima for azocasein hydrolysis, at pH 6.2-7.0 and 7.5-8.2. These pH optima correspond quite closely to those for HPA hydrolysis. The highest rate of substrate hydrolysis was at pH 7.8 in HEPES. The rate of hydrolysis at pH 7.0-7.8 in Tris-HCl however was not significantly different.

3:1b Hydrolyzing activity using peptide-nitroanilides

pH and buffer dependence

An initial screening of all substrates listed in table 3:1 was made using the microtitre assay procedure (section 2:3e). This method was particularly useful as it was fast, sensitive and used small quantities of nitroanilides. All the substrates were assayed by this procedure in the pH range 2-9 using 0.1M McIlvain's buffer and pH 7.0-9.0 using 0.1M Tris-HCl buffer.

It was apparent from this screening procedure that maximum rates of hydrolysis occured in the neutral to alkaline pH range 6.6-8.3. Incubation of substrates at pH 5.0 or below resulted in precipitation of material.

A more quantitative estimation of the rates of hydrolysis of several of the peptide-nitroanilides was then made in the pH range 6-8, table 3:2. The optimum pH for hydrolysis of all the substrates tested was 7.5. Tos-Gly-Pro-Arg-NA was the most rapidly hydrolyzed substrate at this pH although all the substrates were hydrolyzed at a reasonable rate. As there was not a major difference in the rates of hydrolysis in any of the substrates, except where indicated BzArgNA the cheapest substrate was used for further routine analysis.

TABLE 3:2

Hydrolysis of nitroanilide substrates in McIlvain's buffer pH 6-7.5 and Tris-HCl pH 7.5-8.0

Substrate (-NA)	Activity (nmol/min) McIlvain's			Tris-HCl	
pH	6.0	7.0	7.5	7.5	8.0
Bz-Arg	0.14	0.20	0.45	0.35	0.27
Bz-Ile-Glu-Gly-Arg	0.16	0.27	0.40	0.30	0.27
Tos-Gly-Pro-Arg	0.15	0.30	0.52	0.52	0.42
Bz-Pro-Phe-Arg	0.10	0.21	0.44	0.35	0.26
Bz-Phe-Val-Arg	0.12	0.26	0.43	0.34	0.30
Suc-Ala-Ala-Pro-Leu	0.08	0.19	0.44	0.34	0.22
Bz-Tyr	0.06	0.28	0.50	0.41	0.29

All the substrates listed above were assayed as described in section 2:3c. Samples contained 70 g of protein.

Evidence from measurement of peptide hydrolysis in McIlvain's and Tris-HCl buffer suggested that the rates of hydrolysis were also related to the buffering system and not just the pH. For this reason BzArgNA hydrolysis was measured with a variety of buffers over a range of pH's, fig. 3:3. It was apparent that BzArgNA was most rapidly hydrolysed in the pH range 7.3-8.4. Optimum substrate hydrolysis in TAPS, MOPS and Tricine was at pH 8.0. Maximum hydrolysis with HEPES was at pH 7.8 while with Tris-HCl it was at pH 7.6. A second pH optimum for BzArgNA hydrolysis was observed with most of the buffers in the pH range 7-7.2.

3:1c Optimum temperature for hydrolyzing activity

The effects of various incubation temperatures on the extent of BzArgNA hydrolysis after 3 hrs at pH 7.5 were measured, fig. 3:4a. The maximum extent of hydrolysis was observed at 37°C. There was no detectable hydrolysis of BzArgNA at temperatures of 60°C or greater. The loss of activity at the higher temperatures was most likely due to thermodenaturation and precipitation of protein.

The effects of preincubation of whole cell extracts at various temperatures prior to substrate hydrolysis at 37°C were measured, fig. 3:4b. Preincubation of whole cell extracts for 30 min at 30°C resulted in a 20% increase in the extent of BzArgNA hydrolysis compared to extracts that were not preincubated. Preincubation at 37°C resulted in a 10% increase in the extent of substrate hydrolysis at 37°C while preincubation at 20°C had no effect. At temperatures above 37°C preincubation resulted in a decrease in the extent of BzArgNA hydrolysis. Why a preincubation of the whole cell extract, at a temperature lower than the optimal

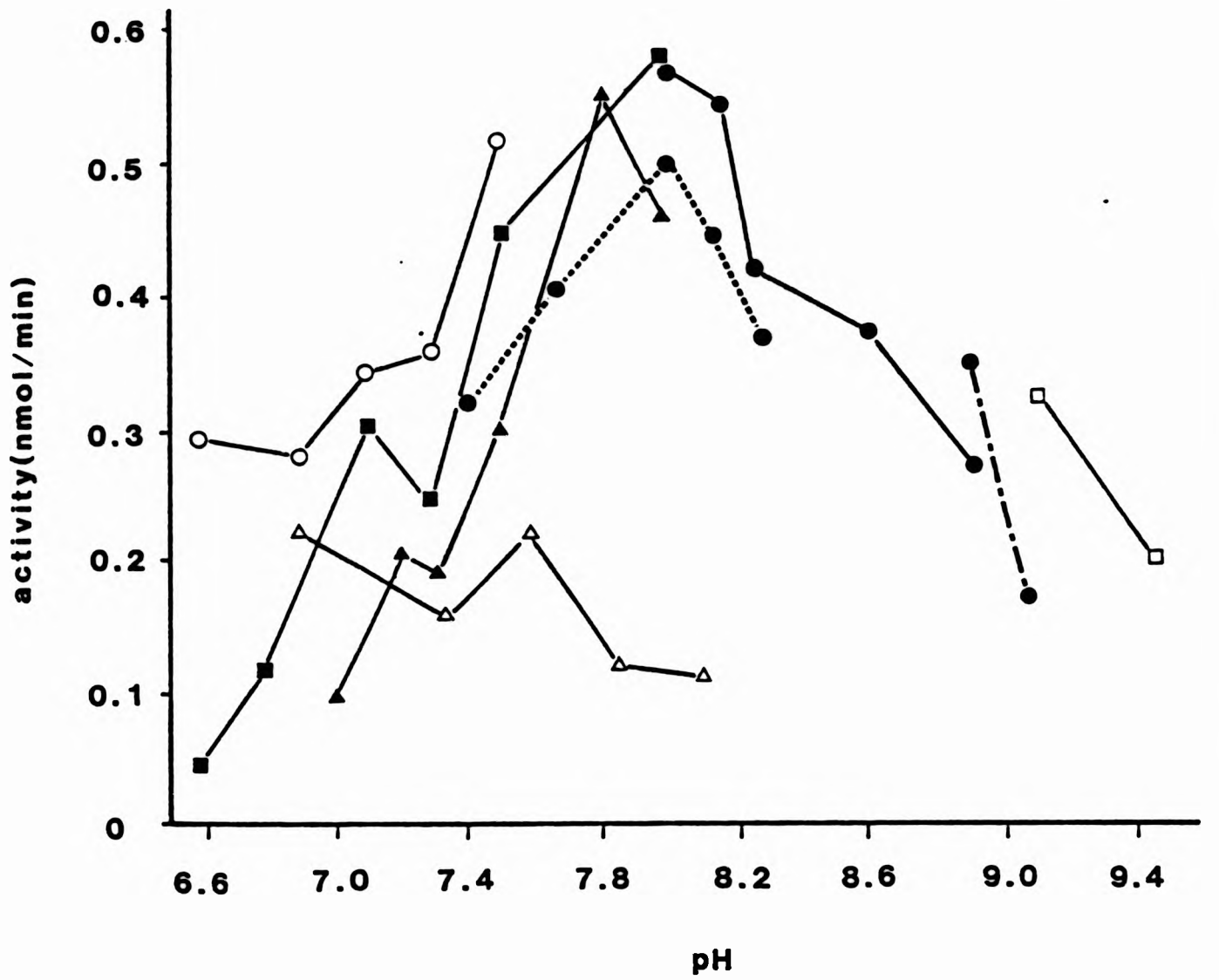
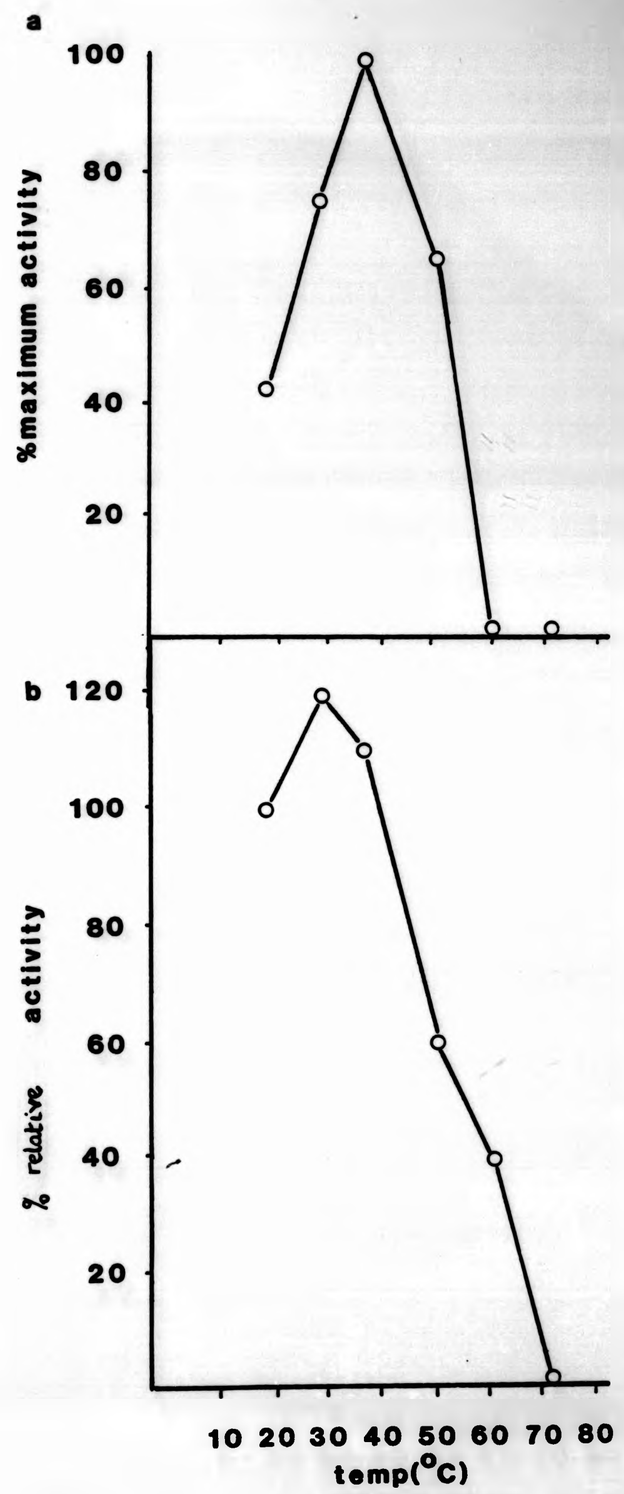


Fig. 3:3 pH dependence and effects of various buffers on the hydrolysis of BzArgNA. Buffers were: 10 mM McIlvaine's buffer (citrate/phosphate) (■——■); 10 mM MOPS (○——○); 10 mM Tricine (●---●); 10 mM TAPS (●——●); 10 mM HEPES (▲——▲); 10 mM glycine (●-·-●); 10 mM CAPS (□——□); 10 mM Tris/HCl (△——△). Samples used contained 70 µg protein.

Fig. 3:4 Optimum temperature for the hydrolysis of BzArgNA by whole cell extracts.

(a) The effect of various incubation temperatures on the extent of BzArgNA hydrolysis after 3 hours in 10 mM Tris/HCl pH 7.5.

(b) The effects of various 30 minute preincubation temperatures on the extent of BzArgNA hydrolysis after 3 hours in 10 mM Tris/HCl pH 7.5 at 37°C. Samples used contained 70 μg protein. Activities were expressed as a percentage of the activity (0.35 nmol/min) of whole cell extracts at 37°C without preincubation.



temperature, should cause an increase in the extent of substrate hydrolysis is unclear. This effect however, has been observed in other bacteria (Car and Woods, 1984).

3:1d The effects of inhibitors on proteinase/peptidase activity

Proteinase inhibitors have proved invaluable in the elucidation of the catalytic mechanism of many proteolytic enzymes (Goldberg et al., 1981; North, 1982a). A number of these proteinase inhibitors have been used in an attempt to characterize the proteolytic enzymes of M. methylotrophus. The effects of inhibitors on the hydrolysis of BzArgNA by whole cell extracts were investigated, table 3:3.

Inhibition of BzArgNA hydrolysis by both EDTA and phenanthroline suggested a specific requirement of metal ions for maximal activity of enzymes in the whole cell extract. Inhibition by PMSF was indicative of serine proteinases in the whole cell extract while inhibition by leupeptin and TLCK was consistent with trypsin-like serine proteinases being present.

Iodoacetic acid is generally regarded as a cysteine proteinase inhibitor, however it is also known to affect the activity of many other enzymes. Since there are few reports of cyteine proteinases in bacterial cells it seems unlikely that inactivation of BzArgNA hydrolysis by iodoacetic acid was due to inhibition of cysteine proteinases. Cysteine proteinases also often require a reducing agent such as DTT for activity, and since there was no effect on the rates of substrate hydrolysis in the presence of this agent it was most unlikely that this type of proteolytic enzyme was present in the whole cell extracts of M. methylotrophus. Inhibition by iodoacetic acid was therefore probably due to its effects on other

TABLE 3:3

The effects of inhibitors on BzArgNA hydrolysis by whole cell extracts

Inhibitor (1 mM unless otherwise indicated)	Inhibition (%)
PMSF*	100
EDTA	56
phenanthroline	81
TLCK*	89
TPCK	0
IAA	21
DTT	0
chymostatin (10 μg/ml)	34
leupeptin (10 μg/ml)	64

* denotes inhibitor dissolved in DMSO.
The extent of inhibition was expressed as a percentage of the rate of hydrolysis of the substrate in the absence of inhibitor. 100% activity was equivalent to 0.35 nmol/min. Samples used contained 70 μg protein.

enzymes involved in proteolysis.

The minimum concentration of an inhibitor required to inactivate an enzyme might also be important in controlling the activity of a single proteinase/peptidase within a complex group of proteolytic enzymes. For this reason the effects of various concentrations of inhibitors on BzArgNA hydrolysis by whole cell extracts was investigated, fig. 3:5.

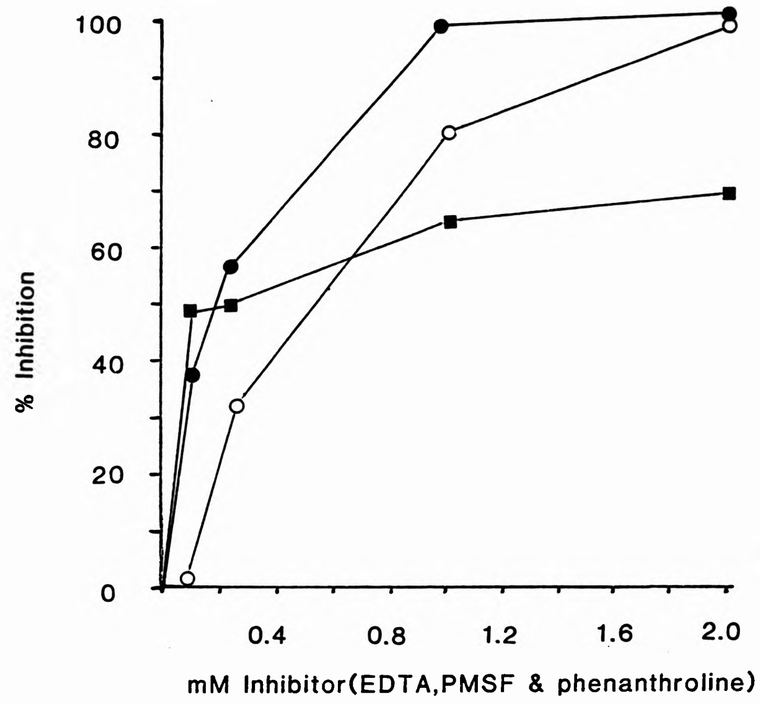
From the results it was apparent that even low concentrations of PMSF caused a significant amount of inhibition of BzArgNA hydrolysis with almost 55% inactivation achieved with 0.2 mM. In the case of EDTA and phenanthroline, maximum inhibition of substrate hydrolysis was apparent at 2 mM, although approximately 50% inactivation was achieved at lower concentrations. Both chymostatin and leupeptin exerted maximum inhibition (36% and 65%, respectively) at approximately 10 μg/ml inhibitor.

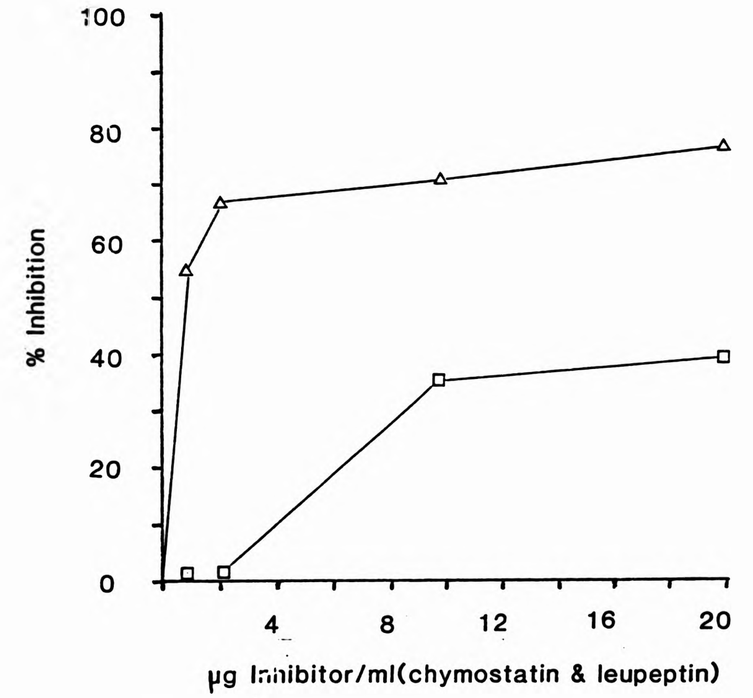
3:1e The effects of divalent cations on proteinase/peptidase activity

The stabilizing and stimulating effects of divalent cations on proteinase activities have been well documented (Holmquist, 1977; Goldberg et al., 1981; Car and Woods, 1984; Mayer and Doherty, 1986). It was therefore of interest to establish the effects of divalent metal ions on proteolytic enzymes from whole cell extracts of M. methylotrophus.

To remove endogenous metal ions extracts were first dialyzed against incubation buffer in the presence of 3 mM EDTA, (section 2:13). EDTA was then removed by dialysis against the incubation buffer. Various concentrations of either Ca^{2+}, Zn^{2+}, Mg^{2+} or Mn^{2+} were then added to the treated extract and the rates of

Fig. 3:5 The effects of various concentrations of inhibitors on the hydrolysis of BzArgNA by whole cell extracts. The extent of inhibition is expressed as a percentage of the rate of hydrolysis of the substrate in the absence of inhibitor. 100% activity is equivalent to 0.35 nmol/min.
Inhibitors: PMSF (●—●); phenanthroline (○—○); EDTA (■—■); chymostatin (□—□); leupeptin (△—△). Samples used contained 70 μg protein.





BzArgNA hydrolysis measured, fig. 3:6. After EDTA treatment and dialysis only 44% of the original BzArgNA hydrolyzing activity of the whole cell extract remained. Total restoration of the EDTA-inhibited extract activity was achieved with either 2 mM Ca^{2+} or 2 mM Zn^{2+}. Extracts made 5 mM with Ca^{2+} exhibited a 160% increase in the rate of substrate hydrolysis while 5 mM Zn^{2+} showed an 80% increase in substrate hydrolysis. Concentrations of Ca^{2+} or Zn^{2+} greater than 5 mM resulted in protein precipitation in assay mixtures. Concentrations of Mg^{2+} or Mn^{2+} up to 5 mM did not restore enzyme activity.

Reports of metal-ion dependent proteolytic enzymes from other bacteria indicated that divalent metal-ions were important for enzyme activity in two ways: (1) in a direct association with the catalytic site of the enzyme and (2) in structural stability of the enzyme. Although it was apparent from the results presented here that Ca^{2+} and Zn^{2+} were required for maximum activity the specific function of these divalent metal-ions was not clear.

3:1f The effects of nucleotides on proteinase/peptidase activity

It has been known for some time that the degradation of proteins in many bacterial and animal cells requires metabolic energy (Herschko and Ciechanover, 1982; Etlinger et al., 1985). An ATP-dependent proteinase has now been isolated and characterized from the whole cell extracts of *E. coli* (Sreedhara Swamy and Goldberg, 1981) (section 1:6d). Attempts were made to locate a comparable energy-dependent protein degrading system within the whole cell extracts of *M. methylotrophus*.

The effects of a number of nucleotides on the hydrolysis of

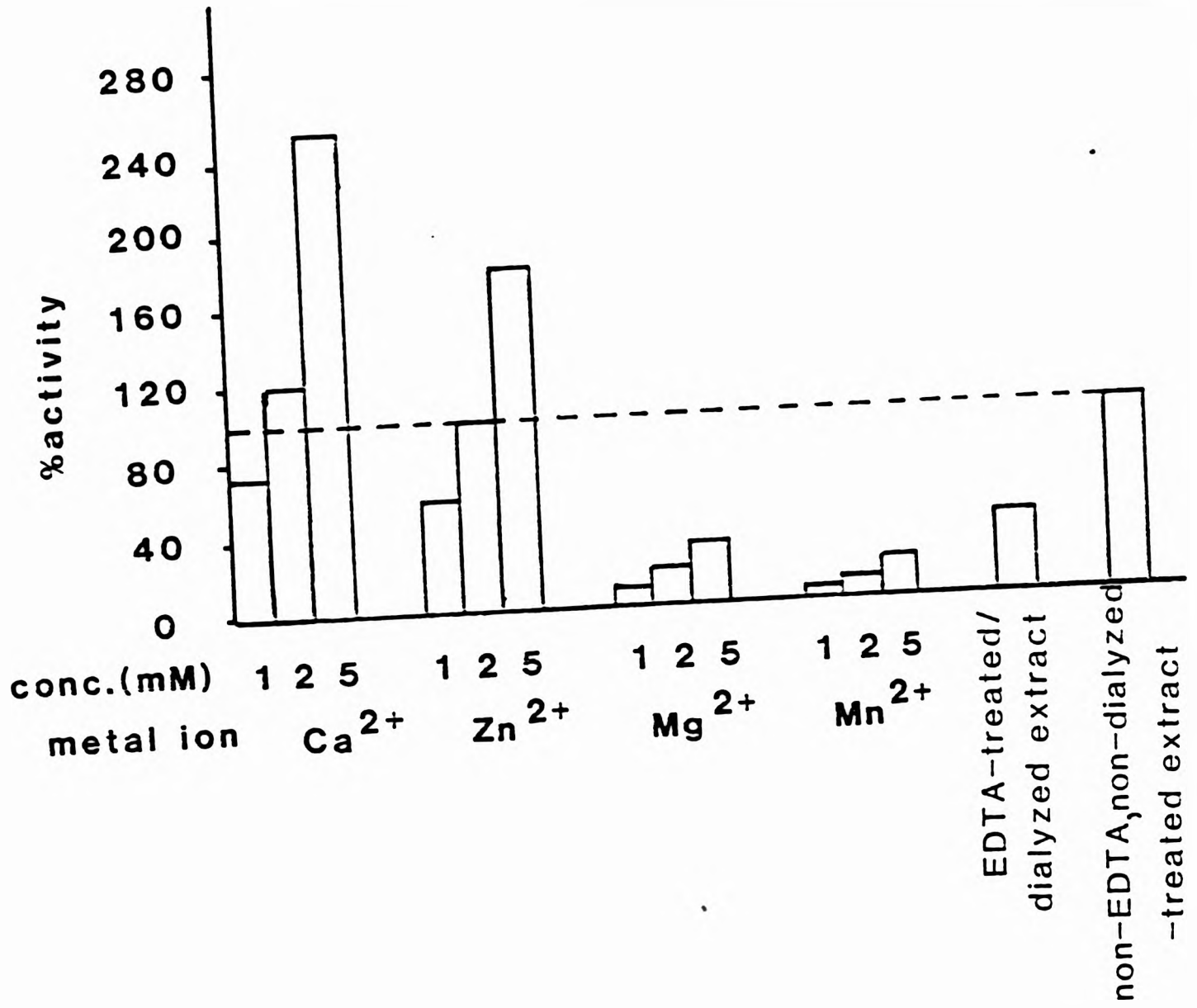


Fig. 3:6 The effects of metal ions on the hydrolysis of BzArgNA by EDTA-treated whole cell extracts (see text for details). Unless indicated,extracts were incubated with 3 mM EDTA then dialyzed against incubation buffer before addition of the metal ions. Enzyme activities were expressed as a percentage of the BzArgNA hydrolyzing activity of untreated whole cell extracts. Untreated extracts (100% activity = 0.35 nmol/min). Samples used contained 70 µg protein assay.

BzArgNA and azocasein by whole cell extracts were investigated, table 3:4. Substrate hydrolysis in the presence of nucleotides was assayed as described in section 2:14.

The results showed that none of the nucleotides tested had any stimulatory effect on the rates of BzArgNA or azocasein hydrolysis. The most noteworthy effect was the apparent 20-30% inhibition of substrate hydrolysis by ATP, ADP, UTP and CTP. These results were reproducible and independent of the substrate hydrolyzed, but the significance of such an effect remains unclear.

3:1g Discussion

The hydrolysis of a broad range of peptide nitroanilides and protein substrates by whole cell extracts indicated that these extracts contained proteinases exhibiting a variety of specificities. Inactivation of substrate hydrolysis by PMSF suggested that certain of the enzymes involved in substrate hydrolysis had a serine-residue at their active site. Inhibition of up to 56% of the total enzyme activity by EDTA implied that divalent metal ions were necessary for maximum enzyme hydrolyzing activity and that these enzymes might be metalloproteinases. Restoration and stimulation of enzyme activity with Ca^{2+} or Zn^{2+} ions implicated these metal ions either in association with the active site or in the structural stability of these enzymes. The moderately broad pH optimum in the neutral to slightly alkaline range, 7.3-8.2, was also consistent with the suggestion that whole cell extracts contained both metallo- and serine-proteinases.

These results are consistent with reports of intracellular proteolytic enzymes from other bacteria (Orlowski and White, 1974;

TABLE 3:4

The effects of nucleotides on substrate hydrolysis by whole cell extracts

Substrate		Activity (%) BzArgNA			Azocasein
Preincubation Temperature	Mg^{2+}	4°C	22°C	30°C	22°C
Nucleotide (3 mM)					
ATP	(+)	79	85	79	80
ATP	(-)	76	89	76	98
ADP	(+)	76	85	71	80
ADP	(-)	84	83	73	85
AMP	(+)	87	100	107	98
AMP	(-)	103	94	108	93
cAMP	(+)	101	104	108	103
cAMP	(-)	108	106	108	104
GTP	(+)	92	100	92	95
GTP	(-)	101	97	95	102
CTP	(+)	72	85	74	80
CTP	(-)	76	90	76	81
UTP	(+)	68	82	70	70
UTP	(-)	69	80	71	72
	(+)	100	100	100	102
Control#	(-)	100	100	100	100

* Activity was expressed as a percentage of the rates of hydrolysis of extracts in the absence of nucleotides and Mg^{2+}.

\# Control is the activity of extracts in the absence of nucleotides and Mg^{2+} expressed as 100%. 100% activity was equivalent to 0.35 nmol/min for BzArgNA hydrolysis and 0.02 A366/min for Azc hydrolysis.

(+) Made 2 mM with Mg^{2+}.

(-) No Mg^{2+} added.

Hiroishi and Kadota, 1976; Miller et al., 1976; Cheng and Aronson, 1977; Goldberg et al., 1981; Car and Woods, 1984; Muro et al., 1985; Kohayashi et al., 1985) (section 1:6c), in which the pH optima for enzyme activities were generally in the pH range 7.0-8.0. The majority of these enzymes have been shown to be serine proteinases. In addition a small number of metalloproteinases have also been isolated from baccerial cells (Hiroishi and Kadota, 1976; Setlow, 1976; Holmquist, 1977; Goldberg et al., 1981). Several of these have been shown to have a direct requirement for Zn^{2+} ions at the active site of the enzyme and for Ca^{2+} ions for enzyme stability (Holmquist, 1977)

The direct involvement of a nucleoside triphosphate (ATP) and a divalent ion (Mg^{2+}) in the initial steps of protein breakdown in the intracellular extracts of E. coli has been widely reported (Goldberg et al., 1985; Goldberg and Waxman, 1985; Waxman and Goldberg, 1986) (section 1:6d). In contrast the proteolytic enzymes from M. methylotrophus have shown no stimulation of substrate hydrolysis with either ATP, ADP, AMP, cAMP, GTP, CTP or UTP in the absence or presence of a divalent cation. However in a complex biological system, such as that of whole cell extracts, the effects of nucleotides, on what might be a single nucleotide-dependent proteolytic enzyme, are likely to be masked by other enzyme activities or energy-dependent pathways.

It was clear therefore, that for further characterization of the enzymes involved in protein and peptide breakdown, the individual proteinases and peptidases would need to be isolated and purified.

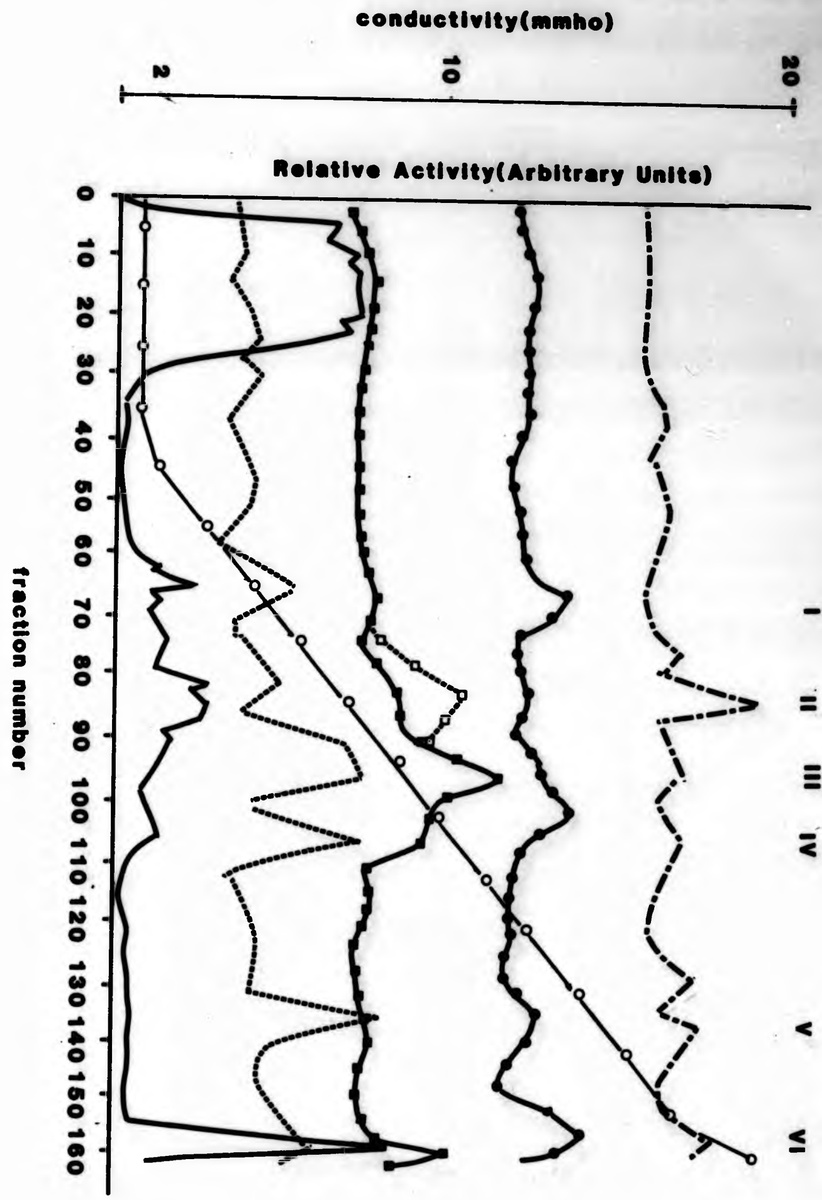
3:2 ISOLATION, PURIFICATION AND CHARACTERIZATION OF PROTEOLYTIC ENZYMES FROM WHOLE CELL EXTRACTS OF M. METHYLOTROPHUS

Having established the methodology and optimal conditions for detecting proteolytic enzymes from the whole cell extract of M. methylotrophus, these enzymes were purified by traditional protein isolation procedures.

The first step in the purification of proteolytic enzymes was based on the selective precipitation of protein using $(NH_4)_2SO_4$. The extract (411 ml) was made 40% (w/v) with solid $(NH_4)_2SO_4$ and precipitated material removed by centrifugation at 11000 g for 15 minutes. The proteinase/peptidase supernatant was then made 80% (w/v) with $(NH_4)_2SO_4$ and the precipitated material recovered by centrifugation at 11000 g for 15 minutes. The pellet, which was enriched for proteolytic enzymes, was then resuspended in column running buffer (section 2:5).

In the second stage of purification the ammonium sulphate fraction was run on a DEAE-cellulose column. Eluted fractions were assayed for hydrolysis of $[^{14}C]$-globin, HPA, azocasein and BzArgNA, fig. 3:7. Six major peaks of $[^{14}C]$-globin-hydrolyzing activity were detected. For convenience, they were designated peaks I - VI, according to their order of elution from the column. Peaks II, III, IV, V and VI all possessed HPA hydrolyzing activity while peaks I, IV, V and VI also hydrolyzed azocasein. BzArgNA hydrolyzing activity was detected in peaks II, III, IV and VI. The BzArgNA hydrolyzing activity from peak II was stimulated in the presence of 3 mM ATP and Mg^{2+}. The amount of ATP stimulation was 25-80%, varying between DEAE-cellulose column runs.

Fig. 3:7 DEAE-cellulose chromatography of proteolytic enzymes from whole cell extracts of M. methylotrophus. Degradation of HPA (•▬▬•); degradation of azocasein (●—●); hydrolysis of BzArgNA measured in the absence (■—■) and presence (□—□) of ATP; degradation of [^{14}C]-globin (▪▪▪▪▪▪▪); A_{280} (———) and conductivity (○—○). Dialyzed ammonium sulphate treated extract was absorbed to a DEAE-cellulose column and eluted with 1500 ml of a linear NaCl gradient (0-0.2M NaCl). The flow rate was 100 ml/hour and 15 ml fractions were collected. Every fourth fraction was assayed. There were no appreciable differences in substrate hydrolysis profiles when DEAE-cellulose chromatography was carried out using either Tris-HCl (pH 7.8) or Tricine (pH 7.8) as column buffer.



A note on the nomenclature of proteinases

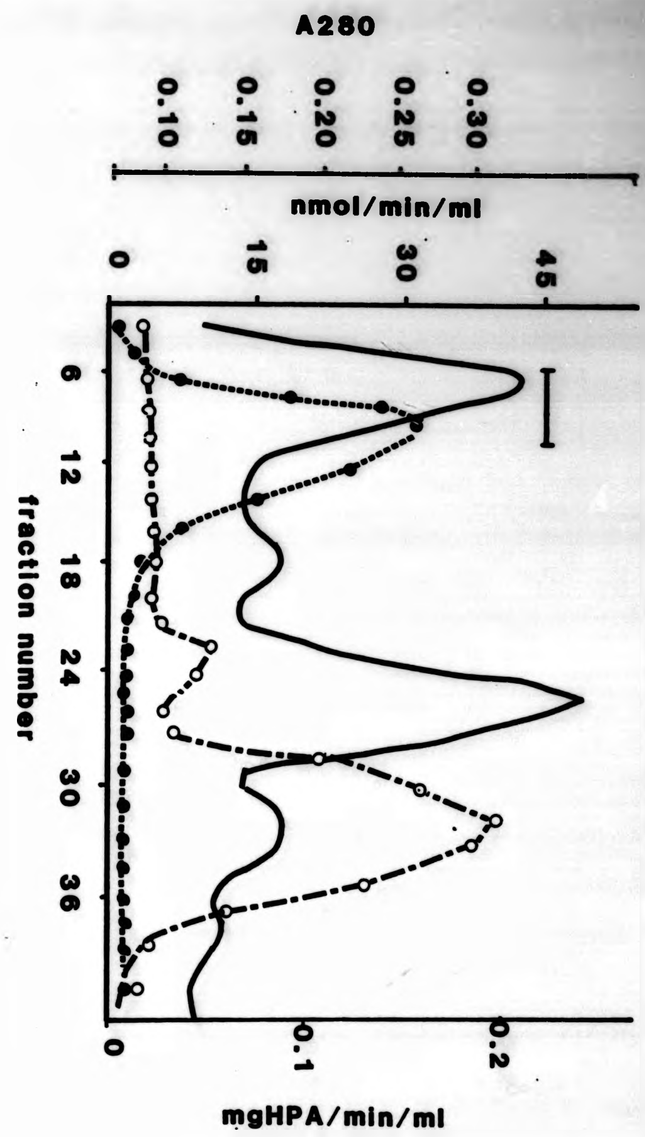
The nomenclature of M. methylotrophus proteinases was as follows: the letter immediately after Proteinase indicated the extract from which the enzyme was purified, W, C and P referring to whole cell extract, cytoplasm and periplasm respectively; the number following the letter indicated the peak fraction of the DEAE-cellulose column from which the enzyme was isolated and the letter following this number indicated individual enzymes extracted from the peak fraction.

3:2a (i) Purification and properties of a BzArgNA hydrolyzing enzyme from peak II

Peak II exhibited BzArgNA-hydrolyzing activity. To isolate the enzyme responsible for this activity peak II fractions were pooled, concentrated and dialyzed against Sephadex G75 column running buffer (section 2:5a). Peak II was then run on a Sephadex G75 column. Eluted fractions were tested for BzArgNA hydrolyzing activity, fig. 3:8. Fractions showing BzArgNA hydrolyzing activity were pooled, concentrated and dialyzed against Sephacryl S300 column running buffer (section 2:5a). The pooled fractions were then run on a Sephacryl S300 column. Eluted fractions were tested for BzArgNA hydrolyzing activity, fig. 3:9. Fractions capable of hydrolyzing BzArgNA were pooled and concentrated.

The BzArgNA hydrolyzing enzyme, referred to as Proteinase W2A was estimated to have a molecular weight of 60000D as determined by gel filtration on Sephadex G75 (although some BzArgNA hydrolysis was also observed in fractions in the void volume), and an apparent molecular weight of approximately 28000D as determined by

Fig. 3:8 Purification of Proteinase W2A by Sephadex G75 column chromatography. Pooled fractions from DEAE-cellulose peak II were concentrated and dialyzed against 10 mM Tris/HCl pH 7.8, 5 mM $MgCl_2$ and applied to a column of Sephadex G75 superfine. Activity was determined using HPA (O---O); BzArgNA (●----●) and gelatin (⊢——⊣) as substrates, A_{280} (——). Activity towards gelatin was determined as described previously (section 2:6c). 3.2 ml fractions were collected and every second fraction assayed.



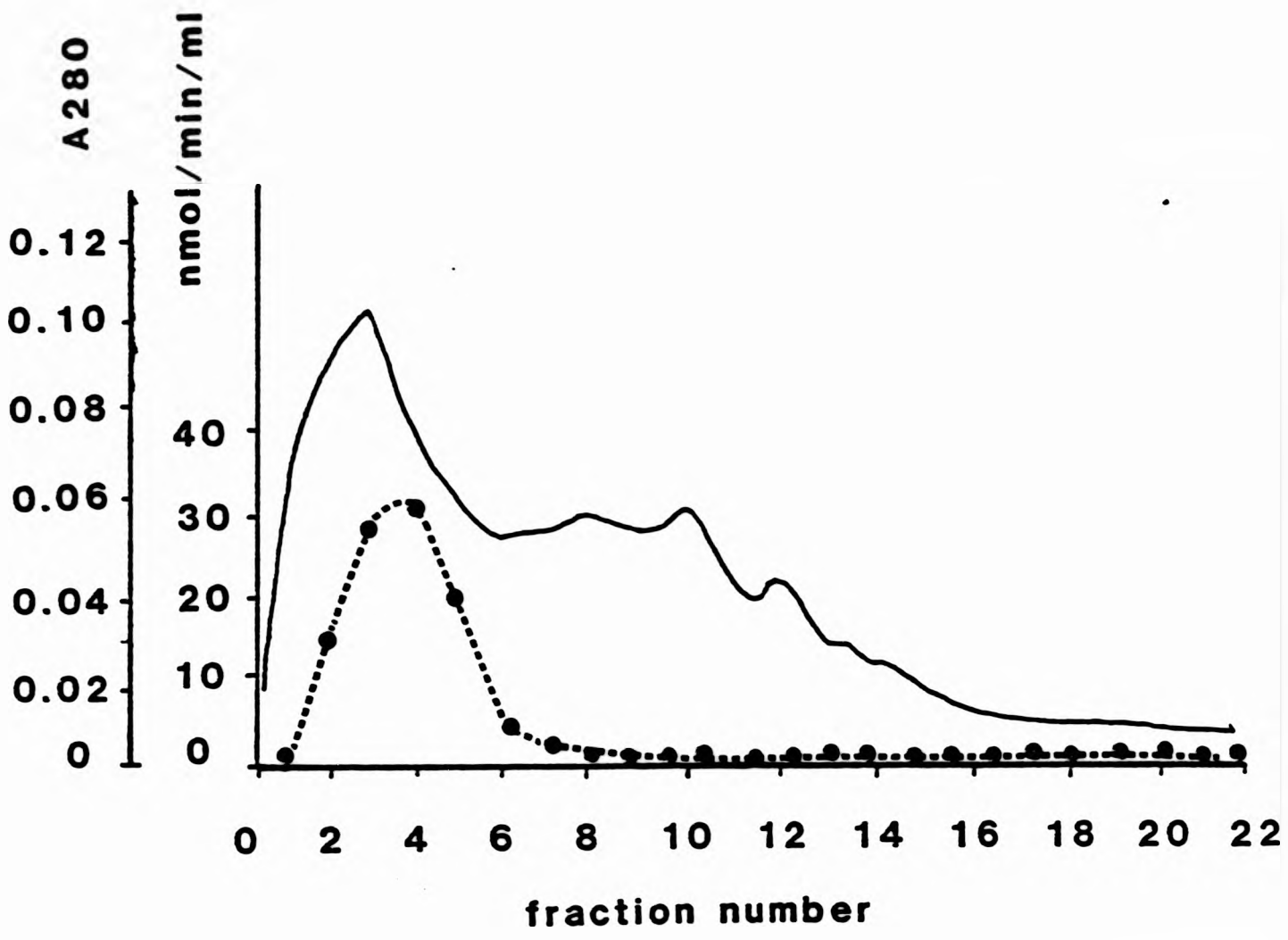


Fig. 3:9 Further purification of Proteinase W2A by Sephacryl S300 column chromatography. Fractions from the Sephadex G75 superfine column able to hydrolyze BzArgNA were pooled, concentrated and dialyzed against 10 mM Tris/HCl pH 7.8, 5 mM $MgCl_2$ and applied to a Sephacryl S300 column. Activity was determined using BzArgNA (●····●) as substrate, A_{280} (———). 2.0 ml fractions were collected and every second fraction assayed.

SDS-polyacrylamide gel electrophoresis, plate 3:1. This suggested that the active enzyme was a dimer of 28000D subunits. A minor contaminant of apparent molecular weight 24000D was also evident on the SDS-polyacrylamide gel and it could not be ruled out that this protein was in fact a component of the enzyme.

Proteinase W2A was not significantly inhibited by either PMSF or EDTA (table 3:5); inhibition by phenanthroline suggested a metal ion requirement other than Ca^{2+} for maximum enzyme activity, however none of the divalent cations tested Ca^{2+}, Zn^{2+}, Mg^{2+}, Mn^{2+} or Co^{2+} restored activity to the phenanthroline inactivated enzyme. Proteinase W2A was also inhibited to some extent by TLCK, TPCK, iodoacetic acid, chymostatin and leupeptin.

The ATP-stimulated increase in BzArgNA hydrolysis observed with peak II fractions from the DEAE-cellulose column, fig. 3:7, was not detected with the purified enzyme, table 3:6. It appeared that the ATP-stimulating activity was lost during purification. In addition to ATP none of the other nucleotides tested were able to stimulate BzArgNA hydrolysis by Proteinase W2A. cAMP however showed reproducibly a 25% inhibition of activity.

The complete purification scheme for Proteinase W2A is shown in table 3:7. It should be pointed out that the fold purifications given for this and other proteinases are likely to be underestimates since these enzymes are responsible for only a portion of the total BzArgNA hydrolyzing activity of the original whole cell extract.

(ii) Hydrolysis of gelatin by Proteinase W2A

A sample of proteinase W2A was run under non-denaturing conditions on an SDS-polyacrylamide gel containing gelatin (section

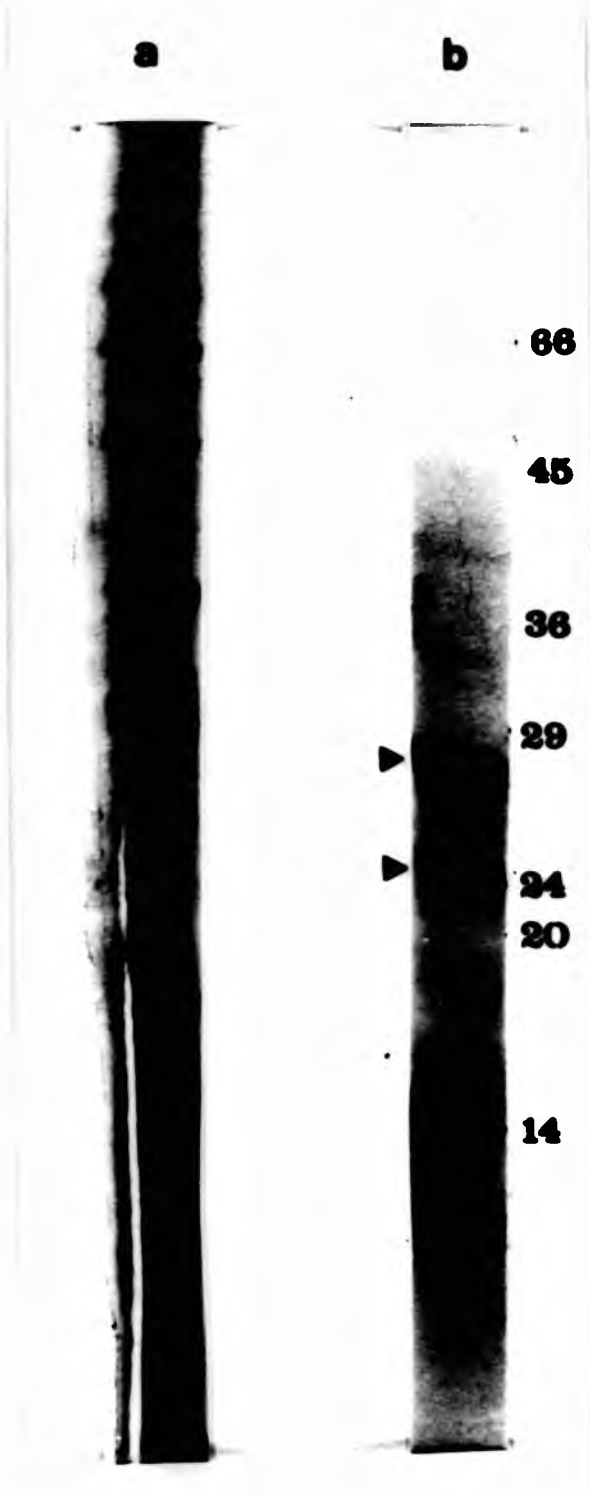


Plate 3:1 SDS-polyacrylamide gel electrophoresis of Proteinase W2A. Lane a, whole cell extract (250 µg protein); lane b, Proteinase W2A from the Sephacryl S300 column (20 µg protein).

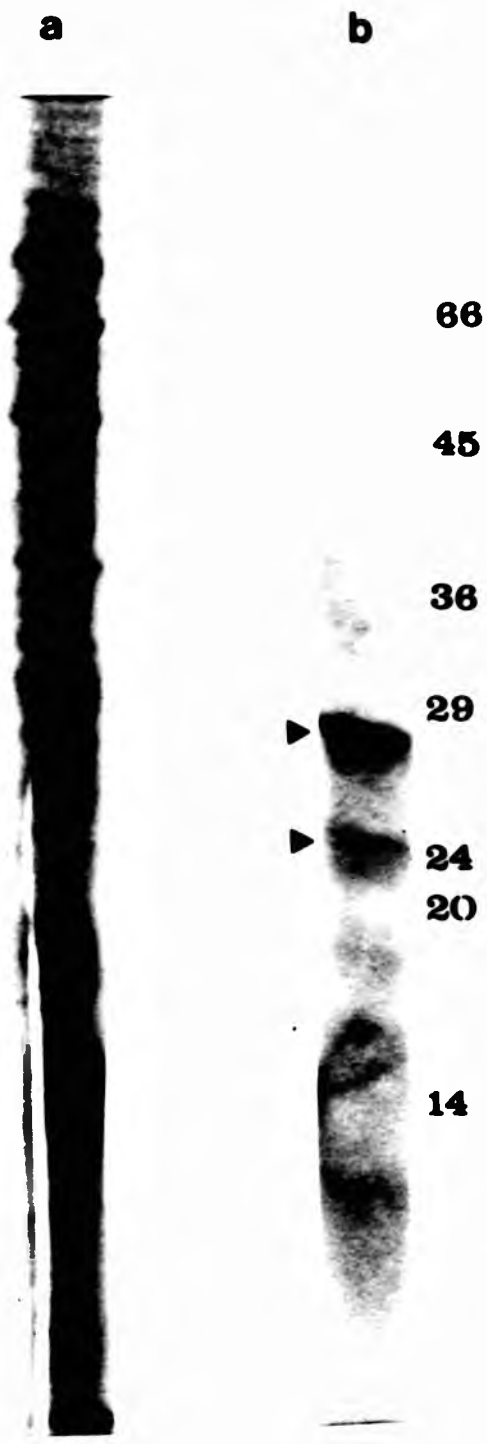


Plate 3:1 SDS-polyacrylamide gel electrophoresis of Proteinase W2A. Lane a, whole cell extract (250 µg protein); lane b, Proteinase W2A from the Sephacryl S300 column (20 µg protein).

TABLE 3:5

Inhibition of Proteinase W2A BzArgNA hydrolyzing activity

Inhibitor (1 mM unless stated otherwise)	Inhibition (%)
PMSF	11
EDTA	6
Phenanthroline	72
TLCK	72
TPCK	21
IAA	33
chymostatin (10 µg/ml)	88
leupeptin (10 µg/ml)	58

Assays were performed as described in section 2:13. 100% activity (2.6 nmol/min) was measured in the absence of inhibitor.

TABLE 3:6

Effects of nucleotides on BzArgNA hydrolysis by Proteinase W2A

Nucleotide (3 mM)	Activity (%)
ATP	93
ADP	119
AMP	100
cAMP	75
UTP	93
GTP	100
CTP	100
No nucleotide	100

Assays were performed as described in section 2:14. 100% activity (2.6 nmol/min) was measured in the absence of nucleotide.

TABLE 3:7

Purification scheme for the BzArgNA hydrolyzing enzyme Proteinase W2A

Extract	Total Protein (mg)	Total Activity (nmol/min)	Sp. Ac. (nmol/min/mg protein)	Purity (fold)	Recovery (%)
Whole cell	288	1440	5	1	100
40-80% $(NH_4)_2SO_4$	160	1920	12	2.4	133
DEAE-cellulose peak II	12	240	20	4.0	17
Sephadex G75 Proteinase W2A	4.5	450	100	20	31
Sephacryl S300	2.0	320	160	32	22

2:6c). The proteinase showed a single gelatin-degrading activity that ran at an apparent molecular weight of 56000D plate 3:2. This result and the fact that Proteinase W2A ran under denaturing conditions as a single protein species of apparent molecular weight 28000D suggested that this enzyme was a dimer made up of 28000D subunits.

(iii) Purification of an HPA-hydrolyzing enzyme from peak II

The enzyme responsible for the HPA-hydrolyzing activity from peak II of the DEAE-cellulose column, fig. 3:7, was separated from Proteinase W2A by gel chromatography on a Sephadex G75 column, fig. 3:8. Fractions that exhibited an HPA-hydrolyzing activity were pooled and concentrated firstly through a PM10 Amicon ultrafiltration unit and then a PM30 unit. The HPA-hydrolyzing enzyme, named Proteinase W2B, had an apparent molecular weight of 18000D as determined by both gel-filtration, fig. 3:8 and SDS-polyacrylamide gel electrophoresis, plate 3:3. The complete purification scheme for Proteinase W2B is shown in table 3:8.

3:2b (i) Purification and properties of BzArgNA hydrolyzing enzymes from peak III

Peak III fractions from the DEAE-cellulose column (fig. 3:7) showed the major BzArgNA-hydrolyzing activities. To isolate the enzymes(s) responsible for this activity, peak III fractions were pooled, concentrated and dialyzed against Sephadex G75 column running buffer. The pooled peak III fractions were then run on a Sephadex G75 column. Eluted fractions were tested for BzArgNA-hydrolyzing activity, fig. 3:10.

2:6c). The proteinase showed a single gelatin-degrading activity that ran at an apparent molecular weight of 56000D plate 3:2. This result and the fact that Proteinase W2A ran under denaturing conditions as a single protein species of apparent molecular weight 28000D suggested that this enzyme was a dimer made up of 28000D subunits.

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3:2b (i) Purification and properties of BzArgNA hydrolyzing enzymes from peak III

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Plate 3:2 Gelatin-hydrolyzing activity of Proteinase W2A.

SDS-polyacrylamide gel electrophoresis of Proteinase W2A run under non-denaturing conditions on gels incorporating gelatin. Lane 1, whole cell extract (250 μg protein); lane 2, periplasmic extract (200 μg protein); lane 3, cytoplasmic extract (200 μg protein); lane 4, peak fraction II from the DEAE-cellulose column (20 μg protein); lane 5, BzArgNA hydrolyzing enzyme from the Sephadex G75 column; lane 6, Proteinase W2A from the Sephacryl S300 column (5 μg protein) (sample boiled for 5 minutes prior to loading); lane 8, molecular weight markers from top to bottom, myosin (205000 D), B-galactosidase (116000 D), phosphorylase b (97400 D), bovine albumin (66000 D), egg albumin (45000 D) and carbonic anhydrase (29000 D); lane 9, molecular weight markers from top to bottom, bovine albumin (66000 D), egg albumin (45000 D), glyceraldehyde-3-phosphate dehydrogenase (36000 D), carbonic anhydrase (29000 D), trypsinogen (24000 D), soybean trypsin inhibitor (20000 D) and ∝ -lactalbumin (14200 D).

Plate 3:2 Gelatin-hydrolyzing activity of Proteinase W2A. SDS-polyacrylamide gel electrophoresis of Proteinase W2A run under non-denaturing conditions on gels incorporating gelatin. Lane 1, whole cell extract (250 μg protein); lane 2, periplasmic extract (200 μg protein); lane 3, cytoplasmic extract (200 μg protein); lane 4, peak fraction II from the DEAE-cellulose column (20 μg protein); lane 5, BzArgNA hydrolyzing enzyme from the Sephadex G75 column; lane 6, Proteinase W2A from the Sephacryl S300 column (5 μg protein) (sample boiled for 5 minutes prior to loading); lane 8, molecular weight markers from top to bottom, myosin (205000 D), B-galactosidase (116000 D), phosphorylase b (97400 D), bovine albumin (66000 D), egg albumin (45000 D) and carbonic anhydrase (29000 D); lane 9, molecular weight markers from top to bottom, bovine albumin (66000 D), egg albumin (45000 D), glyceraldehyde-3-phosphate dehydrogenase (36000 D), carbonic anhydrase (29000 D), trypsinogen (24000 D), soybean trypsin inhibitor (20000 D) and ∝-lactalbumin (14200 D).

