Studies on amino acid assays using Escherichia coli

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STUDIES ON AMINO ACID ASSAYS USING *ESCHERICHIA COLI*.

by

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Thesis submitted in accordance with the requirements for the degree of Doctor Of Philosophy (special regulations) of the University Of Durham.

June 1983

ABSTRACT

The methods most widely used for the assay of amino acids, peptides and proteins are surveyed and the advantages of microbiological assays are discussed. Comparisons are made between the use of 'natural' auxotrophs commonly used for assay and 'artificial' mutant strains of E. coli as assay organisms.

A novel assay is described in which an E.coli Lys^- auxotroph is used to measure Lys-dependent protein synthesis. The response to a given quantity of Lys is constant irrespective of whether it is free, peptide-bound or present in a complex mixture. Since the measured response is enzyme synthesis rather than growth the technique has the advantage of sensitivity and speed. The enzyme measured is the inducible enzyme β-galactosidase, which liberates σ-nitrophenol fron the chromogenic substrate σ-nitrophenol-β-D-galactopyranoside. The number of active enzyme molecules that can be synthesised by the auxotroph depend solely on the Lys present. Thus the amount of available Lys in a digest can be determined by reference to a calibration curve of enzyme activity v. Lys concentration.

Optimisation of the method and the isolation, selection and characterisation of the mutants to assay for available Lys. Met and Trp is described and the sensitivity of the assay is similar for all three amino acids. Application of the technique to measure Lys, Met and Trp present in digests of a variety of pure proteins, feed meals and rice varieties is described and results are compared with published data.

The assay response to Met(O) led to the study of the effects of physiological status of E. coli on oxidation and reduction of Met(O) and on cleavage of $Met(O_2)$ residues.

Finally, since the auxotrophic requirement for the Lys^- Met^- strain could be replaced by B_{12} a preliminary investigation of the use of the technique to assay for this vitamin is reported.

Acknowledgements

I would like to thank Prof. D. Boulter for the use of facilities in the Department of Botany and Dr. M. D. Longfield for the use of facilities at Teesside Polytechnic.

I should like to thank members of the Botany Department at the University of Durham for many stimulating discussions; my special thanks in this respect are due to Dr J.N. Yarwood and to Dr T.M. Nesbit. I should also like to thank my colleagues in the Department of Chemical Engineering, Teesside Polytechnic for their encouragement and understanding on many occasions whilst this work was in progress.

I should also like to thank Mr D Boddy , also of Teesside Poly, for photographic work.

I should like to thank Mrs S. Horrocks for her efficient typing of most of this thesis.

I am extremely grateful to my supervisor, Dr J.W. Payne, for his help and guidance during the course of this work and in particular during the preparation of this manuscript.

Finally, I am indebted to my husband, Geoff, without whose constant support and encouragement during years of part-time study this thesis would not have been possible.

Memorandum

Parts of the work presented in this thesis also appear in the following publications:

Payne J W and Tuffnell J M (1980),
Assays for Amino Acids, Peptides and Proteins.
In : Micro-organisms and Nitrogen Sources , J W Payne (Ed),
J Wiley & Sons, Chichester, England. pp727 - 765.

Agricultural Research Council Protein Evaluation Group, (1980)
A P Williams, (Ed)
6, 7, ARC, Shinfield, Reading.

Tuffnell J M and Payne J W (1981)
Methionine Sulphone Production by Escherichia coli: influence of physiological status on oxidation and reduction of Methionine Sulphoxide.
F E M S Microbiol. Letters , 12, 273-277.

Payne J W and Tuffnell J M (1981)
Cleavage Of Peptide-Bound Methionine Sulphoxide and Methionine Sulphone: Possible Roles In Enhanced Proteolysis in Escherichia coli.
F E M S Microbiol. Letters, 12, 279-282.

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CHAPTER ONE

INTRODUCTION

1-1 Introductory Remarks

"A precise method of evaluating protein quality is important, not only to make possible the estimation of the amount of a given protein mixture required to meet physiological needs for protein but also to guide efforts aimed at improving food crops and food mixtures and to monitor the nutritionally adverse effects of food processing procedures" (Scrimshaw & Young, 1977). Methods for assessment of protein quality, and, perhaps, for ranking foods in these terms would be useful for some applications, but measurement of biological availability of individual amino acids is probably more important.

This thesis describes the development and subsequent applications of an enzymic assay for amino acids using auxotrophic strains of the bacterium Escherichia coli.

Many chemical, biological, and microbiological methods have been described for measuring protein nutritional value and it is clear that no single assay is applicable to all samples for all requirements.

Chemical analyses have advantages of speed and simplicity but when, as is commonly the case, these involve an acid hydrolysis step this destroys certain residues and may liberate residues from linkages that would be resistant to digestion and/or absorption in vivo, hence they will not yield information that strictly correlates with nutritional availability.

Animal feeding tests are prolonged, and require a large quantity of sample, making them unsuitable for many applications, e.g., for screening new plant cultivars, or for quality control during food processing. Also, results depend on the type of animal chosen for the assay since direct comparison of digestion and utilisation between species may not always be justified.

To try and overcome these problems, many microbiological assays have been developed. These usually involve measuring a nutrient-dependent growth response shown by a microorganism that has an auxotrophic requirement for the particular amino acid under test (Section 1-5).

These assays have been widely used and well documented, but results are often difficult to interpret; techniques, including the culture of particularly fastidious organisms, are often relatively complex, and problems caused by the nature of the sample as well as intra and inter-laboratory variations are common.

In the work described here, Escherichia coli is used as the test organism and rather than measuring growth, nutrient-dependent enzyme synthesis is determined. The advantages of Escherichia coli compared with other microorganisms more commonly used for amino acid assays, and the reasons for using an enzymic method are discussed fully in Chapter 3 (Section 3-1-1). In order to put this study into perspective, the literature relating to the nutritional evaluation of amino acids in foods, will first be reviewed.

The survey will be confined to four main areas: Choice of assay method; Chemical methods; Biological methods; Microbiological methods. In this survey, particular attention is paid to the advantages and disadvantages of each type of method and to a comparison of the results obtained from each. It is not intended to be an exhaustive survey of the literature, and further information may be found in the following reviews; (Bodwell, 1977; Bodwell et al., 1981; Evans & Witty, 1978; Ford, 1981; Payne & Tuffnell, 1980; Pellett, 1978; Satterlee et al., 1979; and Reports of the P.A.G., 1975; and I.A.E.A., 1975 and 1977).

1-2 Choice Of Assay

The object of the assay may be to determine the total amino acid complement of the sample or certain individual amino acids. An amino acid of interest may be free or chemically combined. Free amino acids may be in complex mixtures such as human body fluids and tissues, or microbial or plant products. If they are bound, they generally occur as components of proteins and more rarely in the form of small peptides. In this case, it is usually the nutritional evaluation that is of interest i.e., determination of the quantity and/or quality of the entire protein. Quantity can be determined from protein content per unit weight of an animal feed or supplement for example; quality relates to the amino acid composition of the protein, or more particularly to the levels of the nutritionally more important amino acids such as lysine and methionine. The selection of any method will always impose certain limitations and the final choice of method will depend on the type of results required, the nature and size of the sample, facilities available, etc. The nutritional quality of different foodstuffs is to a large extent dependent on their content of Lys, Met and Trp, and to a lesser extent on Thr, Leu and Ile. The amino acid that is most deficient in relation to the standard protein is known as the first limiting amino acid. The standard protein, or reference pattern of amino acids, now most often used is that suggested by the F.A.O. (1973) based on an estimation of the amino acid requirements of young children; whole egg protein has