Plate 3:3 SDS-polyacrylamide gel electrophoresis of Proteinase W2B. Lane 1, whole cell extract (250 μg protein); lane 2, periplasmic fraction (200 μg protein); lane 3, cytoplasmic fraction (200 μg protein); lane 4, peak fraction II from the DEAE-cellulose column (20 μg protein); lane 5, HPA hydrolyzing enzyme from the Sephadex G75 column (5 μg protein); lane 6, Proteinase W2B filtered through a PM30 Amicon filter unit (5 μg protein); lane 7, Proteinase W2B concentrated in a PM10 Amicon filter unit (5 μg protein); lane 9, molecular weight markers from top to bottom, myosin (205000 D), B-galactosidase (116000 D), phosphorylase b (97400 D), bovine albumin (66000 D), egg albumin (45000 D) and carbonic anhydrase (29000 D); lane 10, molecular weight markers from top to bottom, bovine albumin (66000 D), egg albumin (45000 D), glyceraldehyde-3-phosphate dehydrogenase (36000 D), carbonic anhydrase (29000 D), trypsinogen (24000 D), soybean trypsin inhibitor (20000 D) and ∝-lactalbumin (14200 D).

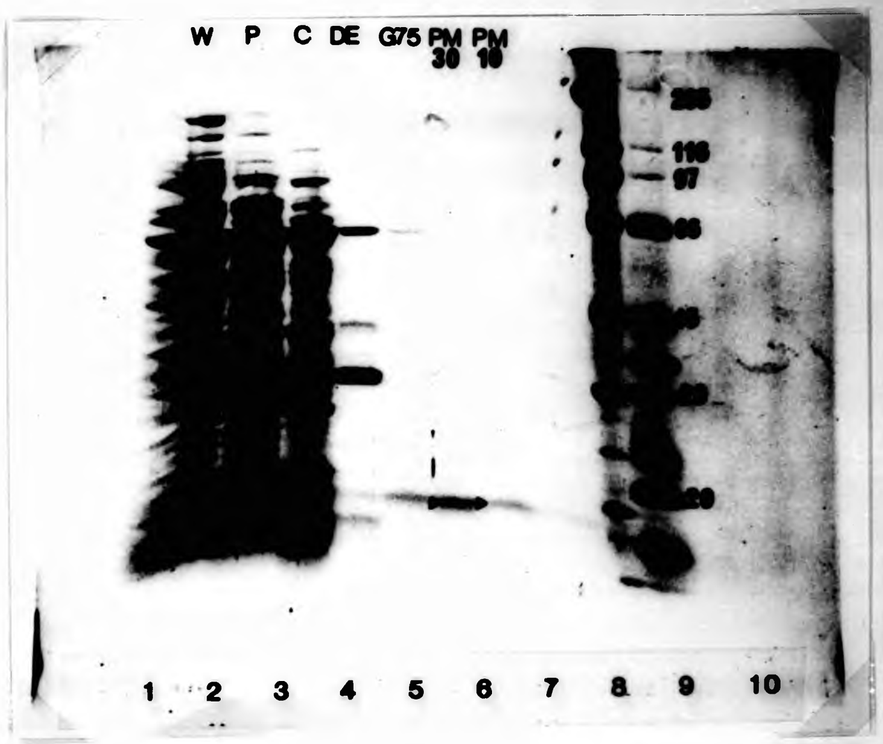


Plate 3:3 SDS-polyacrylamide gel electrophoresis of Proteinase W2B. Lane 1, whole cell extract (250 μg protein); lane 2, periplasmic fraction (200 μg protein); lane 3, cytoplasmic fraction (200 μg protein); lane 4, peak fraction II from the DEAE-cellulose column (20 μg protein); lane 5, HPA hydrolyzing enzyme from the Sephadex G75 column (5 μg protein); lane 6, Proteinase W2B filtered through a PM30 Amicon filter unit (5 μg protein); lane 7, Proteinase W2B concentrated in a PM10 Amicon filter unit (5 μg protein); lane 9, molecular weight markers from top to bottom, myosin (205000 D), B-galactosidase (116000 D), phosphorylase b (97400 D), bovine albumin (66000 D), egg albumin (45000 D) and carbonic anhydrase (29000 D); lane 10, molecular weight markers from top to bottom, bovine albumin (66000 D), egg albumin (45000 D), glyceraldehyde-3-phosphate dehydrogenase (36000 D), carbonic anhydrase (29000 D), trypsinogen (24000 D), soybean trypsin inhibitor (20000 D) and ∝-lactalbumin (14200 D).

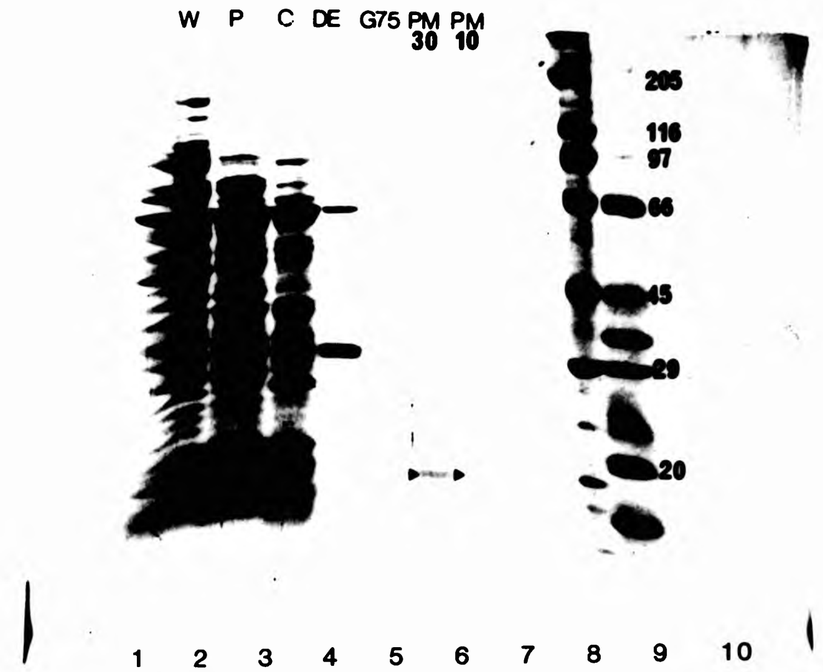
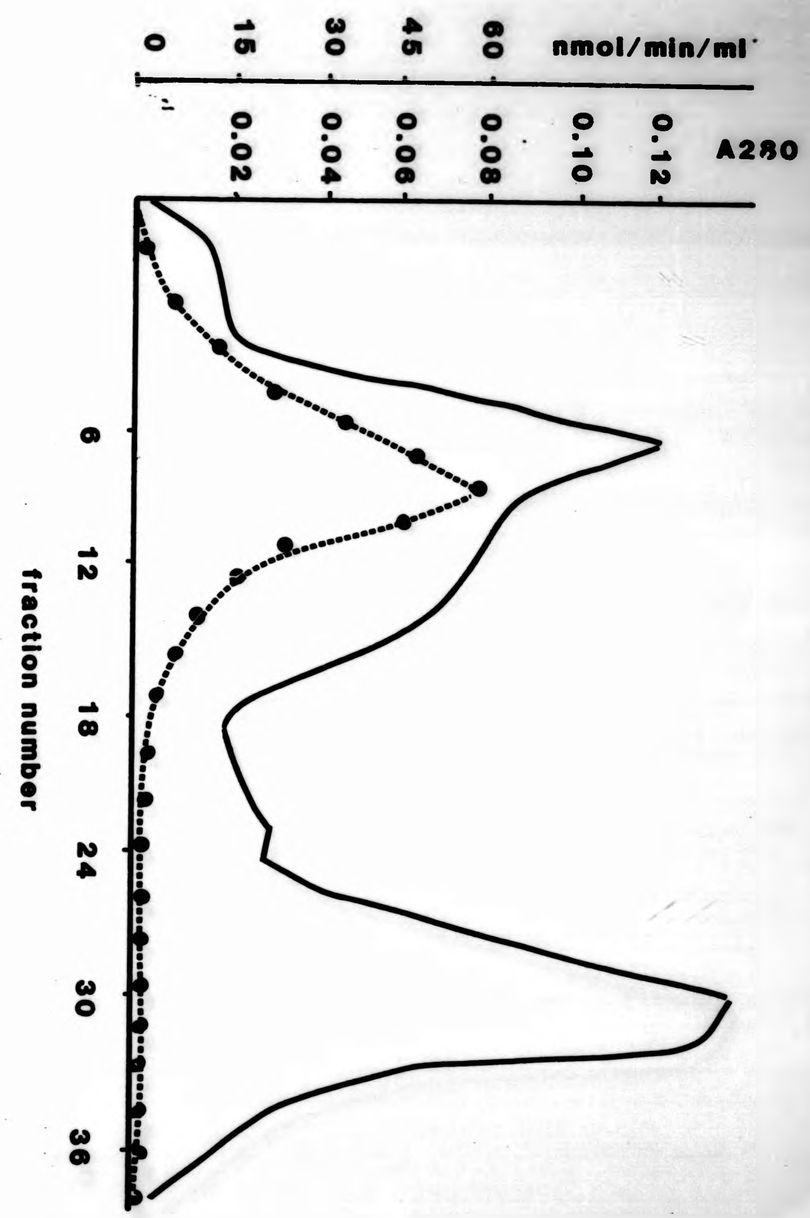


TABLE 3:8

Purification scheme for the HPA hydrolyzing enzyme Proteinase W2B

Extract	Total Protein (mg)	Total activity (mgHPA/min)	Sp. Ac. (mgHPA/min/mg Protein)	Purity (fold)	Recovery (%)
Whole cell	288	61.5	0.21	1	100
40-80% $(NH_4)_2SO_4$	160	67.0	0.42	2	108
DEAE-cellulose peak II	12	16.0	1.33	6.2	26
Sephadex G75 Proteinase W2B	22	27	12.2	57	44
Proteinase W2B (After ultrafiltration)	20	26	12.9	60	42

Fig. 3:10 Purification of BzArgNA-hydrolyzing enzymes from DEAE-cellulose peak III. Pooled fractions from DEAE-cellulose peak III were concentrated and dialyzed against 10 mM Tris/HCl pH 7.5, 5 mM $MgCl_2$ and applied to a column of Sephadex G75 superfine. Activity was determined using BzArgNA (●-----●) as substrate, A_{280} (———). 3.2 ml fractions were collected and every second fraction assayed.



The active fractions were pooled, concentrated and dialyzed against CM52 column buffer (section 2:5b(ii)). CM52 ion-exchange chromatography of the pooled fractions showed two peaks of BzArgNA hydrolyzing activity, fig. 3:11. The enzymes responsible for the hydrolysis of BzArgNA have been named Proteinase W3A and W3B according to their order of elution from this column.

(ii) Purification and characterization of Proteinase W3A

Fractions containing Proteinase W3A were pooled, concentrated and run on a Sephadex G200 superfine column (section 2:5a). Eluted fractions were tested for their BzArgNA hydrolyzing activities, fig. 3:12. Active fractions were pooled and concentrated and stored at -15°C.

According to its elution on a Sephadex G200 superfine column, Proteinase W3A had an apparent molecular weight of greater than 250000 D. SDS-polyacrylamide gel electrophoresis of Proteinase W3A exhibited 3 major protein bands of apparent molecular weights 90000D and 45000D, two minor protein bands of apparent molecular weights 28000D and 27000D and a diffuse low molecular weight protein of approximately 16000D, plate 3:4. The molecular weight of Proteinase W3A cannot be accurately assessed without further purification of this enzyme. Partially purified Proteinase W3A had a pH optimum of approximately pH 7.5 in Tris-HCl buffer. The effects of inhibitors on the hydrolysis of BzArgNA by Proteinase W3A are shown in table 3:9. Inactivation of enzyme activity by PMSF indicated that Proteinase W3A had a serine residue at the active site of the enzyme. Inhibition with both EDTA and phenanthroline suggested a divalent metal ion requirement for enzyme activity or stability.

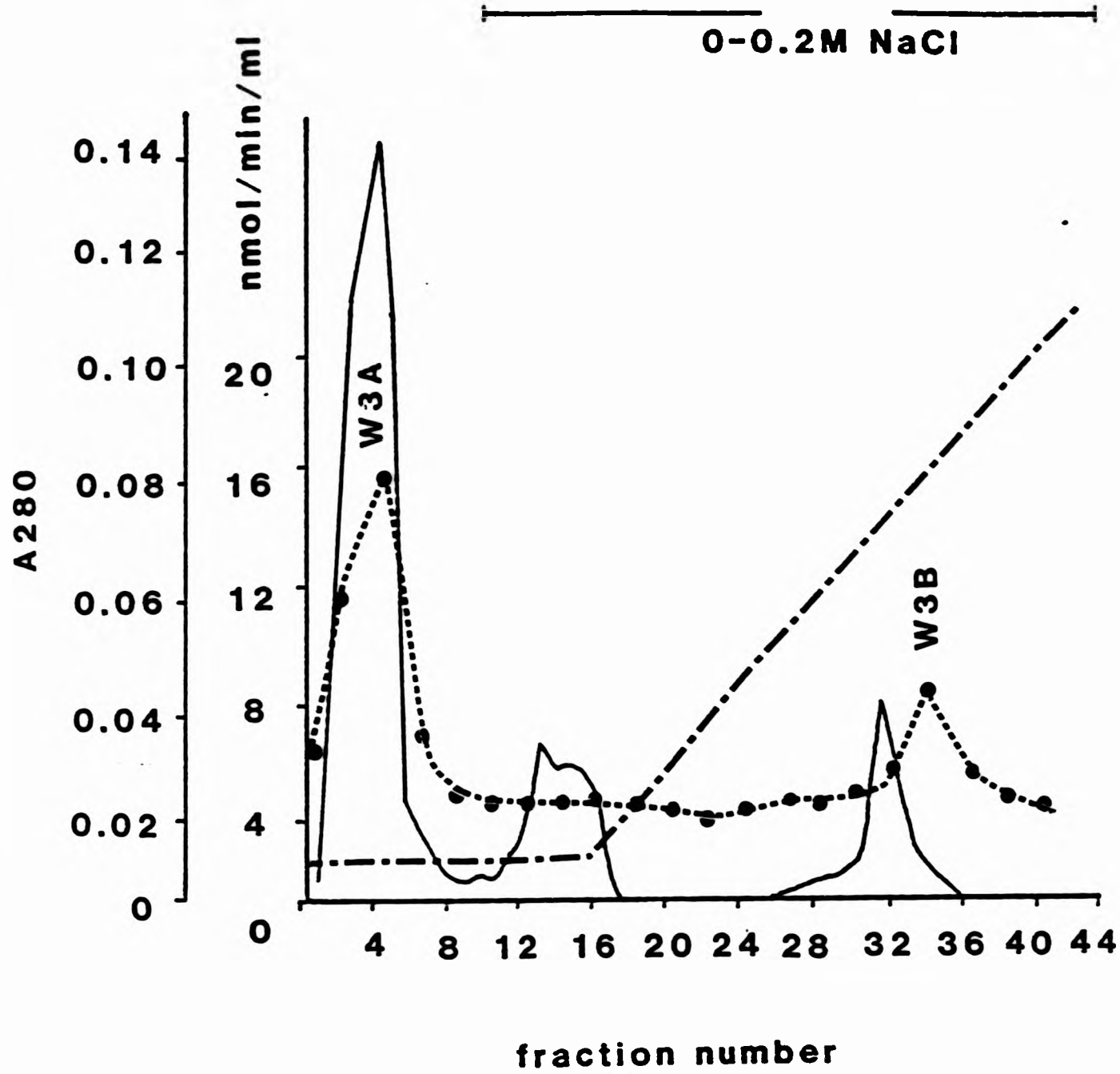


Fig. 3:11 CM-cellulose chromatography of BzArgNA hydrolyzing enzymes from the Sephadex G75 superfine column. Fractions from the Sephadex G75 column were pooled, concentrated and dialyzed against 10 mM Na-acetate, pH 5.5 and applied to a CM-cellulose column. The absorbed proteins were eluted with 400 ml of a 0-0.2M NaCl gradient. The flow rate was 35 ml/hr and 8 ml fractions were collected. Hydrolysis of BzArgNA (●----●), A_{280} (——) and conductivity (··—·).

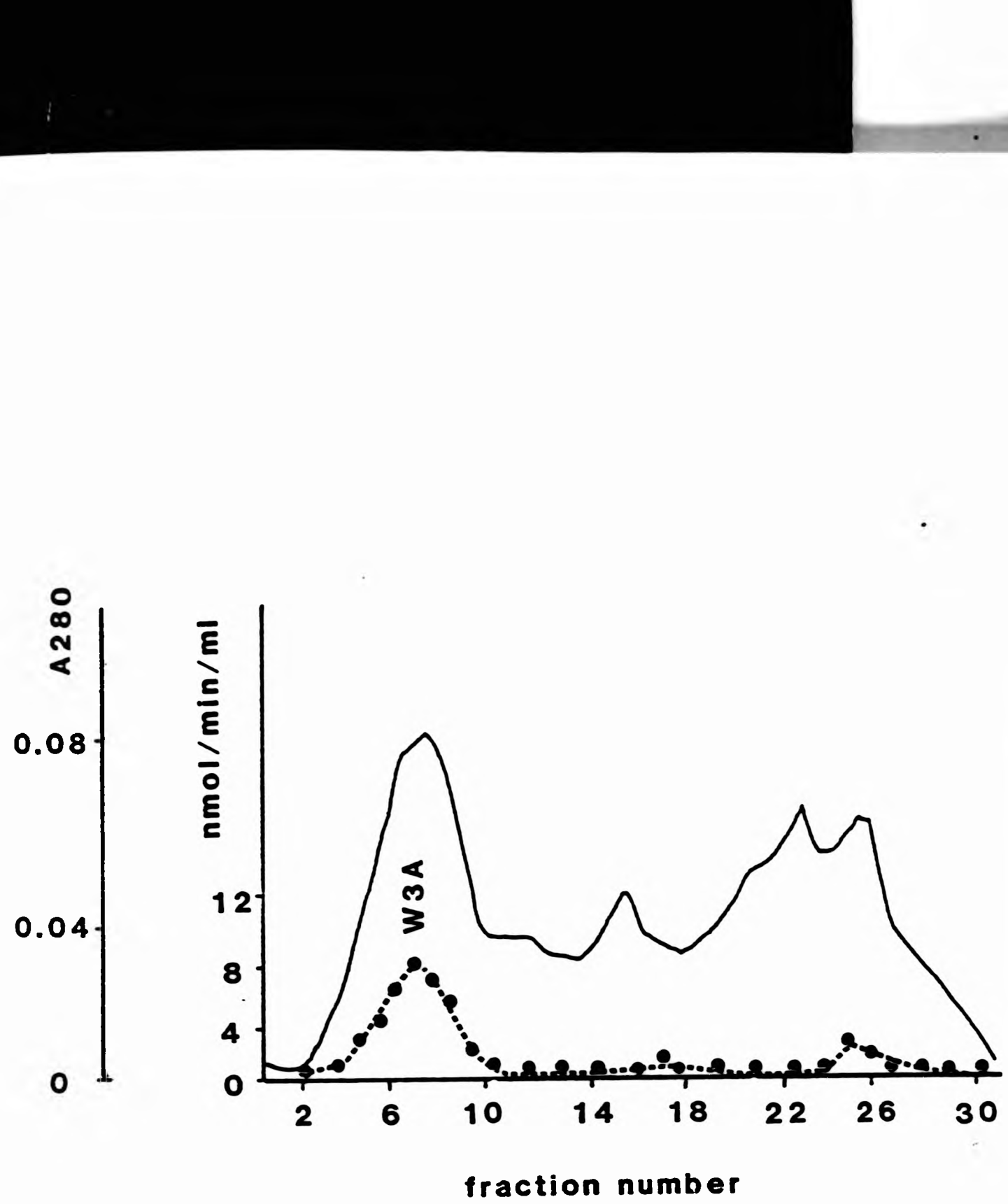


Fig. 3:12 Purification of Proteinase W3A by gel filtration. Fractions from the CM-cellulose column showing Proteinase W3A activity were pooled, concentrated and dialyzed against 10 mM Tris/HCl pH 7.8, 5 mM $MgCl_2$ and applied to a Sephadex G200 column. Activity was determined using BzArgNA (●·····●) as substrate, A_{280} (———). 3.2 ml fractions were collected and every second fraction assayed.

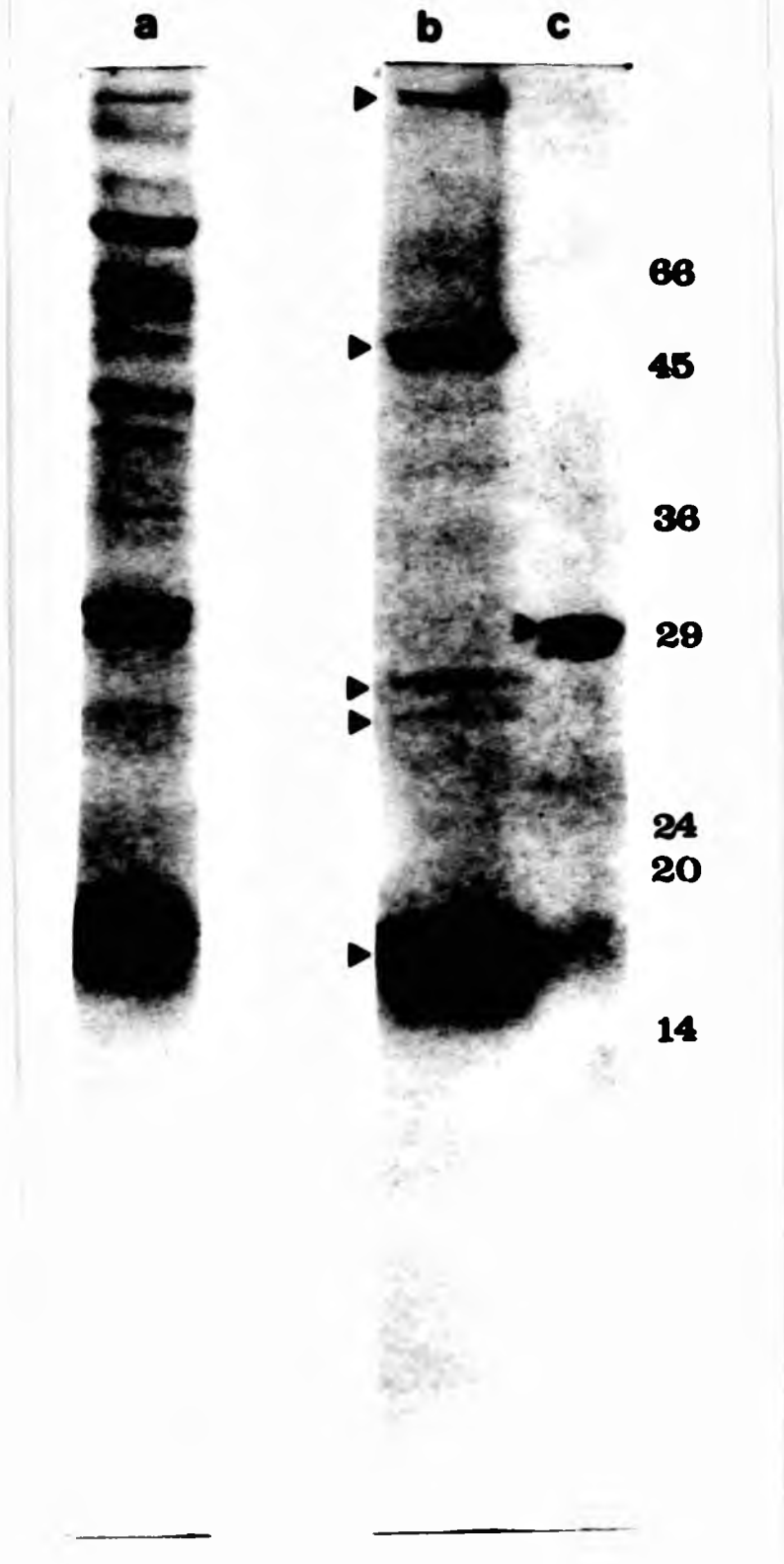


Plate 3:4 SDS-polyacrylamide gel electrophoresis of Proteinases W3A and W3B. Lane a, peak fraction III from the DEAE-cellulose column (100 µg protein); lane b, Proteinase W3A from the Sephadex G200 column (20 µg protein); lane c, Proteinase W3B from the CM52-cellulose column (5 µg protein). Molecular weight markers (kilodaltons) are shown on the right of the plate.

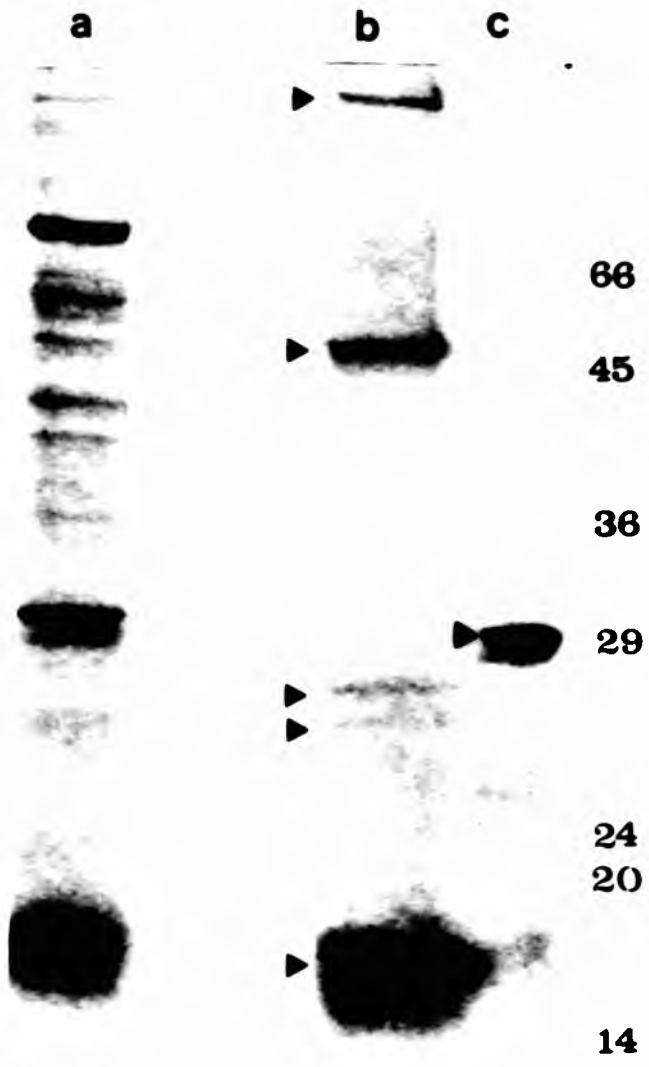


Plate 3:4 SDS-polyacrylamide gel electrophoresis of Proteinases W3A and W3B. Lane a, peak fraction III from the DEAE-cellulose column (100 µg protein); lane b, Proteinase W3A from the Sephadex G200 column (20 µg protein); lane c, Proteinase W3B from the CM52-cellulose column (5 µg protein). Molecular weight markers (kilodaltons) are shown on the right of the plate.

TABLE 3:9

Inhibition of BzArgNA hydrolyzing activity of Proteinase W3A

Inhibitor (1 mM unless stated otherwise)	Inhibition (%)
PMSF	69
EDTA	100
Phenanthroline	83
TLCK	17
TPCK	37
IAA	0
chymostatin (10 µg/ml)	56
leupeptin (10 µg/ml)	78

Assays were performed as described in section 2:13. 100% activity (0.80 nmol/min) was measured in the absence of inhibitor. Samples contained 5 µg of protein per assay.

Proteinase W3A was also inhibited to some extent by TLCK, TPCK, chymostatin and leupeptin.

The restoration of enzyme activity with divalent metal ions was investigated. Divalent metal ions were added back to EDTA-treated Proteinase W3A, fig. 3:13. It was clear that some restoration of enzyme activity was achieved with all the metal ions tested. Co^{2+} (2 mM) restored almost 50% of the enzymes original activity. Concentrations of metal ions greater then 5 mM (1 mM with Zn^{2+}) caused precipitation in assay mixtures. Although the evidence presented suggested metal ions were important in enzyme activity or stability, the metal ion specifically required for these functions was not established.

The effects of nucleotides on the hydrolysis of BzArgNA by Proteinase W3A, were investigated, table 3:10. There was no apparent stimulation of BzArgNA hydrolysis by any of the nucleotides tested. There was however an inhibition of Proteinase W3A activity by 3 mM CTP (52%) and 3 mM UTP (64%). Enzyme extracts that had been inhibited by CTP or UTP could be restored to 90% of their original activity after dialysis against buffer (10 mM Tris-HCl, pH 7.5, 5 mM Mg^{2+}). Inhibition of Proteinase W3A activity by both UTP and CTP was concentration dependent, fig. 3:14. A 50% reduction in BzArgNA hydrolysis was achieved with either 1-2 mM UTP or 3-4 mM CTP. The specificity of the effect was investigated, table 3:10. It was apparent that inhibition only occured in the presence of pyrimidine triphosphates. The significance of this result remains unclear.

(iii) <u>Purification and properties of Proteinase W3B</u>

Proteinase W3B, separated from Proteinase W3A by ion-exchange

Fig. 3:13 The effects of divalent metal ions on the restoration of EDTA-inactivated Proteinase W3A. Unless indicated extracts were incubated with 3 mM EDTA then dialyzed against incubation buffer before addition of the metal ions. The activities were expressed as a percentage of the activity of non-EDTA treated whole cell extracts. Untreated extracts (100% = 0.80 nmol/min). Samples contained 5 μg protein per assay.

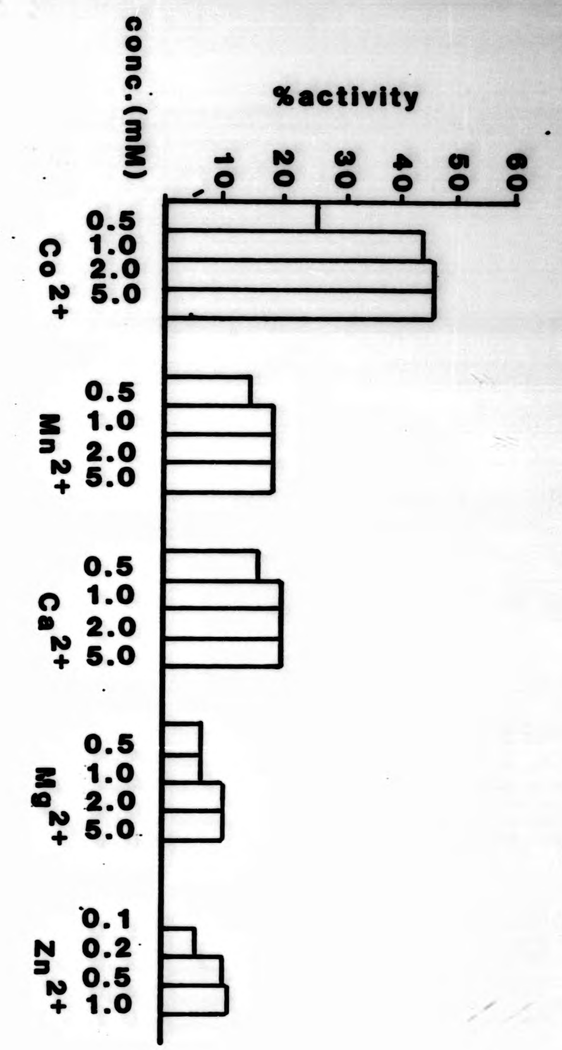


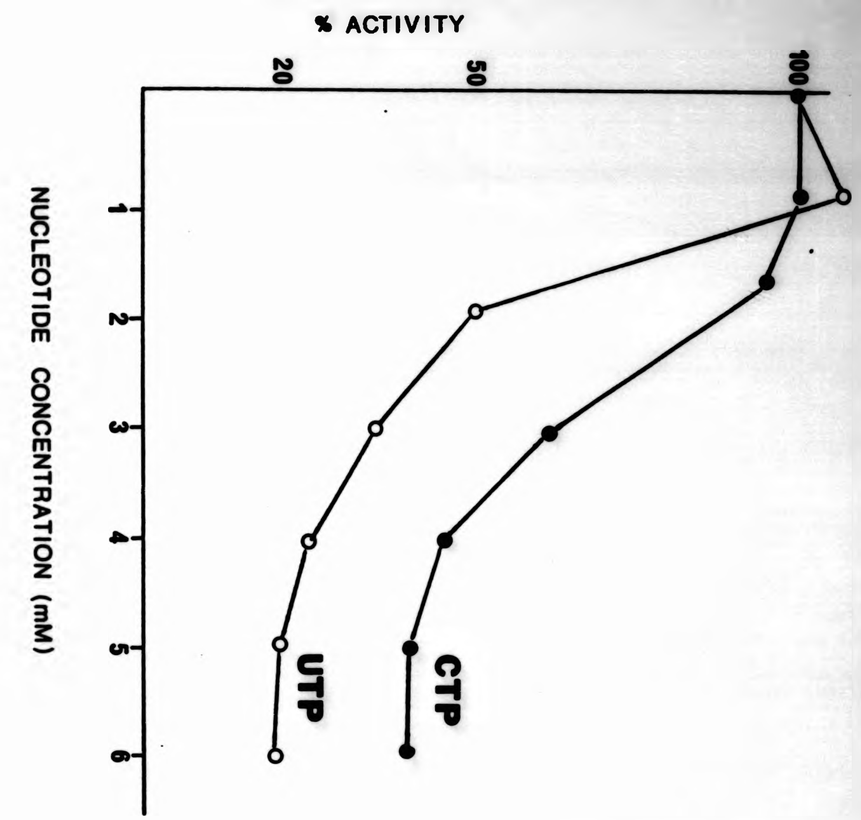
TABLE 3:10

The effects of nucleotide/nucleosides on the Proteinase W3A BzArgNA hydrolyzing activity

Nucleotide/Nucleoside (3 mM)	Activity (%)
ATP	108
ADP	104
AMP	96
cAMP	100
UTP	36
GTP	88
CTP	48
UDP	100
UMP	100
uracil	90
cytosine	97

Assays were performed as described in section 2:14. 100% activity (0.80 nmol/min) was measured in the absence of nucleotide or nucleoside. Samples contained 5 μg of protein per assay.

Fig. 3:14 The concentration dependent effects of UTP and CTP on the hydrolysis of BzArgNA by Proteinase W3A. The activities were expressed as a percentage of the BzArgNA hydrolyzing activity in the absence of the nucleotide triphosphates. 100% activity = 0.80 nmol/min. Samples contained 5 µg protein per assay.



chromatography, fig. 3:11 had an apparent molecular weight of 29000 D as estimated by SDS-polyacrylamide gel electrophoresis, plate 3:4.

Inhibition of BzArgNA hydrolysis by PMSF suggested that Proteinase W3B was a serine-type proteinase, table 3:11. The inability to inactivate the enzyme with either EDTA or o-phenanthroline indicated that metal-ions were not necessary for enzyme activity or stability. Inhibition by both TPCK and leupeptin but not TLCK suggested that Proteinase W3B was trypsin-like in its action.

The effects of nucleotides on Proteinase W3B activity were investigated, table 3:12. Only GTP gave any stimulation of BzArgNA hydrolysis, however this stimulation was not always apparent and varied from one purification of the enzyme to another.

The purification schemes for Proteinase W3A and W3B are shown in table 3:13.

(iv) An alternative procedure for the isolation of BzArgNA hydrolyzing enzymes from peak III

In an attempt to reduce the number of purification steps in the isolation of proteolytic enzymes, an alternative procedure, successfully used in the isolation of a membrane proteinase from E. coli (Régnier, 1981a), was attempted in which the crude extract was run on a preparative SDS-polyacrylamide gel (section 2:6e). This procedure has been used to investigate the proteolytic enzymes from peak III of the DEAE-cellulose column run, fig. 3:7.

Peak III fractions, exhibiting BzArgNA hydrolyzing activity, were pooled and concentrated. The concentrated sample (1.5 ml) was

TABLE 3:11

Inhibition of the BzArgNA hydrolyzing activity of Proteinase W3B

Inhibitor (1 mM unless stated otherwise)	Inhibition (%)
PMSF	100
EDTA	0
phenanthroline	10
TLCK	0
TPCK	21
IAA	33
chymostatin (10 μg/ml)	24
leupeptin (10 μg/ml)	57

Assays were performed as described in section 2:13. 100% activity (0.3 nmol/min) was determined in the absence of inhibitor. Samples contained 0.5 μg protein per assay.

TABLE 3:12

The effects of nucleotides on BzArgNA hydrolysis by Proteinase W3B

Nucleotide (3 mM)	Activity (%)
ATP	101
ADP	91
AMP	96
cAMP	96
UTP	96
GTP	100 - 142
CTP	104
No nucleotide	100

Assays were performed as described in section 2:14. 100% activity (0.30 nmol/min) was measured in the absence of nucleotide. Samples contained 0.5 μg protein per assay.

TABLE 3:13

Purification scheme for BzArgNA hydrolyzing enzymes
Proteinases W3A and W3B

Extract	Total Protein (mg)	Total Activity (nmol/min)	Sp. Ac. (nmol/min/mg protein)	Purity (fold)	Recovery (%)
Whole cell	288	1440	5	1	100
40-80% $(NH_4)_2SO_4$	160	1920	12	2.4	133
DEAE-cellulose peak III	12.6	630	50	10	44
Sephadex G75 BzArgNA hydrolyzing activity	4.0	800	200	40	56
CM52-cellulose					
proteinase W3A	1.9	247	130	26	17
W3B	0.16	93	580	116	6
Sephadex G200 Proteinase W3A	1.0	150	150	30	10

added to an equal volume of solubilization-reduction mix and run on an SDS-polyacrylamide gel prepared as described in section 2:6e. Horizontal slices (5 mm) of the gel, which had been dialyzed against 50 mM Tris-HCl, pH 7.5, were tested for their BzArgNA hydrolyzing activities, fig. 3:15a.

Two sections of the gel showed BzArgNA hydrolyzing activities. The first activity was eluted at an apparent molecular weight of 45000D, the second at an apparent molecular weight of 29000D. The detection of only two BzArgNA hydrolyzing enzymes, by the preparative method, with apparent molecular weights comparable to the two BzArgNA hydrolyzing isolated by gel-chromatography, suggested that the 45000D enzyme was Proteinase W3A (section 3:2b (ii)) and the 29000D enzyme was Proteinase W3B (section 3:2b (iii)).

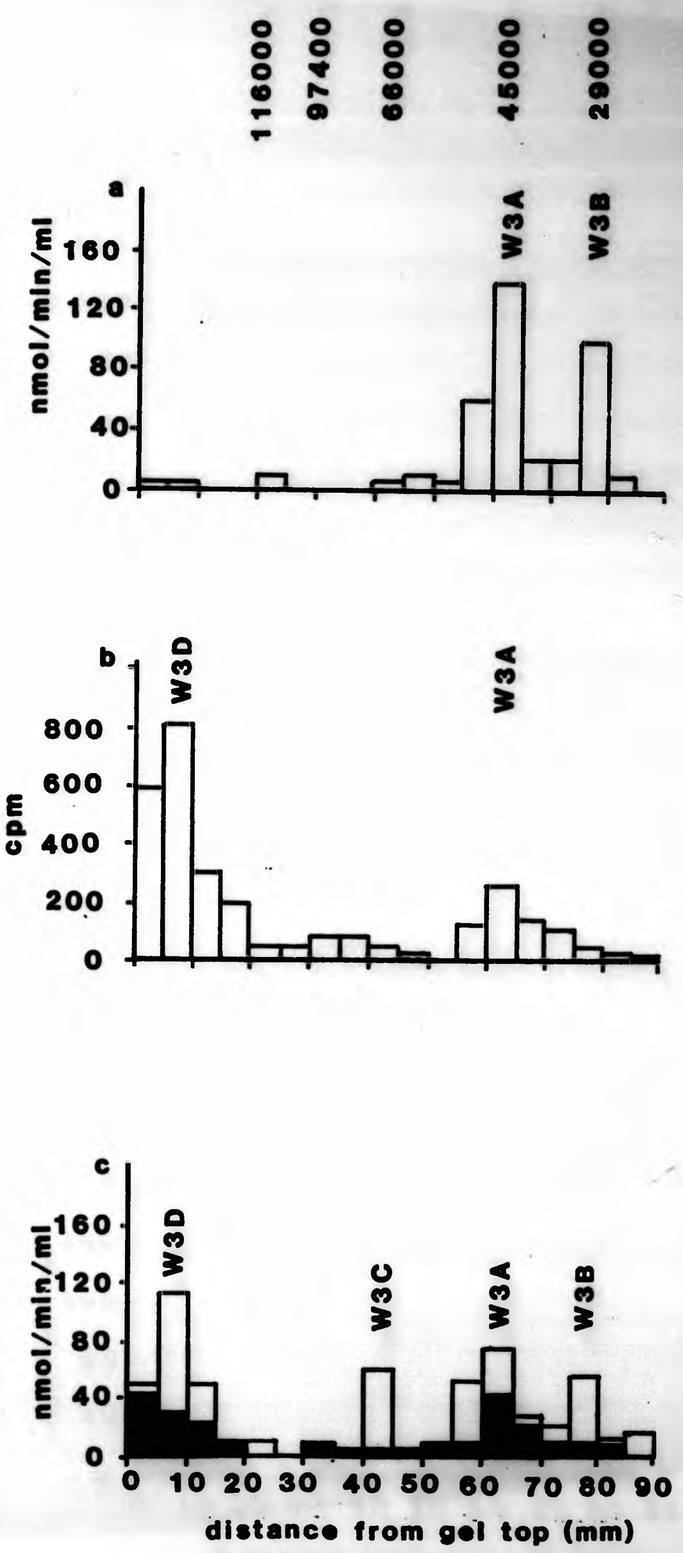
Although further characterization will be necessary before the enzymes isolated by the two procedures can be said unequivocably to be the same, they will, for convenience, be referred to as Proteinases W3A and W3B.

(v) Hydrolysis of other substrates by Proteinases W3A and W3B

Proteinase W3A, isolated by the preparative SDS-polyacrylamide procedure described above, also hydrolyzed [^{14}C]-α_1-casein, fig. 3:15b, and the chymotrypsin substrate succinyl-alaninyl-alaninyl-prolyl-phenylalanine-4-nitroanilide (SAAPP), fig. 3:15c. The rate of nitroaniline release from SAAPP was increased 100% in the presence of aminopeptidase M. These results suggested that Proteinase W3A was in fact an endoproteinase.

Proteinase W3B hydrolyzed both BzArgNA and SAAPP. Hydrolysis of SAAPP however was only detected in the presence of aminopeptidase

Fig. 3:15 Purification of proteolytic enzymes from whole cell extracts by preparative SDS-polyacrylamide gel electrophoresis. 12 mg of protein was loaded on to the gel. Proteins were eluted from 5 mm slices of the gel as described in section 2:6e and assayed with (a) BzArgNA; (b) [^{14}C]-α_1-casein and (c) SAAPP in the absence (▬) and presence (▭) of aminopeptidase M. The position of molecular weight standards are shown at the top of the figure.



M. This result indicated that Proteinase W3B was also an endoproteinase.

(vi) Detection of two more proteolytic enzymes from peak III

The preparative SDS-polyacrylamide gel procedure has allowed two more proteolytic enzymes from peak III of the DEAE-cellulose column to be detected. These enzymes have been called Proteinase W3C and W3D, fig. 3:15b & c.

Proteinase W3C had an apparent molecular weight of 66000 D. There was an aminopeptidase M stimulated release of nitroaniline from SAAPP with this enzyme, it did not however hydrolyze either $[^{14}C]$-α_1-casein or BzArgNA. This enzyme was not characterized any further at this time.

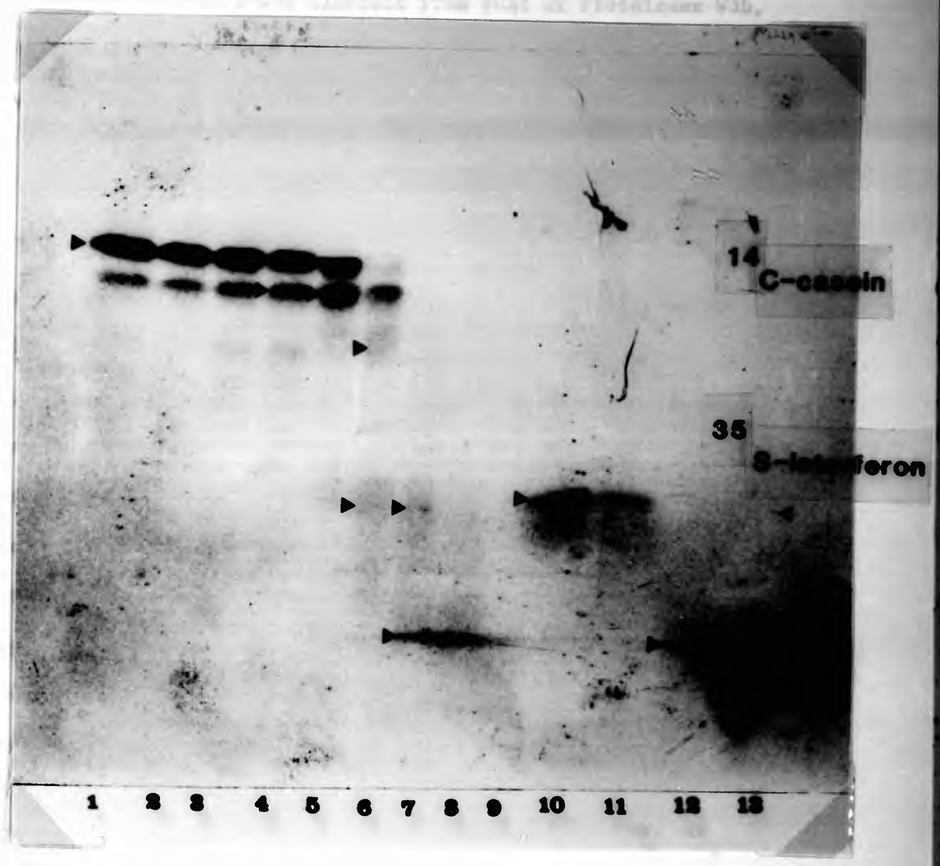
Proteinase W3D had an apparent molecular weight of 140-200000 D. This enzyme hydrolyzed $[^{14}C]$-α_1-casein and showed an aminopeptidase M-stimulated release of nitroaniline from SAAPP. It did not however hydrolyze BzArgNA.

A proteolytic enzyme can be characterized according to the breakdown products formed from particular substrate (Régnier 1981a). Since autoradiography provides a sensitive method of detecting labelled proteins and peptides (section 2:7) the products from the proteolytic degradation of $[^{14}C]$-α_1-casein by Proteinase W3D were investigated.

The degradation products from the hydrolysis of $[^{14}C]$-α_1-casein by Proteinase W3D were observed by autoradiography, plate 3:5. For a comparison the breakdown of $[^{14}C]$-α_1-casein by whole cell extracts was also followed, plate 3:5.

It was clear that the rate of protein degradation by Proteinase

Plate 3:5 Autoradiogram showing the degradation of [^{14}C]-α_1-casein and [^{35}S]-α_2-IFN by whole cell extracts and Proteinase W3D. Lanes 1-4, breakdown of [^{14}C]-α_1-casein (10000 cpm) by whole cell extracts after 0, 30, 90, 120 minutes incubation at 37°C. Assays were performed as described in section 2:3f. [^{14}C]-α_1-casein and breakdown products indicated by closed arrowheads. Lanes 5-8, breakdown of [^{14}C]-α_1-casein by Proteinase W3D after 0, 15, 30 and 60 minutes incubation at 37°C. [^{14}C]-α_1-casein and breakdown products indicated by closed arrowheads. Lanes 10 and 11, breakdown of [^{35}S]-α_2-IFN (3000 cpm) by whole cell extracts after 0 and 180 minutes incubation at 37°C. Assays were performed as described in section 2:3f. [^{35}S]-α_2-IFN and breakdown products indicated by closed arrowheads. Lanes 12 and 13, breakdown of [^{35}S]-α_2-IFN by Proteinase W3D after 30 and 180 minutes incubation at 37°C. Breakdown products of [^{35}S]-α_2-IFN indicated by closed arrowheads.



W3D was much faster than by whole cell extracts. Degradation of [^{14}C]-α_1-casein (35000 D) by Proteinase W3D occured by cleavage of approximately 7000 D polypeptide units or greater. The breakdown of [^{14}C]-α_1-casein by Proteinase W3D to low molecular weight products was achieved in 30 min. Degradation of [^{14}C]-α_1-casein by whole cell extracts was distinct from that of Proteinase W3D, with the initial cleavage of an 8000 D polypeptide and a 10000 D polypeptide. Breakdown of [^{14}C]-α_1-casein by whole cell extracts was not investigated beyond 90 minutes.

In addition to the hydrolysis of [^{14}C]-α_1-casein and SAAPP the degradative effect of Proteinase W3D on a protein encoded by a cloned gene was investigated. The breakdown of [^{35}S]-α_2-IFN (19000 D) by Proteinase W3D was observed by autoradiography, plate 3:5. For a comparison the hydrolysis of [^{35}S]-IFN by whole cell extracts, was also followed, plate 3:5. Incubation of whole cell extract with [^{35}S]-α_2-IFN caused no apparent breakdown of the substrate within 180 min. Incubation of this protein with Proteinase W3D for 30 min resulted in its degradation to polypeptides that ran with the gel front. Further incubation resulted in these polypeptides being further degraded. Although no information on the initial breakdown products of [^{35}S]-α_2-IFN by Proteinase W3D has been determined, it was clear that this enzyme was capable of degrading this substrate to low molecular weight products.

Inhibition of the [^{14}C]-α_1-casein degrading activity of Proteinase W3D by PMSF and the lack of such inhibition by either EDTA or phenanthroline, table 3:14, indicated that this enzyme was a serine proteinase with no specific requirements for a metal ion for

TABLE 3:14

Inhibition of BzArgNA hydrolyzing activity of Proteinase W3D

Inhibitor (1 mM unless stated otherwise)	Inhibition (%)
PMSF (10 mM)	100
PMSF	93
EDTA	17
phenanthroline	10
TLCK	2
TPCK (10 mM)	54
TPCK	19
IAA	0
chymostatin (10 μg/ml)	97
leupeptin (10 μg/ml)	10

Assays were performed as described in section 2:13. 100% activity (12 nmol/min) was determined in the absence of inhibitor.

its activity or stability. In addition inactivation of the enzyme hydrolyzing activity with chymostatin and TPCK and the absence of inhibition with TLCK, suggested that this enzyme was chymotrypsin-like in its activity.

3:2c (i) Purification and properties of a BzArgNA hydrolyzing enzyme from peak IV

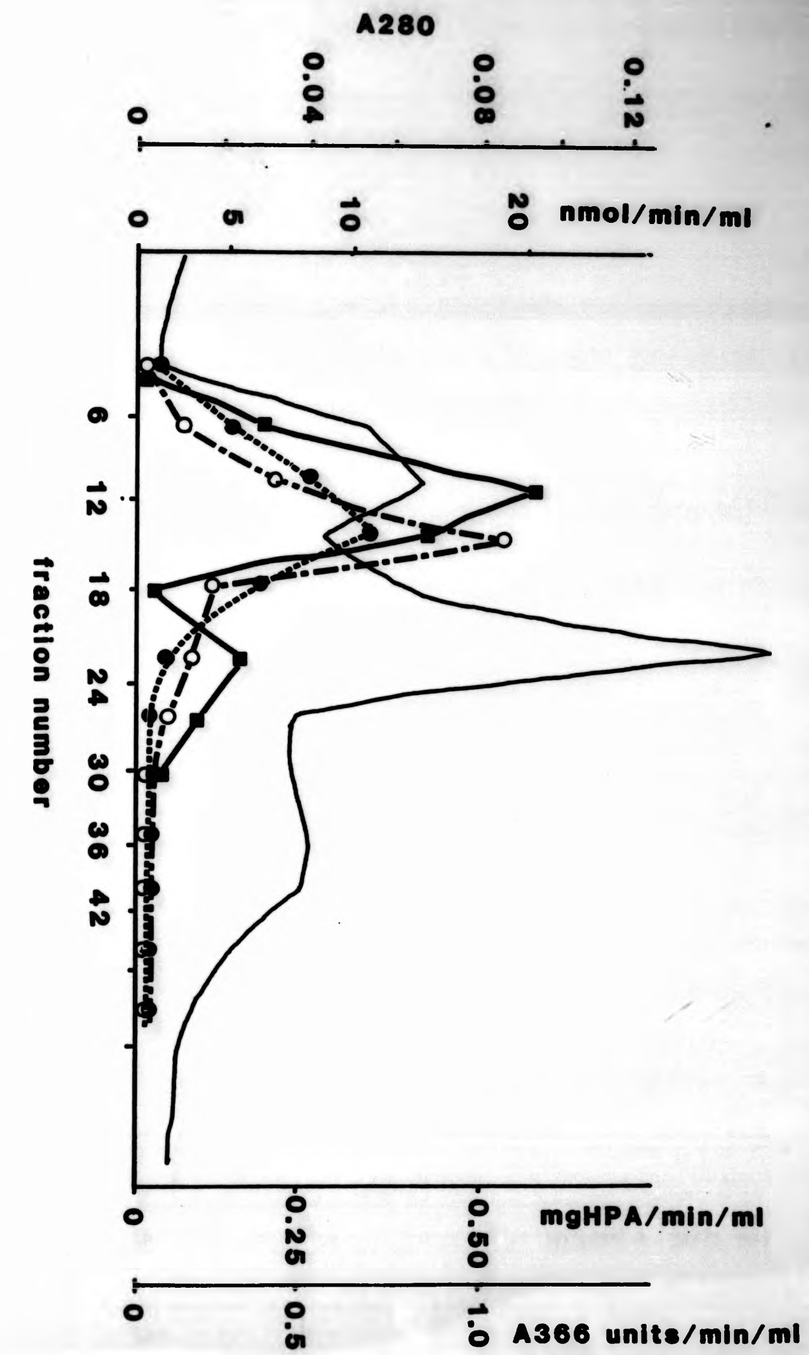
Peak IV fractions from the DEAE-cellulose column, fig. 3:7, were pooled, concentrated and dialyzed against Sephadex G75 column buffer. The concentrated sample was then run on a Sephadex G75 column. Eluted fractions were tested for BzArgNA hydrolyzing activity, fig. 3:16. The active fractions were pooled and concentrated and stored ready for use at $-15^{o}C$.

The enzyme repsonsible for the BzArgNA hydrolysis was referred to as Proteinase W4A. This enzyme had an apparent molecular weight of 38000 D as determined by gel-filtration. On an SDS-polyacrylamide gel Proteinase W4A showed 3 major bands of apparent molecular weights, 58000D, 55000D and 32000D, plate 3:6. Several other minor protein bands were also apparent. From these results it was evident that this enzyme required further purification before its molecular weight could be accurately determined.

The BzArgNA hydrolyzing activity of Proteinase W4A was inhibited by PMSF, table 3:15 indicating that this enzyme had a serine residue at the active site. Inhibition by both EDTA and phenanthroline suggested that metal ions were important for enzyme activity or stability.

The effects of nucleotides on the BzArgNA hydrolyzing enzyme

Fig. 3:16 Purification of Proteinase W4A by Sephadex G75 column chromatography. Pooled fractions from DEAE-cellulose peak IV were concentrated and dialyzed against 10 mM Tris/HCl pH 7.8, 5 mM $MgCl_2$ and applied to a Sephadex G75 superfine column. Activity was determined using BzArgNA (●----●); HPA (○--○) and azocasein (■——■) as substrates, A_{280} (——). 3.2 ml fractions were collected and every second fraction assayed.



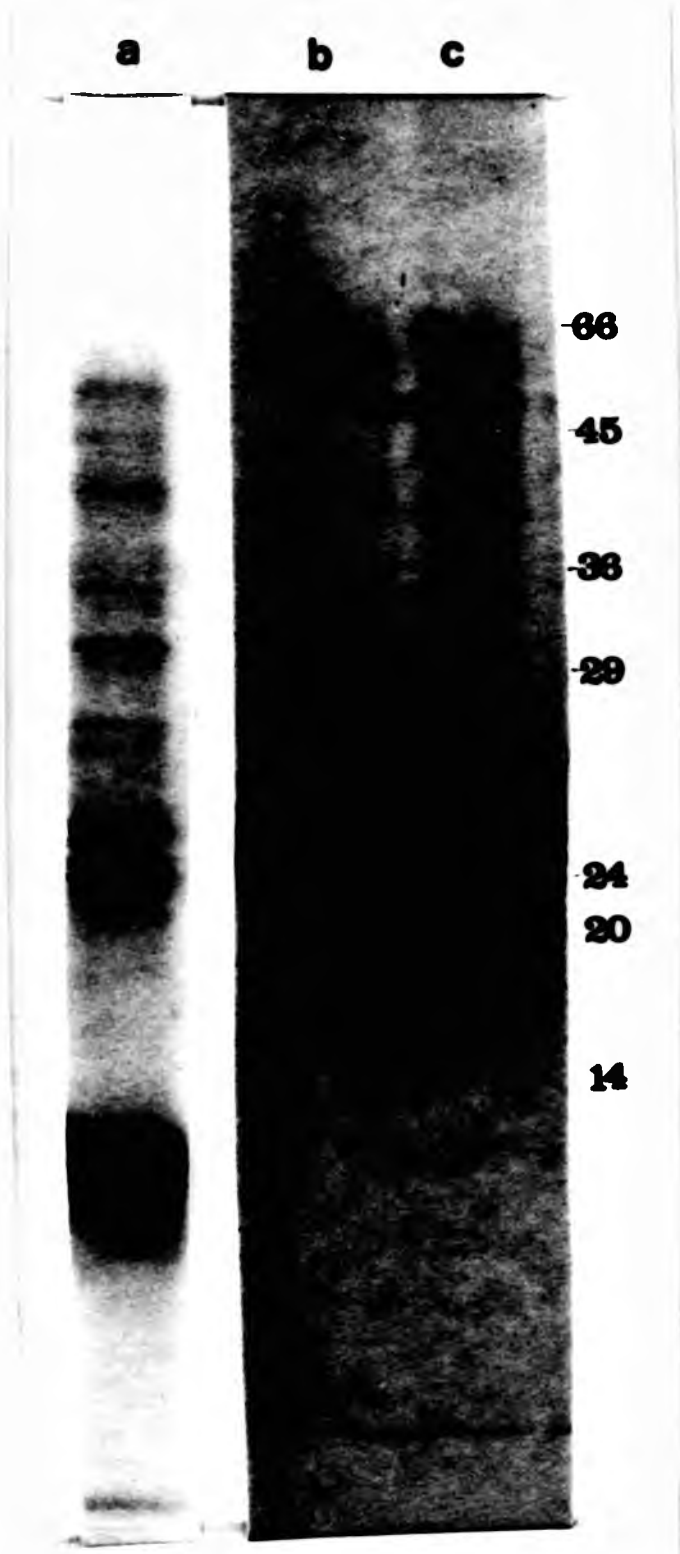


Plate 3:6 SDS-polyacrylamide gel electrophoresis of Proteinases W4A and W4B. Lane a, peak fraction IV from the DEAE-cellulose column (80μg protein); lane b, Proteinase W4A from the Sephadex G75 column (15 μg protein); lane c, Proteinase W4B from the Sephadex G75 column (15μg protein). Arrows indicate the major protein bands. Molecular weight markers (kilodaltons) are shown on the right of the plate.

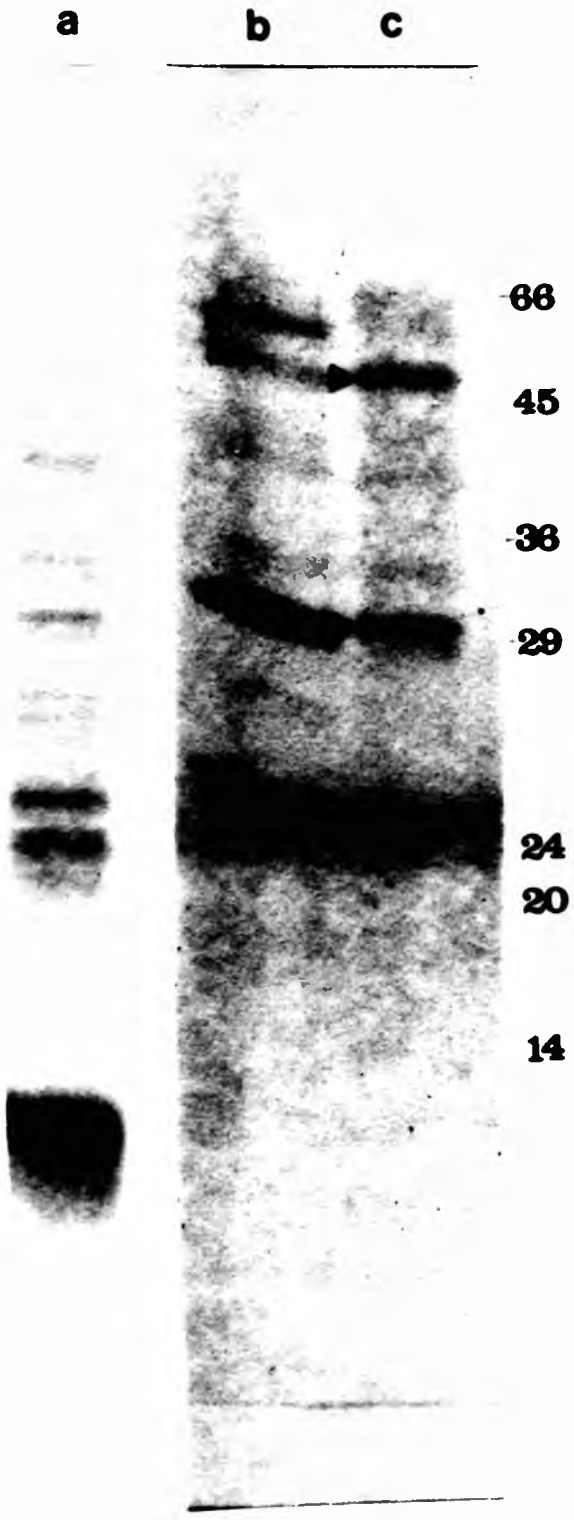


Plate 3:6 SDS-polyacrylamide gel electrophoresis of Proteinases W4A and W4B. Lane a, peak fraction IV from the DEAE-cellulose column (80μg protein); lane b, Proteinase W4A from the Sephadex G75 column (15 μg protein); lane c, Proteinase W4B from the Sephadex G75 column (15μg protein). Arrows indicate the major protein bands. Molecular weight markers (kilodaltons) are shown on the right of the plate.

TABLE 3:15

Inhibition of BzArgNA hydrolyzing activity of Proteinase W4A

Inhibitor (1 mM unless stated otherwise)	Inhibition (%)
PMSF	100
EDTA	93
phenanthroline	59
TLCK	50
TPCK	37
IAA	0
chymostatin (10 µg/ml)	34
leupeptin (10 µg/ml)	11

Assays were performed as described in section 2:13. 100% activity (0.77 nmol/min) was determined in the absence of inhibitor. Samples contained 3 µg protein per assay.

TABLE 3:16

Effects of nucleotides on BzArgNA hydrolyzing activity of Proteinase W4A

Nucleotide (3 mM)	Activity (%)
ATP	78
ADP	84
AMP	98
cAMP	95
UTP	100
GTP	98
CTP	102
No nucleotide	100

Assays were performed as described in section 2:14. 100% activity (0.77 nmol/min) was determined in the absence of nucleotide. Samples contained 3 µg protein per assay.

were investigated, table 3:16. The only notable effect was the small amount of inhibition by ATP and ADP. The partial purificaton scheme for Proteinase W4A is shown in table 3:17.

(ii) Hydrolysis of HPA by Proteinase W4A

The Sephadex G75 fractions exhibiting hydrolysis of BzArgNA also hydrolyzed HPA, fig. 3:16. Without further purification however it cannot be ascertained whether Proteinase W4A was a single enzyme capable of hydrolyzing both these substrates.

(iii) Purification of an azocasein-hydrolyzing enzyme from peak IV

An azocasein-hydrolyzing enzyme, referred to as Proteinase W4B, was separated from Proteinase W4A on a Sephadex G75 column, fig. 3:16. This enzyme was estimated to have a molecular weight of 55000D by gel-filtration. The peak fraction from the Sephadex G75 column showed 2 major proteins on an SDS-polyacrylamide gel, one had an apparent molecular weight of 55000D and the other an apparent molecular weight of 32000D, plate 3:6. Further purification was required to accurately determine the molecular weight of Proteinase W4B. This enzyme has not been characterized further at this time.

3:2d (i) Purification and properties of a BzArgNA hydrolyzing enzyme from peak VI

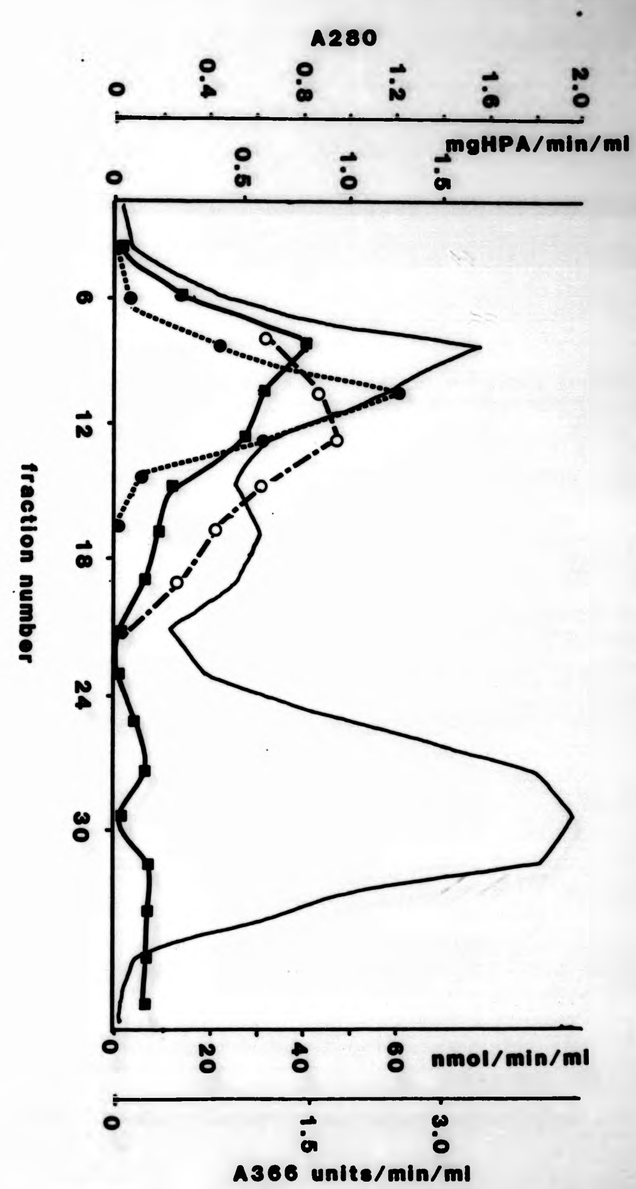
Peak VI fractions eluting with 1M NaCl from the DEAE-cellulose column, fig. 3:7, were pooled, concentrated and dialyzed against Sephadex G75 running buffer. The concentrated sample was then run on a Sephadex G75 column. Eluted fractions were tested for BzArgNA hydrolysis, fig. 3:17. The active fractions were pooled and stored

TABLE 3:17

Purification scheme for the BzArgNA hydrolyzing enzyme Proteinase W4A

Extract	Total Protein (mg)	Total Activity (nmol/min)	Sp. Ac. (nmol/min/mg protein)	Purity (fold)	Recovery (%)
Whole cell	288	1440	5	1	100
40-80% $(NH_4)_2SO_4$	160	1920	12	2.4	133
DEAE-cellulose peak IV	6	480	80	16	33
Sephadex G75 Proteinase W4A	0.54	140	260	52	9.7

Fig. 3:17 Purification of Proteinases W6A, W6B and W6C by Sephadex G75 column chromatography. Pooled fractions from DEAE-cellulose peak VI were concentrated and dialyzed against 10 mM Tris/HCl pH 7.8, 5 mM $MgCl_2$ and applied to a Sephadex G75 superfine column. Activity was determined using BzArgNA (●----●); HPA (○—○) and azocasein (■—■) as substrates, A_{280} (——). 3.2 ml fractions were collected and every second fraction assayed.



at -15°C. The apparent molecular weight of the BzArgNA hydrolyzing enzyme, referred to as Proteinase W6A, was 60000 D as determined by gel filtration.

This partially purified enzyme was inhibited by phenanthroline and to a lesser extent by EDTA, table 3:18. This result suggested a metal ion requirement for enzyme activity or stability. PMSF inactivation of the enzyme was low which suggested that this enzyme was likely a metallo-proteinase.

Nucleotides had no apparent effect on the BzArgNA-hydrolyzing enzyme, table 3:19.

The extent of the purification of Proteinase W6A is shown in table 3:20. It was evident from this result that this enzyme required further purification.

3:2d (ii) Identification of two proteolytic enzymes from peak VI

In addition to the BzArgNA-hydrolyzing enzyme eluted from the Sephadex G75 column, an azocasein hydrolyzing activity and HPA-hydrolyzing activity was also detected, fig. 3:17. The azocasein-degrading enzyme was referred to as Proteinase W6B and had an apparent molecular weight of 75000D as estimated by gel-filtration. The HPA-hydrolyzing enzyme, referred to as Proteinase W6C, had an apparent molecular weight of 56000 D as determined by gel-filtration. Neither of these enzymes has been purified or characterized further at this time.

3:2e Discussion

In total eleven proteolytic enzymes have been separated and partially purified from the whole cell extracts of M.

TABLE 3:18

Inhibition of BzArgNA hydrolyzing activity of Proteinase W6A

Inhibitor (1 mM unless stated otherwise)	Inhibition (%)
PMSF	9
EDTA	19
phenanthroline	40
TLCK	22
TPCK	23
IAA	5
chymostatin (10 μg/ml)	20
leupeptin (10 μg/ml)	20

Assays were perfomed as described in section 2:13. 100% activity (1.8 nmol/min) was determined in the absence of inhibitor. Samples contained 85 μg protein per assay.

TABLE 3:19

The effects of nucleotides on the BzArgNA hydrolyzing activity of Proteinase W6A

Nucleotide (3 mM)	Activity (%)
ATP	103
ADP	103
AMP	100
cAMP	100
UTP	91
GTP	114
CTP	111
No nucleotide	100

Assays were perfomed as described in section 2:14. 100% activity (1.8 nmol/min) was determined in the absence of nucleotide. Samples contained 85 μg protein per assay.

TABLE 3:20

Purification scheme for BzArgNA hydrolyzing enzyme
Proteinase W6A

Extract	Total Protein (mg)	Total Activity (nmol/min)	Sp. Ac. (nmol/min/mg Protein)	Purity (fold)	Recovery (%)
Whole cell	288	1440	5	1	100
40-80% $(NH_4)_2SO_4$	160	1920	12	2.4	133
DEAE-cellulose peak VI	45	540	12	2.4	38
Sephadex G75 Proteinase W6A	3.3	690	21	4.0	48

methylotrophus. A summary of some of their properties are shown in table 3:21.

Although not all these enzymes have been fully characterized, the majority of them appear to be serine proteinases. This result agrees with the findings by other researchers that serine proteinases are the most abundent proteolytic enzymes in bacteria (Goldberg et al., 1981). In addition two metalloproteinases have also been isolated from the whole cell extracts of M. methylotrophus. A similar number of metalloproteinases have been found in E. coli (Goldberg et al., 1981).

The isolation of an ATP-dependent proteolytic enzyme from E. coli prompted a search for such an enzyme in M. methylotrophus. Although an ATP-stimulated hydrolysis of BzArgNA was observed in the peak II fractions of the DEAE-cellulose column, this ATP-stimulated activity was lost when the enzyme was purified further. This result suggests that an ATP-dependent proteinase does exist in M. methylotrophus, however, it will require alternative approaches in order to isolate and purify the labile enzyme responsible.

A notable nucleotide inhibitory effect that was also observed with whole cell extracts was the inactivation of Proteinase W3A BzArgNA hydrolyzing activity by UTP and CTP. Comparable nucleotide effects with other bacterial proteolytic enzymes have not been reported.

The proteinases from the whole cell extracts of M. methylotrophus are capable of hydrolyzing a broad range of substrates, but whether these enzymes function in the breakdown of abnormal proteins or normal cell constituents still remains to be determined. An important clue to the physiological role of these

TABLE 3:21

Proteinases from whole cell extract of M. methylotrophus

Proteinase	Apparent Mol wt (daltons) gel filtration	SDS-PAGE	Inhibitors	Nucleotides + Activation - Inhibition	Purification (fold)	Substrates hydrolyzed
W2A	60000	28000	Phenanthroline, TLCK, IAA, chymostatin leupeptin	-cAMP	32	BzArgNA gelatin
W2B	18000	18000			60	HPA
W3A	250000	45000	EDTA, PMSF, TPCK, chymostatin, leupeptin	-UTP -CTP	30	BzArgNA [^{14}C]-α_1-casein SAAPP (±M)
W3B		29000	PMSF, TPCK, leupeptin, chymostatin	+GTP	116	BzArgNA SAAPP (+M)
W3C		66000				SAAPP (+M)
W3D		140-200000	PMSF, TPCK, chymostatin			[^{14}C]-α_1-casein SAAPP (+M) [^{35}S]-ω_2-IFN
W4A	38000	32000	PMSF, EDTA, TPCK, TLCK, chymostatin	-ATP -ADP	52	BzArgNA HPA
W4B	55000	55000				Azocasein
W6A	60000		phenanthroline		4	BzArgNA HPA azocasein
W6B	75000					Azocasein BzArgNA HPA
W6C	56000					HPA azocasein BzArgNA

(+M) release of nitroaniline only observed in the presence of aminopeptidase M.
(±M) observed nitroaniline release in the absence of aminopeptidase M, but was increased in its presence.

enzymes may be provided from their subcellular location.

3:3 ISOLATION OF SUBCELLULAR FRACTIONS FROM M.METHYLOTROPHUS

The three major components of a bacterial cell are its cytoplasm, periplasm and membranes. These fractions in M. methylotrophus cells were separated from one another by the method of Jones et al., 1982 (section 2:2b). The purity of a particular subcellular fraction was determined using marker enzymes that are specific to a certain fraction.

Methanol dehydrogenase (MDH) was used as the marker enzyme for the periplasmic fraction. It was discovered that this enzyme was loosely associated with the periplasmic side of the respiratory membrane and when the cell was disrupted in the presence of lysozyme and EDTA, the periplasm, including MDH, could be separated from the sphaeroplasts (membrane/cytoplasm) by differential centrifugation. The periplasm was isolated as a pink-orange supernatant.

Glucose-6-phosphate dehydrogenase was used as the marker enzyme for the cytoplasmic fraction. Alternatively 6-phosphogluconate dehydrogenase could be used as the marker enzyme. The cytoplasm was isolated as a straw-coloured supernatant after osmotic shock and differential centrifugation of the sphaeroplasts.

Cytochrome c oxidase, found exclusively in the membranes of M. methylotrophus cells, was used as the marker enzyme for this fraction.

The purities of the isolated subcellular fractions, expressed as the percentage distribution of marker enzymes, are shown in table 3:22. The periplasmic and membrane fractions contained 16% and 3% respectively of the total cytoplasm marker activity, 8% and 2% respectively of the total periplasm marker activity was present in the cytoplasmic and membrane fractions. No membrane marker was

TABLE 3:22

% distribution of marker enzymes

Marker enzymes	Periplasm	Cytoplasm	Membrane
Glucose-6-phosphate dehydrogenase	16	81	3
Methanol dehydrogenase	89	8	2
Ascorbate-TMPD cytochrome c oxidase	0	0	100

detectable in either the cytoplasmic or the periplasmic fractions. The values expressed are a mean of four subcellular fractionations. The percentage distribution of the marker enzymes between the fractions shows close agreement with the values reported by Jones et al., 1982.

The following section details attempts to isolate, purify and characterize proteolytic enzymes from the subcellular extracts of M. methylotrophus, and where appropriate, compare these with those from whole cell extracts.

3:4 ISOLATION AND PURIFICATION OF PROTEOLYTIC ENZYMES FROM THE CYTOPLASMIC FRACTION OF M. METHYLOTROPHUS

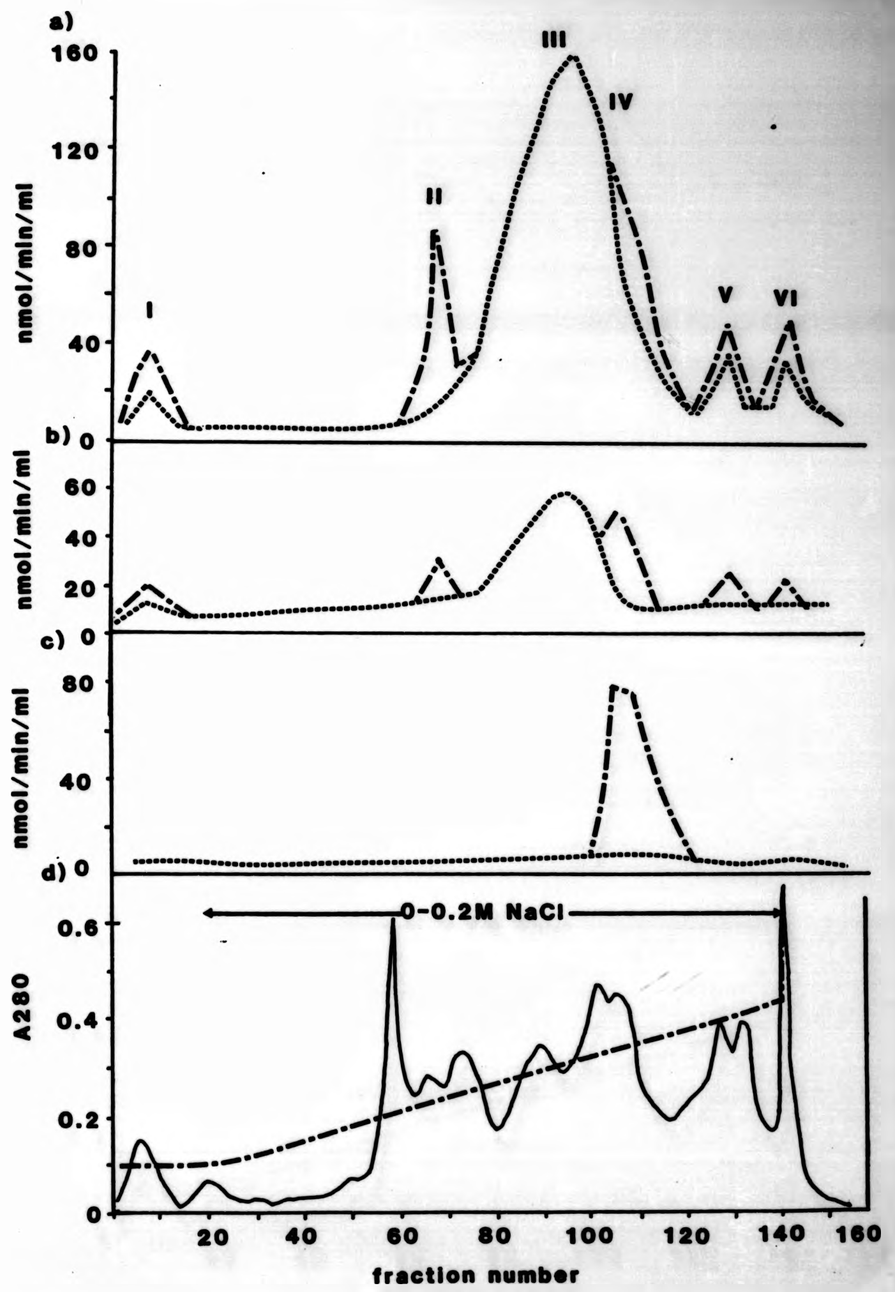
The initial step in the purification of proteolytic enzymes from the cytoplasmic fraction of M. methylotrophus was conducted as for whole cell extracts. The extract was run on a DEAE-cellulose column with proteins eluted with a 0-0.2M NaCl gradient. Eluted fractions were tested for their ability to hydrolyze a variety of peptide nitroanilides and the protein substrates, HPA and gelatin, figs. 3:18, 3:19 and 3:20.

The most rapidly hydrolyzed of the substrates tested was SAAPP, fig. 3:18a. Six major peaks of SAAPP hydrolyzing activity were detected. For convenience they were designated peaks I-VI according to their order of elution from the column. The rate of nitroaniline release from SAAPP, due to enzymes from peaks II and IV, was increased in the presence of aminopeptidase M.

A comparable profile of hydrolyzing activities were detected when the leucine-derivative SAAPL was used as substrate although the rates of nitroaniline release in the presence and absence of aminopeptidase M were as much as four-fold less than with SAAPP, fig. 3:18b. When the alanine-derivative AAAPA was used instead of the Phe- or Leu- derivatives of this peptide, there was a dramatic change in the elution profile of hydrolysis, fig. 3:18c. This substrate was only hydrolyzed by the enzyme(s) from peak IV, and its activity was detected only in the presence of aminopeptidase M.

BzIleGlu-GlyArgNA was hydrolyzed by the enzymes from peaks I and IV only in the presence of aminopeptidase M, fig. 3:19a. Hydrolysis of BzProPheArgNA by peaks IV enzyme(s) was also only detected in the presence of aminopeptidase M, fig. 3:19b. BzArgNA

Fig. 3:18 DEAE-cellulose chromatography of peptide-nitroanilide hydrolyzing enzymes from the cytoplasmic fraction of M. methylotrophus. Hydrolysis of (a) SAAPP; (b) SAAPL and (c) AAAPA in the absence (⋯⋯) and presence (·—··) of aminopeptidase M (d) A_{280} (——). Dialyzed cytoplasmic extract was absorbed onto a DEAE-cellulose column and eluted with 1500 ml of a linear NaCl gradient (0-0.2M NaCl). The flow rate was 100 ml/hr and 15 ml fractions were collected. Every fourth fraction was assayed. Data points are not shown in order to avoid cluttering on the diagram.



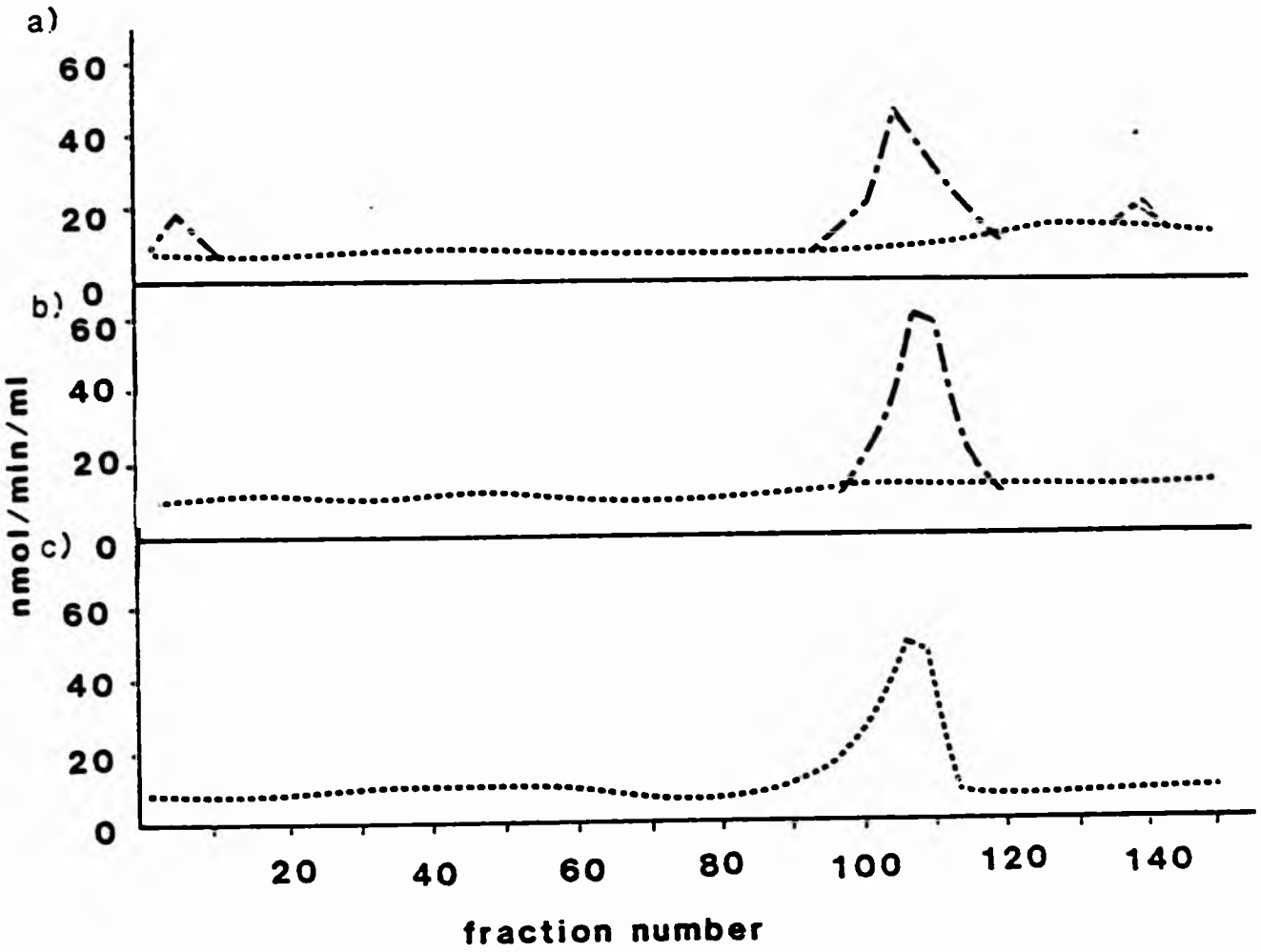


Fig. 3:19 DEAE-cellulose chromatography of peptide-nitroanilide hydrolyzing enzymes from the cytoplasmic fraction of M. methylotrophus. Hydrolysis of (a) BzIleGlu-GlyArgNA and (b) BzProPheArgNA in absence (·····) and presence of (— · —) aminopeptidase M; (c) BzArgNA (·····). Dialyzed cytoplasmic extract was absorbed onto a DEAE-cellulose column and eluted with 1500 ml of a linear NaCl gradient (0-0.2M NaCl). The flow rate was 100 ml/hr and 15 ml fractions were collected. Every fourth fraction was assayed. Data points are not shown in order to avoid cluttering on the diagram.

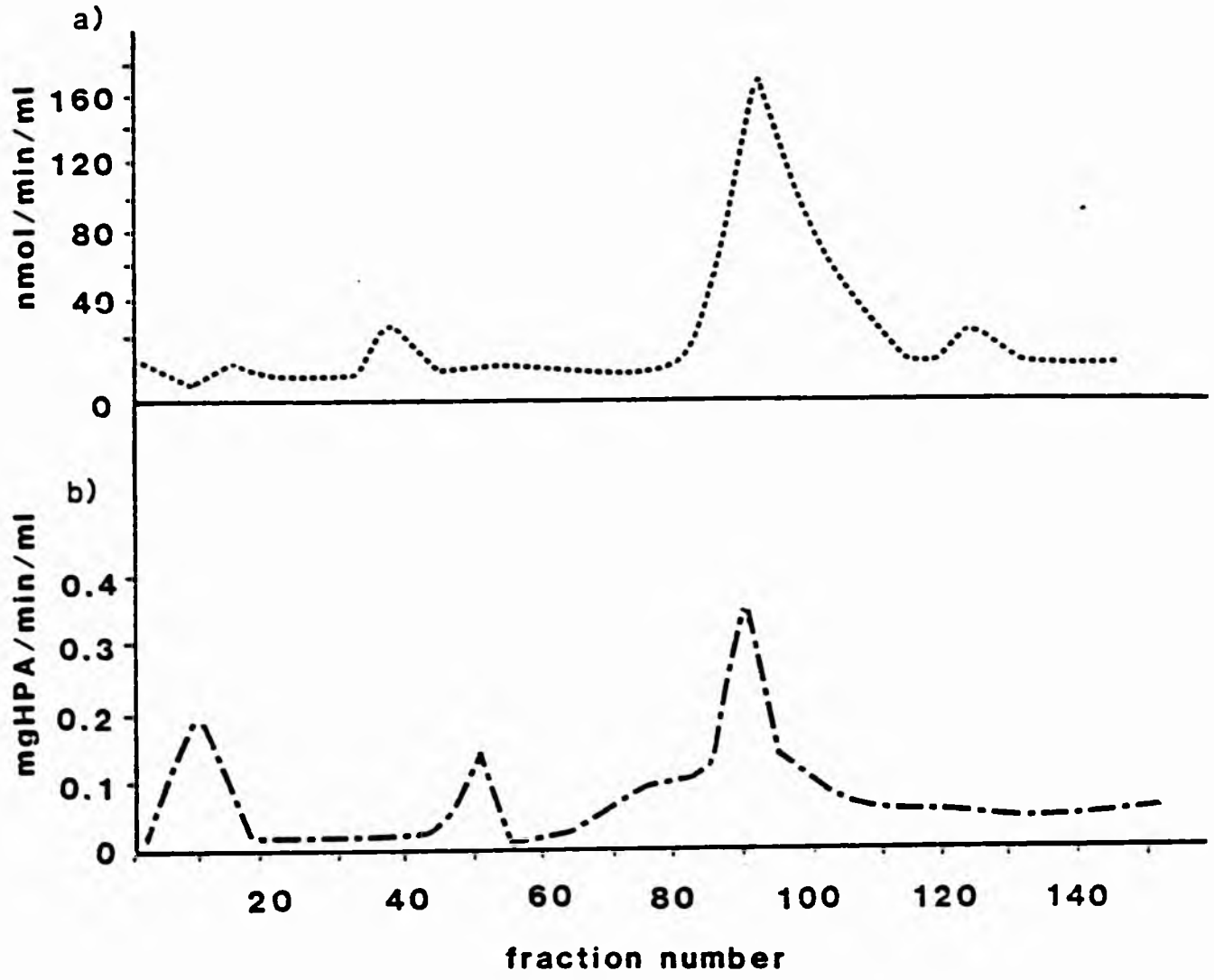


Fig. 3:20 DEAE-cellulose chromatography of aminopeptidase and proteolytic enzymes from the cytoplasmic fraction of M. methylotrophus. Hydrolysis of (a) LeuNA (•••••) and (b) HPA (•—••). Dialyzed cytoplasmic extract was absorbed onto a DEAE-cellulose column and eluted with 1500 ml of a linear NaCl gradient (0-0.2M NaCl). The flow rate was 100 ml/hr and 15 ml fractions were collected. Every fourth fraction was assayed. Data points are not shown in order to avoid cluttering on the diagram.

was hydrolyzed only by fractions from peak IV, fig. 3:19c.

The possibility that the inability to detect an increase in nitroaniline release in the presence of aminopeptidase M was due to endogenous aminopeptidases was investigated. Eluted fractions from the DEAE-column were tested for their ability to hydrolyze LeuNA, fig. 3:20a. Only enzymes from peaks III and IV hydrolyzed this substrate.

The ability of the eluted fractions to hydrolyze the protein substrates HPA and gelatin was also investigated, fig, 3:20b. HPA hydrolysis was observed with fractions from peaks I and III while gelatin was hydrolyzed only by enzymes from peak IV fractions, plate 3:7.

3:4a Purification of a SAAPP hydrolyzing enzyme from peak II

Peak II fractions were pooled, concentrated and dialyzed against Sephadex G75 buffer. The concentrated sample was then run on a Sephadex G75 column. Eluted fractions were tested for SAAPP hydrolyzing activity in the presence and absence of aminopeptidase M, fig. 3:21. A single peak of SAAPP hydrolyzing activity was detected. The release of nitroaniline from SAAPP was almost 3-fold greater in the presence of aminopeptidase M.

The enzyme responsible for the hydrolysis of SAAPP from peak II was referred to as Proteinase C2A. This enzyme had an apparent molecular weight of 32000D as estimated by gel chromatography. The purification scheme for Proteinase C2A is shown in table 3:23. The enzyme has not been purified or characterized any further at this time.

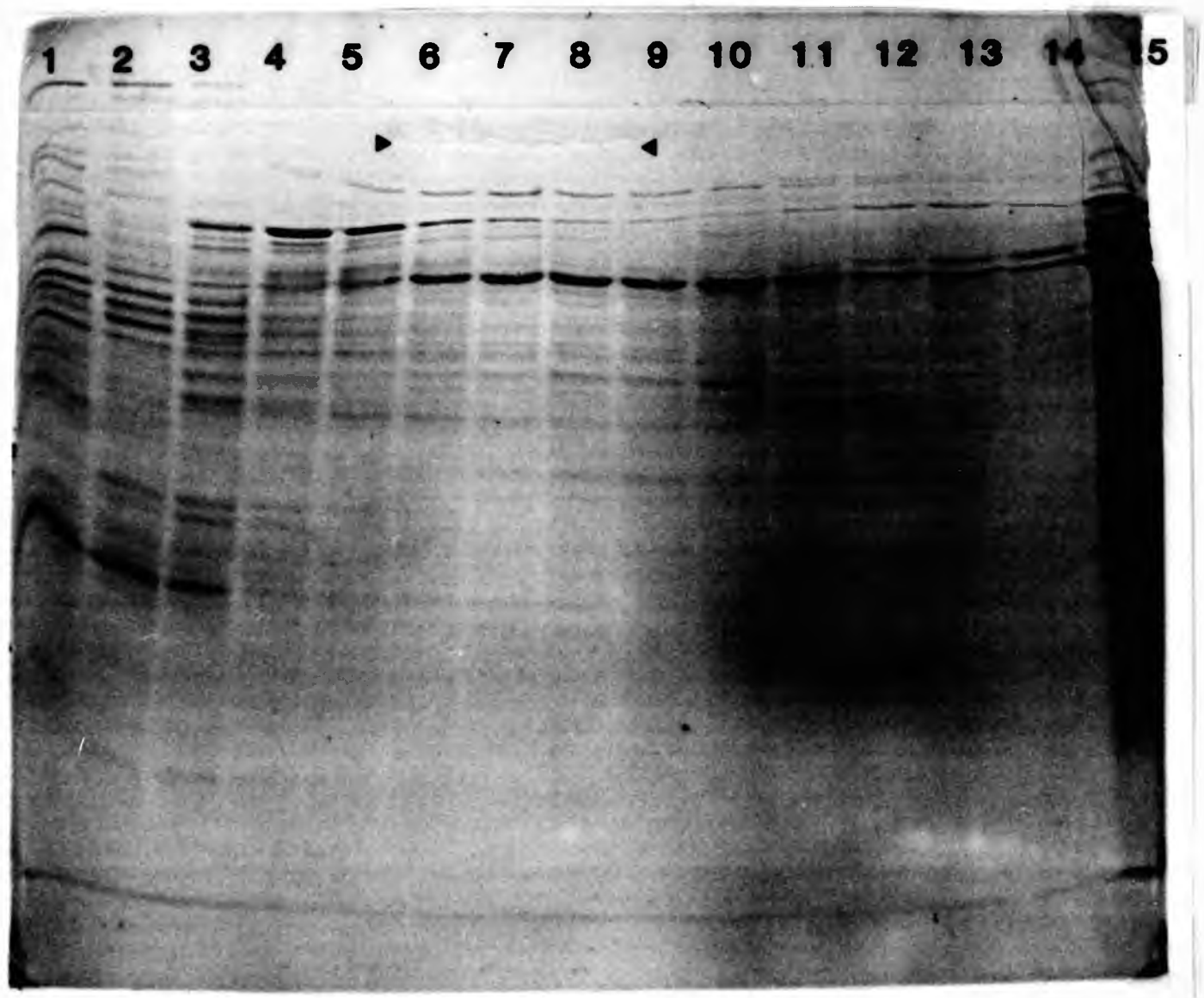


Plate 3:7 Gelatin-hydrolyzing activity of cytoplasmic enzymes eluted from a DEAE-cellulose column. Activities were detected by SDS-polyacrylamide gel electrophoresis under non-denaturing conditions on gels containing gelatin. Lanes 1-14, every fourth fraction from 78-130 eluted from the DEAE-cellulose column; lane 6-8, fractions 98-106 exhibiting gelatin hydrolyzing activities (indicated by closed arrowheads); lane 15, cytoplasmic extract (200μg protein).

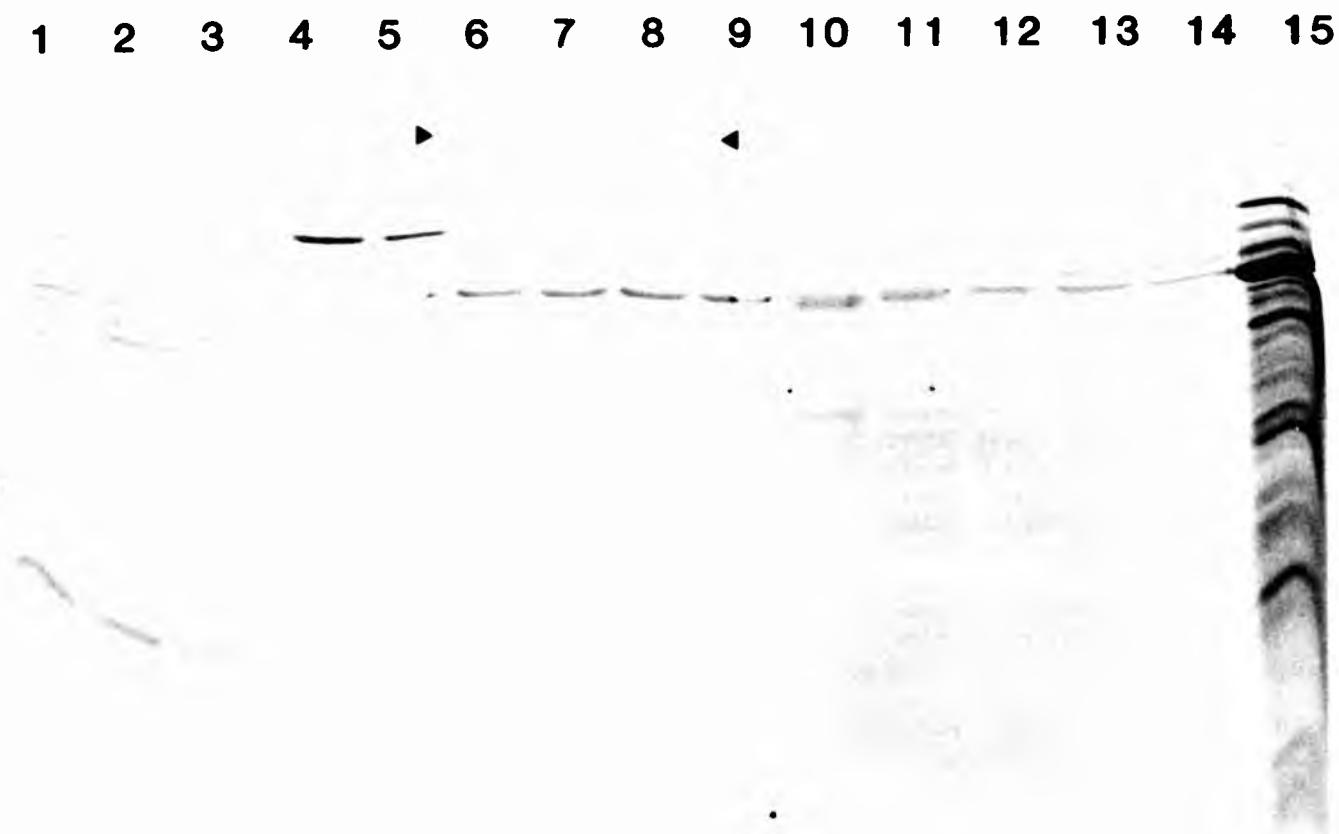


Plate 3:7 Gelatin-hydrolyzing activity of cytoplasmic enzymes eluted from a DEAE-cellulose column. Activities were detected by SDS-polyacrylamide gel electrophoresis under non-denaturing conditions on gels containing gelatin. Lanes 1-14, every fourth fraction from 78-130 eluted from the DEAE-cellulose column; lane 6-8, fractions 98-106 exhibiting gelatin hydrolyzing activities (indicated by closed arrowheads); lane 15, cytoplasmic extract (200μg protein).

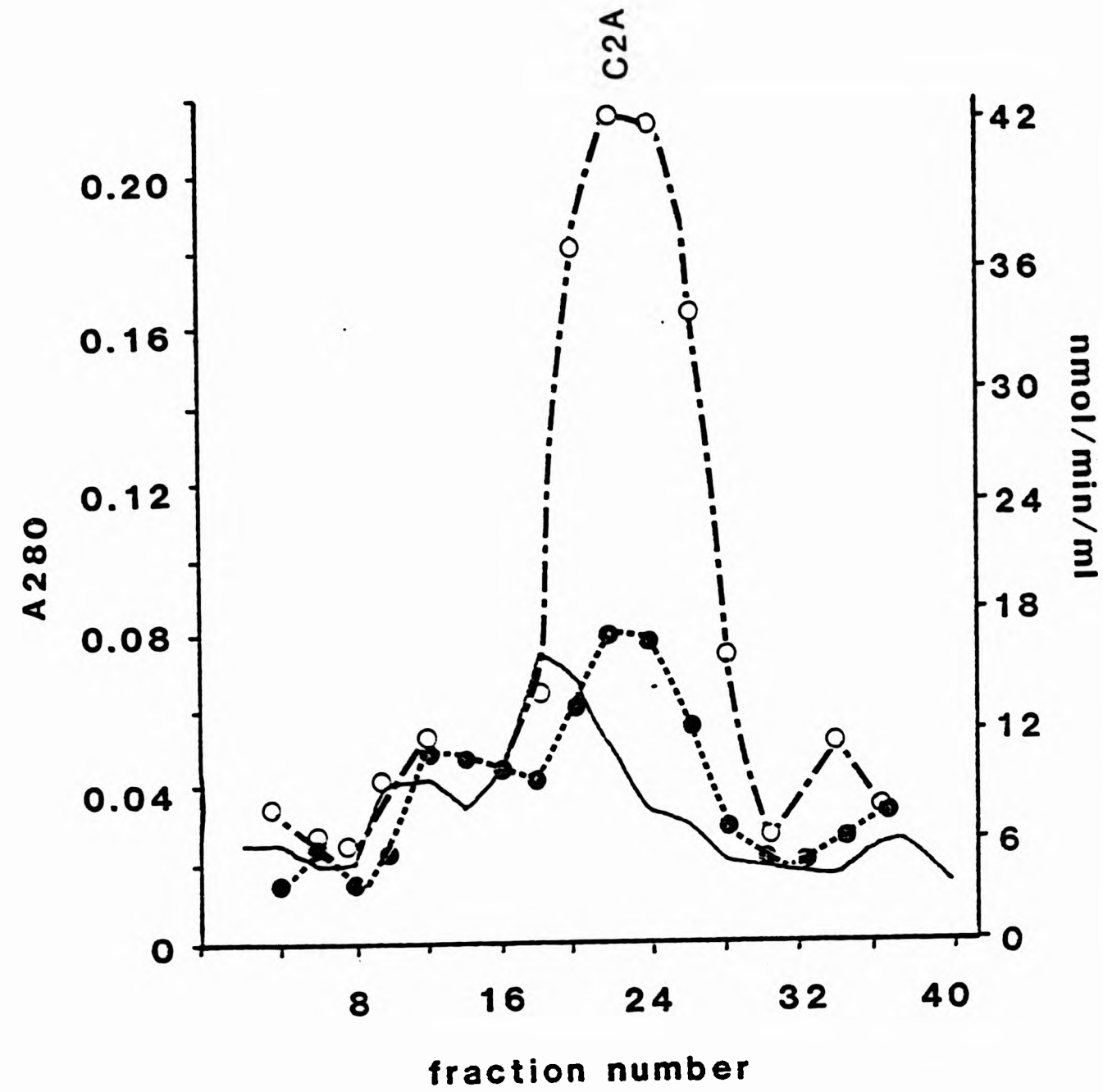


Fig. 3:21 Purification of Proteinase C2A by Sephadex G75 column chromatography. Pooled fractions from DEAE-cellulose peak II were concentrated and dialyzed against 10 mM Tris/HCl pH 7.8, 5 mM $MgCl_2$ and applied to a Sephadex G75 superfine column. Activity was determined using SAAPP in the absence (●····●) and presence (○—·○) of aminopeptidase M; A_{280} (———). 3.2 ml fractions were collected and every second fraction assayed.

TABLE 3:23

Purification scheme for the SAAPP hydrolyzing enzyme Proteinase C2A

Extract	Total Protein (mg)	Total Activity (nmol/min)	Sp. Ac. (nmol/min/mg protein)	Purity (fold)	Recovery (%)
Cytoplasm	690	19320	28	1	100
DEAE-cellulose peak II	26	4990	192	7	26
Sephadex G75 Proteinase C2A	0.9	954	1061	38	5

3:4b (i) Purification of SAAPP-hydrolyzing enzymes from peak III

Peak III fractions were pooled, concentrated and dialyzed against Sephadex G75 column buffer. The concentrated sample was then run on a Sephadex G75 column. Eluted fractions were tested for their ability to hydrolyze SAAPP in the presence and absence of aminopeptidase M, fig 3:22. Two peaks of SAAPP-hydrolyzing activity were observed. Neither of the enzymes responsible for these activities exhibited a significant increase in the release of nitroaniline in the presence of aminopeptidase M. The two enzymes responsible for SAAPP hydrolysis were referred to as Proteinases C3A and C3B. From gel-chromatography the two enzymes had apparent molecular weights of 38000 D and 21000 D respectively. The purification schemes for Proteinases C3A and C3B are shown in table 3:24.

(ii) Hydrolysis of HPA by Proteinases C3A and C3B

In addition to the hydrolysis of SAAPP, the fractions from the Sephadex G75 column corresponding to Proteinases C3A and C3B also hydrolzed HPA, fig. 3:22. Without further purification of these enzymes it cannot be concluded that the enzymes responsible for hydrolysis of SAAPP were the same as those which hydrolyzed HPA.

(iii) Purification of a LeuNA hydrolyzing enzyme from peak III

Eluted fractions from the Sephadex G75 column exhibited a LeuNA hydrolyzing activity that was distinct from the SAAPP hydrolyzing activity, fig. 3:22. The enzyme responsible for this activity, referred to as Aminopeptidase C3A, eluted with the void volume of the column. The apparent molecular weight for this enzyme was

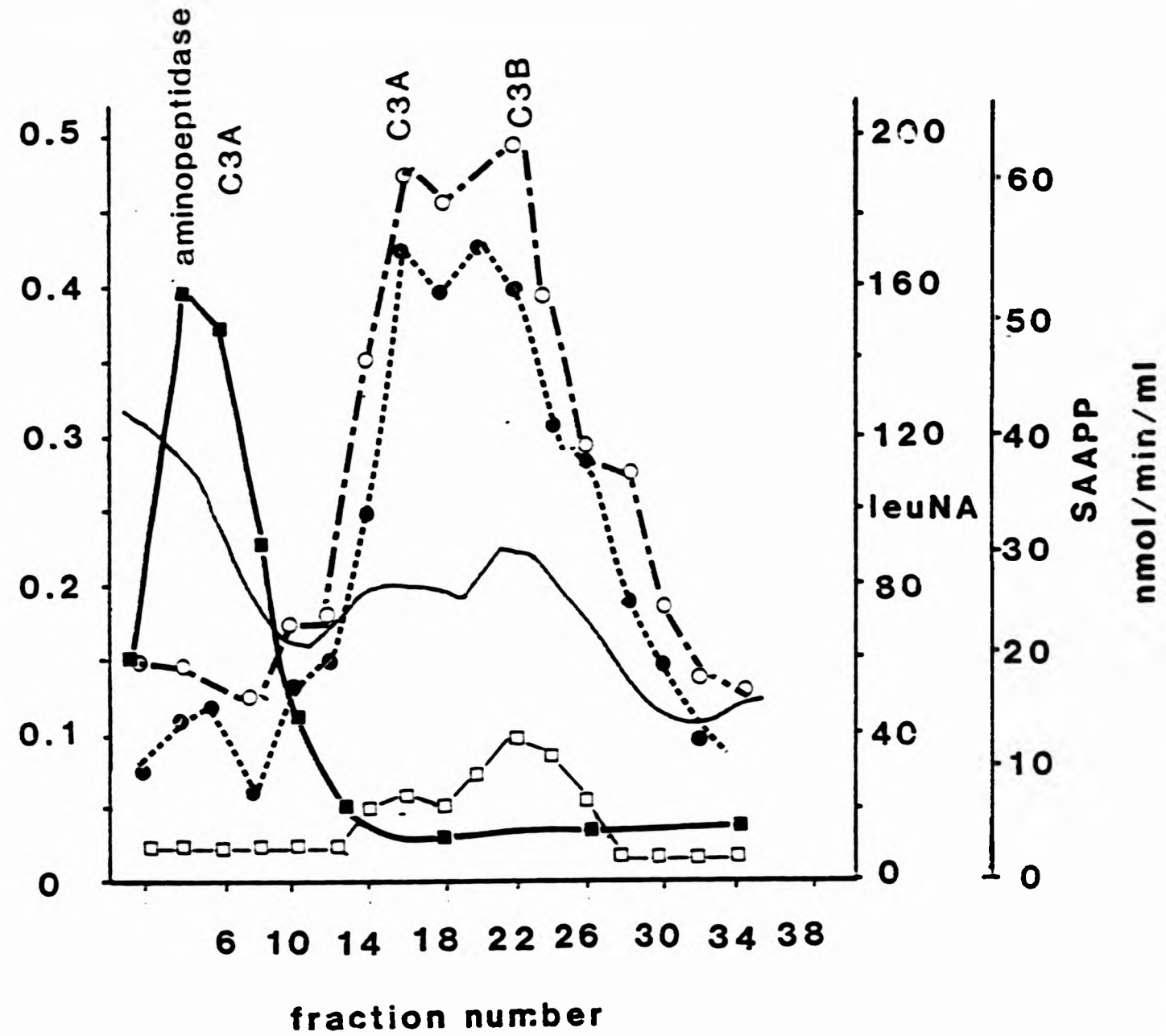


Fig. 3:22 Purification of Proteinases C3A and C3B by Sephadex G75 column chromatography. Pooled fractions from DEAE-cellulose peak III were concentrated and dialyzed against 10 mM Tris/HCl pH 7.8, 5 mM $MgCl_2$ and applied to a Sephadex G75 superfine column. Activity was determined using SAAPP in the absence (●····●) and presence (○—○) of aminopeptidase M; LeuNA (■—■) and HPA (□—□). A_{280} (———). 3.2 ml fractions were collected and every second fraction assayed.

TABLE 3:24

Purification scheme for SAAPP hydrolyzing enzymes
Proteinases C3A and C3B

Extract	Total Protein (mg)	Total Activity (nmol/min)	Sp. Ac. (nmol/min/mg protein)	Purity (fold)	Recovery (%)
Cytoplasm	690	19320	28	1	100
DEAE-cellulose peak III	64	31490	492	18	163
Sephadex G75 Proteinase C3A	1.4	970	695	25	5
C3B	2.2	780	355	13	4

therefore greater than 75000 D. The purification scheme for Aminopeptidase C3A is shown in table 3:25. This enzyme has not been purified or characterized further at this time.

3:4c (i) Purification of SAAPP hydrolyzing enzymes from peak IV

Peak IV fractions were pooled, concentrated and dialyzed against Sephadex G75 column buffer. The concentrated sample was then run on a Sephadex G75 column. Eluted fractions were tested for their ability to hydrolyze SAAPP in the presence and absence of aminopeptidase M, fig. 3:23a. Three peaks of SAAPP hydrolyzing activity were observed. The enzymes responsible for SAAPP hydrolysis have been named Proteinases C4A, C4B and C4C according to their order of elution from the column. There was an increase in nitroaniline release from SAAPP with both Proteinase C4A and C4C in the presence of aminopeptidase M. From gel chromatography, Proteinase C4A, which eluted with the void volume, had an apparent molecular weight of $\geqslant$75000 D; Proteinase C4B and C4C had apparent molecular weights of 30000 D and 20000 D respectively. The purification schemes for Proteinases C4A, C4B and C4C are shown in table 3:26.

Proteinase C4A

Fractions exhibiting Proteinase C4A activity towards SAAPP were pooled and concentrated. This enzyme caused an almost 4-fold increase in the release of nitroaniline in the presence of aminopeptidase M, fig. 3:23a. The effects of inhibitors on Proteinase C4A-catalyzed hydrolysis of SAAPP in the presence of aminopeptidase M was investigated, table 3:27. Inactivation of

TABLE 3:25

Purification scheme for LeuNA hydrolyzing activity of Aminopeptidase C3A

Extract	Total Protein (mg)	Total Activity (nmol/min)	Sp. Ac. (nmol/min.mg protein)	Purity (fold)	Recovery (%)
Cytoplasm	690	46230	67	1	100
DEAE-cellulose peak III	64	24320	380	6	53
Sephadex G75 Aminopeptidase C3A	1.4	2940	2100	31	6.4

Fig. 3:23 Purification of Proteinases C4A, C4B, C4C and C4D by Sephadex G75 column chromatography. Pooled fractions from DEAE-cellulose peak IV were concentrated and dialyzed against 10 mM Tris/HCl pH 7.8, 5 mM $MgCl_2$ and applied to a Sephadex G75 superfine column. Activity was determined using (a) SAAPP in the absence (●----●) and presence (○---○) of aminopeptidase M, A_{280} (——), (b) LeuNA (■—■) and HPA (●—●). 3.2 ml fractions were collected and every second fraction assayed.

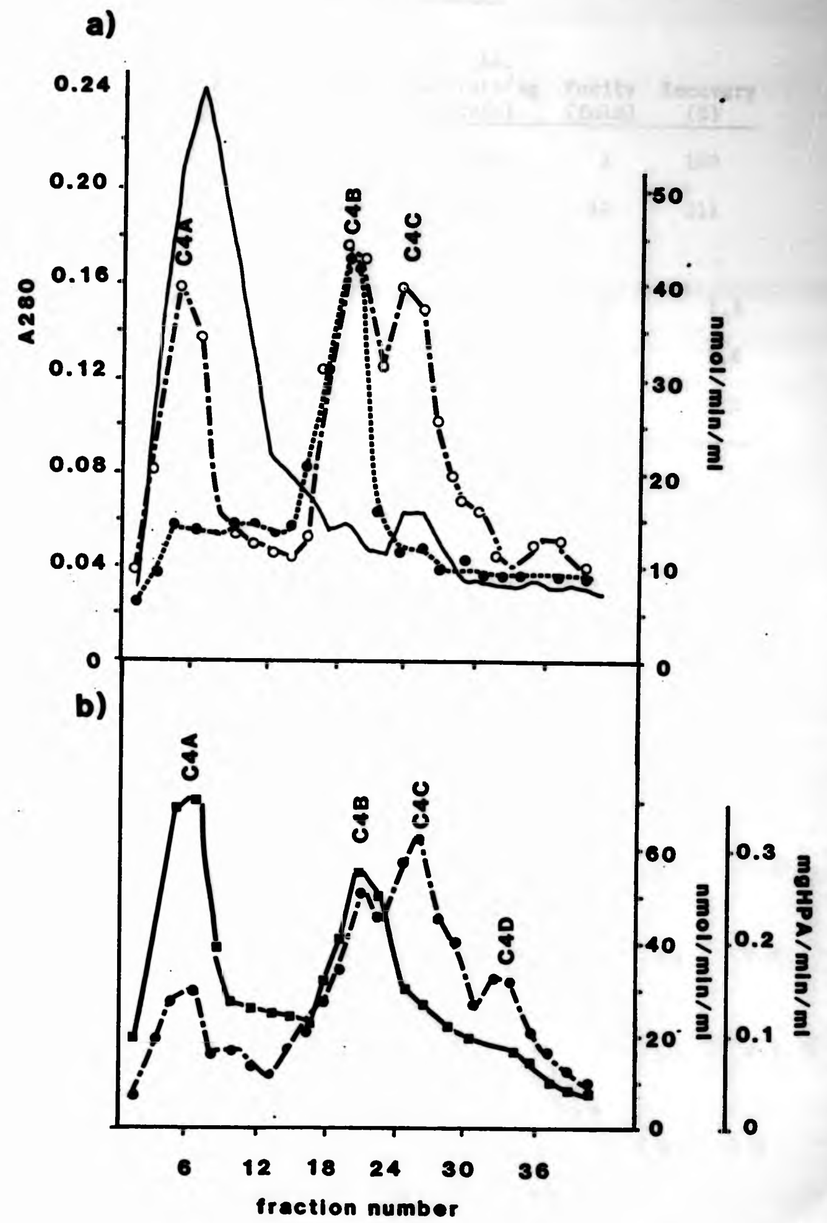


TABLE 3:26

Purification scheme for SAAPP hydrolyzing enzymes
Proteinases C4A, C4B and C4C

Extract	Total Protein (mg)	Total Activity (nmol/min)	Sp. Ac. (nmol.min/mg protein)	Purity (fold)	Recovery (%)
Cytoplasm	690	19320	28	1	100
DEAE-cellulose peak IV	98	40770	416	14	211
Sephadex G75 Proteinase C4A	4.5	450	100	4	2.3
C4B	1.1	510	462	17	2.6
C4C	0.5	230	457	16	1.2

TABLE 3:27

The effects of inhibitors on the SAAPP hydrolyzing activity of Proteinase C4A

Inhibitor (1 mM unless stated otherwise)	Inhibition (%)
PMSF	63
EDTA	100
phenanthroline	63
TLCK	12
TPCK	36
IAA	0
chymostatin (10 µg/ml)	40
leupeptin (10 µg/ml)	58

Assays were performed as described in section 2:13. 100% activity (2.5 nmol/min) was determined in the absence of inhibitor. Samples contained 25 µg protein per assay.

TABLE 3:28

The effects of nucleotides on the SAAPP hydrolyzing activity of Proteinase C4A*

Nucleotide (3 mM)	Activity (%)
ATP	98
ADP	98
AMP	104
cAMP	100
UTP	55
GTP	88
CTP	78
No nucleotide	100

* Release of nitroaniline from SAAPP was measured in the presence of aminopeptidease M.

Assays were performed as described in section 2:14. 100% activity (2.5 nmol/min) was determined in the absence of inhibitor. Samples contained 25 µg protein per assay.

Proteinase C4A by PMSF suggested that this enzyme had a serine residue at the active site of the enzyme. Inhibition by both EDTA and phenanthroline indicated that a divalent metal ion was required for enzyme activity or stability. Proteinase C4A was also inhibited by TPCK, chymostatin and leupeptin. The restoration of enzyme activity with divalent metal ions has not been investigated.

The effects of nucleotides on the ability of Proteinase C4A to hydrolyze SAAPP are shown in table 3:28. The most notable effect was the inhibition of enzyme activity in the presence of UTP and CTP.

Proteinase C4B

The peak fractions showing Proteinase C4B activity from the Sephadex G75 column were pooled and concentrated. Proteinase C4B showed no increase in the release of nitroaniline in the presence of aminopeptidase M, fig. 3:23a. The effects of inhibitors on the ability of Proteinase C4B to hydrolyze SAAPP were investigated, table 3:29. The small amount of inhibition by PMSF in addition to the inactivation by both EDTA and phenanthroline suggested that divalent metal ions were necessary for the maximum activity of the enzyme. Proteinase C4B was also inhibited by TPCK but not by TLCK, chymostatin or leupeptin.

The effects of nucleotides on Proteinase C4B hydrolyzing activity were investigated, table 3:30. A small but reproducible amount of inactivation of enzyme activity was detected with ATP, ADP, AMP and CTP. The significance of this result is not understood at present.

TABLE 3:29

The effects of inhibitors on the hydrolysis of SAAPP by Proteinase C4B

Inhibitor (1 mM unless stated otherwise)	Inhibition (%)
PMSF	24
EDTA	86
phenanthroline	46
TLCK	5
TPCK	100
IAA	8
chymostatin (10 µg/ml)	11
leupeptin (10 µg/ml)	13

Assays were performed as described in section 2:13. 100% activity (2.6 nmol/min) was determined in the absence of inhibitor. Samples contained 6 µg protein per assay.

TABLE 3:30

The effects of nucleotides on the SAAPP hydrolyzing activity of Proteinase C4B

Nucleotide (3 mM)	Activity (%)
ATP	74
ADP	85
AMP	80
cAMP	95
UTP	111
GTP	101
CTP	78
No nucleotide	100

Assays were performed as described in section 2:14. 100% activity (2.6 nmol/min) was determined in the absence of nucleotide. Samples contained 6 µg protein per assay.

Proteinase C4C

The peak fractions from the Sephadex G75 column showing Proteinase C4C activity were pooled and concentrated. Proteinase C4C showed an increase in the release of nitroaniline in the presence of aminopeptidase M, fig. 3:23a. The effects of inhibitors on Proteinase C4C hydrolysis of SAAPP in the presence of aminopeptidase M are shown in table 3:31. Inhibition with PMSF and the inability to inactivate the enzyme with metal-chelators suggested that Proteinase C4C was an enzyme with a specific serine residue at its active site and with no metal ion requirement for either enzyme activity or stability. A small amount of inhibition with IAA (31%) indicated that this enzyme had a thiol group that was necessary for enzyme stability.

There were no apparent effects on the hydrolysis of SAAPP by any of the nucleotides tested, table 3:32.

(ii) Hydrolysis of HPA and LeuNA by fractions containing Proteinase C4A, C4B and C4C.

Proteinase C4A

After elution on a Sephadex G75 column Proteinase C4A coeluted with an enzyme(s) that hydrolyzed both HPA and LeuNA, fig. 3:23b. It is not yet known whether Proteinase C4A was responsible for the hydrolysis of HPA and LeuNA in addition to SAAPP, or whether separate enzymes were involved.

Proteinase C4B

Proteinase C4B coeluted with enzyme(s) that hydrolyzed both HPA and LeuNA, fig. 3:23b. Without further purification it is not

TABLE 3:31

The effects of inhibitors on the hydrolysis of SAAPP by Proteinase C4C

Inhibitor (1 mM unless stated otherwise)	Inhibition (%)
PMSF	98
EDTA	12
phenanthroline	10
TLCK	25
TPCK	35
IAA	31
chymostatin (10 µg/ml)	26
leupeptin (10 µg/ml)	46

Assays were performed as described in section 2:13. 100% activity (2.4 nmol/min) was determined in the absence of inhibitor. Samples contained 5 µg protein per assay.

TABLE 3:32

The effects of nucleotides on the SAAPP hydrolyzing activity of Proteinase C4C

Nucleotide (3 mM)	Activity (%)
ATP	100
ADP	96
AMP	108
cAMP	108
UTP	84
GTP	100
CTP	98
No nucleotide	100

Assays were performed as described in section 2:14. 100% activity (2.4 nmol/min) was determined in the absence of nucleotide. Samples contained 5 µg protein per assay.

possible to say whether Proteinase C4B was capable of hydrolyzing HPA and LeuNA in addition to SAAPP, or whether separate enzymes were involved.

Proteinase C4C

Proteinase C4C coeluted with an enzyme that hydrolyzed HPA but not LeuNA, fig. 3:23b. As with both Proteinase C4A and C4B it could not be concluded whether the coeluting activities were due to a single or individual enzymes.

In addition to the 3 SAAPP hydrolyzing enzymes, a fourth enzyme from peak IV of the DEAE-cellulose column, was detected, that appeared only to hydrolyze HPA, fig. 3:23b. This enzyme has been called Proteinase C4D. This enzyme has not been characterized any further at this time.

Enzymes from peaks I, V and VI of the DEAE-cellulose column have been shown to hydrolyze SAAPP and SAAPL, fig. 3:18. In addition an enzyme(s) from peak I hydrolyzes BzIleGlu-GlyArgNA and HPA, fig. 3:19 and 3:20. These enzymes have not been purified or characterized further at this time.

3:4d Discussion

Eight enzymes have been partially purified and characterized from the cytoplasmic fraction of M. methylotrophus. A summary of the properties of these enzymes is shown in table 3:33. Five of these enzymes hydrolyzed a protein substrate in addition to SAAPP, two only hydrolyzed SAAPP and one only hydrolyzed the aminopeptidase substrate LeuNA. Of the enzymes characterized two were serine proteinases and one was a metalloproteinase. A similar number of

TABLE 3:33

Proteinases from the cytoplasmic fraction of M. methylotrophus cells

Proteinase	Apparent Mol wt (daltons) gel chromatography	Inhibitors	Nucleotides + Activation - Inhibition	Purification (fold)	Substrates hydrolyzed
C2A	32000			38	SAAPP (+M)
C3A	38000			25	SAAPP (±M) HPA
C3B	21000			26	SAAPP (±M) HPA
C4A	400000	PMSF, EDTA, TPCK, chymostatin Leupeptin	-UTP -CTP	11	SAAPP (+M) HPA LeuNA
C4B	30000	EDTA, phenan-throline, TPCK	-ATP -ADP -AMP -CTP	17	SAAPP (±M) HPA LeuNA
C4C	20000	PMSF		16	SAAPP (+M)
C4D	14000				HPA
Aminopeptidase					
C3A	75000			31	LeuNA

(+M) release of nitroaniline only observed in the presence of aminopeptidase M.
(±M) nitroaniline release observed in the absence of aminopeptidase M, but was increased in its presence.

proteinases have been reported to be associated with the cytoplasmic fraction of E. coli cells, (Goldberg et al., 1981), and as with M. methylotrophus, these enzymes were either serine or metallo-proteinases. In contrast to E. coli, the cytoplasmic proteinases of M. methylotrophus generally had much lower molecular weights. The notable exception was Proteinase C4A which had a high molecular weight comparable to Protease La from E. coli. Proteinase C4A activity, unlike that of Protease La, was not stimulated in the presence of ATP (Sreedhara Swamy and Goldberg, 1981; Goldberg et al., 1985). Proteinase C4A activity was however inactivated to some extent by UTP and CTP.

The cytoplasmic enzyme Proteinase C4A also appeared to be equivalent to Proteinase W3A isolated from whole cell extracts. Both these enzymes showed similarities in molecular weight, inhibition by PMSF, EDTA, TPCK, chymostatin and leupeptin, hydrolysis of protein substrates and SAAPP and in the novel inactivation by UTP and CTP. In addition Proteinase C4B from the cytoplasmic fraction showed some similarities to Proteinase W4A from whole cell extracts. Both these enzymes had similar molecular weights, inhibition by EDTA and TPCK, hydrolysis of HPA and inactivation with ATP and ADP.

3:5 ISOLATION AND PARTIAL PURIFICATION OF PROTEOLYTIC ENZYMES FROM THE PERIPLASMIC FRACTION OF M. METHYLOTROPHUS

As an intial stage of purification the periplasmic fraction was run on a DEAE-cellulose column. Fractions eluted with a 0-0.2M NaCl gradient were assayed for their ability to hydrolyze SAAPP, SAAPL, AAAPA and LeuNA and the protein substrates, HPA, and gelatin, figs. 3:24 and 3:25.

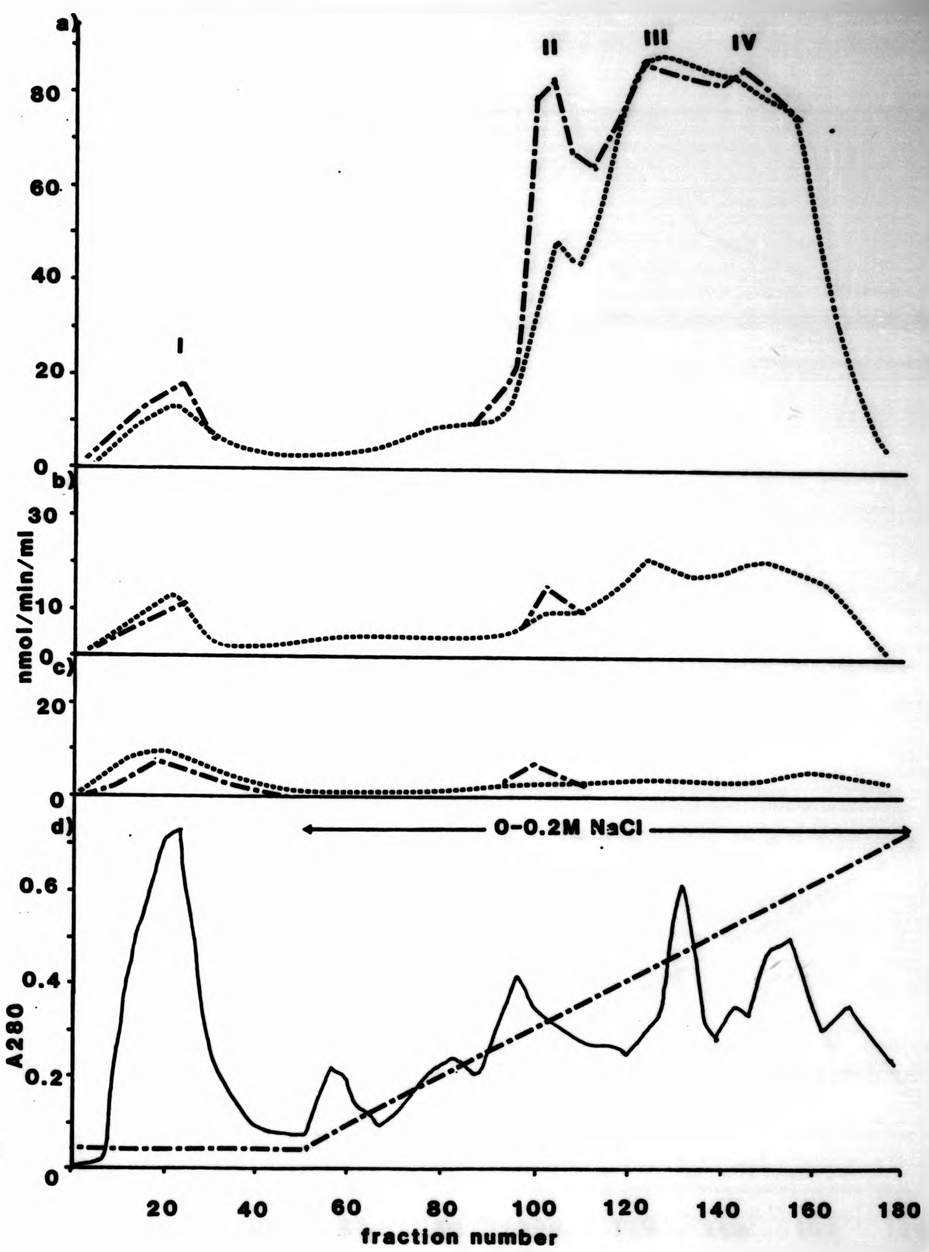
The most rapidly hydrolyzed of the substrates tested was SAAPP, fig. 3:24a. Four major peaks of SAAPP hydrolyzing activity were identified. For convenience these have been numbered peaks I-IV according to their order of elution from the column. The release of nitroaniline from SAAPP by enzyme(s) from peak II was increased in the presence of aminopeptidase M.

A comparable profile of hydrolyzing activities was detected when the Leu-derivative was used as substrate, fig, 3:24b, although the release of nitroaniline from this substrate in the absence or presence of aminopeptidase M was as much as five-fold less than with SAAPP. When the Ala-derivative was used as substrate there was a reduction in the rates of hydrolysis compared to either SAAPP or SAAPL, fig, 3:24c.

Endogenous aminopeptidase activity in the periplasmic fraction was detected using the amino-nitroanilide LeuNA. Three peaks showing LeuNA hydrolysis were observed, two of which coeluted with peaks I and III while the other eluted as a separate hydrolyzing activity, fig. 3:25a.

The ability of the fractions eluted from the DEAE-column to hydrolyze the protein substrates HPA and gelatin was also investigated, fig. 3:25b. HPA hydrolysis was observed with

Fig. 3.24 DEAE-cellulose chromatography of peptide-nitroanilide hydrolyzing enzymes from the periplasmic fraction of M. methylotrophus. Hydrolysis of (a) SAAPP; (b) SAAPL and (c) AAPPA in the absence (•••••) and presence (—••) of aminopeptidase M (d) A_{280} (———). Dialyzed periplasmic extract was absorbed onto a DEAE-cellulose column and eluted with 1500 ml of a linear NaCl gradient (0-0.2M NaCl). The flow rate was 100 ml/hr and 15 ml fractions were collected. Every fourth fraction was assayed. Data points are not shown in order to avoid cluttering on the diagram.



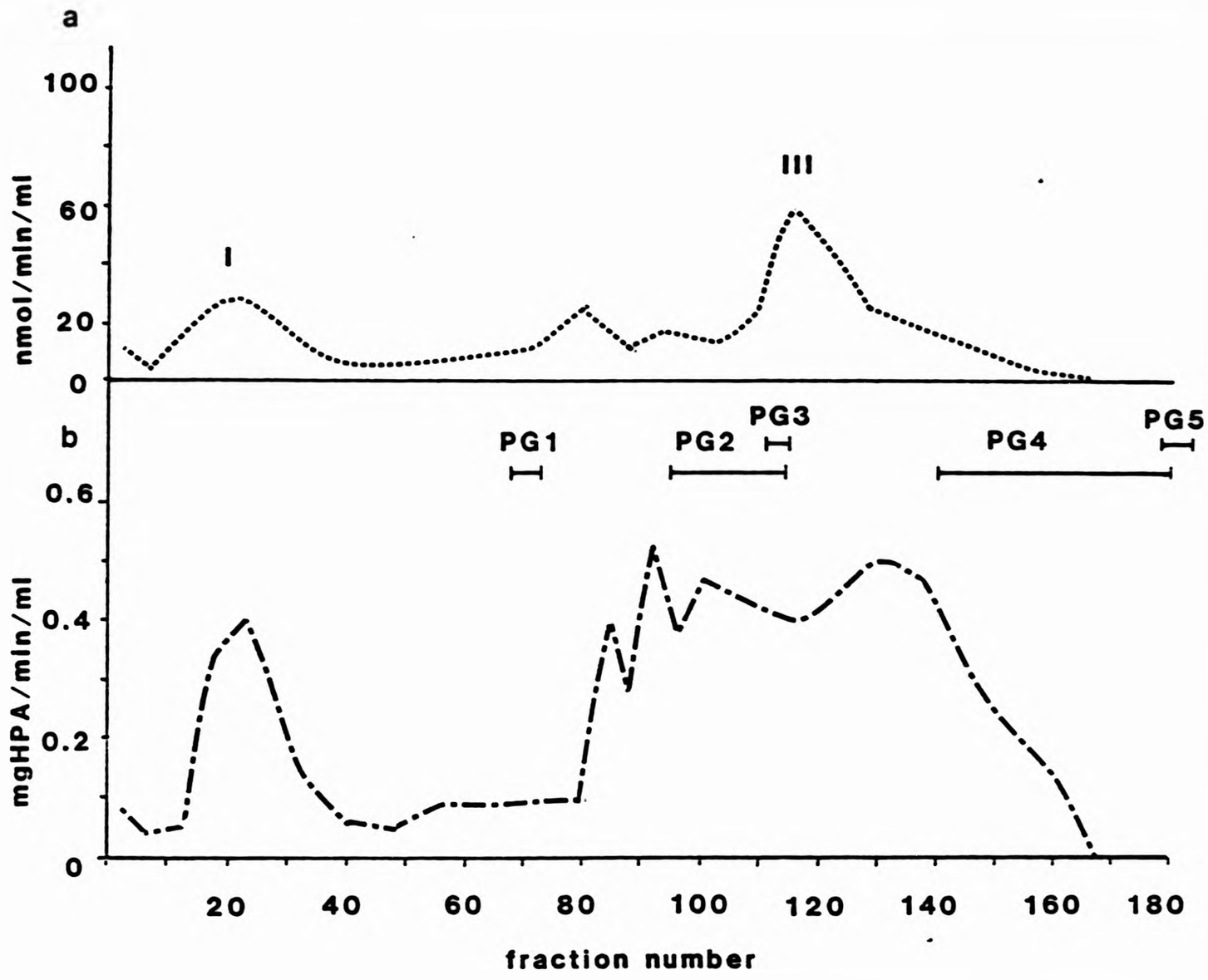


Fig. 3:25 DEAE-cellulose chromatography of aminopeptidase and proteolytic enzymes from the periplasmic fraction of M. methylotrophus. Hydrolysis of (a) LeuNA (····) (b) HPA (—·—) and gelatin. Gelatin degradation was assayed as described in section 2:6c. Dialyzed periplasmic extract was absorbed onto a DEAE-cellulose column and eluted with 1500 ml of a linear NaCl gradient (0.-0.2M NaCl). The flow rate was 100 ml/hr and 15 ml fractions were collected. Every fourth fraction was assayed.

fractions from peaks I-IV, however a distinction between separate hydrolyzing activities was not made. Five gelatin hydrolyzing activities were identified, plate 3:8a & b. These have been named, Proteinases PG1-5, according to their order of elution from the DEAE-cellulose column. Their apparent molecular weights are shown in table 3:34. Proteinases PG2 and PG3 eluted in peak II, while Proteinase PG4 eluted in peak IV. Proteinases PG1 and PG5 eluted separately from other hydrolyzing activities, fig. 3:25b. The gelatin-hydrolyzing enzymes have not been purified or characterized further at this time, although certain of these enzymes may have been responsible for HPA and peptide-nitroanilide hydrolysis.

3:5a (i) Purification of SAAPP hydrolyzing enzymes from peak II

Fractions corresponding to peak II from the DEAE-cellulose column were pooled, concentrated and dialyzed against Sephadex G75 column buffer. The concentrated sample was then run on a Sephadex G75 column. Eluted fractions were tested for their ability to hydrolyze SAAPP, fig. 3:26. Two peaks of SAAPP hydrolyzing activity were detected, both of which showed an increased release of nitroaniline in the presence of aminopeptidase M. The enzyme responsible for these activities have been named, Proteinase P2A and P2B, according to their order of elution from the column. Proteinase P2A and P2B had apparent molecular weights of 45000 D and 25000 D respectively as estimated by gel chromatography.

(ii) Hydrolysis of protein substrates by Proteinases P2A and P2B

The fractions eluted from the Sephadex G75 column were tested for their ability to hydrolyze $[^{14}C]$-α_1-casein, fig. 3:26. Two

Plate 3:8 Gelatin-hydrolyzing activities of periplasmic enzymes eluted from a DEAE-cellulose column. Activities were detected by SDS-polyacrylamide gel electrophoresis under non-denaturing conditions on gels containing gelatin. (a) Lanes 1-15, every fifth fraction from 15-25 and 60-115 eluted from the DEAE-cellulose column; lane 6, Proteinase PG1 fraction 70; lanes 11-14, Proteinase PG2 fractions 95-110; lane 15, Proteinase PG3, fraction 115. (b) Lanes 1-14, every fifth fraction from 120-185 eluted from the DEAE-cellulose column; lanes 6-13, Proteinase PG4 fraction 140-180; lanes 13 and 14, Proteinase PG5 fraction 180 and 185; lane 15, periplasmic fraction (200 µg protein).

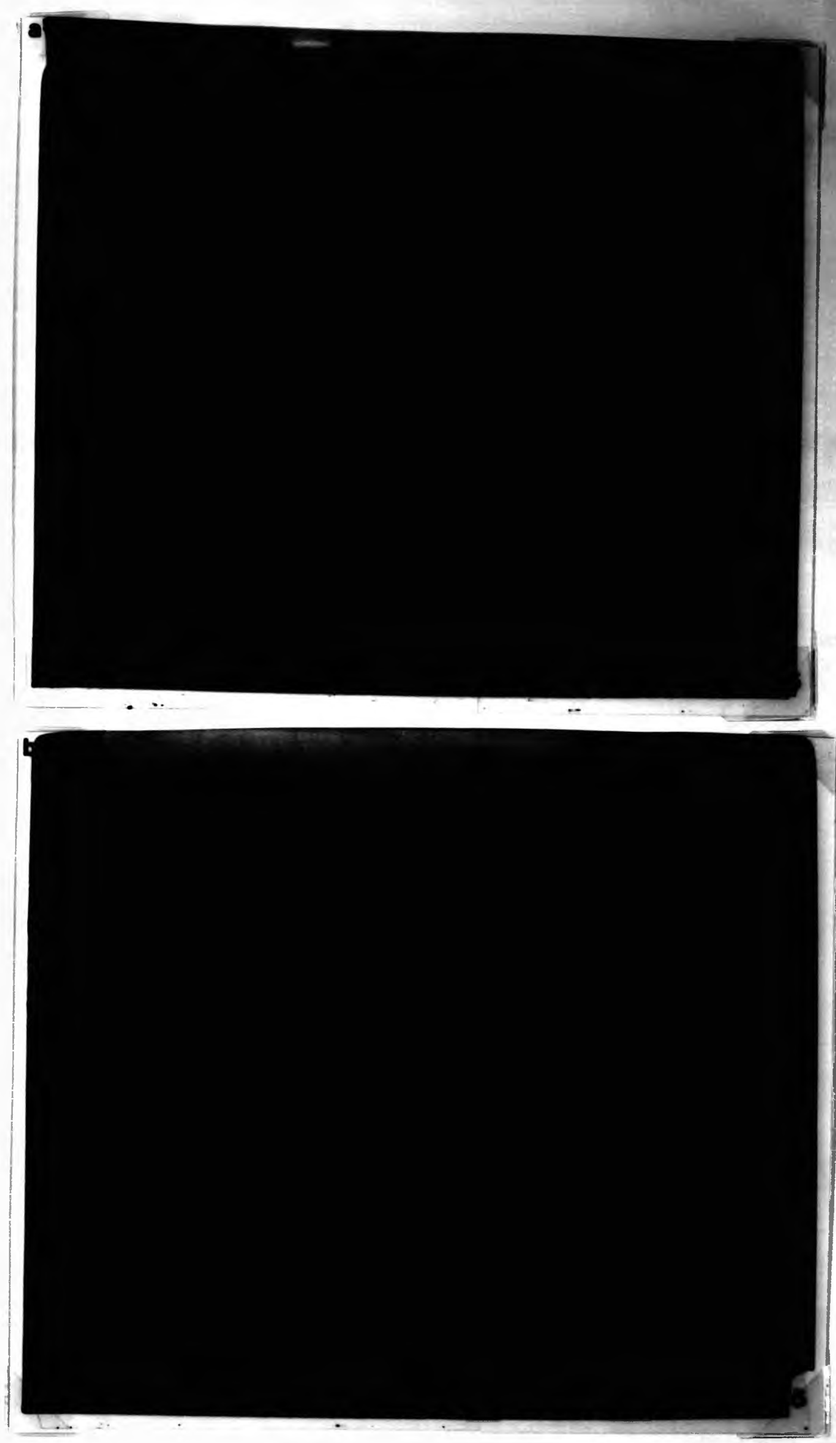


Plate 3:8 Gelatin-hydrolyzing activities of periplasmic enzymes eluted from a DEAE-cellulose column. Activities were detected by SDS-polyacrylamide gel electrophoresis under non-denaturing conditions on gels containing gelatin. (a) Lanes 1-15, every fifth fraction from 15-25 and 60-115 eluted from the DEAE-cellulose column; lane 6, Proteinase PG1 fraction 70; lanes 11-14, Proteinase PG2 fractions 95-110; lane 15, Proteinase PG3, fraction 115. (b) Lanes 1-14, every fifth fraction from 120-185 eluted from the DEAE-cellulose column; lanes 6-13, Proteinase PG4 fraction 140-180; lanes 13 and 14, Proteinase PG5 fraction 180 and 185; lane 15, periplasmic fraction (200 µg protein).

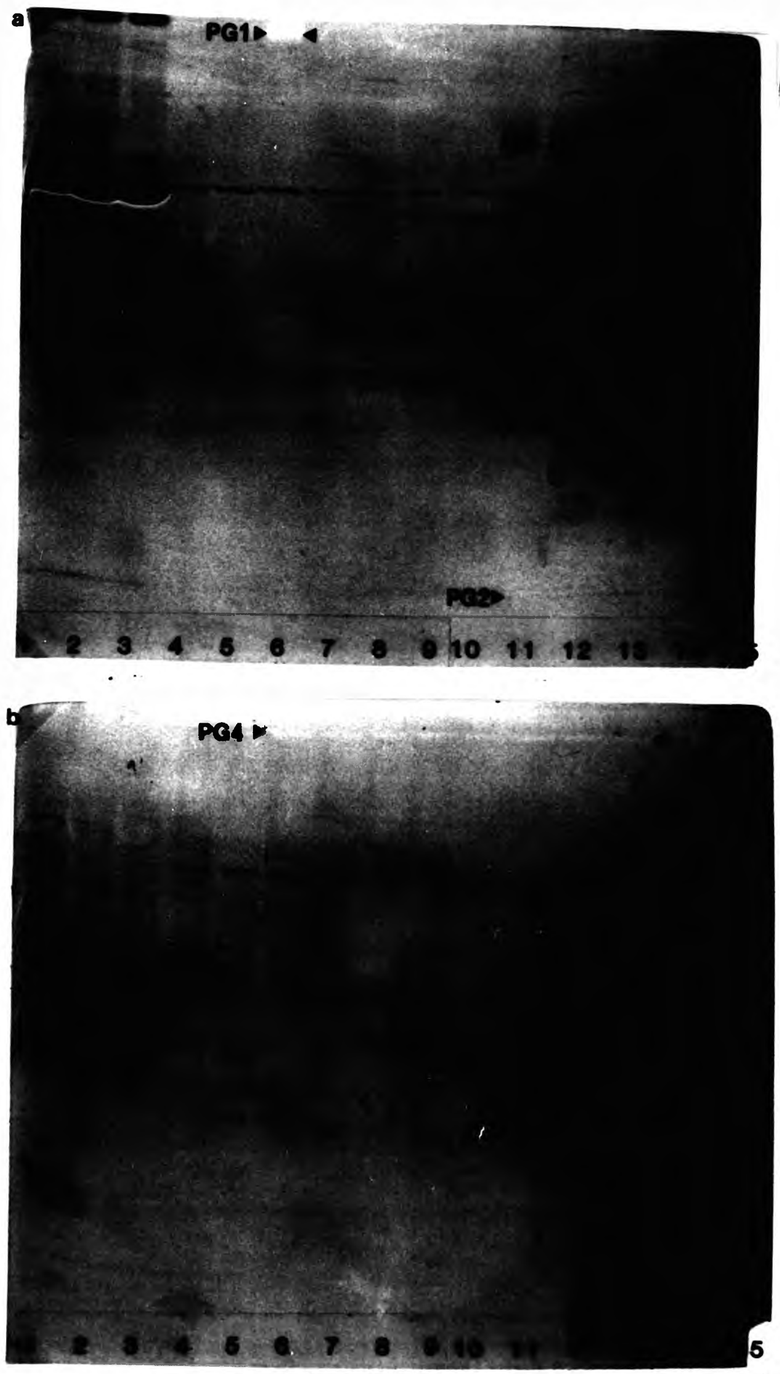


TABLE 3:34

Apparent molecular weight of gelatin-hydrolyzing enzymes

Proteinase	Apparent mol wt (daltons)
PG1	200000
PG2	3000
PG3	55000
PG4	150000
PG5	50000

TABLE 3:34

Apparent molecular weight of gelatin-hydrolyzing enzymes

Proteinase	Apparent mol wt (daltons)
PG1	200000
PG2	3000
PG3	55000
PG4	150000
PG5	50000

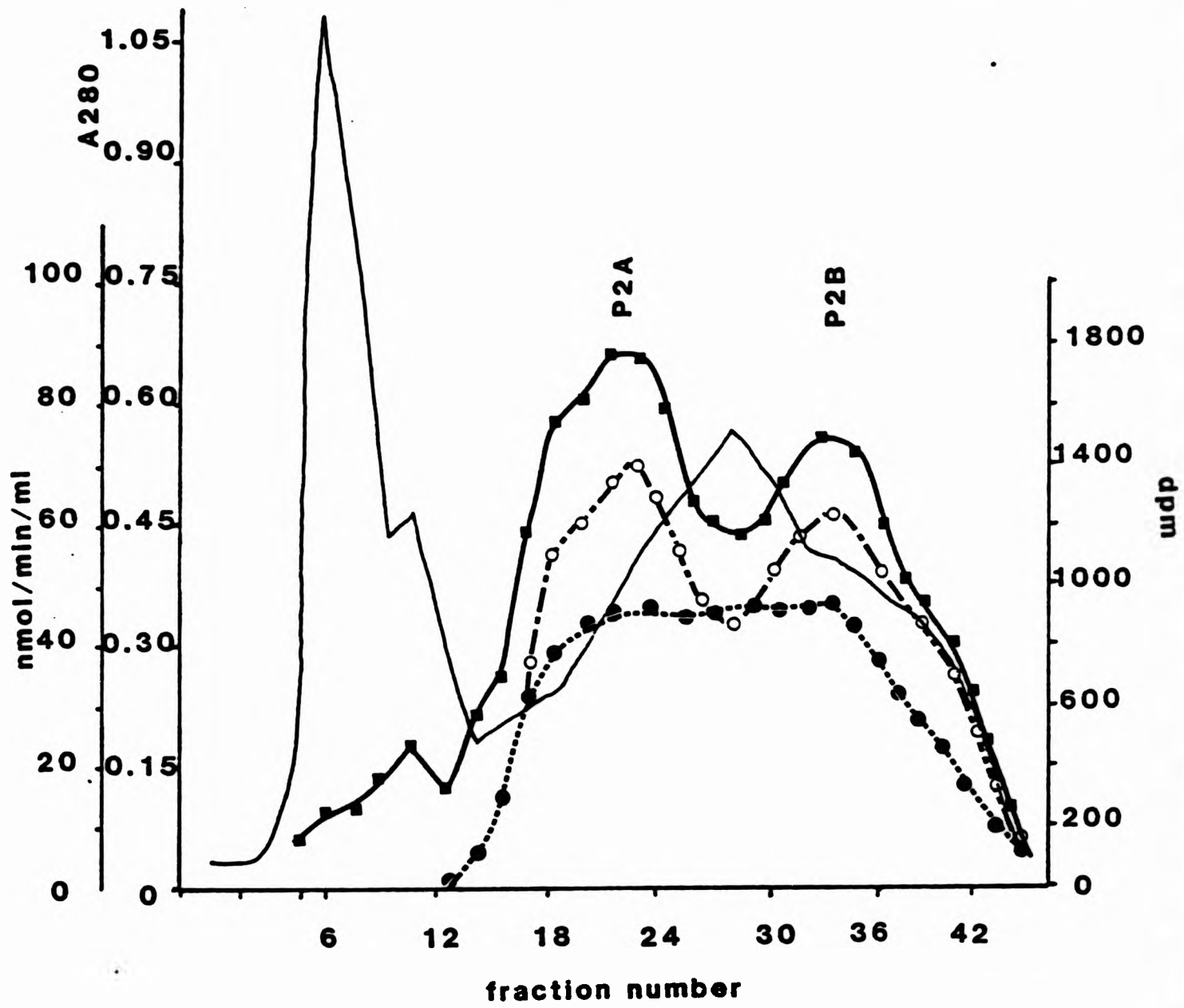


Fig. 3:26 Purification of Proteinases P2A and P2B by Sephadex G75 gel chromatography. Pooled fractions from DEAE-cellulose peak II were concentrated and dialyzed against 10 mM Tris/HCl pH 7.8, 5 mM $MgCl_2$ and applied to a Sephadex G75 superfine column. Activity was determined using SAAPP in the absence (●----●) and presence (○—○) of aminopeptidase M and [^{14}C]-α_1-casein (■——■). A_{280} (———). 3.2 ml fractions were collected and every second fraction assayed.

peaks of [^{14}C]-α_1-casein hydrolyzing activity were observed that coeluted with Proteinases P2A and P2B. Further purification of these enzymes would be necessary to determine whether they hydrolyze both SAAPP and [^{14}C]-α_1-casein.

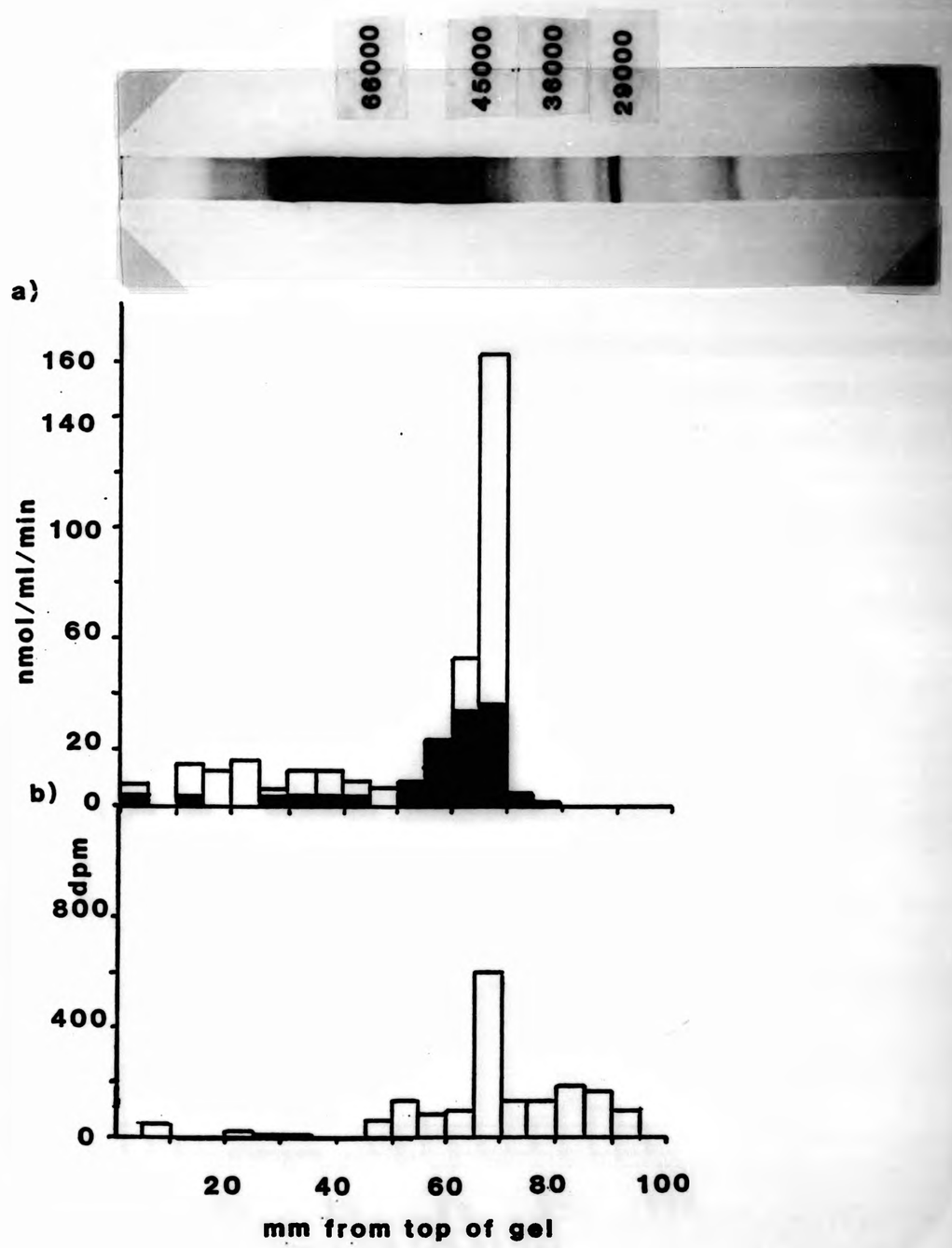
(iii) Further purification of Proteinase P2A

Fractions from the Sephadex G75 column that showed Proteinase P2A activity were pooled, concentrated and run on a preparative SDS-polyacrylamide gel (section 2:6e). 5 mm horizontal sections were taken from the gel and tested for their ability to hydrolyze [^{14}C]-α_1-casein and SAAPP, fig. 3:27. The major [^{14}C]-α_1-casein and SAAPP hydrolyzing activities were eluted from the gel at an apparent molecular weight of 45000 D. The purification scheme for Proteinase P2A is shown in table 3:35. This enzyme has not been characterized further at present.

3:5b (i) Purification of SAAPP hydrolyzing enzymes from peak IV

Fractions from DEAE-cellulose peak IV were pooled, concentrated and dialyzed against Sephadex G75 column running buffer. The concentrated sample was then run on a Sephadex G75 column. Eluted fractions were assayed for SAAPP hydrolyzing activities, fig. 3:28. Two major peaks exhibiting SAAPP hydrolysis were detected and the enzymes responsible for these activities have been named, Proteinases P4A and P4B, according to their order of elution from the column. From gel chromatography Proteinases P4A and P4B had apparent molecular weights of $\geqslant$75000 D and 25000 D respectively.

Fig. 3:27 Purification of proteolytic enzymes from M. methylotrophus periplasmic fractions by preparative SDS-polyacrylamide gel electrophoresis. Fractions corresponding to Proteinase P2A from the Sephadex G75 superfine column were pooled and concentrated and run on an SDS-polyacrylamide gel as described in section 2:6e. Proteins were eluted from 5 mm slices of the gel and assayed for activity towards (a) SAAPP in the absence (■) and presence (□) of aminopeptidase M and (b) [^{14}C]-α_1-casein. Molecular weight standards (daltons) are shown at the top of the figure along with the protein profile for Proteinase P2A from the Sephadex G75 column.



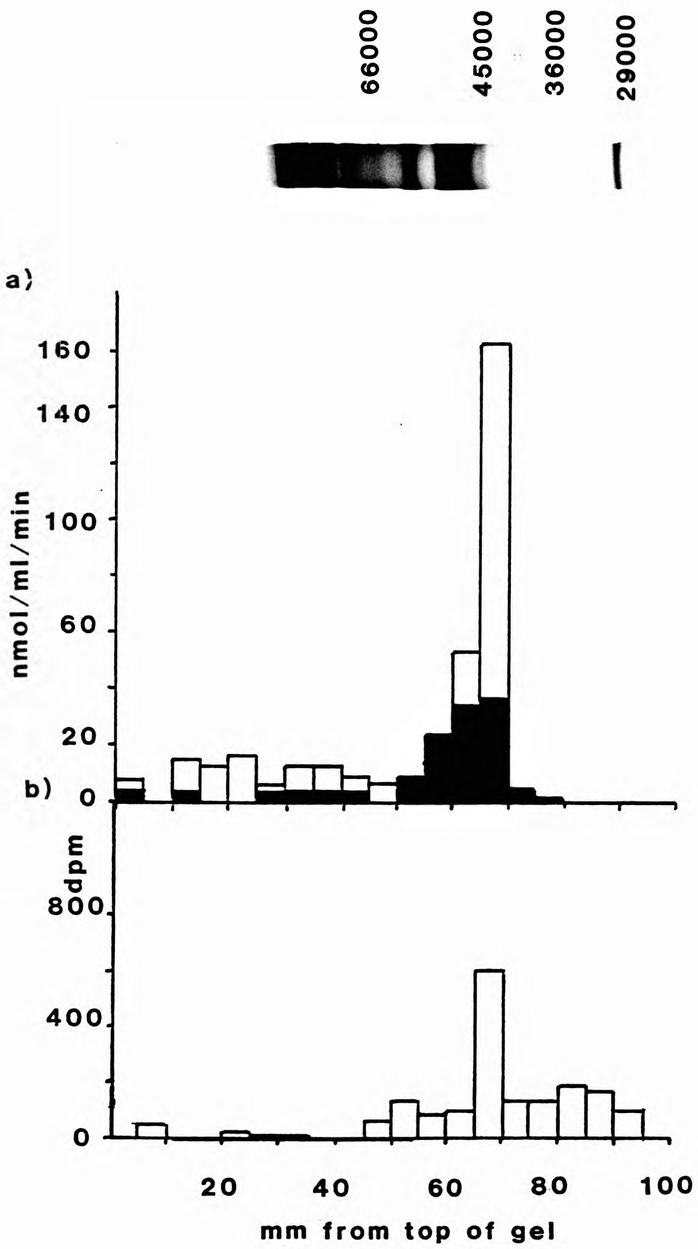


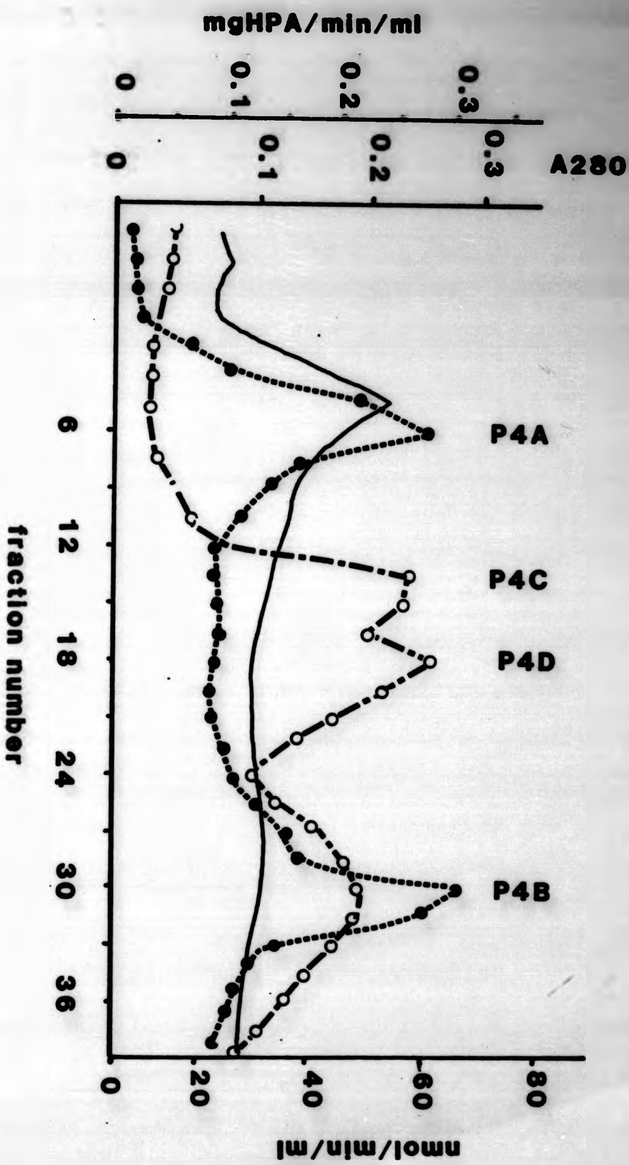
Fig. 3:27 Purification of proteolytic enzymes from M. methylotrophus periplasmic fractions by preparative SDS-polyacrylamide gel electrophoresis. Fractions corresponding to Proteinase P2A from the Sephadex G75 superfine column were pooled and concentrated and run on an SDS-polyacrylamide gel as described in section 2:6e. Proteins were eluted from 5 mm slices of the gel and assayed for activity towards (a) SAAPP in the absence (▬) and presence (▭) of aminopeptidase M and (b) [^{14}C]-α_1-casein. Molecular weight standards (daltons) are shown at the top of the figure along with the protein profile for Proteinase P2A from the Sephadex G75 column.

TABLE 3:35

Purification scheme for the SAAPP hydrolyzing enzyme Proteinase P2A

Extract	Total protein (mg)	Total Activity (nmol/min)	Sp. Ac. (nmol/min/mg protein)	Purity (fold)	Recovery (%)
Periplasm	600	27600	46	1	100
DEAE-cellulose peak II	112	8512	76	1.7	31
Sephadex G75 Proteinase P2A	9.6	2880	300	6.5	10
Preparative gel Section# 14 Proteinase P2A	0.02	1600	8000	174	5.8

Fig. 3:28 Purification of Proteinases P4A, P4B, P4C and P4D by Sephadex G75 column chromatography. Pooled fractions from DEAE-cellulose peak IV were concentrated and dialyzed against 10 mM Tris/HCl pH 7.8, 5 mM $MgCl_2$ and applied to a Sephadex G75 superfine column. Activity was determined using SAAPP (●----●) and HPA (○--○) as substrates, A_{280} (———). 3.2 ml fractions were collected and every second fraction assayed.



(ii) Purification of HPA hydrolyzing enzymes from peak IV

In addition to the SAAPP hydrolyzing activities detected in the eluted fractions from the Sephadex G75 column, three HPA hydrolyzing activities were also observed, fig. 3:28. Two of the enzymes responsible for HPA hydrolysis were distinct from the SAAPP hydrolyzing enzymes, Proteinase P4A and P4B, and have been named Proteinase P4C and P4D. Proteinase P4C and P4D had apparent molecular weights of 65000 D and 45000 D respectively. A third HPA hydrolyzing activity was also detected which coeluted with Proteinase P4B. Further purification of this enzyme was necessary to determine whether it was capable of hydrolyzing both HPA and SAAPP.

3:5c Discussion

Six enzymes, in addition to five gelatin-hydrolyzing enzymes (table 3:34), have been identified from the periplasmic fraction of M. methylotrophus cells, table 3:36. Five of these enzymes hydrolyzed protein substrates, three of which also hydrolyzed SAAPP, while the sixth enzyme only hydrolyzed SAAPP. Only three proteolytic enzymes have been reported to be associated with the periplasmic fraction of E. coli (Goldberg et al., 1981).

Without further characterization of the periplasmic enzymes of M. methylotrophus comparisons with the individual proteinases isolated from either the cytoplasmic or whole cell extracts were very difficult to make.

A comparison can be made however between the total hydrolyzing activity of the subcellular fractions. The total SAAPP hydrolyzing activity of the periplasmic fractions (2.76×10^4 nmol/min) (table

TABLE 3:36

Properties of periplasmic proteinases

Proteinase	Apparent Mol wt (daltons)	Substrates hydrolyzed
P2A	45000	[^{14}C]- α_1-casein SAAPP (±M)
P2B	25000	[^{14}C]- α_1-casein SAAPP (±M)
P4A	75000	SAAPP
P4B	25000	SAAPP HPA
P4C	65000	HPA
P4D	45000	HPA

(±M) Nitroaniline release was observed in the absence of aminopeptidase M, but was increased in its presence.

3:35) was 40% greater than that of the cytoplasmic fractions (1.93 x 10^4 nmol/min) (table 3:23). This high proportion of hydrolyzing activity in the periplasm in relation to the cytoplasm, in addition to the higher apparent levels of HPA and gelatin hydrolyzing activities eluted from the DEAE-cellulose column (fig 3:25b cf. fig. 3:20b) may indicate that the periplasm of this bacteria plays an integral role in the overall proteolysis of the cell.

3:6 EXTRACTION OF MEMBRANE-BOUND PROTEOLYTIC ENZYMES FROM M. METHYLOTROPHUS CELLS

Membrane-bound or associated proteolytic enzymes and esterases have been isolated and characterized from a number of bacterial cells (Régnier and Thang, 1973; Thomas et al., 1974; Miller and Becker, 1978; Régnier, 1981a; Régnier 1981b; Voellmy and Goldberg, 1981; Pacaud, 1982a; Pacaud, 1982b) (section 1:6e). It was of some interest to determine whether such proteolytic enzymes were associated with membranes of M. methylotrophus cells.

Previous attempts to characterize particulate enzymes of other bacteria have been hampered by the lack of suitable solubilization procedures, instability of the solubilized enzymes, and unknown substrate requirements (Pacaud, 1982a). Many of these problems have now been resolved in the isolation of membrane-bound enzymes in E. coli. In the case of M. methylotrophus, substrate requirements were not a problem as proteolytic enzyme substrates had been identified by earlier research (section 3:1a & 3:1b). Of particular use for this purpose were the nitroanilide substrates BzArgNA and SAAPP. The instability of solubilized enzymes was not considered at this time.

The actual lysis, solubilization and delipidation of membranes is an extremely complex process which appears to be dependent on the physiological make-up of the cellular membrane and on the concentration and type of detergent used. (For a review of the solubilization of membranes by detergents see Helenius and Simons (1975). The membranes of M. methylotrophus have been reported to have a characteristic thick cell envelope due to an unusually high lipid-content (Anthony, 1982). This is likely to mean that

solubilization of these membranes will require unique procedures. Initially therefore it was necessary to determine the optimum conditions for solubilization of these membranes.

3:6a Optimum conditions for the solubilization of membranes

Membrane fractions isolated by the procedure of Jones et al, 1982 (section 2:2b) were prepared at a protein concentration of 2 mg/ml, 10 mg/ml and 100 mg/ml. The final protein concentration for extractions were 0.4 mg/ml, 2 mg/ml and 20 mg/ml respectively. Membrane fractions were extracted with a type A soluble amphiphile, an anionic detergent sodium dodecyl sulphate (SDS) and a type B soluble amphiphile, sodium deoxycholate (DOC). Prior to detergent extractions, the membranes were washed twice with 50 mM Tris-HCl, pH 7.5 buffer. Wash and detergent extractions required spinning down the membrane pellet at 13000 g. The supernatants were then used to determine BzArgNA and SAAPP hydrolyzing activities.

From the results (fig. 3:29) it was apparent that the highest activity for both BzArgNA- and SAAPP-hydrolyzing enzymes was obtained using 0.2% (w/v) DOC or 0.2% (w/v) SDS in conjunction with the lowest concentration of membrane (0.4 mg/ml). The BzArgNA-hydrolyzing enzyme was solubilized equally well by both DOC and SDS, (fig. 3:29 (a) and (c)) while the SAAPP hydrolyzing enzyme was preferentially released by DOC, (fig. 3:29 (b) and (d)). The initial inference from this result was that the types of enzymes released from the membrane were dependent on the type of detergent used in the solubilization process. A difference in the solubilization properties of a particular detergent may prove to be invaluable when localizing a proteolytic enzyme associated with

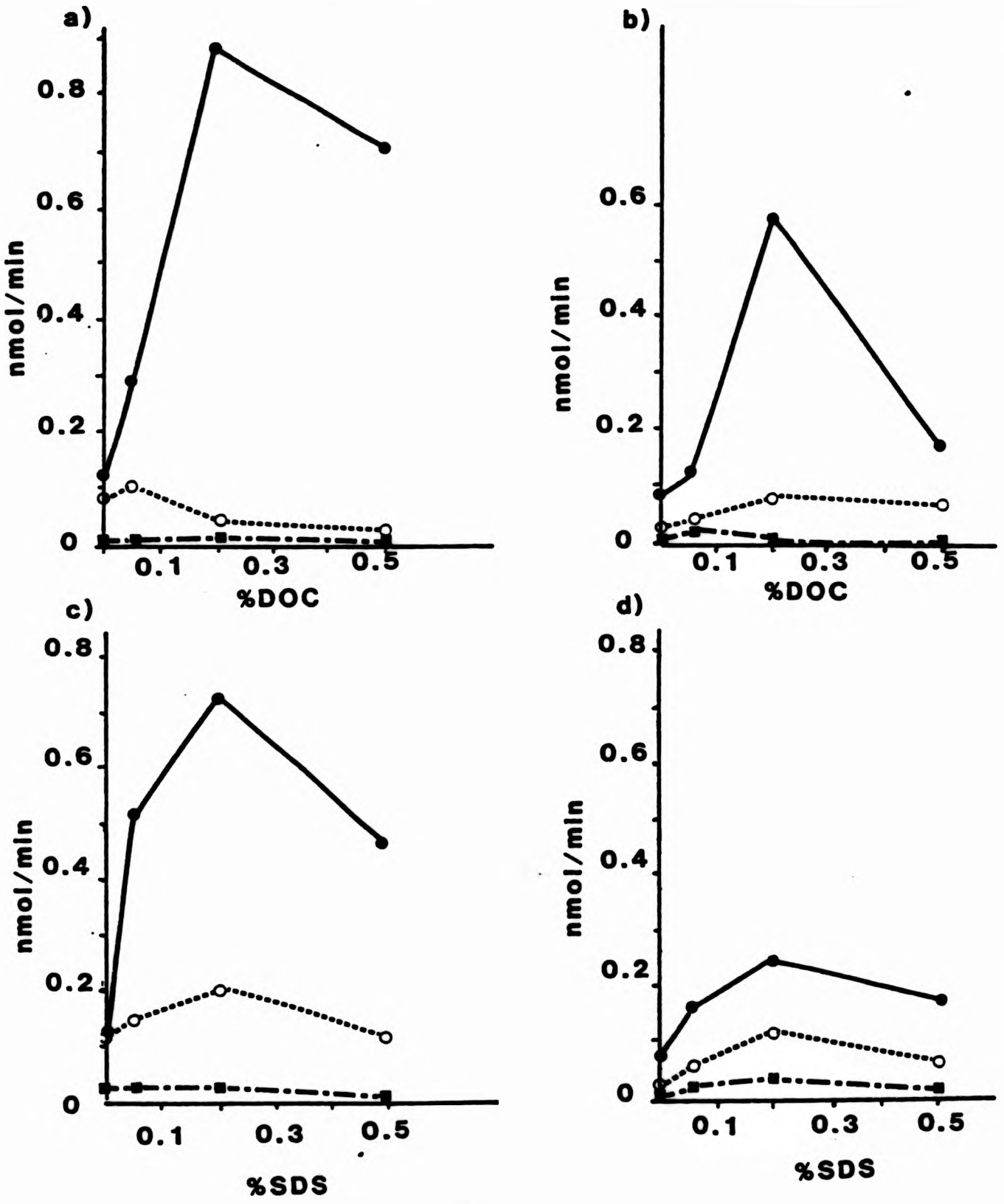


Fig. 3:29 Extraction of peptide-nitroanilide hydrolyzing enzymes from membranes of M. methylotrophus. Membrane fractions at protein concentrations, 0.4 mg/ml (●—●); 2 mg/ml (○····○) and 20 mg/ml (■-·-■) were extracted with, 0-0.5% DOC (a & b) and 0-0.5% SDS (c & d). Activities of eluted enzymes were determined using BzArgNA (a & c) and SAAPP (b & d).

either the inner or outer membrane of a bacterial cell.

3:6b (i) The detection of proteolytic enzymes released from membranes

Further experiments were required in order to ascertain whether the activity detected in the supernatants after detergent extraction was due to membrane-bound enzymes.

(1) SAAPP and BzArgNA hydrolyzing activities were still detectable in supernatants after 2 washes prior to membrane solubilization, fig. 3:29. Unless the buffer actually caused membrane dissociation, which was most improbable, the most likely explanation for the presence of hydrolyzing activities in the wash supernatants was contamination from cytoplasmic and/or periplasmic proteolytic enzymes. It was therefore necessary to wash the membranes until no further hydrolyzing activity could be detected in the supernatants.

(2) An additional procedure for membrane protein extraction was used. This was based on the solubilization of cytochrome oxidases from the membranes of M. methylotrophus cells using Triton X-100 (section 2:15) (Froud and Anthony, 1984). The rationale was that a procedure that had been used successfully to isolate an active enzyme from membranes of this bacteria might also be successful for isolating proteolytic enzymes.

(3) Enzymes extracted by solubilization of membranes were tested for their ability to hydrolyze protein substrates in addition to BzArgNA and SAAPP.

(4) Because of the known complicated effects of detergents on membranes (Helenius and Simons, 1975), there was the additional worry that detergents might inhibit the ability to detect the

release of certain proteolytic enzymes. For this reason all supernatants were extensively dialyzed to remove detergents prior to measuring their activities.

The results for all the above changes are shown in fig. 3:30, 3:31 and 3:32. All activities resulting from contamination of the membranes with cytoplasmic or periplasmic proteinases/peptidases were removed by four washes of the memebranes with 50 mM Tris-HCl, pH 7.5. The enzymes responsible for the activities solubilized by DOC, SDS and Triton X-100 are discussed below.

(ii) BzArgNA-hydrolyzing enzyme(s) released by detergent extraction

BzArgNA-hydrolyzing enzymes were released from membranes by all three detergents, with the maximum activity solubilized using SDS, fig. 3:30. Dialysis of DOC and Triton X-100 extracts caused some loss of activity while SDS extracts showed an increase in activity. The increase in activity of SDS extracts after dialysis was likely to be due to the removal of the inhibitory effects of SDS.

(iii) SAAPP-hydrolyzing enzyme(s) released by detergent extraction

SAAPP-hydrolyzing enzymes were released from membranes by all three detergents with the maximum activity solubilized using DOC, fig. 3:31. After dialysis the highest activity was found in the DOC extracts although the amount of activity released by all extracts was similar.

(iv) [^{14}C]-α_1-casein degrading enzyme(s) released by detergent extraction

[^{14}C]-α_1-casein degrading enzymes were released from

Fig. 3:30 Extraction of BzArgNA hydrolyzing enzymes from membranes of M. methylotrophus. Membranes (0.4 mg protein/ml) were resuspended in 20 mM Tris/HCl, pH 7.8 and washed four times in the same buffer. BzArgNA hydrolyzing enzymes were extracted from the washed membranes with 0.2% (w/v) DOC; 0.2% (w/v) SDS or 2.5% (v/v) Triton X-100. The protein concentration (▬); BzArgNA hydrolyzing activity (▭) and specific activity (▨) was determined for the membrane supernatant, all four washes and detergent extractions, (a) before dialysis and (b) after extensive dialysis.

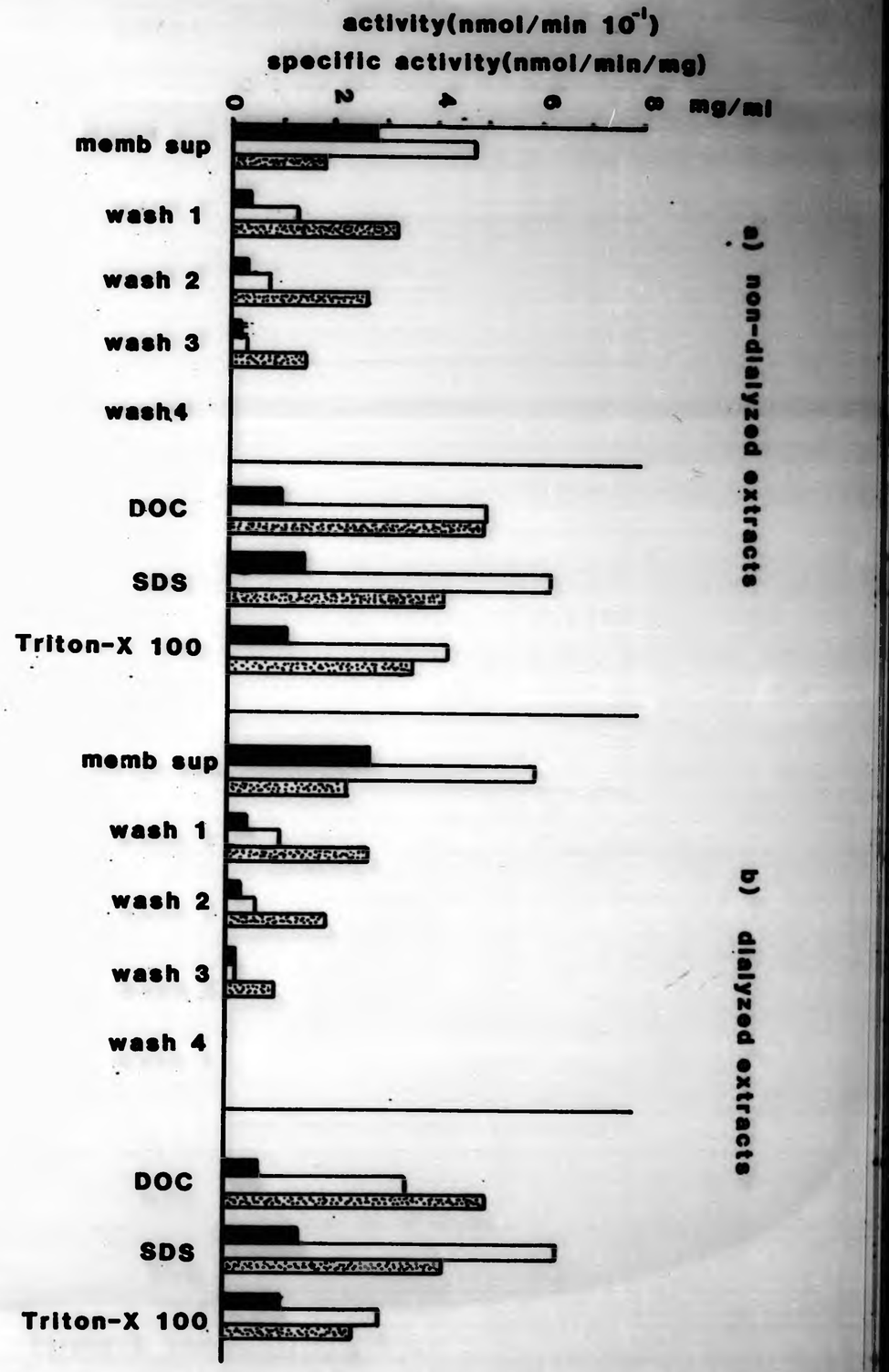


Fig. 3:31 Extraction of SAAPP hydrolyzing enzymes from membranes of M. methylotrophus. Membranes (0.4 mg protein/ml) were resuspended in 20 mM Tris/HCl, pH 7.8 and washed four times in the same buffer. SAAPP hydrolyzing enzymes were extracted from the washed membranes with 0.2% (w/v) DOC; 0.2% (w/v) SDS or 2.5% (v/v) Triton X-100. The protein concentration (▬); SAAPP hydrolyzing activity (▭) and specific activity (▨) was determined for the membrane supernatant, all four washes and detergent extractions, (a) before dialysis and (b) after extensive dialysis.

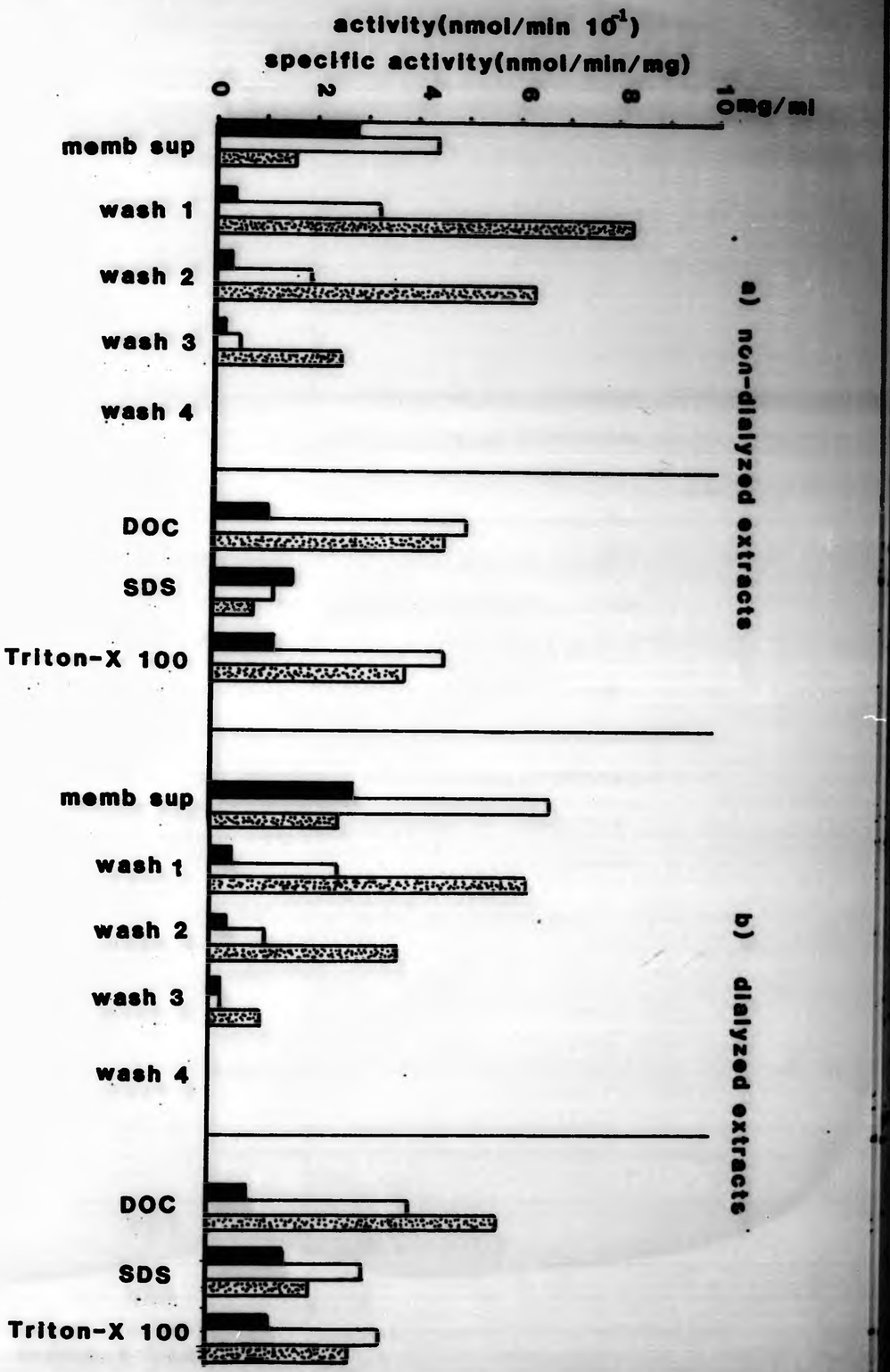
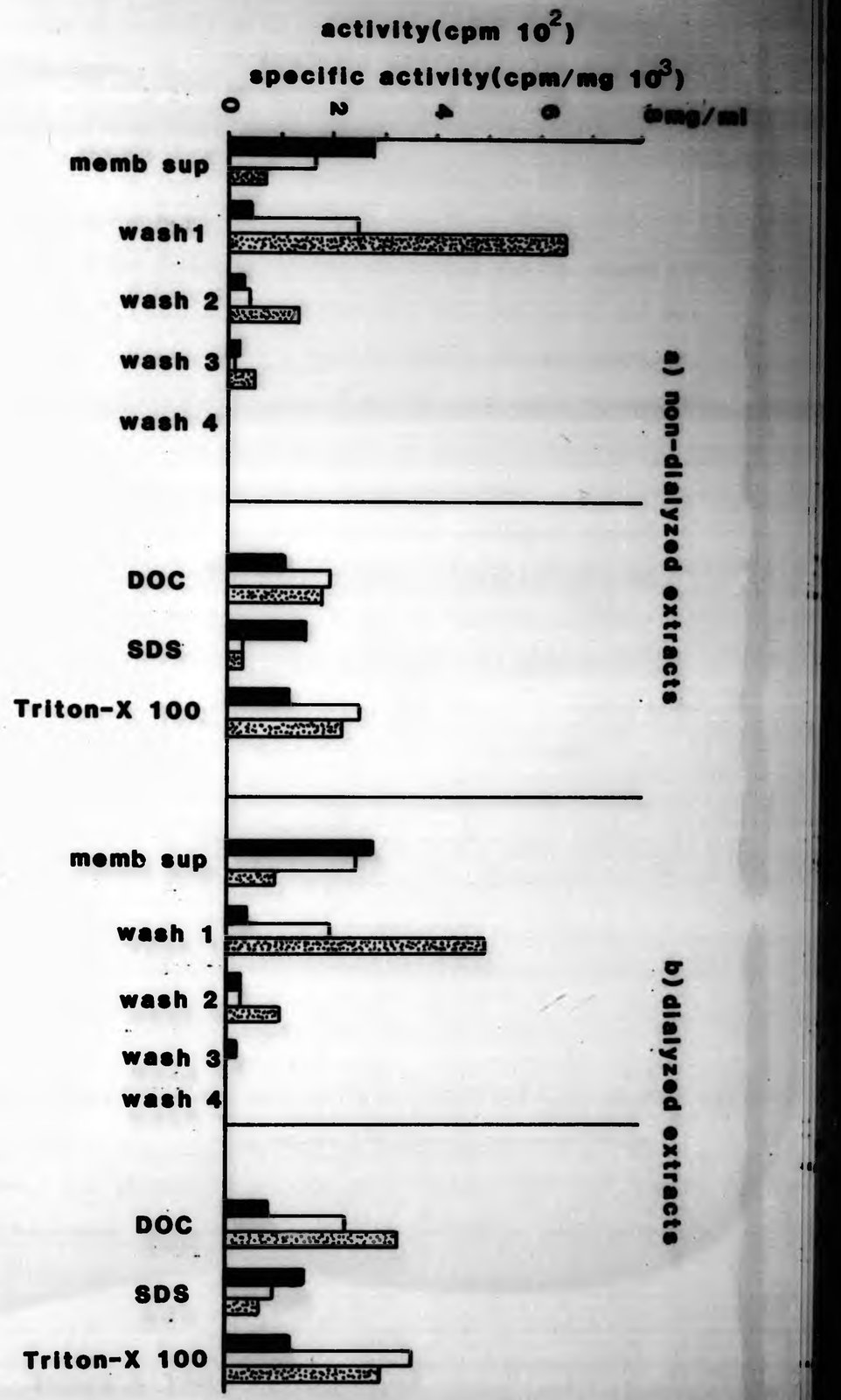


Fig. 3:32 Extraction of [^{14}C] α_1-casein degrading enzymes from membranes of M. methylotrophus. Membranes (0.4 mg protein/ml) were resuspended in 20 mM Tris/HCl, pH 7.8 and washed four times in the same buffer. [^{14}C] α_1-casein degrading enzymes were extracted from the washed membranes with 0.2% (w/v) DOC; 0.2% (w/v) SDS or 2.5% (v/v) Triton X-100. The protein concentration (▬); [^{14}C] α_1-casein degrading activity (▭) and specific activity (▩) was determined for the membrane supernatant, all four washes and detergent extractions, (a) before dialysis and (b) after extensive dialysis.



membranes by all three detergents, with the maximum activity solubilized using Triton X-100, fig. 3:32. Dialysis led to an increase in $[^{14}C]$-α_1-casein degrading activities for all three detergent extractions.

The results from sections 3:6b (ii), (iii) and (iv) showed that each detergent solubilized membranes to a different extent and that the greatest release of enzyme activity towards all substrates tested in relation to the amount of protein solubilized was with DOC.

(v) Characterization of membrane-bound proteinases

Attempts were made to characterize the proteolytic enzymes released by solubilization of membranes. Detergent extracts and supernatants from the washing steps were incubated with $[^{14}C]$-α_1-casein and the breakdown products visualized by fluorography as described previously, (section 2:7), (plate 3:9). For both the unwashed membrane supernatant and the first membrane wash supernatant, the major cleavage products of α_1-casein, native molecular weight of (35000 D), in 2 hours consisted of 31000 D and 25000 D species. This result suggested that a proteinase capable of cleaving a~5000 D segment of protein was associated with the membrane in the unwashed state, but was gradually removed during the washing stages.

The results for the detergent extracted membranes were not as clear. In the incubation time used the initial cleavage products for SDS and Triton X-100 extracts appeared distinct from the washed membrane supernatants. This suggested proteinase(s) were released with both these detergents. The results for the DOC-extracted supernatant was not as easy to interpret. It was clear that this

Plate 3:9 Autoradiogram showing the degradation of $[^{14}C]$-α_1-casein by membrane extracts. Extracts; unwashed membrane supernatant (270 μg protein) (lanes 1-3); first wash membrane supernatant (40 μg protein) (lanes 4-6); DOC-extracted membrane supernatant (100 μg protein) (lanes 7-9); SDS-extracted membrane supernatant (180 μg protein) (lanes 10-12) and Triton X-100 extracted membrane supernatant (120 μg protein) (lanes 13-15) incubated with $[^{14}C]$-α_1-casein (10000 cpm) at 37°C for 0 minutes (lanes 1, 4, 7, 10 and 13), 30 minutes (lanes 2, 5, 8, 11 and 14) and 120 minutes (lanes 3, 6, 9, 12 and 15). $[^{14}C]$-α_1-casein breakdown products are indicated with closed arrowheads.

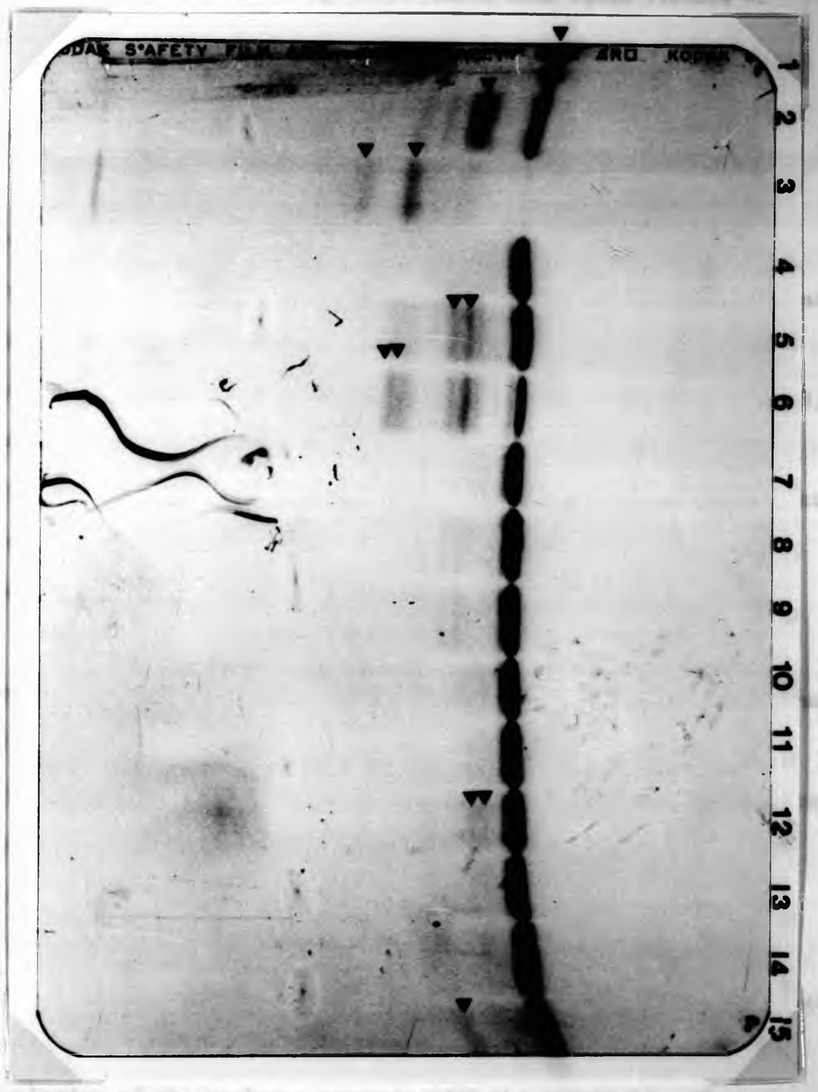
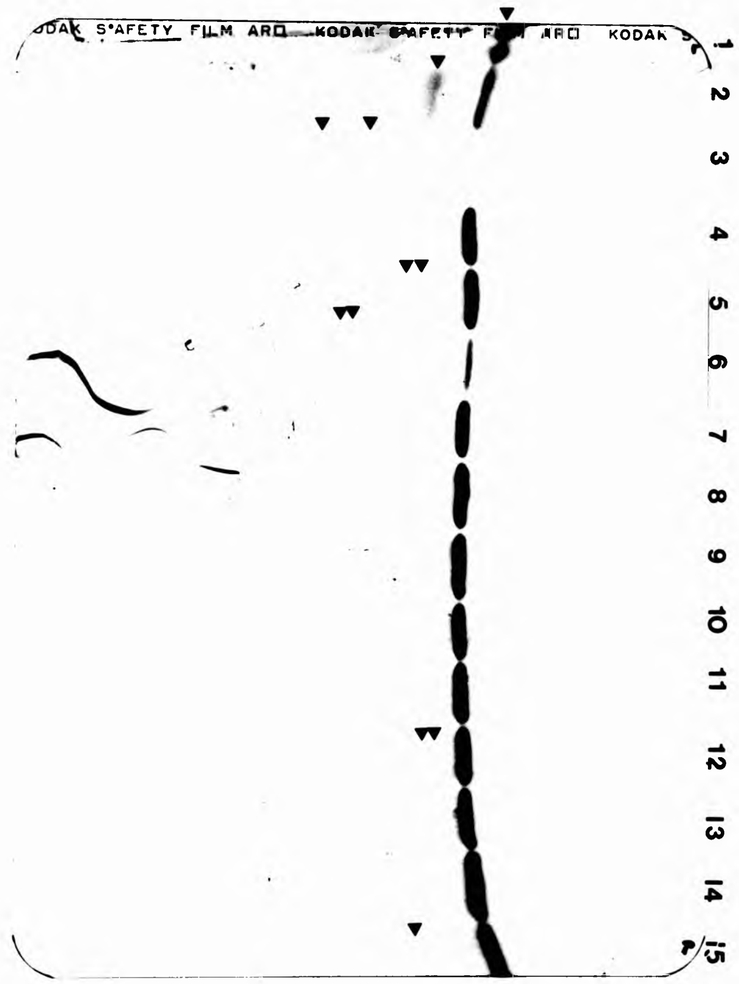


Plate 3:9 Autoradiogram showing the degradation of $[^{14}C]$-α_1-casein by membrane extracts. Extracts; unwashed membrane supernatant (270 µg protein) (lanes 1-3); first wash membrane supernatant (40 µg protein) (lanes 4-6); DOC-extracted membrane supernatant (100 µg protein) (lanes 7-9); SDS-extracted membrane supernatant (180 µg protein) (lanes 10-12) and Triton X-100 extracted membrane supernatant (120 µg protein) (lanes 13-15) incubated with $[^{14}C]$-α_1-casein (10000 cpm) at 37°C for 0 minutes (lanes 1, 4, 7, 10 and 13), 30 minutes (lanes 2, 5, 8, 11 and 14) and 120 minutes (lanes 3, 6, 9, 12 and 15). $[^{14}C]$-α_1-casein breakdown products are indicated with closed arrowheads.



method of detecting proteolytic enzymes may prove very useful for identification procedures, but in this case much longer periods of incubation would be necessary to evaluate the breakdown products. For more comparable results it would also have been better to incubate equal amounts of protein rather than equal volumes of samples with the substrate.

3:6c Discussion

There is now considerable evidence to suggest that bacteria have membrane-bound proteolytic enzymes in addition to soluble proteinases and peptidases (section 1:6e). Although membrane-bound proteinases/peptidases have been reported to be involved in such processes as transfer of secreted proteins (Di Rienzo et al., 1978), membrane biogenesis (Ito et al., 1977), catabolism of abnormal proteins (Voellmy and Goldberg, 1981) and penetration of toxins into cells (Sherrat, 1979), the physiological significance of these enzymes and their specific cleavages are still unknown.

The results reported in this section demonstrate that proteolytic enzymes and peptidases are also present in the membranes of M. methylotrophus cells. The evidence indicates that these enzymes are strongly associated with the membrane and are therefore not likely to be located on outer membrane surfaces. Attempts to characterize the proteolytic enzymes responsible for $[^{14}C]\alpha_1$-casein breakdown by observing the peptide products of proteolysis on SDS-polyacrylamide gels, were not successful. However it is believed that this could be achieved by longer periods of incubation of the membrane extracts with the substrate. The specific activities of the enzymes extracted from the membranes of

M. methylotrophus were generally 5-10-fold less than those isolated from either cytoplasmic or periplasmic fractions. The specific activities of the enzymes extracted from M. methylotrophus membrane were compared with the specific activities of both cytoplasmic and periplasmic extracts before they were purified. The low activity of the membrane-bound proteolytic enzymes may also account for the difficulty in detecting the breakdown products of [^{14}C]-α_1-casein. The specific activities of these enzymes however are comparable with those reported for membrane bound proteinases Protease IV and V of E. coli at a similar stage of purification (Pacaud 1982b). Further characterization of the enzymes associated with the membranes of M. methylotrophus will necessitate large scale preparations of membrane fractions and purification procedures similar to those described for the isolation of enzymes from the soluble fractions (section 3:2, 3:4 and 3:5).

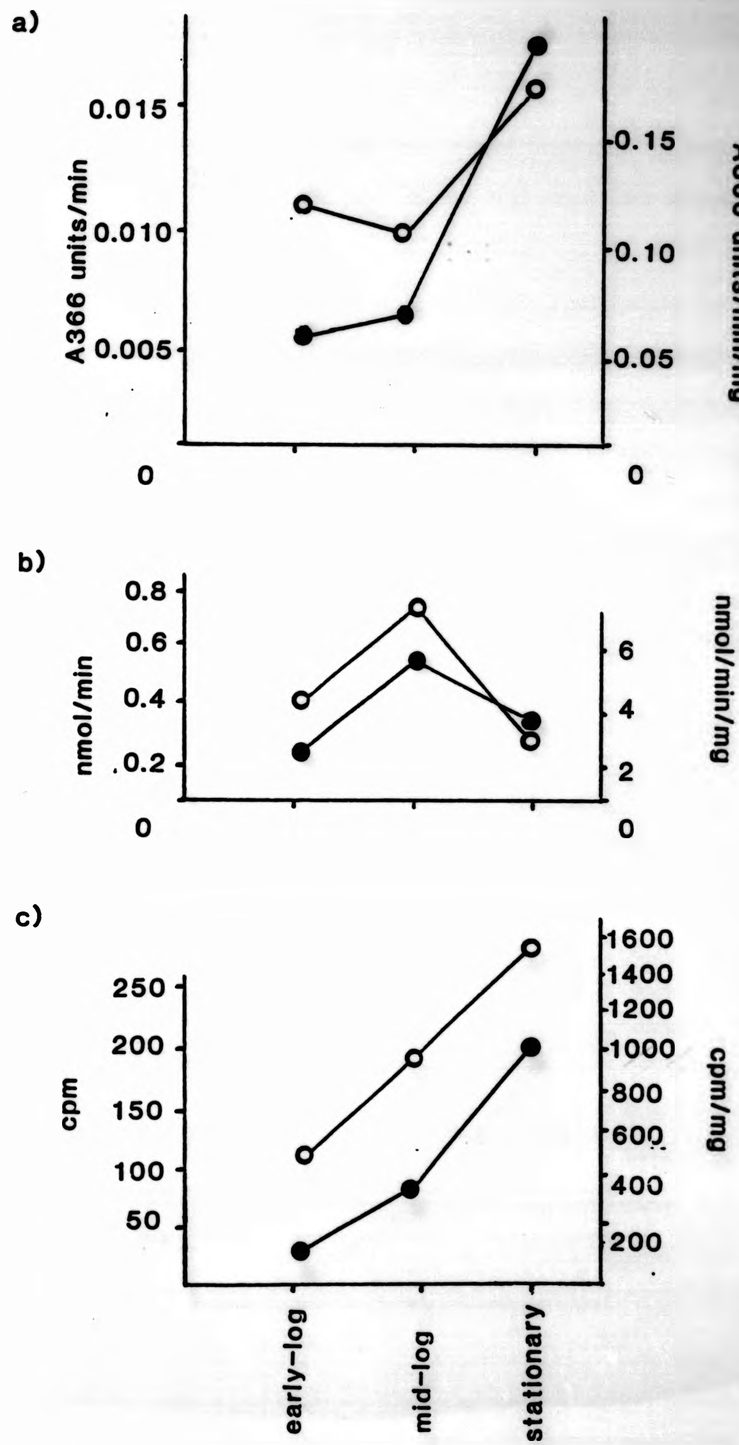
3:7 THE RELATIONSHIP BETWEEN PROTEIN DEGRADATION AND CELL GROWTH

It has previously been observed that soluble extracts of exponentially grown E. coli cells degrade proteins relatively slowly and most of the soluble proteins at this phase of growth are stable (Cocucci and Davies, 1983). If however the rate of growth of these cells is reduced, for instance by shortage of nutrients, protein degradation is enhanced (Mandelstam, 1957; Mandelstam, 1960; Pine, 1972). In all cases the experiments described in sections 3:1-3:6 M. methylotrophus cells were grown to the same stage (mid-log phase). It was of interest to determine what effects the stage of cell growth had on the stability of intracellular proteins and on the ability of whole cell extracts isolated from these cells to degrade exogenous protein and peptide-nitroanilide substrates.

3:7a The hydrolysis of non-physiological substrates at various stages of growth

Whole cell extracts isolated from cells grown to early-log (entering exponential), mid-log (exponential) and stationary phase, were tested for their ability to hydrolyze the protein substrates, azocasein and $[^{14}C]$-α_1-casein and the nitroanilide substrate, BzArgNA, fig. 3:33a-c. The hydrolyzing activity towards azocasein and $[^{14}C]$-α_1-casein increased slightly from early-log to mid-log stages of growth. The most dramatic effect towards these substrates however was observed with whole cell extracts from cells grown to stationary phase. Under these conditions there was a four-fold increase in substrate hydrolyzing activities. BzArgNA hydrolyzing activity also increased from early to mid-log phase, but this activity dropped on entering the stationary phase of growth.

Fig. 3:33 The relationship between the stage of growth of M. methylotrophus ASI cells and the levels of proteolysis. Whole cell extracts were isolated from cells grown to early-log, mid-log and stationary phase (section 2:2a(i)). The activities (●—●) and specific activities (○—○) of whole cell extracts towards (a) azocasein; (b) BzArgNA and (c) [^{14}C]-α_1-casein were determined.



3:7b The stability of intracellular proteins during various stages of growth

The effects of incubation on the stability of intracellular proteins from early-, mid- and stationary phases of growth were investigated. The protein profiles of the incubated extracts were examined by SDS-polyacrylamide gel electrophoresis, plate 3:10.

The majority of intracellular proteins appeared to be stable over the 3 hour period of incubation. (The Coomassie Blue staining procedure will detect the most abundant proteins and will therefore be biased towards the more stable proteins.) In addition, the protein profiles did not appear to be affected by the growth stage. The only difference between the protein profiles was observed with stationary phase extracts. In this case a large molecular weight protein (150000 D) was observed after 15 min incubation. The origin of this protein is unclear although it was most likely the cleavage product of a large molecular weight protein that did not enter the gel at time 0 min. The concentration of this protein appeared to increase over 180 min. A further protein of apparent molecular weight 96000 D was also observed after 30 and 60 min of incubation, but had disappeared within 180 min.

3:7c Discusssion

The results presented here indicate that there may be a difference in the ability of cells grown to stationary phase to degrade intracellular proteins as opposed to non-physiological substrates. The 4-fold increase in the rates of hydrolysis of azocasein and $[^{14}C]$-α_1-casein , fig. 3:33a and b, suggest that the levels of proteolytic activity increase substantially on

Plate 3:10 Stability of intracellular protein at various stages of cell growth. Extracts of Cells grown to early-log phase (150 μg protein) (lanes 1-5); mid-log phase (350 μg protein) (lanes 6-10) and stationary phase (600 μg protein) (lanes 11-15) incubated at 37°C for 0 minutes (lanes 1, 6 and 11); 10 minutes (lanes 2, 7 and 12); 30 minutes (lanes 3, 8 and 13); 60 minutes (lanes 4, 9 and 14) and 180 minutes (lanes 5, 10 and 15). Molecular weight markers (kilodaltons) are shown on the right of the plate.

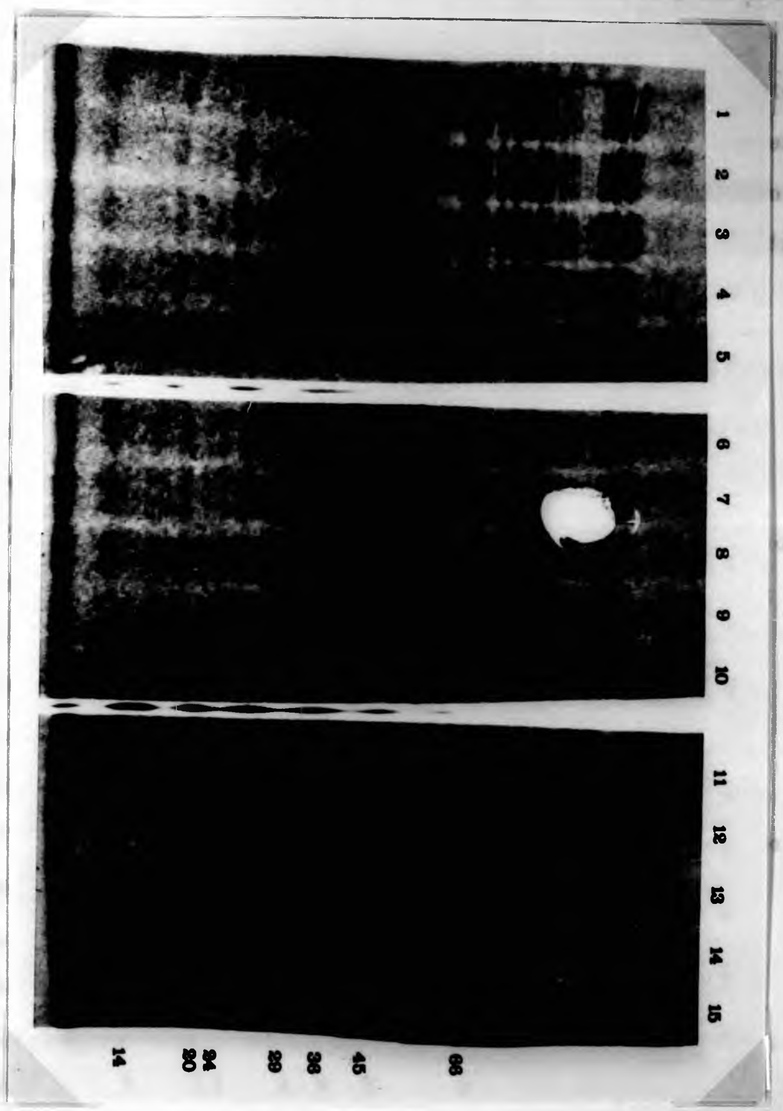
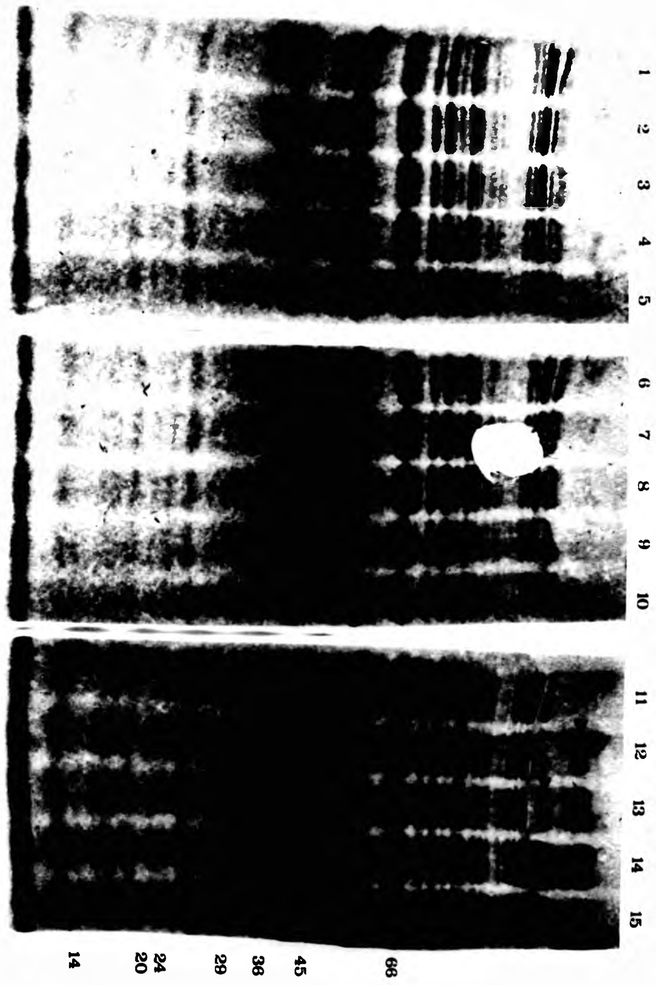


Plate 3:10 Stability of intracellular protein at various stages of cell growth. Extracts of Cells grown to early-log phase (150 μg protein) (lanes 1-5); mid-log phase (350 μg protein) (lanes 6-10) and stationary phase (600 μg protein) (lanes 11-15) incubated at 37°C for 0 minutes (lanes 1, 6 and 11); 10 minutes (lanes 2, 7 and 12); 30 minutes (lanes 3, 8 and 13); 60 minutes (lanes 4, 9 and 14) and 180 minutes (lanes 5, 10 and 15). Molecular weight markers (kilodaltons) are shown on the right of the plate.



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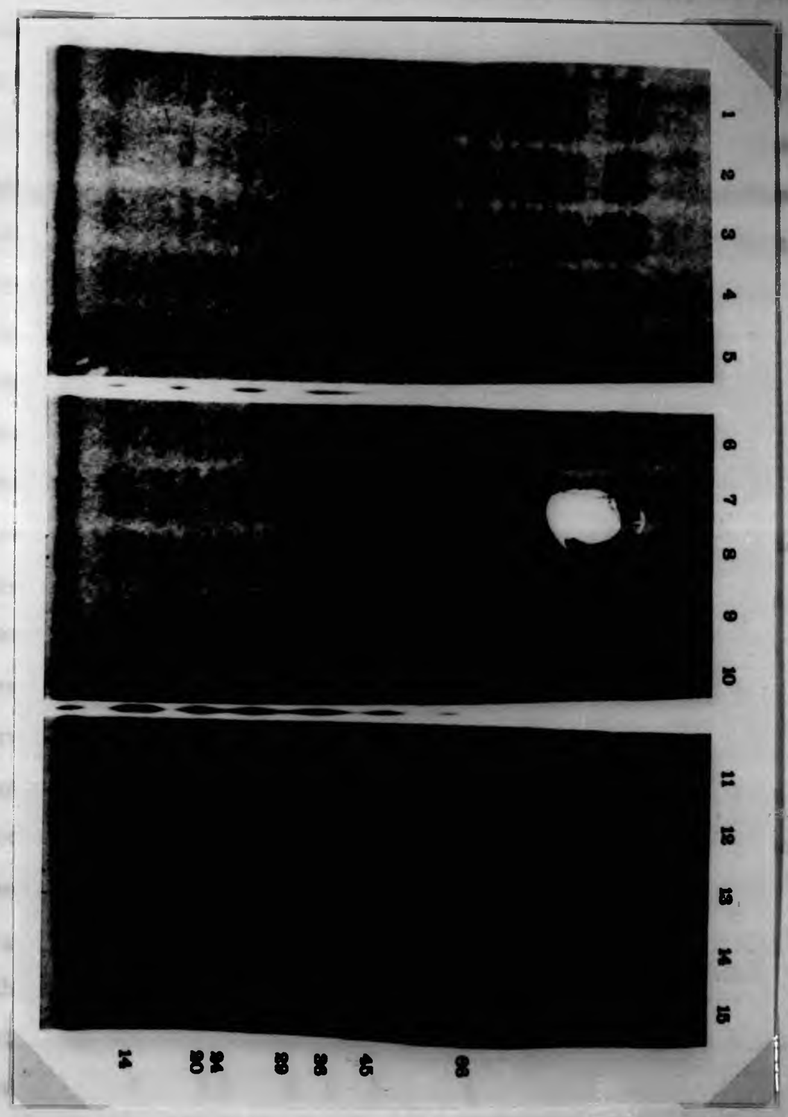
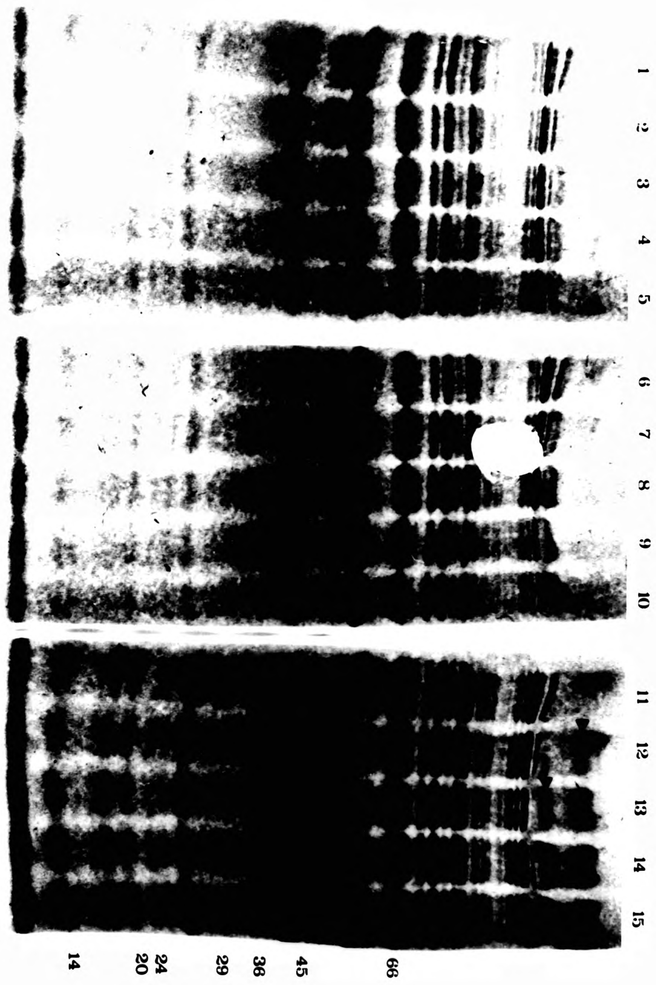


Plate 3:10 Stability of intracellular protein at various stages of cell growth. Extracts of Cells grown to early-log phase (150 µg protein) (lanes 1-5); mid-log phase (350 µg protein) (lanes 6-10) and stationary phase (600 µg protein) (lanes 11-15) incubated at 37°C for 0 minutes (lanes 1, 6 and 11); 10 minutes (lanes 2, 7 and 12); 30 minutes (lanes 3, 8 and 13); 60 minutes (lanes 4, 9 and 14) and 180 minutes (lanes 5, 10 and 15). Molecular weight markers (kilodaltons) are shown on the right of the plate.



shifting to stationary phase conditions. However in contrast to this intracellular protein remains comparatively stable. This would suggest that the degradative system of these cells can distinguish between 'self' and 'non-self' protein. One can immediately see the advantage of such a system in the self-preservation of the cell. This is further substantiated by the fact that intracellular protein was not 'non-specifically' degraded on entering the stationary phase of growth, in fact the results would suggest that protein breakdown might remain selective under these conditions. These results are in contrast to reports with E. coli which suggest that there is a marked decrease in the stability of intracellular proteins on entering conditions unfavourable for growth (Pine, 1972). The increase in proteolysis in E. coli cells on going from exponential to stationary phase is believed to be a consequence of proteinase induction due to abnormal protein production and/or the necessity to produce free amino acids no longer available in the media (Goldberg and Dice, 1974; Goldberg and St John, 1976; Goldberg et al., 1985). Abnormal protein production during the stationary phase of cell growth in M. methylotrophus might result in increased proteolysis. An indication that this might be the case is shown by the increased ability to degrade non-physiological substrates, however there is no evidence to suggest that abnormal proteins are produced in situ during this stage of growth. In the case of spore-forming bacteria the change to stationary phase is believed to cause the specific induction of proteinases involved in the inactivation or degradation of enzymes required in protein synthesis or nucleotide formation (Switzer et al., 1975; Nara and Morioka, 1986). Such a specific induction system in M. methylotrophus might also account for the

relative stability of the majority of intracellular proteins during the stationary phase of growth. Some indication that a selective induction of enzymes may occur on entering the stationary phase was shown by the increase in azocasein and [^{14}C]-α_1-casein-degrading activity, fig. 3:33a and b, while the BzArgNA hydrolyzing activity dropped to levels approaching that of early-log cells, fig. 3:33c. Whether such an elaborate induction mechanism exist in M. methylotrophus cells however remains to be determined.

3:8 THE EFFECTS OF STORAGE ON THE PROTEOLYTIC ACTIVITY OF WHOLE CELL EXTRACTS

It has been observed in a number of microorganisms that storage of their soluble extracts results in an increased ability of these extracts to degrade protein and peptide substrates relative to freshly prepared extracts. This increase in proteolytic activity is related to both temperature and time of storage (Fischer and Thomson, 1979; Meusdoerffer et al., 1980; Stevens et al., 1981). An increase in protein degrading activity during storage is believed to be due to the breakdown of inhibitors specifically associated with proteolytic enzymes (Wolf and Holzer, 1980). The effects of storage and temperature on the protein degrading activity of whole cell extracts from M. methylotrophus cells were investigated.

3:8a Proteolytic activity of whole cell extracts stored at 22°C

(i) Hydrolysis of exogenous substrates

The ability of whole cell extracts, stored at 22°C for up to 8 days, to hydrolyze azocasein and BzArgNA was investigated, fig. 3:34. There was a 28% increase in the rate of hydrolysis of both substrates by whole cell extracts stored for up to 1 day at 22°C. Extracts stored for 3 days showed a 260% increase in their ability to hydrolyze the substrates. Although whole cell extracts that had been stored from 5-8 days hydrolyzed both substrates at twice the rate of freshly prepared extracts, the actual rate of substrate hydrolysis had fallen compared to extracts stored for up to 3 days. Bacterial contamination during extended storage of extracts did not appear to be a contributing factor in substrate hydrolysis since there were no apparent differences in the results of the experiment

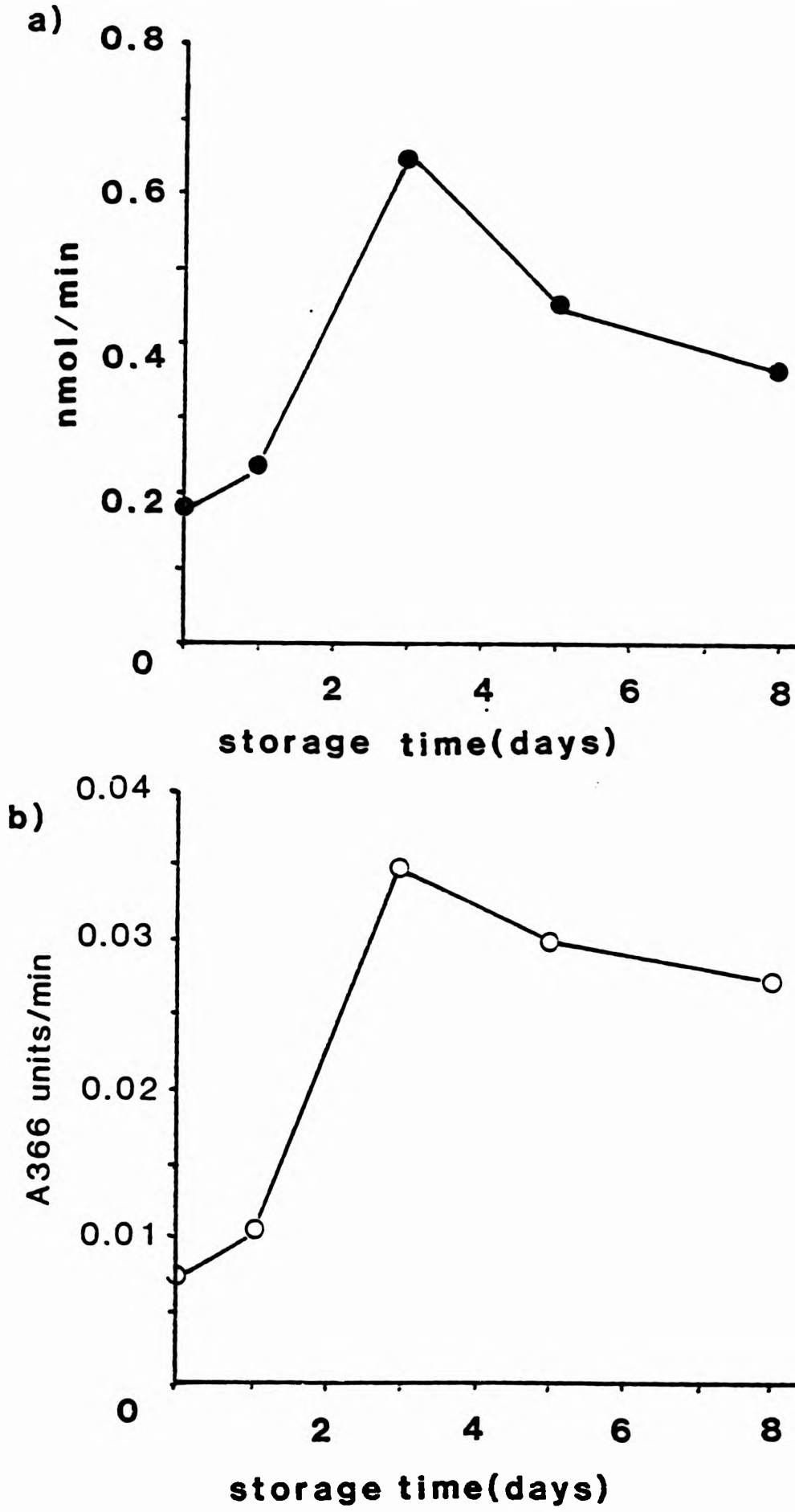


Fig. 3:34 Activation of intracellular proteinase during storage. Whole cell extracts were incubated for 1, 3, 5 and 8 days at 22°C. Activity was measured using (a) BzArgNA (●—●) and (b) azocasein (○—○).

performed in the presence or absence of the bacterial electron-transport inhibitor sodium azide. A comparable experiment to that described above was performed at 37°C. The results for this experiment were not appreciably different from those for 22°C and as such served only to confirm the data reported above.

(ii) Degradation of intracellular protein during storage at 22°C

The degradation of whole cell extract proteins, stored at 22°C for up to 8 days, was investigated by comparing protein profiles on SDS-polyacrylamide gels, plate 3:11. Breakdown of individual protein was apparent within 1 day of storage at 22°C. It was clear that the majority of proteins were susceptible to degradation. Four proteins however appeared particularly resistant to proteolysis, the major protein species of apparent molecular weight 60000 D and 3 minor proteins with apparent molecular weights of 55000 D, 30000 D and 26000 D (indicated by closed arrow-heads lane 2). The absence of new bands produced from the "susceptible" proteins suggested that their degradation was fairly extensive. Storage of the whole cell extract for 3 days at 22°C resulted in further degradation of the susceptible proteins, in some cases to the extent that they could no longer be detected on SDS-polyacrylamide gels. At this time there was also the first evidence of limited cleavage of the major 60000 D protein to a 36000 D breakdown product (indicated by a closed arrow-head, lane 8). From 5-8 days of storage of the whole cell extract there was further evidence although circumstantial for the limited cleavage of the 60000 D protein species, with an increase in the concentration of the 36000 D protein corresponding to a decrease in the

Plate 3:11 Degradation of intracellular proteins at 22°C.
Untreated extracts (lanes 2, 5, 8, 11 and 14);
PMSF-treated extracts (lanes 3, 6, 9, 12 and 15) and
EDTA-treated extracts (lanes 4, 7, 10, 13 and 16).
Extracts (250 μg protein) stored at 22°C for 0 days
(lanes 2-4); 1 day (lanes 5-7); 3 days (lanes 8-10); 5
days (lanes 11-13) and 8 days (lanes 14-16). Lane 1,
molecular weight markers from top to bottom, bovine
albumin (66000 D), egg albumin (45000 D),
glyceraldehyde-3-phosphate dehydrogenase (36000 D),
carbonic anhydrase (29000 D), trypsinogen (24000 D),
soybean trypsin inhibitor (20000 D) and α-lactalbumin
(14200 D).

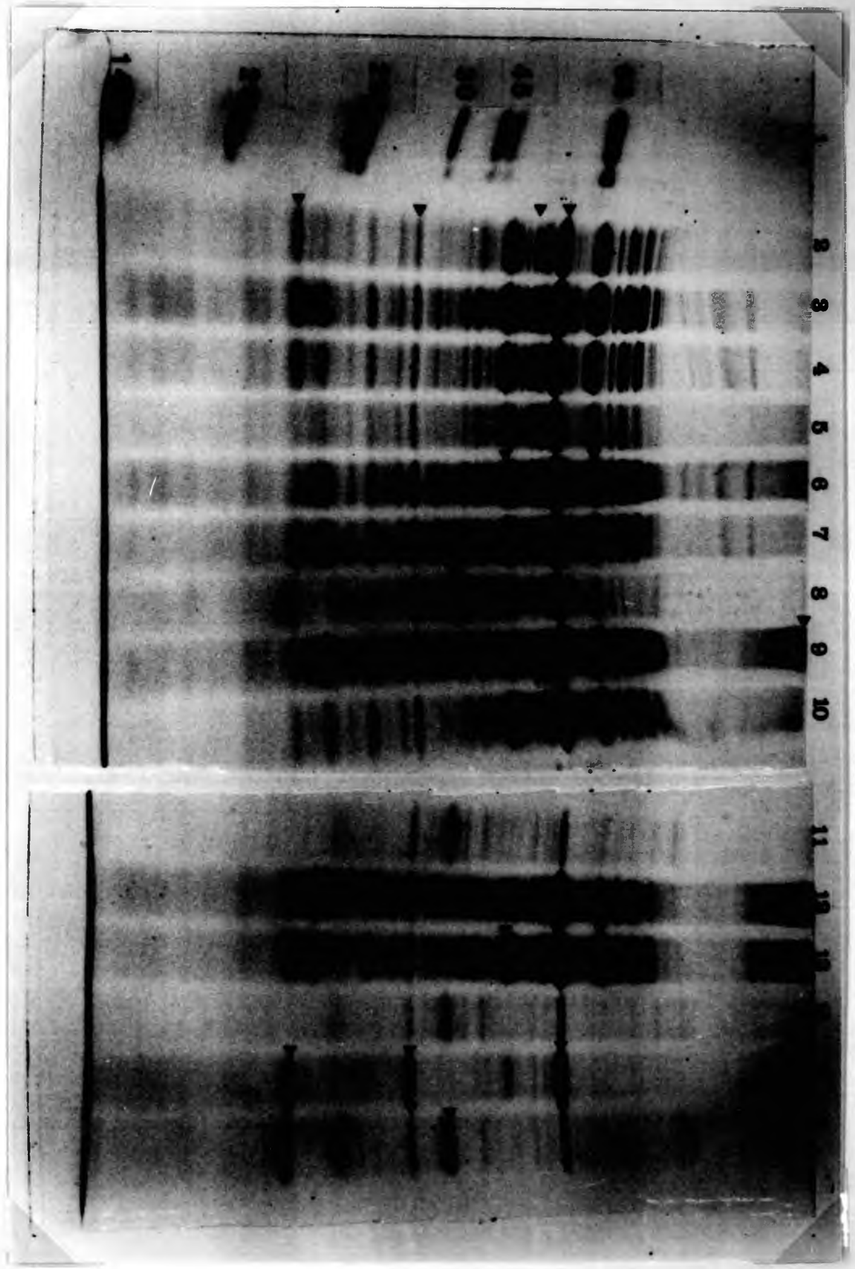


Plate 3:11 Degradation of intracellular proteins at 22°C.

Untreated extracts (lanes 2, 5, 8, 11 and 14);

PMSF-treated extracts (lanes 3, 6, 9, 12 and 15) and

EDTA-treated extracts (lanes 4, 7, 10, 13 and 16).

Extracts (250 μg protein) stored at 22°C for 0 days

(lanes 2-4); 1 day (lanes 5-7); 3 days (lanes 8-10); 5

days (lanes 11-13) and 8 days (lanes 14-16). Lane 1,

molecular weight markers from top to bottom, bovine

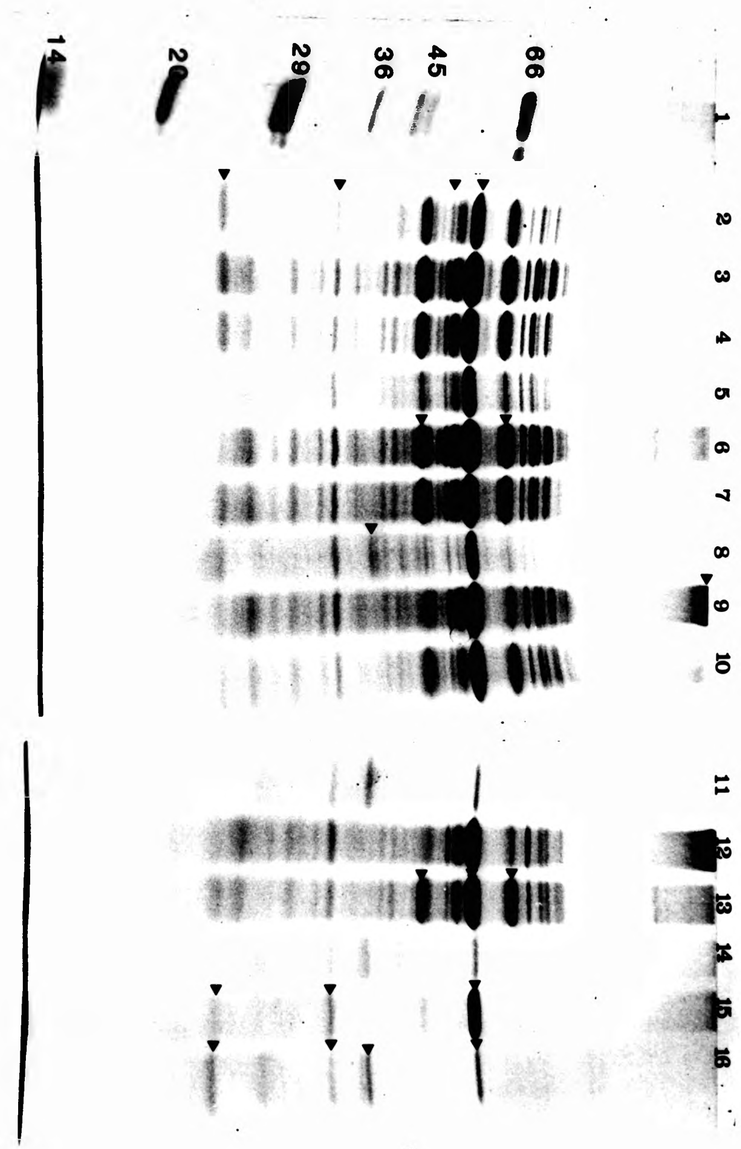
albumin (66000 D), egg albumin (45000 D),

glyceraldehyde-3-phosphate dehydrogenase (36000 D),

carbonic anhydrase (29000 D), trypsinogen (24000 D),

soybean trypsin inhibitor (20000 D) and ∝-lactalbumin

(14200 D).



concentration of the 60000 D protein. After 8 days of storage of the extract at 22°C the only proteins still evident in the protein profile were what remained of the 60000 D protein and its 36000 D breakdown product and the proteinase resistant 30000 D protein species.

3:8b (i) <u>The effects of PMSF and EDTA on proteolysis by whole cell extracts stored at 22°C.</u>

By observing the effects of inhibitors on the extent of substrate hydrolysis and endogenous protein breakdown by stored extracts it should be possible to learn something about the specificity of proteinase(s) responsible for proteolytic degradation during storage at 22°C.

(ii) <u>The effect of inhibitors on the hydrolysis of exogenous substrates</u>

<u>PMSF</u> : The effects of PMSF on the ability of whole cell extracts, stored at 22°C for up to 8 days, to hydrolyze BzArgNA were investigated, fig. 3:35. On immediate addition of PMSF to freshly prepared extracts there was a 61% inhibition of BzArgNA hydrolyzing activity. PMSF treated extracts stored for 3 days at 22°C showed a 100% increase in the ability to hydrolyze BzArgNA compared to extracts at day 0. This rate of substrate hydrolysis however was still more than 6-fold less than for untreated whole cell extracts. After 5-8 days of storage at 22°C the whole cell extracts hydrolysed BzArgNA at a rate approaching that of untreated extracts stored for the same length of time.

<u>EDTA</u> : The effects of EDTA on the ability of whole cell

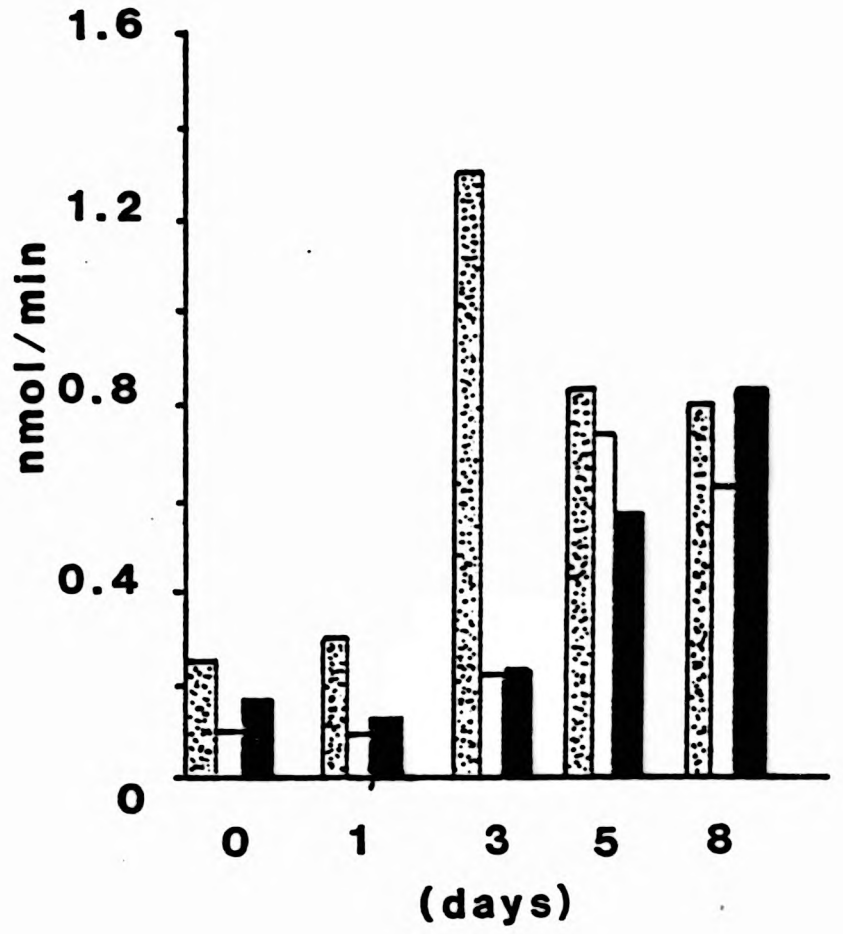


Fig. 3:35 Inhibition of intracellular proteinase activity during storage. Whole cell extracts were incubated for 1, 3, 5 and 8 days at 22°C in the absence of inhibitor (▒▒▒); in the presence of PMSF (▭) and in the presence of EDTA (■■■). Activity was measured using BzArgNA.

extracts, stored at 22°C for up to 8 days, to hydrolyze BzArgNA were investigated, fig. 3:35. On immediate addition of EDTA to freshly prepared extracts there was a 50% inhibition of BzArgNA hydrolyzing activity. There was a 40% increase in the rate of hydrolysis of BzArgNA by EDTA treated extracts stored for up to 3 days when compared to treated extracts at day 0. This rate of substrate hydrolysis however was 6-fold less than for untreated extracts. After 5-8 days of storage the EDTA-treated extracts hydrolyzed BzArgNA at a rate approaching that of untreated extracts stored for the same length of time.

(iii) The effects of inhibitors on the breakdown of intracellular proteins

A comparison was made between the protein profiles of whole cell extracts stored in the absence and presence of PMSF or EDTA, plate 3:11.

PMSF appeared to prevent degradation of all the proteins of the whole cell extract stored at 22°C for 1 day. After storage of the PMSF-treated extract for 3 days the majority of proteins still appeared resistant to proteolytic degradation. The notable exceptions were two of the major protein species of the whole cell extract. These proteins had apparent molecular weights of 66000 D and 50000 D (indicated by closed arrow-heads, lane 9). The major protein species (60000 D) appeared to be resistant to breakdown after 3 days of storage. This was further indicated by the obvious lack of the 36000 D breakdown product which has been apparent in extracts not treated with PMSF, lane 8 (section 3:8a (ii)). There appeared to be a general decrease in protein content after 5 days of

storage suggesting that the proteins within the extract were no longer resistent to proteolysis. At this stage of storage there was the first appearance of a protein band at the interface of the separating and stacking gel (indicated by a closed arrow-head, lane 12). The origin of this protein is not clear although it could have been a breakdown product of a much larger protein that had not previously entered the gel. After 8 days of storage of the whole cell extract in the presence of PMSF the majority of proteins had been degraded extensively. The only proteins that were not degraded were the major 60000 D species and the 30000 D and 26000 D proteins that were not degraded even in the absence of inhibitors (closed arrow-heads indicate the 3 proteins that were not degraded, lane 15).

EDTA appeared to protect the majority of intracellular protein from degradation for up to 5 days of storage at 22°C. It was clear that EDTA prevented extensive degradation of the 3 major whole cell extract proteins (66000 D, 60000 D and 50000 D) (indicated by closed arrow-heads, lane 13) which had been rapidly broken down in untreated extracts (lanes 5, 8 and 11). After 8 days of storage at 22°C in the presence of EDTA the majority of whole cell extract proteins had been degraded and this included limited cleavage of the 60000 D protein species, producing a 36000 D breakdown product (indicated by closed arrow-heads, lane 16), previously observed in untreated extracts (lanes 8, 11 and 14). Proteins of apparent molecular weights 30000 D and 26000 D were still not degraded after 8 days of storage (indicated by closed arrow-heads, lane 16).

3:8c Discussion

The degradation of proteins during storage of whole cell

extracts and the increased ability of these extracts to hydrolyze exogenous protein and peptide substrates (section 3:8a (i) and (ii)) suggests that the breakdown of intracellular protein is somehow linked to the increased proteolytic activity of these extracts. This increase in proteinase activity could be the result of either activation of a zymogen or the degradation of endogenous substrates and/or inhibitors (Wolf and Holzer, 1980; Ansari and Stevens. 1983). The results presented here are comparable to those reported by other researchers, using fungi and yeast (Fischer and Thomson, 1979; Meusdoerffer et al., 1980; Wolf and Holzer, 1980; Stevens et al., 1981; Ansari and Stevens, 1983), from which it was concluded that the increase in protein hydrolyzing activity was infact due to the digestion of endogenous inhibitors.

The increase in proteolytic activity of stored whole cell extracts lasted for 3 days, after which time there was a slow decrease. This observation has also been reported in other microorganisms (Ansari and Stevens, 1983). Although no explanation of this effect has been given it is likely that the decrease in hydrolyzing activity was the result of self degradation of proteolytic enzymes after longer periods of incubation.

The inhibition of both exogenous protein and peptide hydrolysis and degradation of intracellular protein by PMSF and EDTA indicated that the individual enzymes responsible for proteolytic degradation during storage at 22^{o}C included both serine- and metallo- type proteinases. The different protein profiles seen with extracts treated with PMSF and EDTA (section 3:8b (ii)), suggested that although both the serine- and metallo- proteinases exhibited broad specificities for substrates they also showed some specificity. The

stability of the 60000 D protein only in PMSF-treated extracts indicated that this protein was digested only by a serine-proteinase. The degradation of the 60000 D protein by a serine-proteinase resulted in the production of a 36000 D breakdown product that was resistent to proteolysis in the presence of EDTA. This indicated that the 36000D protein was only digested by a metalloproteinase. The two other major protein species (66000 D and 50000 D) were both relatively resistant to degradation in the presence of EDTA, but were digested quickly in untreated and PMSF-treated extracts. It was therefore most likely that these proteins were only susceptible to degradation by metalloproteinases.

The results also showed that there was an increase in the hydrolyzing activity of whole cell extracts stored for longer than 3 days in the presence of PMSF and EDTA. This increase in hydrolysis in the presence of PMSF could be due to an increase in metallo-proteinase activity. Alternatively this increase could be the result of inactivation of PMSF during storage. A report on the instability of PMSF in aqueous solutions by Gordon (1978), supports this explanation. Unlike PMSF, EDTA is stable in aqueous solutions and therefore an increase in hydrolysis in the presence of this inhibitor is most likely the result of an increase in serine-proteinase activity.

3:9 ISOLATION OF ENDOGENOUS INHIBITORS

The indirect evidence for the presence of endogenous inhibitors and for their involvement in maintaining minimum levels of protein degradation in fresh whole cell extracts of M. methylotrophus was presented in section 3:8. A more direct approach to isolating and identifying endogenous inhibitors is described below.

A non-dialyzable, heat-stable factor that inhibited hydrolysis of casein by trypsin and chymotrypsin has been identified in whole cell extracts of E. coli. Similar inhibitory factors have also been confirmed in a variety of other gram-negative bacteria (Green and Ryan, 1971; Imada et al., 1985), gram-positive bacteria (Millet, 1976), fungi (Stevens et al., 1985) and yeast (Meussdoerffer et al., 1980).

The next step therefore was to isolate and characterize putative inhibitors from the cells of M. methylotrophus. The rationale for this work was based on the observation that the majority of inhibitors from a variety of organisms share certain properties (Chung et al., 1983), (1) they are generally small proteins ($\sim$20000 D); (2) have isoelectric points between pH 4.0 and pH 5.0 and (3) are stable at low pH and high temperature.

A non-dialyzable, heat- and acid-stable factor was prepared from the whole cell extracts of M. methylotrophus as described in section 2:16. The concentrated supernatant prepared by this process was called the 'Inhibitor extract' (Ie).

3:9a (i) The effect of Ie on commercial proteinases

The inhibitory activity of Ie was assayed by measuring its effect on the rates of hydrolysis of azocasein by trypsin and

chymotrypsin.

(1) The effects of substrate concentration on inhibition by Ie

As substrates may compete with inhibitors for sites on the proteolytic enzyme, the inhibitory effect of Ie on the hydrolyzing activity of trypsin and chymotrypsin was tested over a range of azocasein concentrations, fig. 3:36. Maximum inhibition of hydrolysis by trypsin (67%) and chymotrypsin (82%) was achieved with the lowest concentration of substrate tested. The inhibitory capacity of Ie decreased as the substrate concentration was increased. At substrate concentrations above 5 mg/ml there was an apparent activation of proteinase hydrolyzing activity.

(2) The effects of Ie concentration on inhibition of azocasein hydrolysis

The minimum concentration of Ie required to cause inhibition of trypsin and chymotrypsin was determined, fig. 3:37. Maximum inhibition of both trypsin and chymotrypsin-hydrolyzing activity was achieved with 32 µg inhibitor extract (Chung et al., 1983) when the enzyme concentration was 0.01 mg/ml and the substrate concentration was 0.1 mg/ml. (The concentration of inhibitor extract was given as the protein content/ml. However the protein content was not necessarily entirely due to inhibitor protein.) The optimum inhibitor extract concentration determined in this experiment was coincidently the same as that used to determine the optimum substrate concentration.

(ii) The effect of Ie on proteolytic enzymes from M. methylotrophus

The effects of Ie on the hydrolyzing activities of whole cell

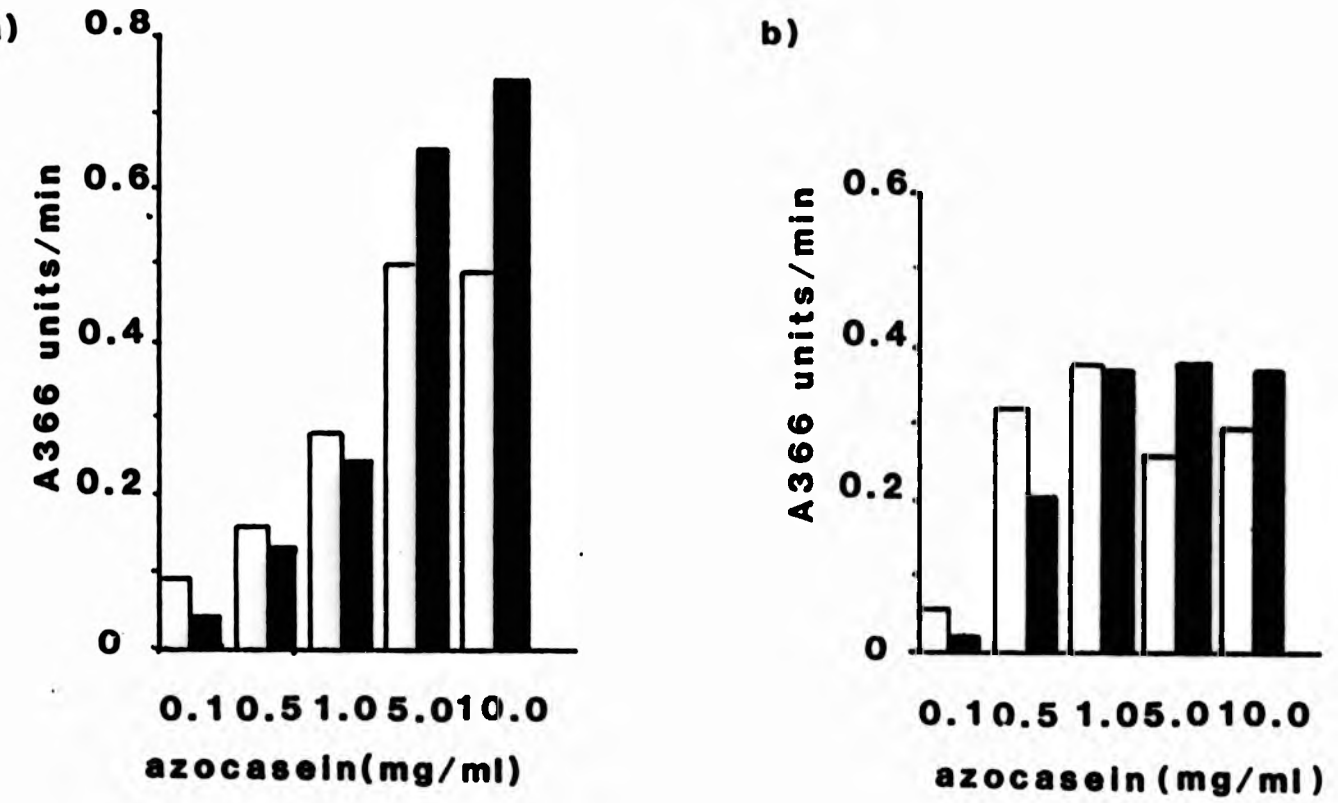


Fig. 3:36 The effects of substrate concentration on inhibition by inhibitor extract. Hydrolysis of azocasein (0.1-10 mg/ml) by (a) trypsin (0.1 mg/ml) and (b) chymotrypsin (0.01 mg/ml) in the absence (▭) and presence (▬) of 'Inhibitor extract' (0.32 mg protein/ml).

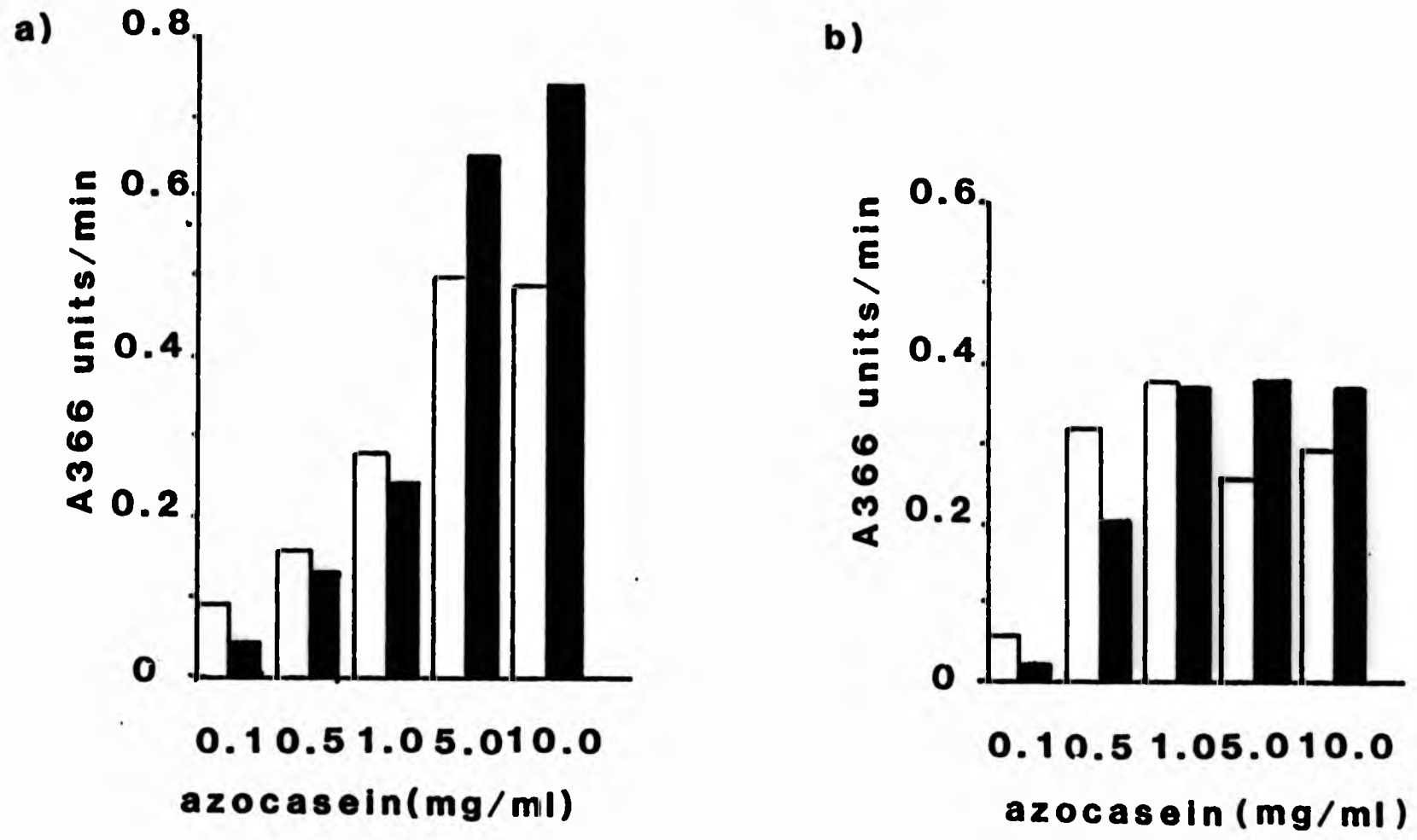


Fig. 3:36 The effects of substrate concentration on inhibition by inhibitor extract. Hydrolysis of azocasein (0.1-10 mg/ml) by (a) trypsin (0.1 mg/ml) and (b) chymotrypsin (0.01 mg/ml) in the absence (□) and presence (■) of 'Inhibitor extract' (0.32 mg protein/ml).

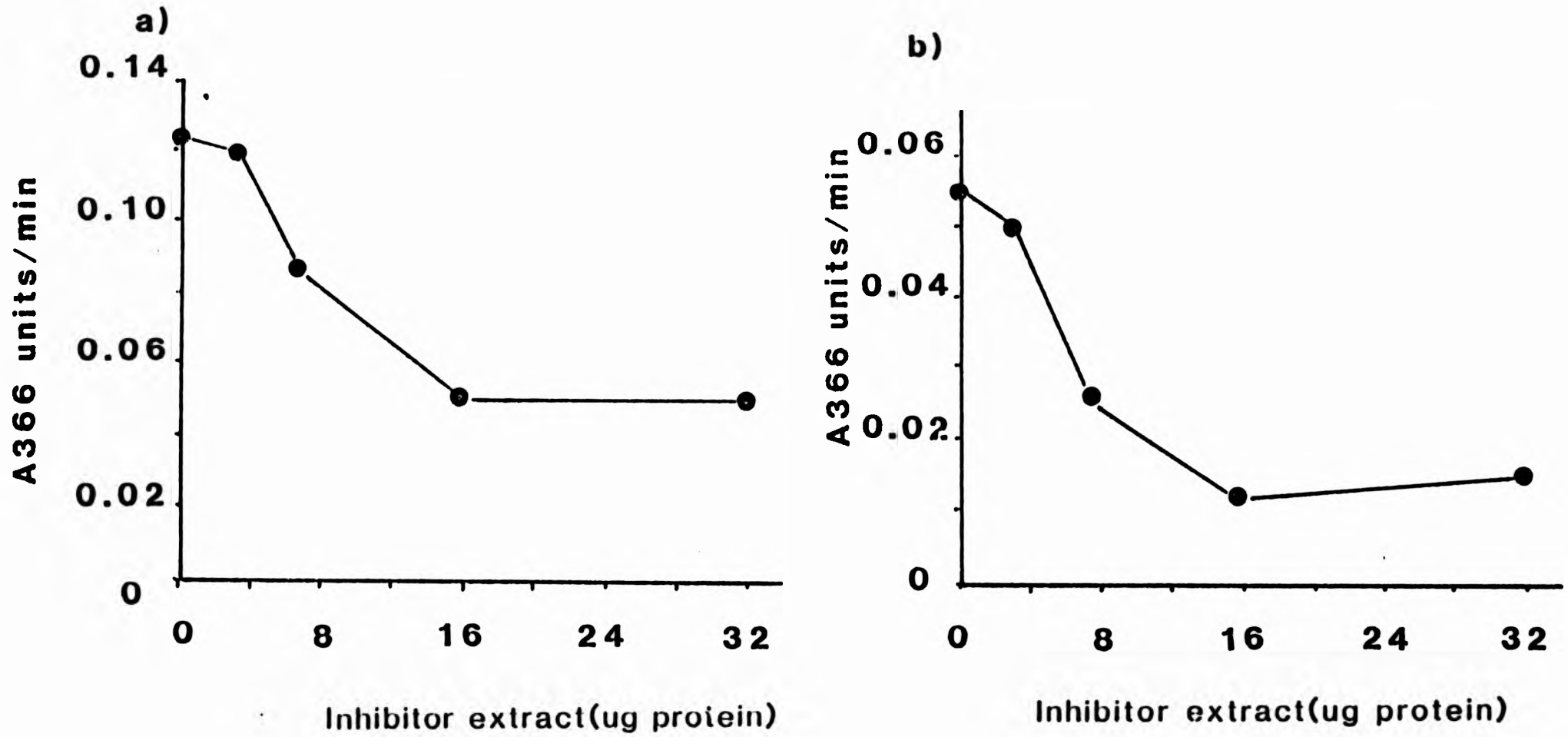


Fig. 3:37 The effects of 'Inhibitor extract' concentration on the inhibition of proteolytic activity. Azocasein (0.1 mg/ml) was hydrolyzed by (a) trypsin (0.1 mg/ml) and (b) chymotrypsin (0.1 mg/ml) in the presence of 0-32 μg of 'Inhibitor extract'.

extracts, periplasmic and cytoplasmic fractions and certain purified proteinases of M. methylotrophus were investigated, table 3:37. The hydrolysis of SAAPP and BzArgNA was assayed as described in section 2:3c. Ie showed no inhibition of the SAAPP hydrolyzing activity of the whole cell extract, it did however inhibit hydrolysis by the cytoplasmic (18%) and periplasmic (32%) fractions. The increased inhibitory activity of Ie with the subcellular fractions could have been due to the removal of endogenous inhibitors or substrates during isolation thus freeing proteolytic enzymes that could then be partly or wholly inactivated.

The inhibitory effect of Ie on a number of BzArgNA hydrolyzing enzymes isolated from whole cell extracts was tested, table 3:37. Proteinase W2A (purified 32-fold, section 3:2a) was not inhibited significantly by the inhibitor extract. Proteinase W3A (purified 30-fold) and Proteinase W3B (purified 116-fold, section 3:2a & b) were both inhibited to a small extent, 24% and 19% respectively. The most significant inhibitory effect of Ie (81%) was towards Proteinase W4A (purified 52-fold, section 3:2c).

3:9b (i) Purification of endogenous inhibitors

The evidence presented in section 3:9a indicated that endogenous inhibitors were presented in the "Inhibitor extract" isolated from M. methylotrophus whole cell extracts. Attempts were then made to isolate and characterize these inhibitors further. Three different approaches were made to purify these putative endogenous inhibitors; (1) separation according to molecular weight by gel-chromatography making use of the apparent small size of these molecules; (2) separation by affinity chromatography based on the

Table 3:37

Inhibition of the hydrolyzing activities of cellular extracts and proteinases from M. methylotrophus by Ie

Cellular extract	Inhibition of SAAPP hydrolyzing activities (%)*	Sp. Ac. (in the absence of inhibitor) (nmol/min/mg of protein)
Whole cell	0	5
Cytoplasm	18	28
Periplasm	32	46
	Inhibition of BzArgNA hydrolyzing activites (%)	
Proteinase W2A	2	162
Proteinase W3A	24	150
Proteinase W3B	19	580
Proteinase W4A	81	260

* Inhibition was calculated as the activity remaining after treatment with Ie given as a percentage of the activity.

evidence that the inhibitor extract contains inhibitors that inactivated trypsin and (3) separation according to molecular weight by SDS-polyacrylamide gel electrophoresis in the presence of gelatin.

(ii) Separation of endogenous inhibitors by gel-chromatography

Inhibitor extract (7.74 mg) was run on a Sephadex G75 column as described in section 2:5a. The inhibitor activity of the eluted fractions was assayed by measuring the rate of hydrolysis of SAAPP or BzArgNA by the commercial proteinases trypsin and chymotrypsin and the subcellular fractions isolated from M. methylotrophus cells. An inhibitor was assessed to be present in a particular eluted fraction if the rate of substrate hydrolysis by a proteinase or subcellular fraction in the presence of the eluted fraction was significantly less than in its absence. Although several protein peaks were detected in the eluted fractions of the column there was no evidence to suggest that any of these contained inhibitory activity.

(iii) Isolation of endogenous inhibitors by affinity-chromatography

In a more direct approach to isolating endogenous inhibitors Ie was run on a Trypsin-Sepharose affinity column prepared as described in section 2:5c. The inhibitory activity of eluted fractions was assayed by measuring their rate of hydrolysis of BzArgNA and $[^{14}C]$ α_1-casein by trypsin. Although a single peak of protein was eluted from the column at a NaCl concentration of 0.025M there was no evidence for inhibitor activity in any of the eluted fractions.

(iv) Identification of endogenous inhibitors by SDS-polyacrylamide gel electrophoresis in the presence of gelatin

An electrophoretic procedure for assaying proteinase inhibitors was reported by Pellegrini et al. (1984). In this method fibrinogen and agarose were incorporated into an SDS-polyacrylamide gel. Putative proteinase inhibitors from the whole cell extracts of M. methylotrophus were investigated by an adaptation of this procedure in which gelatin was incorporated into the gels in place of fibrinogen (section 2:6d).

Attempts were made to directly identify inhibitors from whole cell extracts (0.25 mg), subcellular fractions (0.25 mg) and from the inhibitor extract (Ie) (0.03 mg). In addition commercially available proteinase inhibitors, bovine trypsin inhibitor (BTI), soybean trypsin inhibitor (STI) and Ovoinhibitor (all 5 µg) were used to standardize the procedure. After electorphoresis of cellular extracts and inhibitors the gels were incubated with various commerically available proteinases: trypsin (13 µg/ml), chymotrypsin (20 µg/ml) and elastase (26 µg/ml), all in 0.2M Tris-HCl, pH 7.5, 20 mM $MgCl_2$. Theoretically these proteinases should digest away all the gelatin and proteins in the gel except where inhibitors are present. On staining with Coomassie Blue these proteins should be the only visible bands.

With the exception of BTI which showed a faint single low molecular weight band after incubation with trypsin, none of the other extracts or inhibitors produced any bands that may have indicated that endogenous inhibitors were present.

3:9c Discussion

A decrease in the inhibitory capacity of Ie with increased substrate concentration was most likely due to a preference of the proteolytic enzymes for the substrate. At lower concentrations of substrate there was less competition for the enzyme active site allowing binding of the inhibitor. As the substrate concentration was increased the active site on the enzymes became saturated with the substrate preventing binding of the inhibitor. At this stage the inhibitor may have caused an activation of the enzyme (Goldberg et al., 1985). This activation of the enzyme is believed to be due to the fact that proteins may alter or activate a proteinase towards a particular substrate without being degraded itself therefore effectively increasing the levels of substrate hydrolysis. Since Ie was an extract that contained a number of proteins other than inhibitor proteins it was possible also that it was these proteins that resulted in the increased rates of hydrolysis at the higher substrate concentrations.

Although there was evidence for inhibitors of M. methylotrophus being capable of inactivating trypsin and chymotrypsin and endogenous proteolytic enzymes, attempts to purify these inhibitors were unsuccessful. The possible problems associated with the techniques used to isolate inhibitors from the inhibitor extract were as follows; (1) the assay procedures for detecting inhibitors may not have been sensitive or specific enough to detect individual inhibitors separated by column chromatography; (2) a Trypsin-Sepharose column may not have been specific enough for the inhibitors of M. methylotrophus. Alternatively the method employed to bind the inhibitor to the column or elute it from the column may

not have been the most suitable and (3) the concentration of an individual inhibitor may have been too low to detect by SDS-polyacrylamde gel electrophoresis. Alternatively the proteinase used to digest gelatin may not have been specific enough for the inhibitors of M. methylotrophus or the conditions for digestion may have not been appropriate.

Although all the techniques described above have been used successfully in the isolation and purification of inhibitors by other researchers, the conditions of isolation used for each and every type of inhibitor may be as individual as the inhibitors themselves and therefore their isolation may be more fortuitous than rational.

3:10 THE HEAT-SHOCK PHENOMENON IN M. METHYLOTROPHUS

The phenomenon now known as heat-shock was first described about 25 years ago during a study on the temperature effects on the salivary gland chromosomes of the fruitfly, Drosophila busokii (Ritossa, 1962). It was observed that there was a change in the puffing pattern of polytene chromosomes, and this was later shown to be the result of very active gene transcription that led to the synthesis of a small set of proteins (Schlesinger, 1986).

It was not until 1973 that Alfred Tissiéres, using SDS-slab gel electrophoresis to analyze radiolabelled proteins from heat-shocked Drosophila salivary glands, demonstrated that heat-shock induced the synthesis of a small number of polypeptides and at the same time repressed the synthesis of most other cellular proteins (Schlesinger et al., 1982). For almost 15 years this selective induction of proteins was thought to be unique to the fruit fly. In 1978 however an analagous response was detected in avian and mammaliam tissue culture as well as in E. coli (Lemeaux et al., 1978; Yamamori et al., 1978) and Tetrahymena cells (Fink and Zeuther, 1978). We now recognise that virtually all organisms from bacteria to man have a heat-shock response and furthermore these shock proteins have been strongly conserved in structure throughout evolution (Schlesinger et al., 1982; Pelham, 1985), clearly indicating that they play a key role in the survival of the organism (Neidhardt et al., 1984; Pelham, 1985; Lindquist, 1986;).

The so called heat-shock proteins (hsps) have also been shown to be induced by other changes in environmental conditions suggesting that activation of hsp genes is part of a more general stress response phenomenon (Pelham, 1985; Lindquist, 1986). This

response is now more accurately termed 'stress-shock'.

The stress-shock phenomenon has now been extensively studied in many organisms (Neidhardt et al., 1984; Lindquist, 1986). The best studied bacterial system is that of the gram-negative bacterium E. coli. At least 17 proteins have been shown to be induced in E. coli after stress-shock treatment (Yamamori et al., 1978; Schlesinger et al., 1982; Neidhardt et al., 1984). Of particular interest was the discovery that one of these hsps was a proteolytic enzyme (Goff and Goldberg, 1985). Furthermore E. coli mutants with an altered heat-shock response have been shown to have a reduced turnover of aberrant polypeptides (Carr et al., 1985; Ho et al., 1986). Proteolytic enzymes or proteins directly involved with proteolysis have been demonstrated to be induced in other organisms after stress-shock treatment (Bond and Schlesinger, 1985; Gross and Schulz-Harder, 1986).

As part of a study of the control of proteolysis and in particular the factors that might affect the stability of foreign gene products synthesized in M. methylotrophus the effects of stress-shock in this bacterium were investigated.

3:10a The effect of heat-shock on cell growth

The effects of heat-shock on the growth of M. methylotrophus cells were investigated, fig. 3:38.

Within the first 15 minutes of heat-shock there were no apparent differences between the rates of growth of cells kept at 30°C (non-shocked cells) and those shifted to 40°C (heat-shocked cells). Within 120 minutes cells shifted to 40°C had increased in turbidity by 0.3 A650 units compared to 0.25 A650 units for cells

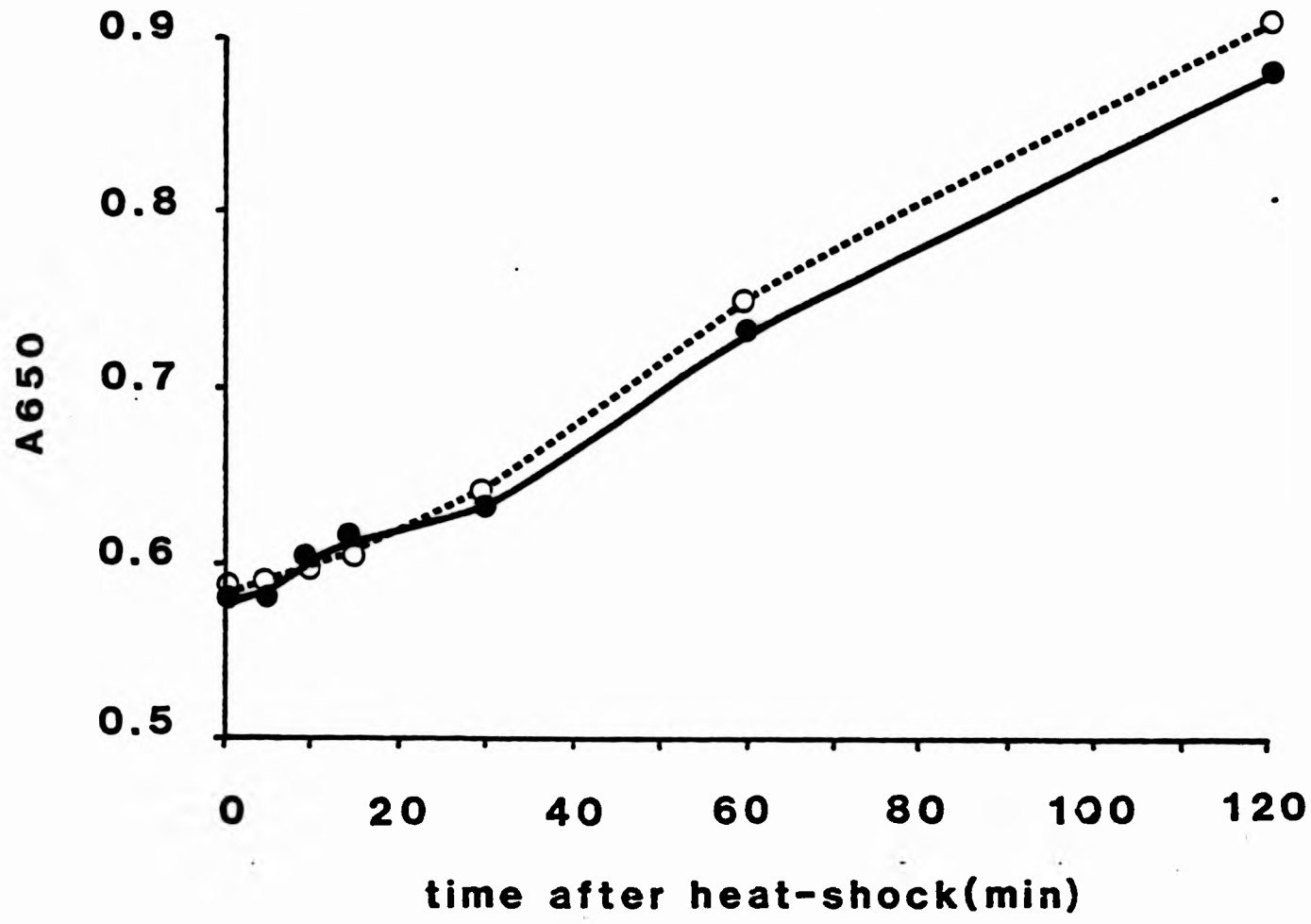


Fig. 3:38 The effect of heat-shock on the growth of M. methylotrophus ASI cells. Cells grown at 30°C to an A_{650} of 0.58 were either kept at 30°C (●——●) or shifted to 40°C (O····O) for 120 minutes.

maintained at 30°C.

3:10b The effect of heat-shock on total protein content of cells

The effects of heat-shock on the total protein content of M. methylotrophus cells were investigated, plate 3:12. The results showed that there were no obvious differences between the total protein content of non-shocked and heat-shocked cells when analyzed by this procedure. This result suggested that either (1) no heat-shock phenomenon occured in M. methylotrophus cells or (2) this method was not sensitive enough to detect minor changes in protein. Since the heat-shock phenomenon has been reported to be ubiquitous (Schlesinger et al., 1982), and since heat-shock proteins are generally induced within the first 30 minutes after heat-shocking, it was unlikely that this phenomenon was absent from this bacteria.

3:10c The effect of heat-shock on the synthesis of proteins

The sensitivity for detecting newly synthesized proteins after heat-shock was increased by the incorporation of [^{35}S]-methionine ([^{35}S]-met) into growing cells. M. methylotrophus cells grown to mid-log phase at 30°C were labelled with [^{35}S]-met and immediately split into two.

One half was kept at 30°C while the other was shifted to 40°C. Total protein was extracted from both cultures after 0 and 30 minutes and run on an SDS-polyacrylamide gel. An autoradiogram to the gel was prepared plate 3:13 (section 2:7). (Hsps were indicated with closed arrow-heads). A shift to 40°C was accompanied by a dramatic reduction in the overall rate of synthesis of cellular proteins . In addition there was the production of a

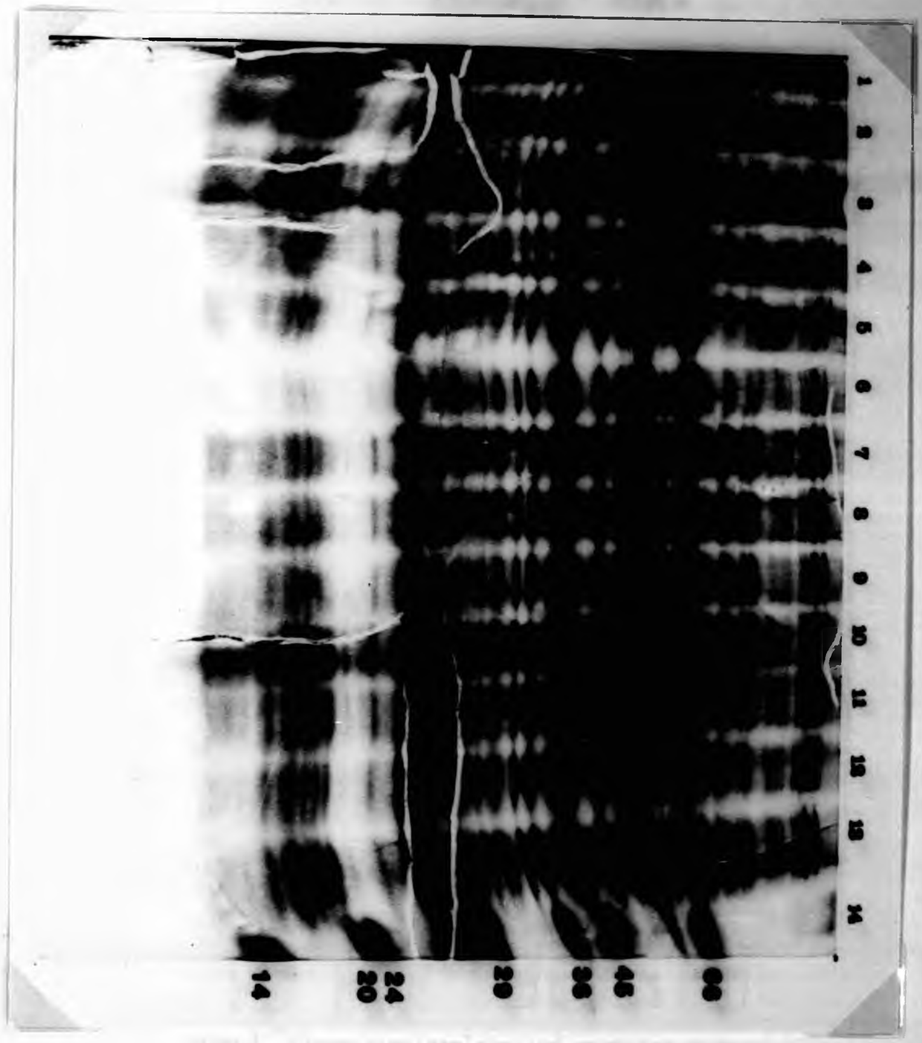
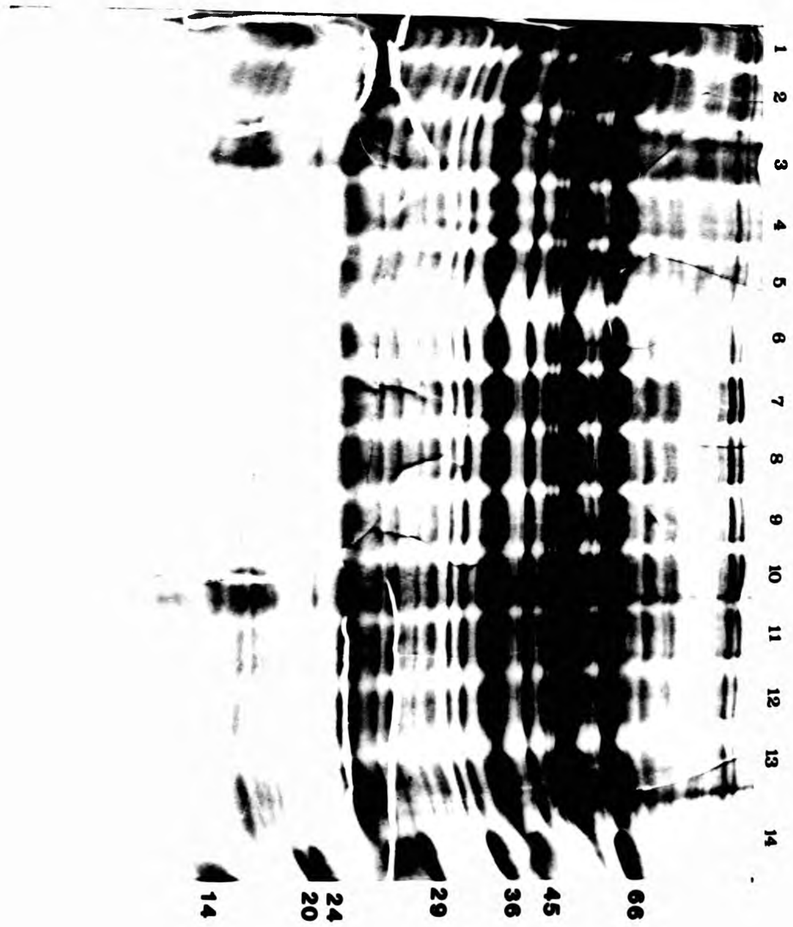


Plate 3:12 The effects of heat-shock on the total protein content of cells. Total protein from cells grown at 30°C for 0, 1, 5, 15, 30, 60 and 120 minutes (lanes 1-7 respectively) and heat-shocked cells grown for 1, 5, 15, 30, 60 and 120 minutes (lanes 8-13 respectively). Total protein was extracted as described in section 2:17. The amount of protein loaded was related to the A650 of the cells. Lane 14, molecular weight markers from top to bottom, bovine albumin (66000 D), egg albumin (45000 D), glyceraldehyde-3-phosphate dehydrogenase (36000 D), carbonic anhydrase (29000 D), trypsinogen (24000 D), soybean trypsin inhibitor (20000 D) and ∝ -lactalbumin (142000 D).

Plate 3:12 The effects of heat-shock on the total protein content of cells. Total protein from cells grown at 30°C for 0, 1, 5, 15, 30, 60 and 120 minutes (lanes 1-7 respectively) and heat-shocked cells grown for 1, 5, 15, 30, 60 and 120 minutes (lanes 8-13 respectively). Total protein was extracted as described in section 2:17. The amount of protein loaded was related to the A650 of the cells. Lane 14, molecular weight markers from top to bottom, bovine albumin (66000 D), egg albumin (45000 D), glyceraldehyde-3-phosphate dehydrogenase (36000 D), carbonic anhydrase (29000 D), trypsinogen (24000 D), soybean trypsin inhibitor (20000 D) and α-lactalbumin (142000 D).



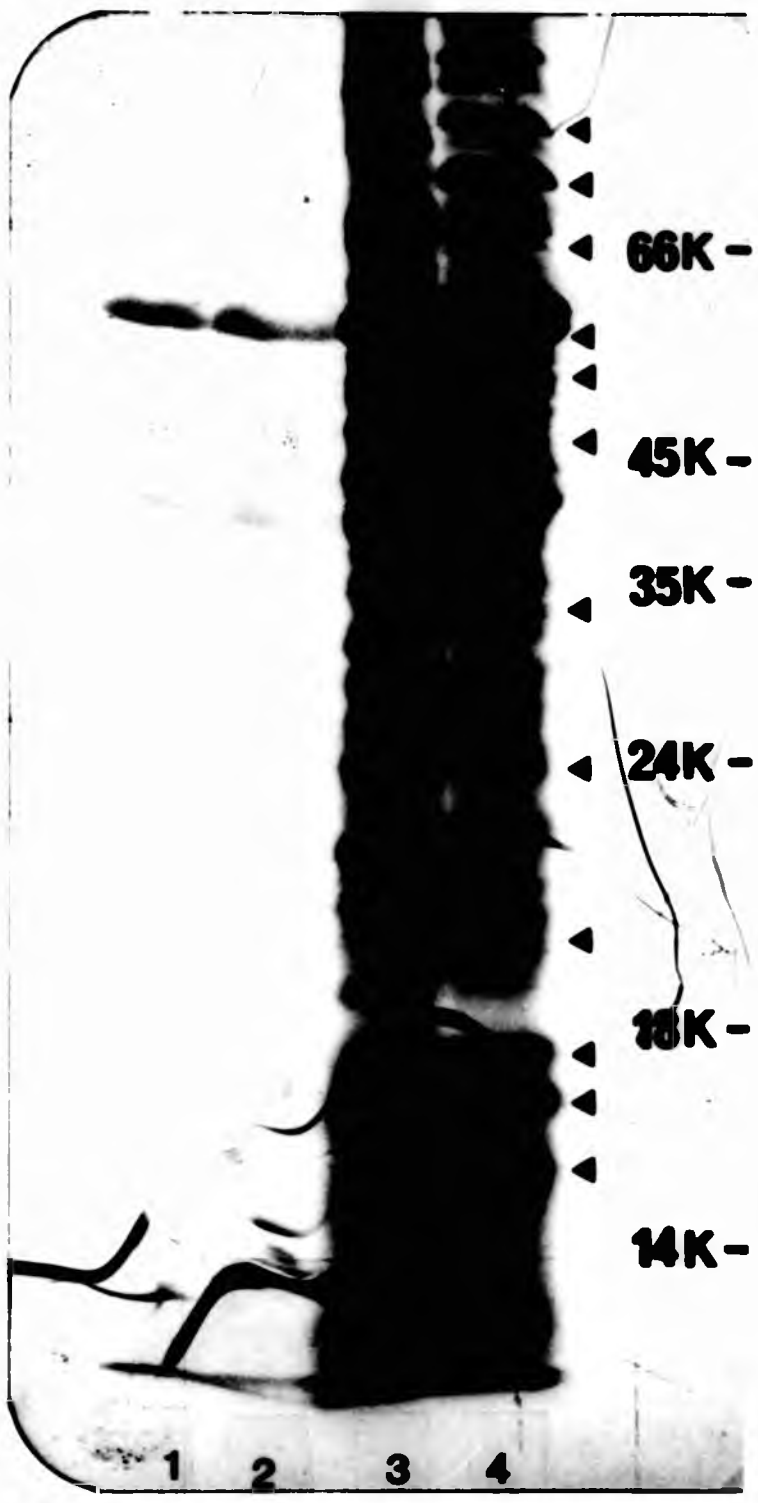


Plate 3:13 [^{35}S]-methionine incorporation into proteins in whole cell extracts of heat-shocked cells. Samples were prepared after 0 and 30 minutes treatment as described in section 2:17. Lanes 1 and 3 show [^{35}S]-methionine-labelled proteins from cells grown at 30^{o}C for 0 and 30 minutes respectively. Lanes 2 and 4 show [^{35}S]-methionine-labelled proteins from heat-shocked cells after 0 and 30 minutes respectively. The position of protein standards is indicated (Mr in daltons) on the right of the plate. The heat-shock proteins are arrowed.

certain number of novel proteins not observed in cells grown at 30°C. These novel proteins have been called 'heat-shock proteins' (hsps) after similar proteins observed in other species. Twelve hsps were identified in M. methylotrophus heat-shocked cells. Their apparent molecular weights and nomenclature are shown in table 3:38.

In addition to shocking cells by a temperature shift of 30-40°C, the effects of growing cells at 40°C and a temperature shift of 40-30°C were investigated (section 2:17). Total protein was extracted from the cells and run on an SDS-polyacrylamide gel. An autoradiogram towards the gel was prepared, plate 3:14. The principle of this experiment was to determine whether proteins induced during a shift-up in temperature were the consequence of the actual shift in temperature or the result of cells growing at elevated temperatures.

Although the quality of the autoradiogram was poor this experiment still served to demonstrate that cells grown and maintained at 40°C or shifted down from 40-30°C did not induce hsps observed after a 30-40°C shift. In fact there did not appear to be any difference in the proteins synthesized at 30°C, 40°C or after a 40-30°C shift down. A more detailed account of the synthesis of stress-shock proteins with time is given in section 3:10(g).

3:10d The effects of other shocking agents on cell growth

In addition to heat-shock the effects of a 30 minute treatment with either methanol, ethanol or puromycin on cell growth were investigated (section 2:17) (fig. 3:39). Both methanol and ethanol reduced the rate of cell growth during the 30 minute period after

Table 3:38

Apparent molecular weights and nomenclature of M. methylotrophus heat-shock proteins

Heat-Shock protein	Apparent mol. wt (D)
Hsp 83	83000
Hsp 78	78000
Hsp 66	66000
Hsp 60	60000
Hsp 56	56000
Hsp 48	48000
Hsp 33	33000
Hsp 27	27000
Hsp 20	20000
Hsp 17	17000
Hsp 16	16000
Hsp 14	14000

Plate 3:14 [^{35}S]-methionine incorporation into proteins in whole cell extracts after various temperature-shifts. Samples were prepared after 0, 5, 15 and 30 minutes treatment as described in section 2:17. Lanes 1-4, [^{35}S]-methionine labelled cells kept at 30°C; lanes 5-8 cells grown at 30°C and shifted to 40°C; lanes 9-12, cells grown at 40°C and lanes 13-16, cells grown at 40°C and shifted down to 30°C. The position of protein standards is indicated (Mr in daltons) on the left of the plate. The heat-shock proteins are arrowed.

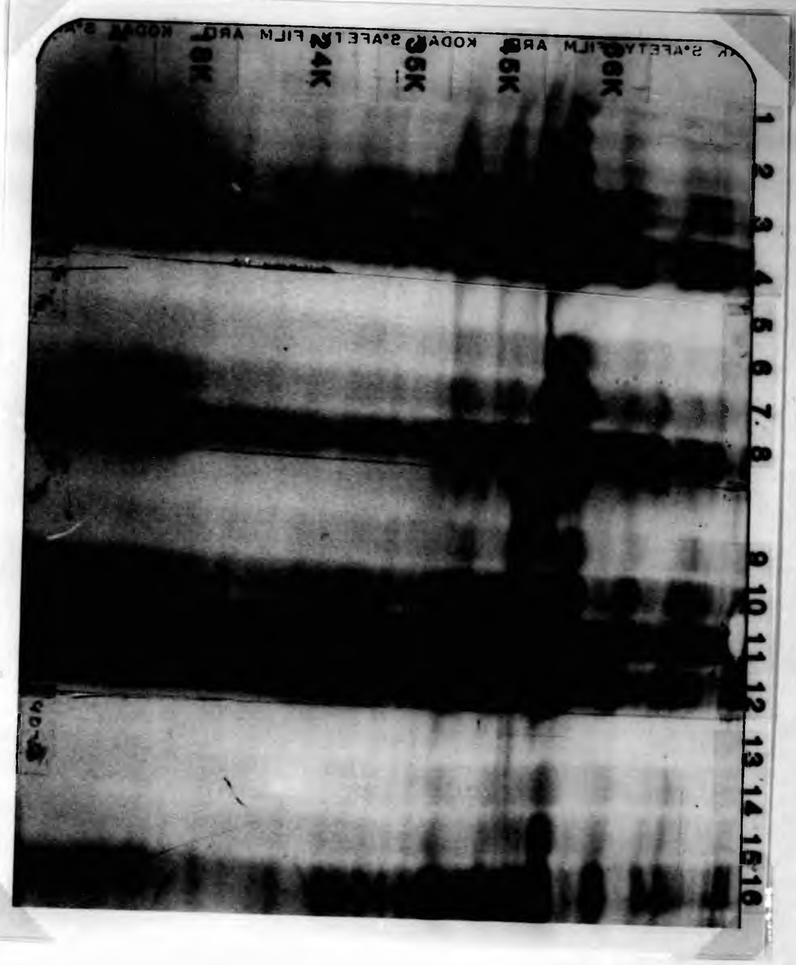
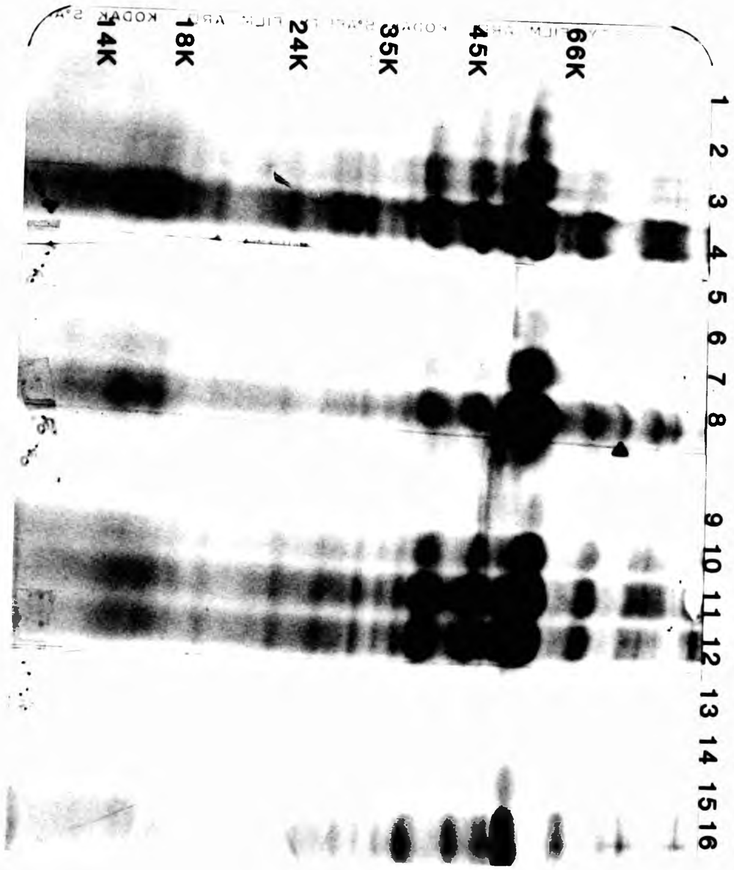


Plate 3:14 $[^{35}S]$-methionine incorporation into proteins in whole cell extracts after various temperature-shifts. Samples were prepared after 0, 5, 15 and 30 minutes treatment as described in section 2:17. Lanes 1-4, $[^{35}S]$-methionine labelled cells kept at 30°C; lanes 5-8 cells grown at 30°C and shifted to 40°C; lanes 9-12, cells grown at 40°C and lanes 13-16, cells grown at 40°C and shifted down to 30°C. The position of protein standards is indicated (Mr in daltons) on the left of the plate. The heat-shock proteins are arrowed.



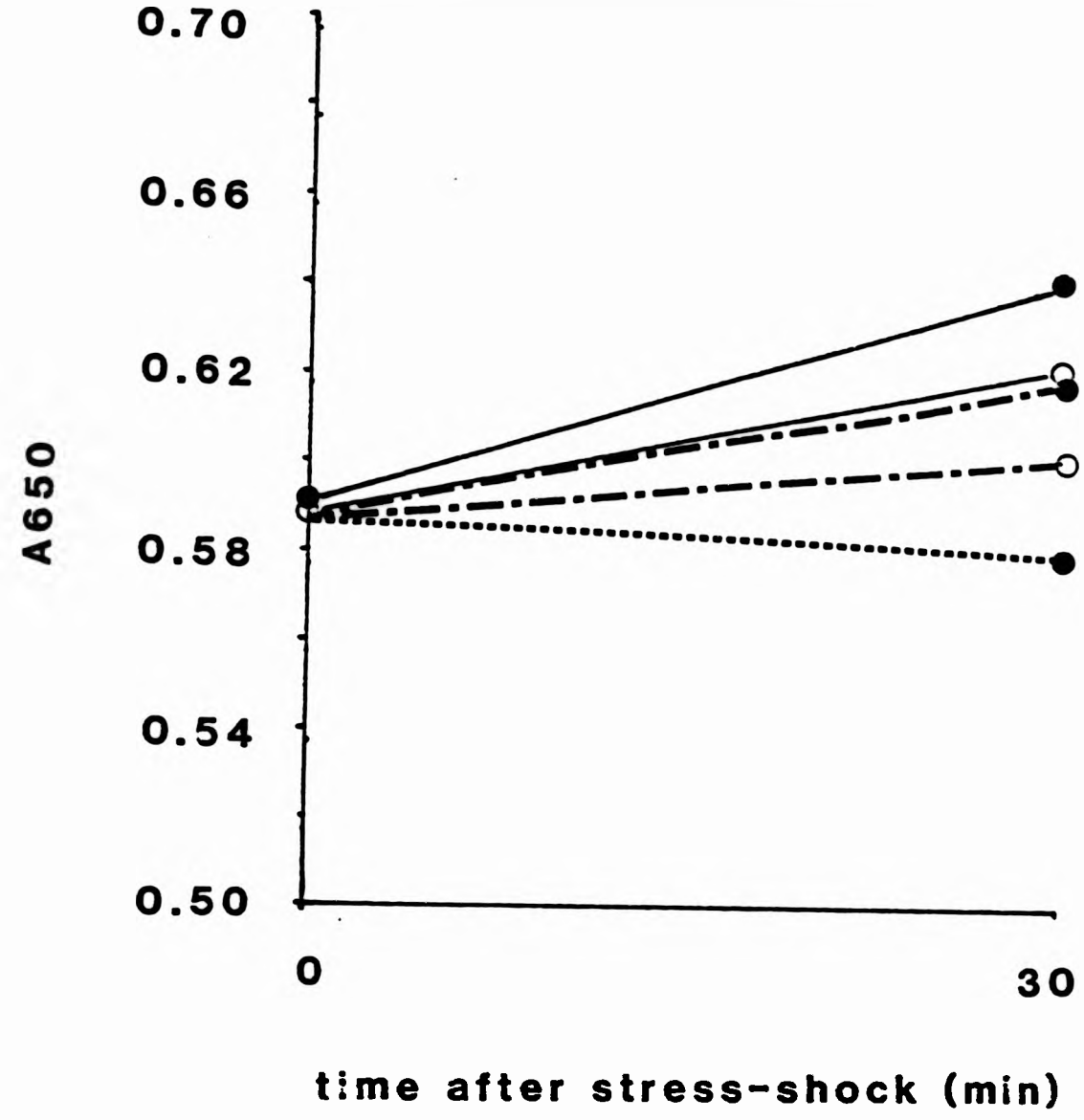


Fig. 3:39 The effect of stress-shock on the growth of M. methylotrophus ASI cells. Cells grown at 30°C to an A_{650} of 0.59 were kept at 30°C (O—O); shifted to 40°C (●—●); treated with methanol (5% v/v) (O-··O); treated with ethanol (5% v/v) (●····●) or treated with puromycin (100 µg/ml) (●—●).

stress-shocking (Chu and Papoutsakis, 1987). (The term stress-shock refers to the effect caused by all agents, including heat, that result in the induction of hsps comparable to heat-shock). The addition of puromycin to cells growing at 30°C did not appear to affect the rate of cell growth over the 30 minute period tested.

3:10e The effect of other stress-shock treatments on protein synthesis - a comparison with heat-shock treatment

M. methylotrophus AS1 cells grown to mid-log phase at 30°C were labelled with [^{35}S]-met and split into 5 separate cultures. One culture was kept at 30°C, another was shifted to 40°C and the other 3 cultures were made either, 5% with methanol, 5% with ethanol or 100 μg/ml with puromycin. Total protein was extracted from all cultures after 30 minutes and run on an SDS-polyacrylamde gel. An autoradiogram to the gel was prepared as described previously, plate 3:15.

Hsps 83, 78, 66, 60, 48, 27, 20, 16 and 14 were observed after heat-shocking. The apparent absence of Hsp 56, 33 and 17 when compared to plate 3:13 was likely due to a difference in the times of exposure of the autoradiograms. Treatment of cells with methanol, or ethanol appeared to have a comparable effect to that for heat-shocked cells, with the production of a novel set of proteins within 30 minutes of the stress-shock. It was notable however that although certain of the hsps produced by heat-shock were also induced by methanol, namely Hsps 83, 78, 63, 27, 20 and 14 and ethanol, namely Hsps 78 and 20, certain of the hsps were not present in the alcohol-treated samples. In additon, methanol induced three hsps, Hsp 94, 36 and 29 not observed in the heat-shock

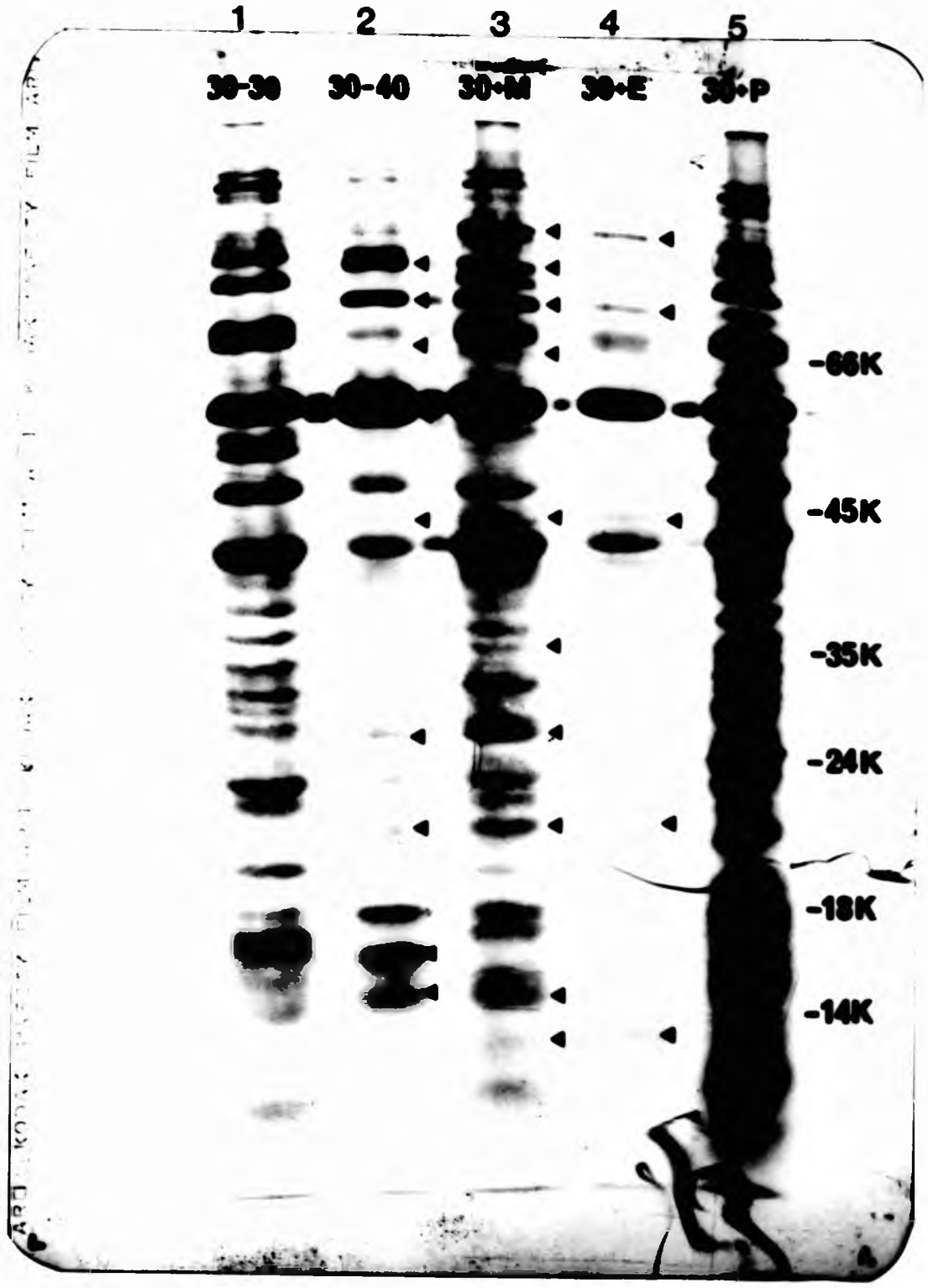


Plate 3:15 [^{35}S]-methionine incorporation into proteins in whole cell extracts of stress-shocked cells. Samples were prepared after 30 minutes treatment as described in section 2:17. The cells were kept untreated at 30°C, lane 1; shifted to 40°C, lane 2; treated with methanol (5% v/v), lane 3; treated with ethanol (5% v/v), lane 4; and treated with puromycin (100 µg/ml), lane 5. The position of protein standards is indicated (Mr in daltons) on the right of the plate. The stress-shock proteins are arrowed.

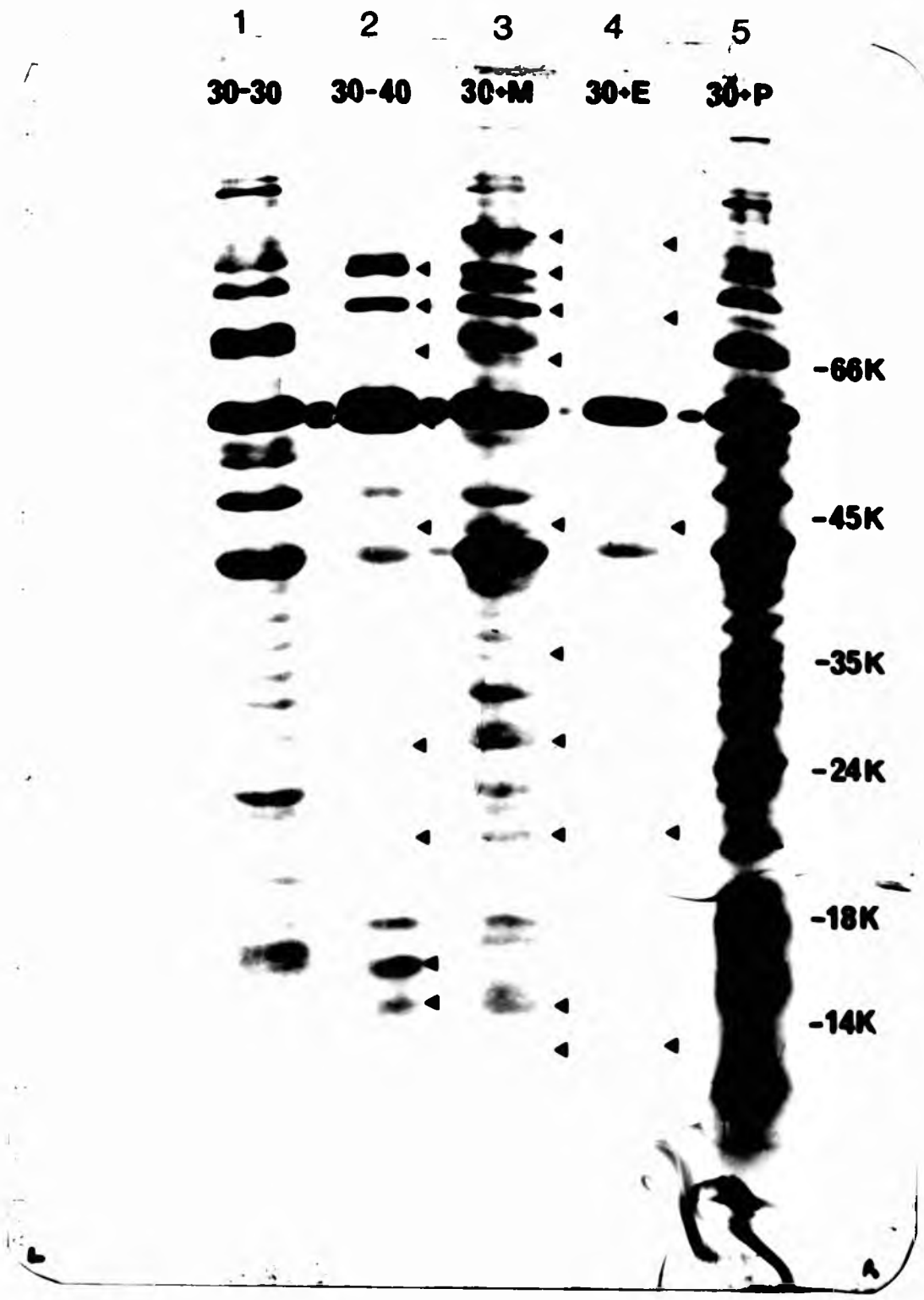


Plate 3:15 [^{35}S]-methionine incorporation into proteins in whole cell extracts of stress-shocked cells. Samples were prepared after 30 minutes treatment as described in section 2:17. The cells were kept untreated at 30°C, lane 1; shifted to 40°C, lane 2; treated with methanol (5% v/v), lane 3; treated with ethanol (5% v/v), lane 4; and treated with puromycin (100 µg/ml), lane 5. The position of protein standards is indicated (Mr in daltons) on the right of the plate. The stress-shock proteins are arrowed.

experiment. A similar effect was apparent when ethanol was used in this case Hsp 94 and 36 were induced. An additional hsp, Hsp 13 was observed only with ethanol-shocked cells. It was of some interest that both Hsp 94 and 36 were unique to the alcohol stress-shock. The effects caused by treatment with puromycin were less apparent. The overall protein profile, although higher in concentration, did not appear to be dissimilar to that of non-shocked cells. The previously observed relatively poor uptake of other materials, such as amino-acids by this bacteria (M. J. Worsey, personal communication) might account for the apparent lack of the stress-shock phenomenon when puromycin was used. If this was the case one might expect both a reduction in cell growth and the production of hsps over longer periods of incubation. This possibility has not been investigated further.

3:10f Time course for the synthesis of stress-shock proteins

The induction of hsps in M. methylotrophus cells described in sections 3:10c and e was determined after 30 min of stress-shock. This is a long period of shock-treatment compared with systems in which the induction of hsps has been reported to occur within 5 min.

The early effects of stress-shock on the production of hsps in M. methylotrophus cells were investigated. Total protein extracted from stress-shocked cells was run on an SDS-polyacrylamide gel. An autoradiogram was prepared towards the gel as described previously, plate 3:16.

The majority of stress-shock proteins were synthesized within 15 minutes of stress-shocking. Hsp 78 was synthesized as early as 5 minutes under all stress-shock conditions. In addition an hsp of

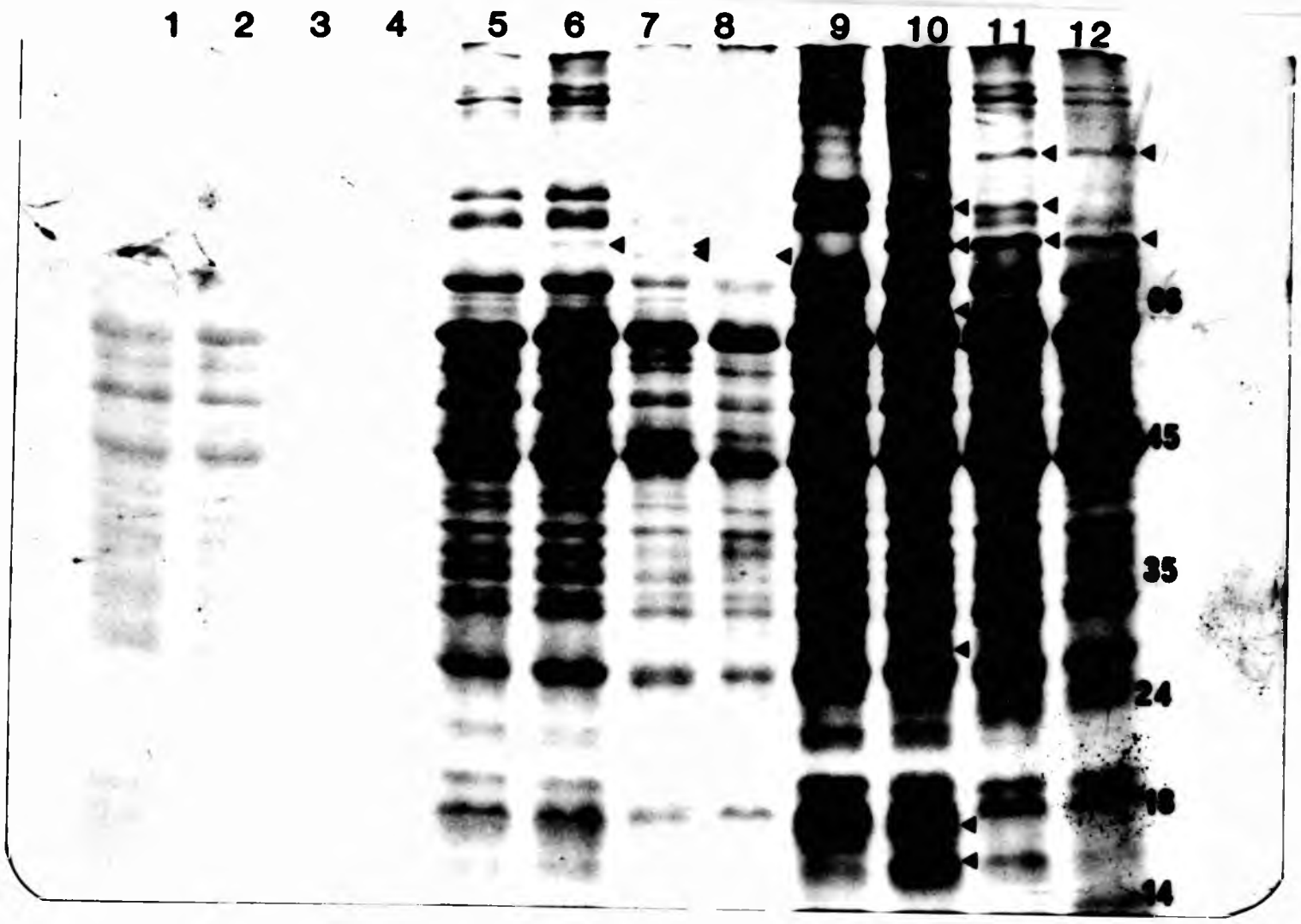


Plate 3:16 Time course of [^{35}S]-methionine incorporation into proteins in whole cell extracts of stress-shocked cells. Samples were taken after treatment and labelling for 1 minute (lanes 1-4), 5 minutes (lanes 5-8) or 15 minutes (lanes 9-12). The cells were kept untreated at 30°C (lanes 1, 5 and 9), shifted to 40°C (lanes 2, 6 and 10), treated with methanol (lanes 3, 7 and 11) or treated with ethanol (lanes 4, 8 and 12). The position of protein standards is indicated (Mr in kdaltons) on the right of the plates. The stress-shock proteins are arrowed.

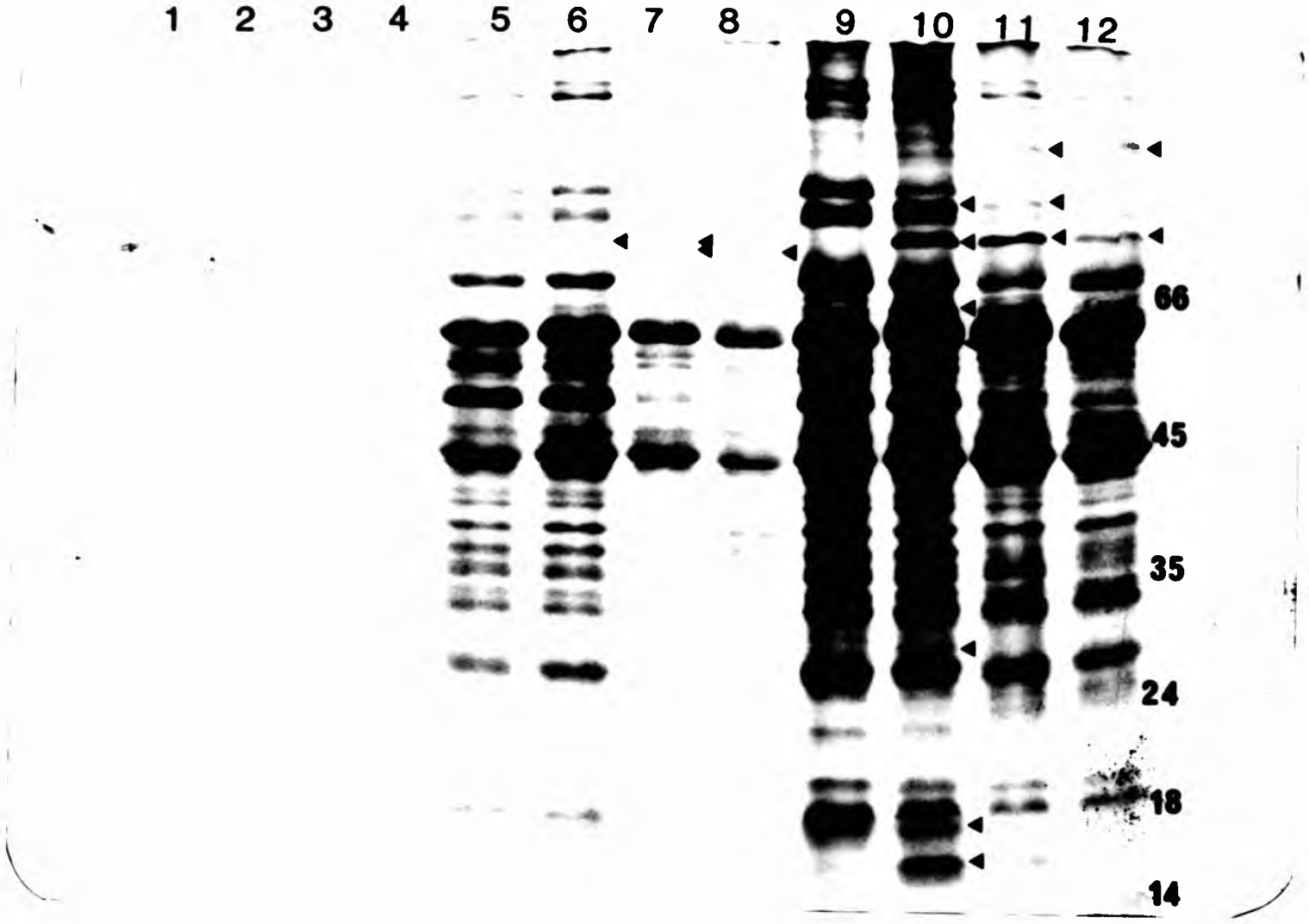


Plate 3:16 Time course of $\{^{35}S\}$-methionine incorporation into proteins in whole cell extracts of stress-shocked cells. Samples were taken after treatment and labelling for 1 minute (lanes 1-4), 5 minutes (lanes 5-8) or 15 minutes (lanes 9-12). The cells were kept untreated at 30°C (lanes 1, 5 and 9), shifted to 40°C (lanes 2, 6 and 10), treated with methanol (lanes 3, 7 and 11) or treated with ethanol (lanes 4, 8 and 12). The position of protein standards is indicated (Mr in kdaltons) on the right of the plates. The stress-shock proteins are arrowed.

apparent molecular weight 77000 was also detected after 5 min in methanol- and ethanol-shocked extracts. This previously unobserved hsp was not synthesized after 5 minutes and could not be detected at 15 minutes.

3:10g The stability of stress-shock proteins

Since newly synthesized proteins were induced in response to a particular physiological change, attempts were made to establish the effects of removing these stress-conditions. Heat-shock treatment was the most convenient method for following the turnover of hsps.

Cells that had been heat-shocked for 30 min by a 30-40°C temperature shift (section 2:17) were shifted back down to 30°C for a further 30 minutes. Total protein was extracted from the cells and examined by SDS-polyacrylamide gel electrophoresis and autoradiography, plate 3:17.

The results showed that hsps induced by heat-shock treatment were stable within the cell for at least 30 minutes. There was not therefore a rapid turnover of these proteins.

3:10h The effect of two simultaneous stress-shocks on protein synthesis

The induction of proteins that are unique to a particular stress condition, (section 3:10e) challenges previous concepts of a single modulator controlling the stress-shock response (Pelham, 1985; Lanks, 1986). If a single effector were responsible for this response and differences in protein profiles were therefore a secondary effect of the particular stress-condition, due to induction or repression of certain proteins, then one would expect

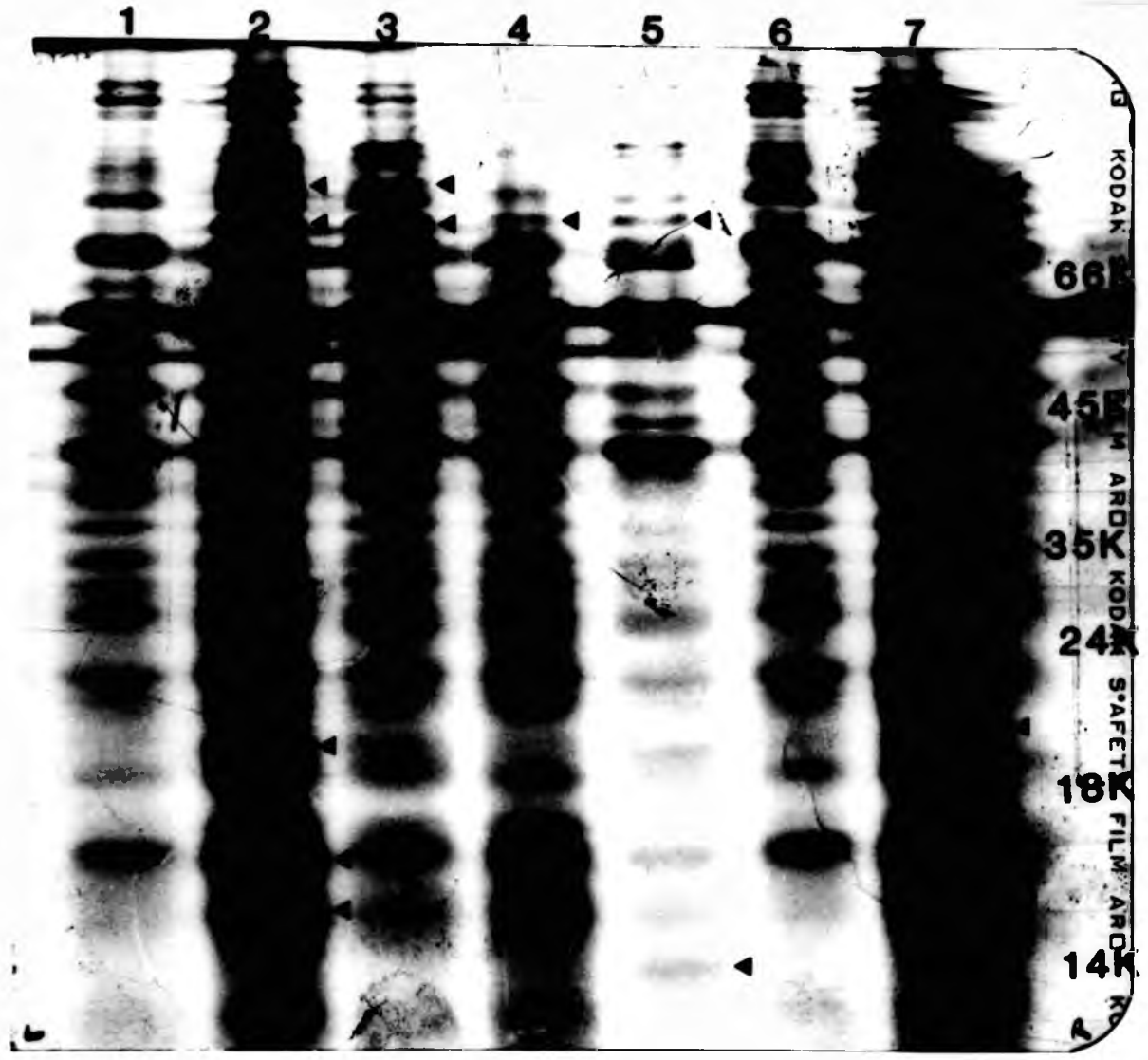


Plate 3:17 The stability of [^{35}S]-methionine-labelled stress-shock proteins after removal of stress conditions. Samples were prepared after 30 minutes treatment as described in section 2:17. The cells were kept untreated at 30°C, lane 1; shifted to 40°C, lane 2; treated with methanol (5% v/v), lane 3; treated with ethanol (5% v/v), lane 4; treated with puromycin (100 μg/ml), lane 5; cells grown at 30°C kept at this temperature for a further 30 minutes, lane 6; cells shifted to 40°C shifted back down to 30°C for a further 30 minutes, lane 7. The position of protein standards is indicated (Mr in daltons) on the right of the plate. The stress-shock proteins are arrowed.

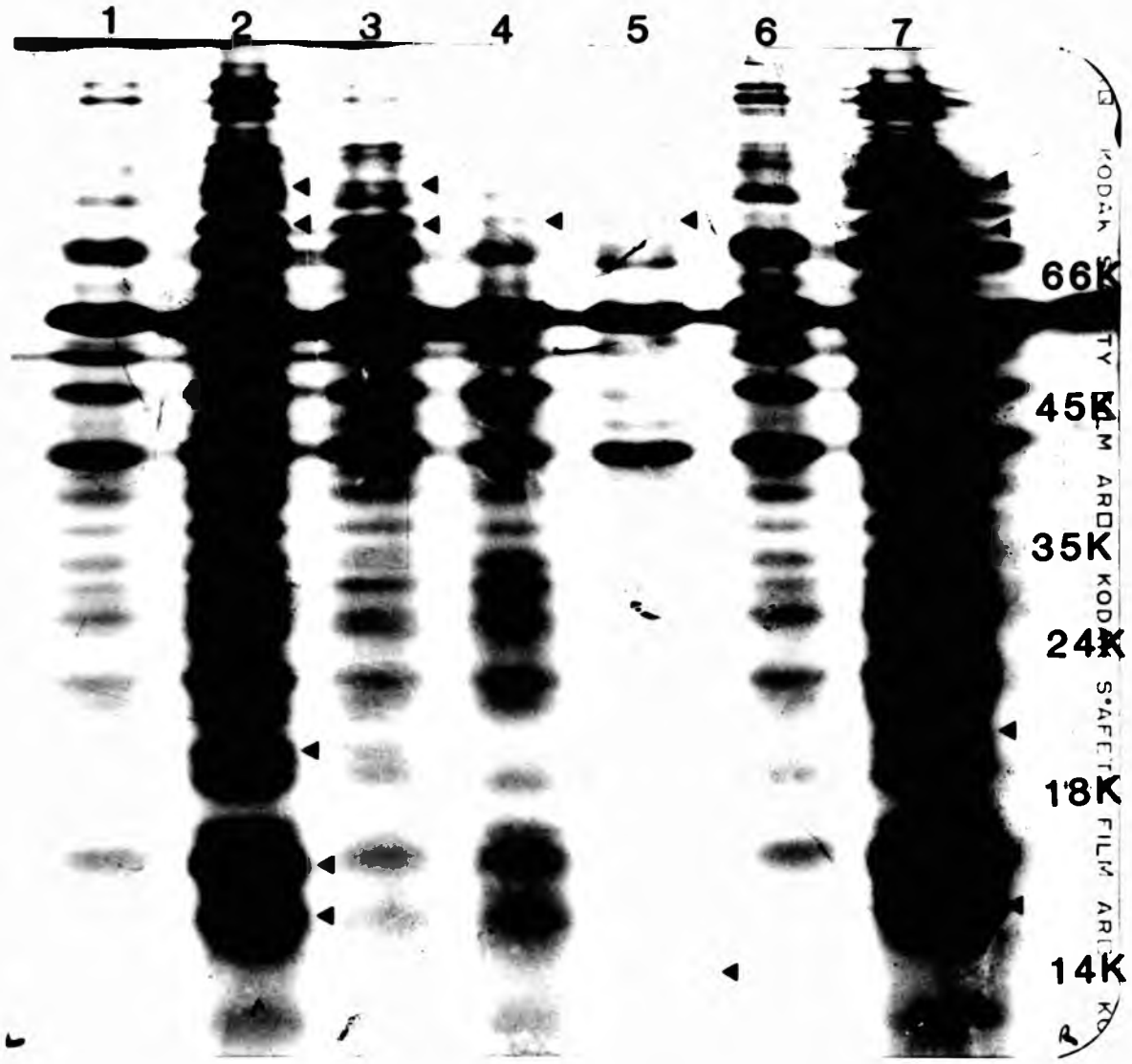


Plate 3:17 The stability of $[^{35}S]$-methionine-labelled stress-shock proteins after removal of stress conditions. Samples were prepared after 30 minutes treatment as described in section 2:17. The cells were kept untreated at 30°C, lane 1; shifted to 40°C, lane 2; treated with methanol (5% v/v), lane 3; treated with ethanol (5% v/v), lane 4; treated with puromycin (100 μg/ml), lane 5; cells grown at 30°C kept at this temperature for a further 30 minutes, lane 6; cells shifted to 40°C shifted back down to 30°C for a further 30 minutes, lane 7. The position of protein standards is indicated (Mr in daltons) on the right of the plate. The stress-shock proteins are arrowed.

that a simultaneous shock by two stress-conditions would result in the repression of one or other set of stress-shock proteins.

To investigate this possibility heat-shocked cultures were simultaneously shocked with either methanol or ethanol while methanol-shocked cultures were simultaneously shocked with ethanol, plate 3:18.

From the results it was apparent that both sets of hsps were induced after the mixed shocked treatment and there was no repression of the synthesis of any of the hsps induced by separate treatments.

3:10i Subcellular location of stress-shock proteins

To analyze the stress-shock response further, the pattern of induced proteins in the subcellular fractions of M. methylotrophus was determined. Subcellular fractions were isolated from [^{35}S]-met labelled stress-shocked cells as described in section 2:2b. Total protein was extracted from the fractions and run on an SDS-polyacrylamide gel. An autoradiogram towards the gel was prepared, plate 3:19.

The location of the proteins differed significantly, although the nature of the stressing agent had no effect on the distribution of individual proteins. The 94 kD protein induced by methanol and the 20 kD hsp were detected only in the membrane fraction. Some of the 83 kD hsp was associated with the cytoplasmic fraction, but it too was a major protein in the membrane fraction. The 78 kD hsp was detected in all three fractions, but there was significantly less in the cytoplasmic than in the other two fractions. Because of the presence of a major consitutive protein of similar molecular weight

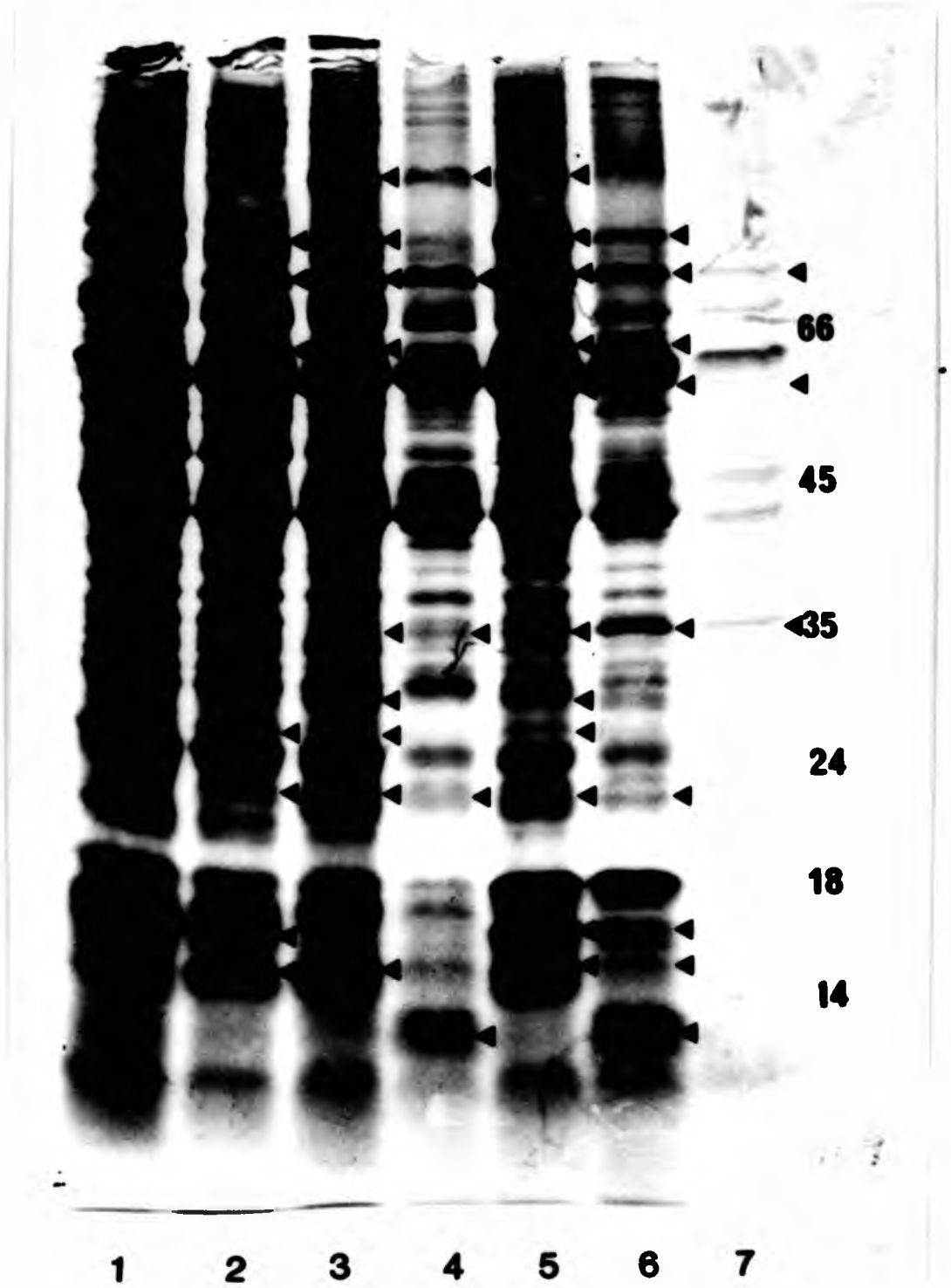


Plate 3:18 $[^{35}S]$-methionine incorporation into proteins in whole cell extracts after two simultaneous stress-shock treatments. Samples were prepared after 30 minutes treatment as described in section 2:17. The cells were kept untreated at 30°C, lane 1; shifted to 40°C, lane 2; treated with methanol (5% v/v), lane 3; treated with ethanol (5% v/v), lane 4; shifted to 40°C and treated with methanol, lane 5; shifted to 40°C and treated with ethanol, lane 6 or treated with methanol and ethanol (both 5% v/v), lane 7. The position of protein standards is indicated (Mr in kdaltons) on the right of the plate. The stress-shock proteins are arrowed.

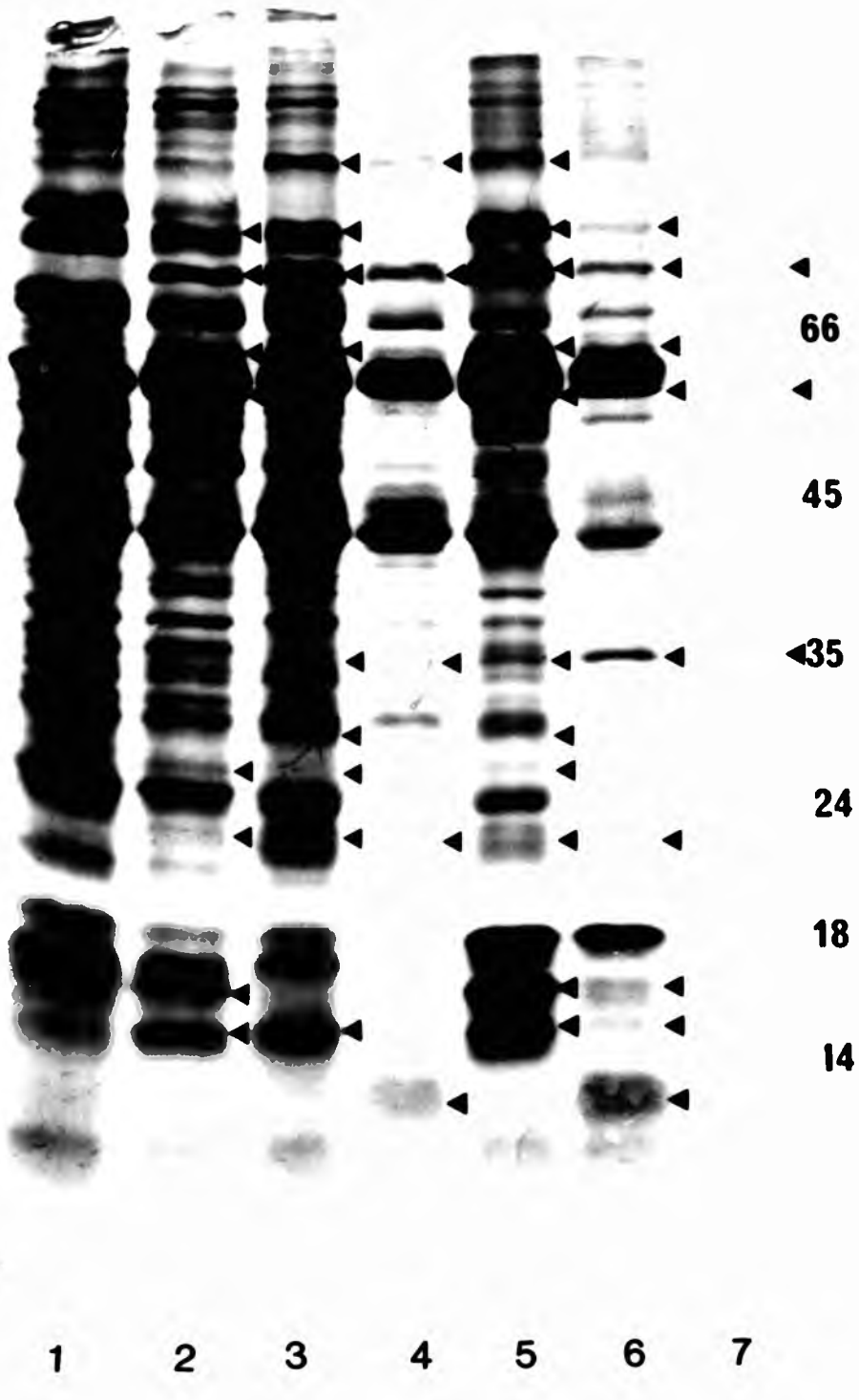


Plate 3:18 $\left[^{35}S\right]$-methionine incorporation into proteins in whole cell extracts after two simultaneous stress-shock treatments. Samples were prepared after 30 minutes treatment as described in section 2:17. The cells were kept untreated at 30°C, lane 1; shifted to 40°C, lane 2; treated with methanol (5% v/v), lane 3; treated with ethanol (5% v/v), lane 4; shifted to 40°C and treated with methanol, lane 5; shifted to 40°C and treated with ethanol, lane 6 or treated with methanol and ethanol (both 5% v/v), lane 7. The position of protein standards is indicated (Mr in kdaltons) on the right of the plate. The stress-shock proteins are arrowed.

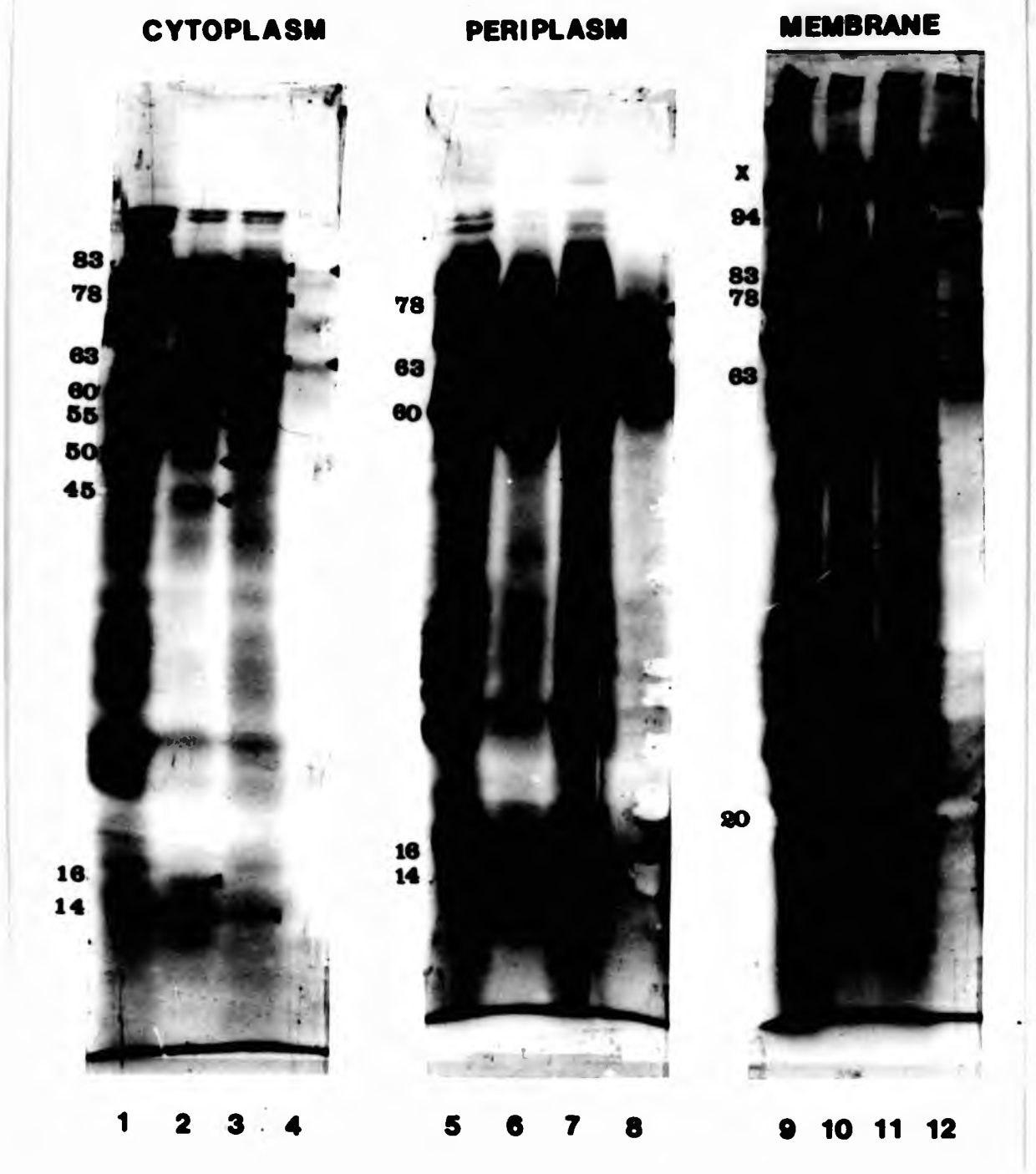


Plate 3:19 $[^{35}S]$-methionine incorporation into proteins in subcellular fractions prepared from stress-shocked cells. Samples were taken after 30 minutes treatment and cytoplasmic, lanes 1-4; periplasmic, lanes 5-8 and membrane, lanes 9-12, fractions prepared. The cells were kept untreated at 30°C, lanes 1, 5 and 9; shifted to 40°C, lanes 2, 6, and 10; treated with methanol, lanes 3, 7 and 11 or treated with ethanol, lanes 4, 8 and 12. The position and size (Mr in kD) of the stress-shock proteins are indicated: the molecular weight of one protein (x in lane 10) was too high to be estimated accurately.

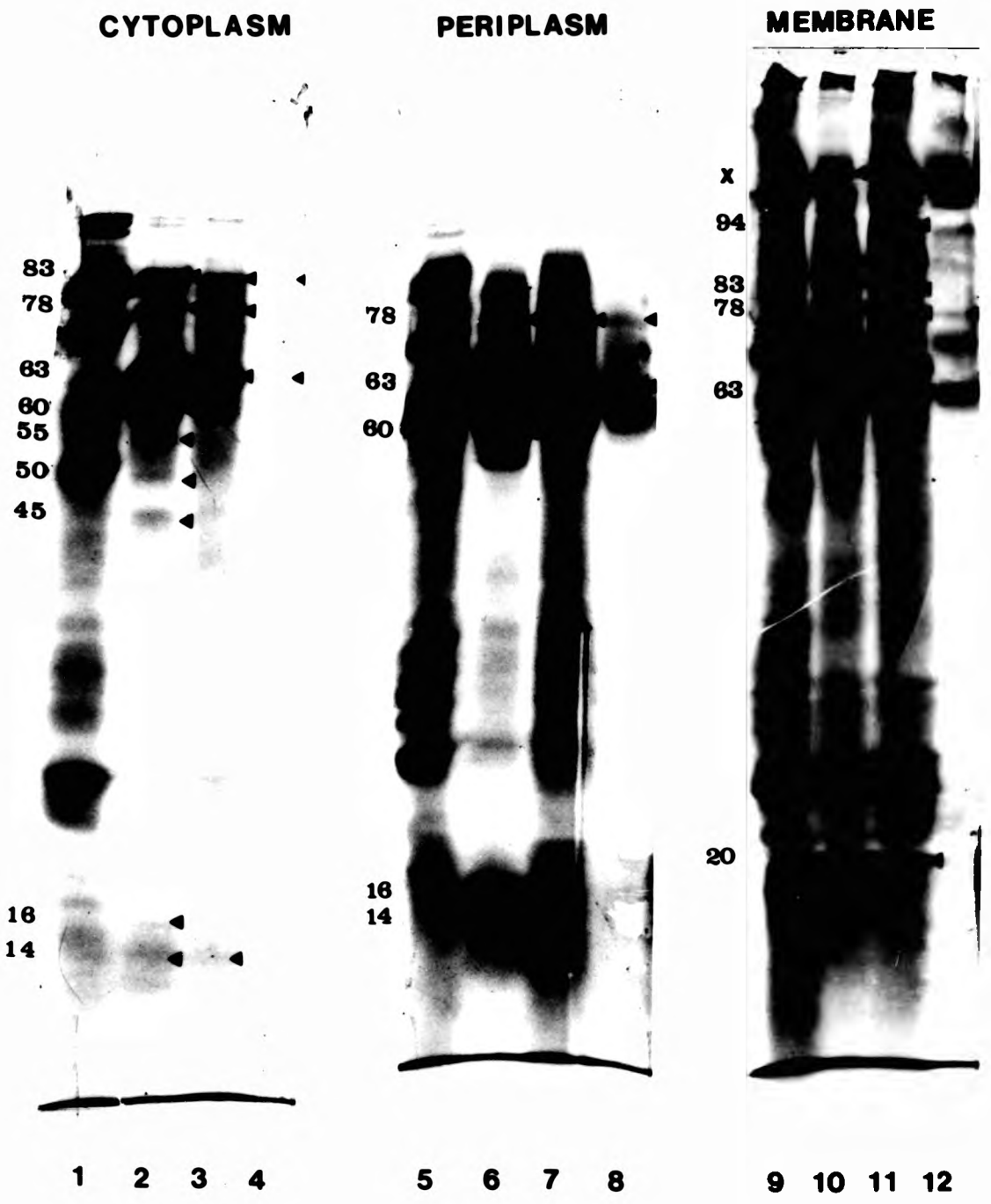


Plate 3:19 $\{^{35}S\}$-methionine incorporation into proteins in subcellular fractions prepared from stress-shocked cells. Samples were taken after 30 minutes treatment and cytoplasmic, lanes 1-4; periplasmic, lanes 5-8 and membrane, lanes 9-12, fractions prepared. The cells were kept untreated at 30°C, lanes 1, 5 and 9; shifted to 40°C, lanes 2, 6, and 10; treated with methanol, lanes 3, 7 and 11 or treated with ethanol, lanes 4, 8 and 12. The position and size (Mr in kD) of the stress-shock proteins are indicated: the molecular weight of one protein (x in lane 10) was too high to be estimated accurately.

it was difficult to assess precisely the distribution of the 63 and 60 kD hsps, although the latter was apparently absent from the membranes. The 16 and 14 kD proteins were predominantly in the periplasmic fraction. The fractionation revealed additional hsps which were not detected in whole cell extracts, namely a high molecular weight protein exclusive to membrane preparations and three other (45, 50 and 55 kD) which were present only in the cytoplasmic fraction. In contrast, some of the minor stress proteins detected in whole cell extracts were not apparent in any of the fractions.

3:10j The effects of stress-shock on the levels of proteolytic activity -

Whole cell extracts were isolated from cells grown at 30°C and from cultures exposed to the four stress-shocks. The ability of these extracts to hydrolyze (1) [^{14}C]-α_1-casein and (2) BzArgNA was tested, table 3:39. From the results it was clear that there was a small increase in the level of hydrolyzing activities towards both substrates with heat-shocked, methanol-shocked and ethanol-shocked extracts. Extracts from puromycin treated cultures did not show an increase in the rates of hydrolysis with any of the substrates tested. This result was in accordance with previous findings for puromycin-treated cultures (section 3:10d & e).

3:10k Comparison of whole cell extracts from non-shocked and stress-shocked cultures - analysis after running on DEAE-Sephacel columns.

A comparison of the effects of stress-shock treatment on whole

Table 3:39

Comparison of proteolytic activities of the whole cell extracts from M. methylotrophus after stress-shock treatment

Stress-shock	Concentration (mg/ml)	[^{14}C]-α_1-casein Sp. Ac cpm/min/mg	BzArgNA Sp. Ac nmol/min/mg
30-30°C	1.80	8.6	1.6
30-40°C	1.75	12.4	1.88
30 + Methanol	1.45	17.6	1.92
30 + Ethanol	1.00	14.9	1.91
30+ Puromycin	1.65	8.1	0.72

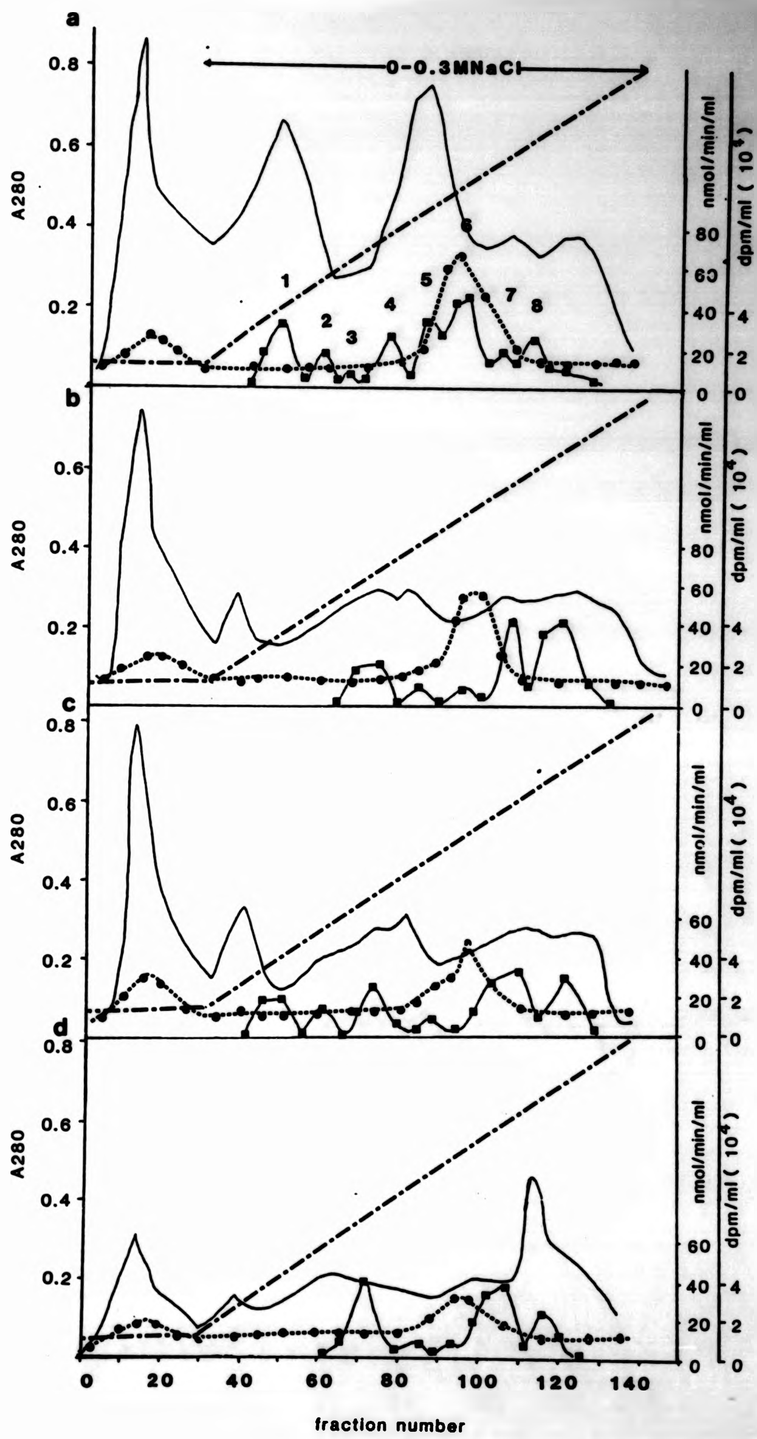
Whole cell extracts were isolated after 30 minutes shock or non-shock treatment.

cell extract proteolysis provides little information on the actual effect on individual proteolytic enzymes or groups of enzymes. Therefore extracts, isolated after various shock treatments, were run on DEAE-Sephacel columns and a comparison made between the hydrolytic activities of the eluted fractions, fig. 3:40. (The protein and hydrolyzing activity profiles for whole cell extracts from cells grown at 30°C shown in fig. 3:40a were comparable to those for whole cell extracts isolated from cells grown at 37°C and run on a DEAE-cellulose column, fig. 3:7).

Comparison of the protein profiles (A280) indicated that there were major differences in the amount of protein eluted at various concentrations of NaCl. Two major protein peaks which eluted at low and medium NaCl concentrations with non-shocked whole cell extracts were drastically reduced in stress-shocked samples. The protein profiles were similar for both the heat-shocked and methanol treated samples, figs. 3:40b & c. Ethanol-treated extracts exhibited a unique protein peak at the higher concentration of NaCl, fig. 3:40d. Generally it appeared that protein profiles were unique to the type of shock treatment used.

Fractions eluted from non-shocked whole cell extracts showed eight peaks of $[^{14}C]\alpha_1$-casein degrading activity. These peaks of activity have been numbered 1-8 according to their order of elution from the column. Heat-shock treatment resulted in the loss of activities in peaks 1 and 2, a reduction in activities in peaks 5 and 6 and an increase in $[^{14}C]\alpha_1$-casein degrading activities in peaks 7 and 8. A comparable result was observed after ethanol and methanol treatment although with methanol-shocked cells activities in peaks 1 and 2 were also observed.

Fig. 3:40 DEAE-Sephacel chromatography of proteolytic enzymes from whole cell extracts of M. methylotrophus after stress-shock. Degradation of [^{14}C]-α_1-casein (■—■); hydrolysis of BzArgNA (●···●); A_{280} (——) and conductivity (·—·). Proteins were eluted from whole cell extracts isolated from cells grown at 30°C and (a) kept at 30°C; (b) shifted to 40°C; (c) treated with methanol (5% v/v) and (d) treated with ethanol (5% v/v). Dialyzed whole cell extracts were absorbed to DEAE-sephacel columns and eluted with 1500 ml of a linear NaCl gradient (0-0.2M NaCl). The flow rate was 100 ml/hr and 15 ml fractions were collected. Every fourth fraction was assayed.



BzArgNA-hydrolyzing activities present in non-shocked extracts co-eluted with [^{14}C]-α_1-casein hydrolyzing activities in peaks 5, 6 and 7. The single peak of BzArgNA-hydrolyzing activity was reduced with all stress-shocked extracts. This result is difficult to explain if compared to increased levels of activity of whole cell extracts (table 3:39).

3:101 Discussion

In common with other organisms M. methylotrophus synthesizes a characteristic set of hsps after heat-shock treatment. The molecular weights of these hsps are similar, although not identical, to those of E. coli and other bacteria (Schlesinger et al., 1982). In contrast to other organisms however a sudden shift-down in temperature of growing M. methylotrophus cells did not result in an induction of hsps.

A broad range of factors (modulators) are known to induce (or suppress) the production of heat-shock proteins in many organisms (Neidhardt et al., 1984; Lanks, 1986). The treatment of M. methylotrophus cells with methanol or ethanol induced the synthesis of some, although not all the proteins observed after heat-shock: in addition other unique proteins that were characteristic of the stress-shock treatment were also produced. Simultaneous heat- and alcohol-shock of M. methylotrophus cells resulted in the induction of all the hsps common to both shock treatments. This indicates that each stress condition induces its own set of hsps under the control of an individual regulator. This view is now closer to that reported for other organisms including E. coli and S. typhimurium in which variations in response to different stress-stimuli are

apparent (Groat et al., 1986, Jones et al., 1986; Spector et al., 1986).

In many organisms the synthesis of hsps occurs within 5 min of the stress-shock (Yamamori et al., 1978; Lindquist, 1986). The majority of hsps synthesized in M. methylotrophus cells were only apparent after 15 minutes of stress-shock treatment. Hsp 78 and 77 however were observed as early as 5 minutes . Hsp 77 was only induced in response to methanol or ethanol shock and unlike all the other hsps it was not synthesized after 5 minutes. The significance of the novel behaviour of this hsp is unclear. The term hsp referring to proteins induced by methanol or ethanol but not by heat may be slightly confusing. 'Stress-shock proteins' (ssps) may in fact be a more accurate expression. However this terminology is not widely accepted at present and therefore for convenience all these proteins will be called 'heat-shock proteins' (hsps).

On return of a cell to normal conditions after shock, normal protein synthesis is resumed while hsp production is halted (Lindquist, 1986). The hsps of M. methylotrophus however are still synthesized 30 minutes after a return to normal growth temperature. It has been reported in other organisms that the speed of a cell's return to normal conditons after heat-shock is related to the severity of the temperature change (Lindquist, 1986). The continued synthesis of hsps in M. methylotrophus cells even after a 30 minute return to normal conditions suggests that the heat-shock response was severe.

Hsps of M. methylotrophus were found in all three of its subcellular fractions. Certain hsps were unique to one fraction while others were found in all three or just two fractions. Although

it is not possible, without a time course for the synthesis of hsps in subcellular fractions, to say whether they are synthesized in one fraction and then transported to another or produced in individual fractions, it is clear that the stress-shock response affects the entire cell. Determination of the subcellular localization of individual hsps may prove invaluable in establishing the role of these proteins in response to survival or stress, however, with the exception of certain eukaryotes (Schlesinger et al., 1982), this aspect of the stress-shock phenomenon has received little attention in other organisms.

There is now a great deal of evidence to implicate denatured or abnormal protein as modulators of the stress-shock response (Carr et al., 1985; Goff and Goldberg, 1985; Ananthan et al., 1986) and one of the consequences of the stress-shock response is an increased capacity to degrade abnormal proteins (Ananthan et al., 1986 & Goff and Goldberg, 1985). The study of the effects of stress-shock on the level of proteolysis in M. methylotrophus cells have shown that it causes an increase in the hydrolyzing activity of whole cell extracts but a decrease in the total number of enzymes reponsible for these activities (as determined by DEAE-Sephacel chromatography). This effect on hydrolyzing activities however appears to be specific with loss of certain peaks of activity accompanied by an increase in the activity of other peak fractions. It still remains to be determined whether the effects on the levels of hydrolyzing activity associated with stress-shock in M. methylotrophus cells are the result of the induction of a particular proteolytic enzyme(s) as has been shown to be the case in yeast (Gross and Schulz-Harder, 1986), E. coli (Goff and Goldberg, 1985;

Ho et al., 1986) and certain eukaryotic species (Bond and Schlesinger, 1985).

3:11 THE HEAT-SHOCK PHENOMENOM IN E. COLI

Despite the vast amount of work involving the hsps of E. coli (Neidhardt et al., 1984) and the discovery that stress-shock results in an increase in the levels of protein degradation by whole cell extracts (Carr et al., 1985) and that at least one of these hsps is a proteolytic enzyme (Phillips et al., 1984), little work has been done on the effects of stress-shock on the actual numbers of protein degrading enzymes induced (or suppressed) in this response.

Some evidence of the effects of stress-shock on the number of hydrolyzing enzymes induced (or suppressed) in M. methylotrophus was provided by separation of these enzymes by DEAE-Sephacel chromatrography. The effects of stress-shock on the number of hydrolyzing enzymes of E. coli were investigated in a comparable fashion.

3:11a The effects of stress-shock on cell growth

The effects of stress-shock on the growth of E. coli K12 cells were investigated as described in section 2:17, fig. 3:41. After 30 minutes of heat-shock there was a small (0.15 A_{650}) increase in cell turbidity. Stress-shock with methanol, ethanol and puromycin resulted in a decrease in the rate of cell growth. Ethanol treatment actually caused a decrease in the level of turbidity (likely to be due to cell lysis).

3:11b The effects of stress-shock on protein synthesis

The effects of various stress-shock treatments on protein synthesis in E. coli cells were investigated (section 2:17). Total protein was extracted from the cells (section 2:17) and run on an

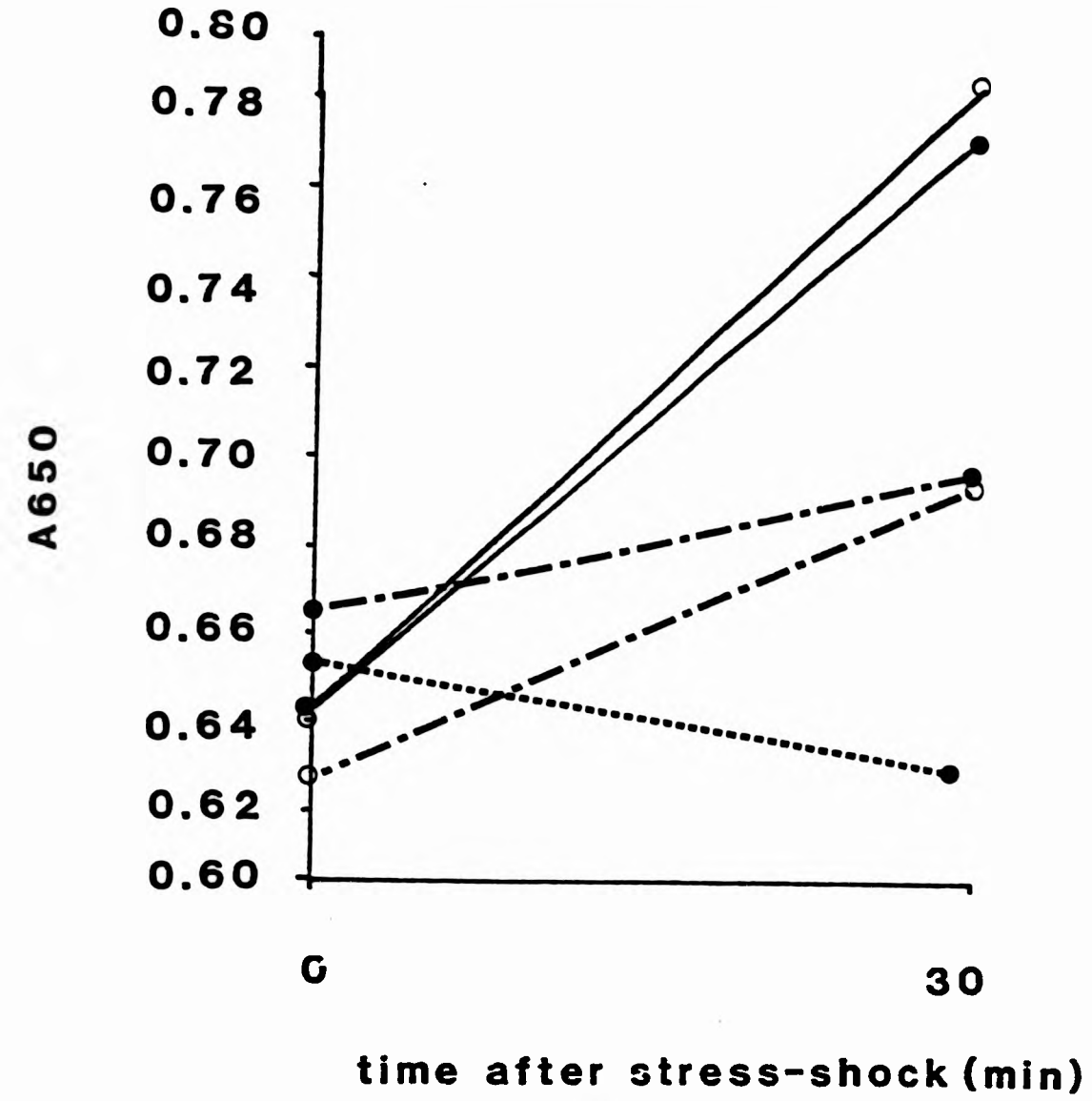


Fig. 3:41 The effects of stress-shock on the growth of E. coli K12 cells. Cells grown at 30°C to an A_{650} of 0.63 and 0.66 were kept at 30°C (●—●); shifted to 42°C (○—○); treated with methanol (5% v/v) (●-—●); treated with ethanol (5% v/v) (●···●) or puromycin (100 µg/ml) (○—·○).

SDS-polyacrylamide gel (section 2:6a). An autoradiogram was prepared towards the gel (section 2:7), plate 3:20.

A temperature shift to 42°C or stress-shock by addition of methanol, ethanol or puromycin resulted in a reduction in the overall rate of synthesis of cellular proteins. Although the production of novel proteins after stress-shocking was not absolutely conclusive, certain proteins were selectively over-produced. The apparent molecular weights of these proteins are shown in table 3:40. Ten hsps were identified after heat-shock treatment, Seven hsps after methanol treatment, six hsps after ethanol treatment and one hsp after puromycin treatment.

3:11c The effect of stress-shock on the levels of proteolytic activity

Whole cells were isolated from cells grown at 30°C and from heat-shocked and ethanol-shocked cultures. The ability of these whole cell extracts to hydrolyze (1) $[^{14}C]$-α_1-casein, (2) BzArgNA and (3) SAAPP in the presence and absence of aminopeptidase M was tested, table 3:41. The levels of activity towards all the substrates tested increased after stress-shock treatment. The increase in hydrolyzing activities ranged from 80-130% depending on the substrate tested.

3:11d Comparison of whole cell extracts from non-shocked and stress-shocked cultures - analysis after running on DEAE-Sephacel columns.

Whole cell extracts from non-shocked, heat-shocked and ethanol-shocked cells were run on DEAE-Sephacel columns and eluted

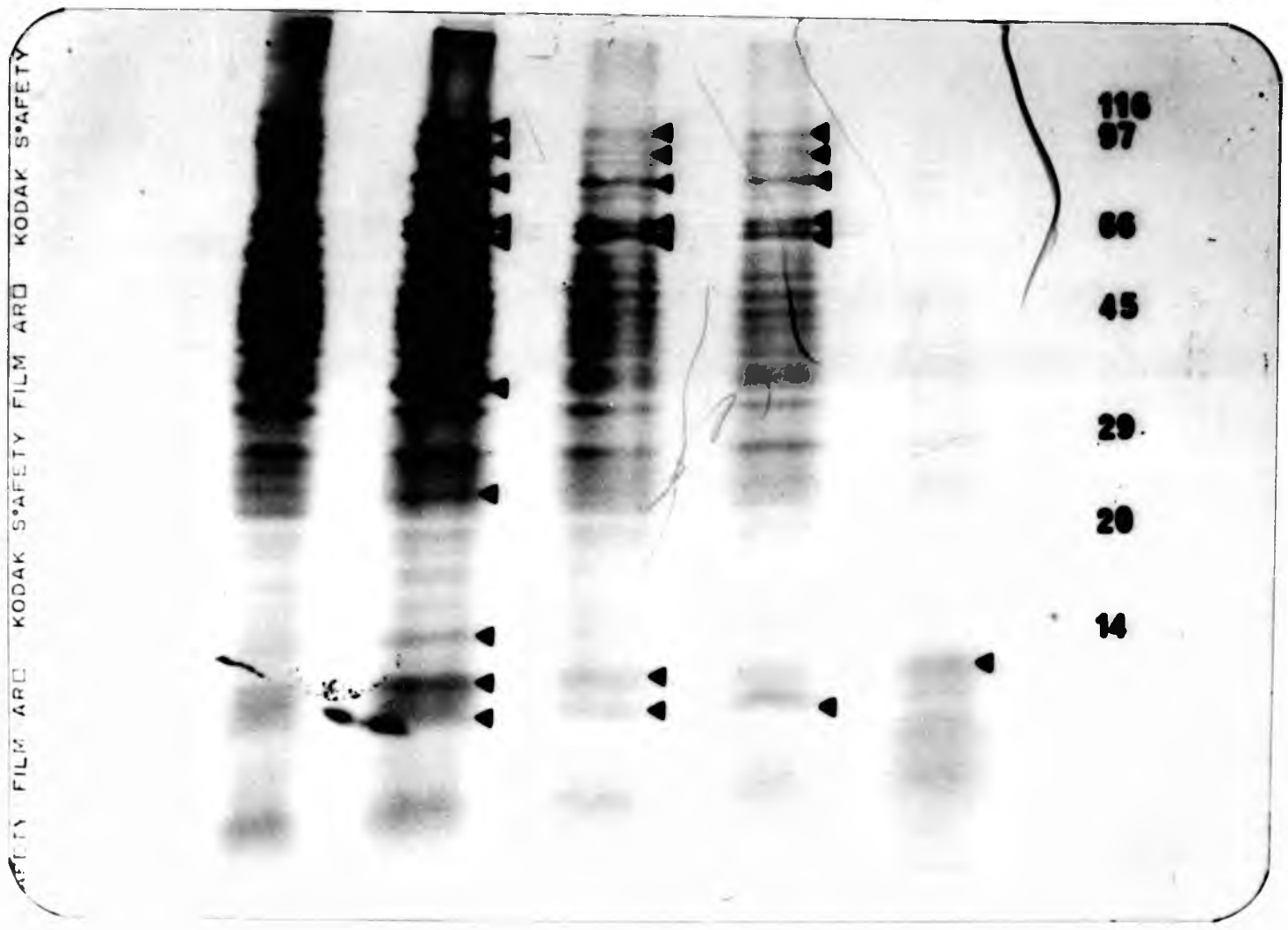


Plate 3:20 $[^{35}S]$-methionine incorporation into proteins in whole cell extracts of stress-shocked E. coli cells. Samples were prepared after 30 minutes treatement as described in section 2:17. The cells were kept untreated at 30°C, lane 1; shifted to 42°C, lane 2; treated with methanol (5% v/v), lane 3; treated with ethanol (5% v/v), lane 4 or treated with puromycin (100 μg/ml), lane 5. The position of protein standards is indicated (Mr in kdaltons) on the right of the plate. The stress-shock proteins are arrowed.

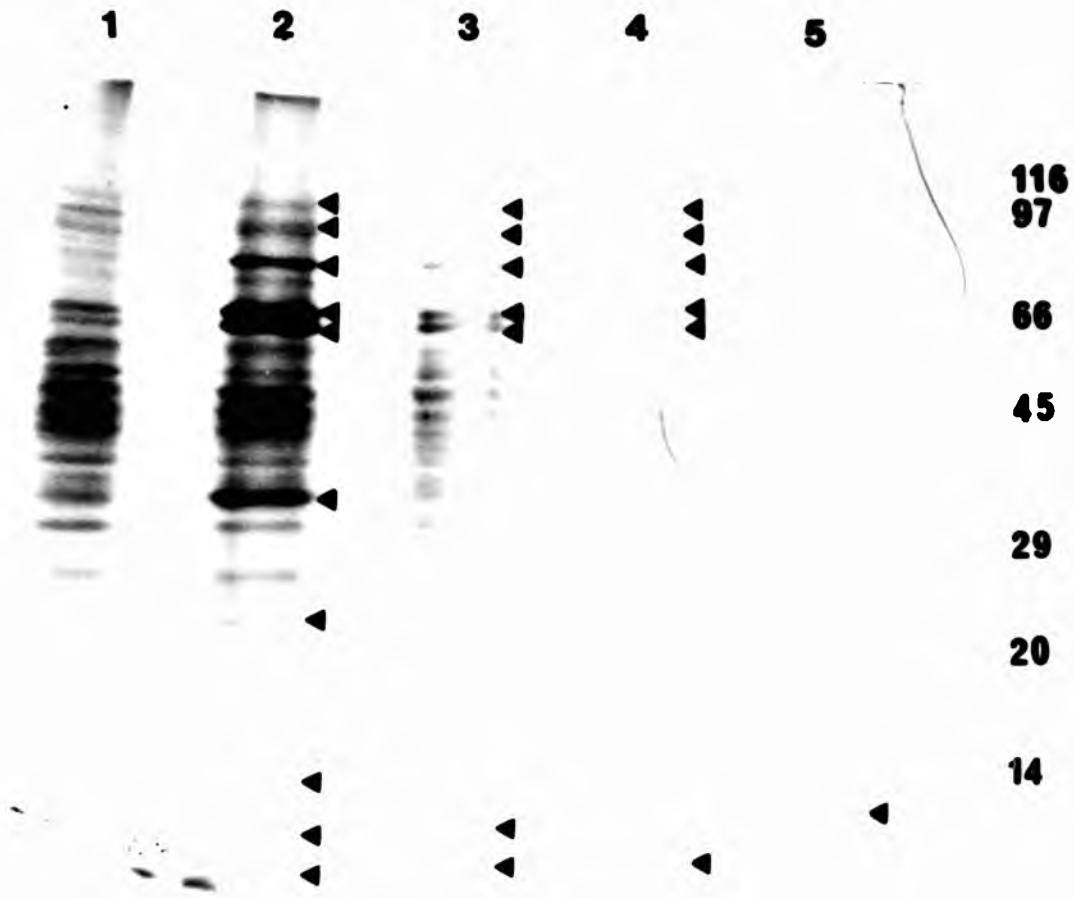


Plate 3:20 $[^{35}S]$-methionine incorporation into proteins in whole cell extracts of stress-shocked E. coli cells. Samples were prepared after 30 minutes treatement as described in section 2:17. The cells were kept untreated at 30°C, lane 1; shifted to 42°C, lane 2; treated with methanol (5% v/v), lane 3; treated with ethanol (5% v/v), lane 4 or treated with puromycin (100 μg/ml), lane 5. The position of protein standards is indicated (Mr in kdaltons) on the right of the plate. The stress-shock proteins are arrowed.

Table 3:40

Apparent molecular weights of E. coli heat-shock proteins

Heat-Shock protein	Apparent mol. wt (D)
Hsp 92	92000
Hsp 85	85000
Hsp 74	74000
Hsp 63	63000
Hsp 60	60000
Hsp 32	32000
Hsp 21	21000
Hsp 12	12000
Hsp 11	11000
Hsp 9	9000

The hsps above were detected by the method described in section 2:17. The molecular weights of these proteins were estimated from plate 3:20.

Table 3:41

Comparison of proteolytic activities of the whole cell extract from E. coli after stress-shock treatment

Stress-shock	Concentration (mg/ml)	[^{14}C]-α_1-casein Sp. Ac cpm/min/mg	BzArgNA Sp. Ac. nmol/min/mg	SAAPP(-M) Sp. Ac. nmol/min/mg	SAAPP(+M) Sp. Ac. nmol/min/mg
30-30°C	3.2	24.4	3.5	2.4	1.9
30-42°C	3.0	26.3	4.1	2.9	4.4
30 + Ethanol	2.7	28.7	3.8	3.7	3.6

Whole cell extracts were isolated after 30 minutes shock or non-shock treatment.

(-M) Specific activity in the absence of Aminopeptidase M.
(+M) Specific activity in the presence of Aminopeptidase M.

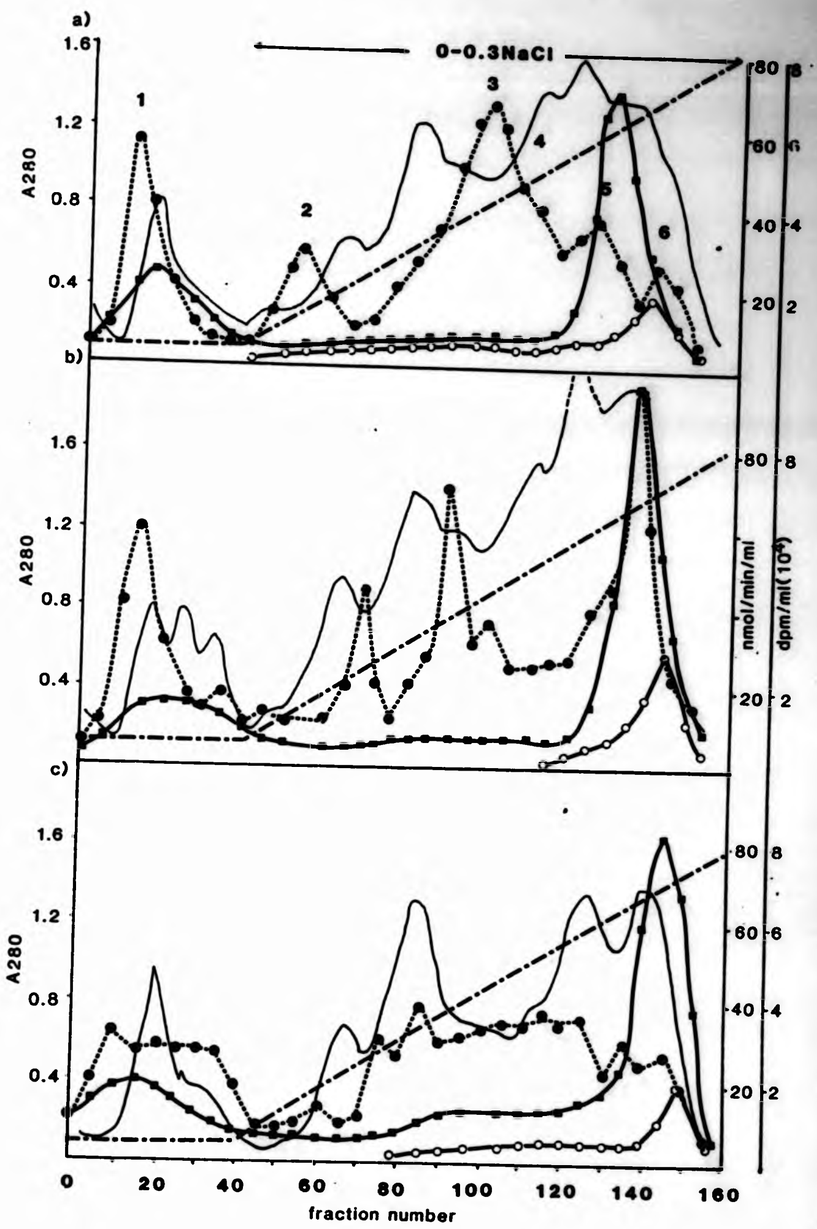
fractions assayed for their ability to hydrolyze [^{14}C]-α_1-casein and SAAPP, fig. 3:42. The protein profiles (A_{280}) of eluted fractions from the various treatments showed a great deal of similarity but with certain significant increases or decreases in the levels in various fractions.

At least six [^{14}C]-α_1-casein degrading activities were detected in the eluted fractions obtained with non-shocked cells. These peaks of activity were numbered 1-6 according to their order of elution from the column. Heat-shock resulted in the loss of activity of enzymes from peak 2 and a reducton in the hydrolyzing activity of enzymes in peaks 3 and 4. In addition this treatment caused an increase in the rate of [^{14}C]-α_1-casein degrading activity of enyzmes in peaks 5 and 6.

The most noticable effect of ethanol-shock was that there was an overall decrease in [^{14}C]-α_1-casein degrading activity although it was not possible to distinguish between individual peaks of activity as in non-shocked and heat-shocked extracts.

A further comparison of the effects of stress-shock on proteolysis by whole cell extracts of E. coli was made using SAAPP, fig. 3:42. The results showed that two SAAPP hydrolyzing activities were eluted from the column. The first activity was eluted from the column with the unbound protein and was only detected in the presence of aminopeptidase M. Heat- and ethanol-shock resulted in a decrease in the hydrolyzing activity observed in these unbound fractions. The second SAAPP hydrolyzing activity of whole cell extracts of non-shocked cells was eluted at a higher salt concentration. Although this activity was detected in the absence of aminopeptidase M there was a 4-fold increase in the release of

Fig. 3:42 DEAE-Sephacel chromatography of proteolytic enzymes from whole cell extracts of E. coli after stress-shock. Degradation of [^{14}C]α_1-casein (●----●); hydrolysis of SAAPP in the absence (○—○) and presence (■—■) of aminopeptidase M; A_{280} (——) and conductivity (·—··). Proteins were eluted from whole cell extracts isolated from cells grown at 30°C and (a) kept at 30°C; (b) shifted to 42°C; (c) treated with ethanol (5% v/v). Dialyzed whole cell extracts were absorbed to DEAE-sephacel columns and eluted with 1500 ml of a linear NaCl gradient (0-0.2M NaCl). The flow rate was 100 ml/hr and 15 ml fractions were collected. Every fourth fraction was assayed.



nitroaniline in its presence. Heat- and ethanol-shock resulted in an increase in this SAAPP hydrolyzing activity.

3:11e Discussion

The results presented here for the number and molecular weights of hsps induced after stres-shock in E. coli are in close agreement with those reported by other researchers (Schlesinger et al., 1982; Neidhardt et al., 1984). The two most abundant hsps identified, Hsp 63 and 60, correspond to B56.5 (apparent molecular weight 62883 D) and B66.0 (apparent molecular weight 69121 D) identified previously by Schlesinger and his colleagues (Schlesinger et al., 1982).

Reports by other researchers on the effects of stress-shock on proteolysis in E. coli cells have generally dealt with the effects on the protein-degrading activities of whole cell extracts and not on individual enzymes (Yamamori et al., 1978). The obvious exception to this is the vast amount of work that has been done on Protease La, the product of the lon gene, that has now been shown to be a heat-shock protein (Goff and Goldberg, 1985; Carr et al., 1985). Although it is known that this enzyme is induced during stress-shock little is known about the effects of stress-shock on the other proteolytic enzymes of the whole cell extract.

The results from DEAE-sephacel chromatography shown above indicate that although at least one hydrolyzing enzyme is induced after stress-shock, the majority of the other enzymes responsible for proteolytic degradation appear to be suppressed. This is apparently the first such observation on this aspect of the stress-shock response.

A comparable induction and suppression of individual

proteolytic enzymes after stress-shock in M. methylotrophus cells (section 3:10k) suggests that this effect, at least in bacteria, might be part of a more general response to adverse conditions.

3:12 ATTEMPTS TO ISOLATE PROTEOLYTIC DEFICIENT MUTANTS OF M. METHYLOTROPHUS

The isolation of mutants of a variety of organisms deficient in a number of proteolytic enzymes has enabled a certain of these enzymes to be characterized and as such be attributed specific roles in intracellular proteolysis (Miller et al., 1976; Jones, 1977; Wolf and Ehmann, 1978; Yamori and Yura, 1982; Jenkinson and Lord, 1983; Carr et al., 1985; Ciechanover et al., 1985; Bond et al., 1987). Furthermore the advantage of proteolytic-deficient mutants for the production of foreign proteins in bacteria is self-evident (Fahnestock and Fisher, 1987). It was therefore of some interest to attempt to isolate such mutants in M. methylotrophus.

The rationale behind the isolation of these mutants was based on that used for the isolation of proteolytically-deficient mutants from S. cerevisiae (Jones, 1977; Wolf and Ehmann, 1978) and E. coli (Miller et al., 1976). In these procedures, selection after mutagenesis was made for Ts mutants that lyse at the non-permissive temperature. Lysis resulted in the release of intracellular contents whose activity could then be detected. The release of proteolytic enzymes was detected on agar substrate plates as zones of clearance around lyzed colonies (section 2:19). Alternatively release of APNE hydrolyzing activities could be detected (section 2:19). Mutagenised Ts lysis mutants were then prepared and screened in order to detect those mutants that did not release proteolytic enzymes and hence did not produce zones of clearing around colonies on substrate plates. If these mutants were not revertants, then they would be proteolytically-deficient mutants.

3:12a Ts mutants of M. methylotrophus

A total of 44 Ts mutants were isolated after mutagenesis of M. methylotrophus cells. Of these mutants 28 were prepared and supplied by Dr. M.J. Worsey (I.C.I.). The remaining 16 Ts mutants were isolated after screening 3600 colonies (~100 colonies/plate).

3:12b Identification of Ts lysis mutants and proteinase deficient mutants

Each of the 44 Ts mutants were tested for lysis by replica plating onto agar plates containing either HPA, azocasein or skimmed milk, or by replica plating onto Whatman filter paper no 1 and testing for APNE-hydrolyzing activity. No zones of clearing or APNE-hydrolyzing activities were apparent for any of the Ts mutants, suggesting that none of them were lysis mutants. It was possible that the release of the intracellular contents was simply not detected with the substrates used, or as has been suggested in previous experiments, because whole cell extracts have relatively low levels of protein-degrading activity.

The inability to detect Ts lysis mutants therefore made progress to the next step in the isolation of proteolytically-deficient mutants impossible. It was clear that many more Ts mutants would need to be isolated and screened in order to be fortuitous enough to isolate lysis mutants. Alternatively other methods of isolating the mutants must be devised.

' I know why there are so many people
who love chopping wood. In
this activity one immediately
sees the results '

Albert Einstein

CHAPTER FOUR

4:1 CONCLUDING REMARKS

4:2 THE PROTEOLYTIC SYSTEM OF M. METHYLOTROPHUS

Although there are major differences between M. methylotrophus and other bacteria with respect to their biochemistry and physiology, the controls of proteolysis effected by various growth and storage conditions and the type of proteolytic enzymes present in these microorganisms are markedly similar. This is best illustrated by the following examples: (1) their intracellular proteolytic enzymes are exclusively serine- and metalloproteinases maximally active in the pH range 7.2-8.3 (sections 3:1, 3:2, 3:4 and 3:5) (Orlowski and White, 1974; Hiroishi and Kadota, 1976; Miller et al., 1976; Cheng and Aronson, 1977; Dignam and Setlow, 1980; Goldberg et al., 1981; Bromme and Kleine, 1984; Car and Woods, 1984; Kohayashi et al., 1985); (2) several of these enzymes require Ca^{2+} or Zn^{2+} for maximum activity or stability (section 3:1, 3:2, 3:4 and 3:5) (Goldberg et al., 1981); (3) these enzymes have been found in all subcellular fractions, cytoplasm, periplasm and membranes (sections 3:4, 3:5 and 3:6) (Goldberg et al., 1981; Regnier, 1981a; Pacaud, 1982a); (4) their protein is relatively stable in exponentially growing cells but is less so during stationary phase (section 3:7) (Mandelstam, 1957; Pine, 1972; Cocucci and Davies, 1985). Soluble extracts from cells harvested at mid-log phase degrade non-physiological substrates much more slowly than those from stationary phase cells (section 3:7); (5) proteolytic degradation in freshly-prepared cellular extracts is at relatively low levels owing to the presence of endogenous proteinase inhibitors (section 3:8) (Green and Ryan, 1971; Imada et al., 1985). Storage

of extracts results in degradation of intracellular protein and increased levels of proteolytic activity towards non-physiological substrates. Increases in proteolytic degradation are due to breakdown of endogenous inhibitors (Wolf and Holzer, 1980). In addition to these similarities it has also been shown that exponentially growing cells subjected to stress-shock either by heat, methanol or ethanol (and other factors) results in an induction of synthesis of a novel set of proteins known as 'heat-shock proteins' (Hsps) (sections 3:10 and 3:11) (Schlesinger, 1986). In M. methylotrophus cells, as in other bacteria, stress-shock results in a stimulation of the intracellular levels of proteinase activity (sections 3:10 and 3:11) (Goff and Goldberg, 1985; Bishai et al., 1987).

These similarities suggest that protein degrading systems of bacteria and the related phenomenon of the stress-shock response have all been highly conserved. It is likely therefore that aspects of proteolysis and the stress-shock response not demonstrated in other bacteria but apparent in M. methylotrophus, e.g. (1) inhibition of proteolytic degradation by pyrimidine nucleotides (sections 3:1 and 3:2); (2) selective degradation of intracellular protein during stationary phase growth (section 3:7); (3) specific repression of proteolytic enzymes in response to stress-shock (sections 3:10 and 3:11); (4) induction of hsps that are synthesized for much shorter periods than others (section 3:10); (5) induction of hsps unique to the type of stress-shock (section 3:10) and (6) localization of hsps in all subcellular compartments (section 3:10), may all also be found to apply to many other microorganisms. It is clear that a study of proteolysis and the heat-shock response

in the gram-negative bacterium M. methylotrophus may also contribute to our general understanding of these systems in other bacteria.

4:3 M. METHYLOTROPHUS AS A HOST FOR FOREIGN GENE EXPRESSION

The ability to express eukaryotic cDNA in M. methylotrophus cells (Hennam et al., 1982; De Maeyer et al., 1982) and the numerous advantages offered by this bacterium for the manufacture of SCP on an industrial scale (Anthony, 1982; Large, 1983; Wiseman, 1983; Smith, 1985) suggested a likely role for its use in the production of commercially important proteins by recombinant DNA technology. However proteolytic degradation of foreign gene products expressed in bacterial hosts can present a major stumbling block to the commercial production of these proteins (Itakura et al., 1977; Goeddel et al., 1979a; Taniguchi et al., 1980; Talmadge and Gilbert, 1982; Wang and Novich, 1987).

The results presented in this thesis on the nature of the proteolytic enzymes, proteolysis in general and the stress-shock response of M. methylotrophus, in addition to the literature reviewed on proteinases of other microorganisms, allow certain suggestions to be made about possible ways of controlling proteolytic degradation during eukaryotic gene expression and product isolation.

4:3a Controlling protein degradation during the isolation of foreign gene products

In the absence of any secretory proteins in M. methylotrophus isolation of the foreign gene product from the cell necessitates cellular disruption. Based on the results presented in this thesis

the best way to minimize protein degradation during this process would be to disrupt cells by sonication in a buffer containing serine- and metalloproteinase inhibitors such as PMSF and EDTA. In addition the extraction buffer should be kept at a temperature around 4°C and be at a pH 1-2 units below the optima for these enzymes (assuming the pH is not critical for foreign protein integrity). The high levels of proteolytic activity resulting from even short periods of storage of cell extracts emphasize the need to remove the foreign protein from the cellular contents as quickly as possible.

4:3b Controlling proteolysis of eukaryotic protein in vivo

Protein degradation is not only a problem associated with isolation of the foreign gene product from the cell. It also occurs in vivo during or directly after expression of the eukaryotic protein (Wang and Novich, 1987; Bishai et al., 1987). The suppression of proteinase activity under these conditions requires a different approach to that described above.

One important pointer to a means of controlling protein degradation in vivo arises from the evidence indicating that endogenous proteinase inhibitors are present in the soluble fraction of M. methylotrophus cells and that they control levels and/or the activity of proteolytic enzymes. Isolation and purification of these inhibitors may lead to the development of cDNAs which could then be inserted into plasmids. Overexpression of these inhibitors during expression of the foreign gene may effectively reduce hydrolysis of the eukaryotic protein. Alternatively cDNA could be inserted in tandem with the foreign gene. A promoter controlling

the expression of the eukaryotic gene could then simultaneously switch on the expression of inhibitors, preventing degradation of the foreign protein. Since overexpression of endogenous proteinase inhibitors during normal cell growth would probably result in the death of the cell a method of controlling this expression would be desirable. One possible method of doing this has already been used to control the expression of eukaryotic genes in E. coli (Bishai et al., 1987). This method, known as temperature induction, involves inserting the transriptional promoter and repressor from bacteriophage into a plasmid preceeding the genes to be expressed. It is then possible to turn gene expression on and off at appropriate temperatures. Such a control also results in a more stable plasmid at lower temperatures. This method would therefore allow cells to be grown to a high biomass at the lower temperature with a shift to the higher temperature resulting in the expression of the desired genes.

There are certain inherent problems associated with the control of gene expression by temperature induction. It has been shown in the work presented here on M. methylotrophus that a sudden shift-up in temperature (equivalent to temperature induction) results in a heat-shock response (section 3:10). This response results in the specific induction of several unique proteins, several of which appear to be proteolytic enzymes, while suppressing the synthesis of many other cellular proteins. The problems associated with this response are as follows: (1) it results in the wasteful utilization of energy in a process of no consequence for foreign gene expression; (2) the turning off of the transcription of the majority of cellular proteins may also reduce transcription of the desired genes and (3)

proteolytic degradation observed to accompany stress-shock would only serve to magnify the original problem. It is clear that in the light of these problems other means of controlling foreign protein degradation may be necessary. One possible way of doing this is by the use of bacterial mutants defective in the heat-shock response or in the production of active proteinases (see below).

4:4 CONTROLLING PROTEIN DEGRADATION BY USING BACTERIAL MUTANTS

If the technique of temperature induction is to be maximally effective, the problems arising from heat-shock response must be overcome. One possible way of doing this is by using mutant strains of the bacterium defective in the heat-shock response. This type of mutant has already been isolated from E. coli (Goff and Goldberg, 1985). Alternatively strains of the bacterium defective in the production of the proteolytic enzymes responsible for degradation of eukaryotic proteins could be used. Several such mutants have been used successfully in the expression of eukaryotic genes in other bacteria (Goff and Goldberg, 1985; Fahnestock and Fisher, 1987). Attempts to isolate proteinase-deficient mutant strains of M. methylotrophus proved unsuccessful, however, such mutants, if developed may prove extremely advantageous for the commercial production of eukaryotic protein.

4:5 ALTERNATIVES TO M. METHYLOTROPHUS

It is clear that protein degradation could be a major problem in the production of foreign proteins in M. methylotrophus. However it is important to realise that this may not be the only limiting factor in the use of this bacterium for the production of these

proteins. Inability to export the foreign gene product, lack of suitable enzymes for posttranslational modification, loss of plasmids through growth dilution and lack of knowledge about desired genes and suitable vectors are all important aspects to be considered if this bacterium is to be used for eukaryotic protein production on an industrial scale (Caulcott and Rhodes, 1986). The net effect of this may make M. methylotrophus less attractive than other bacteria (Reid et al., 1986; Fahnestock and Fisher, 1987) or even eukaryotic organisms (Ingram, 1986) that have well established protein export systems, enzymes capable of the necessary posttranslational modifications and well characterised mutants defective in either the stress-shock response or the production of active proteolytic enzymes.

' It is very pleasent to be written up, even for a writer '

Joyce Cary

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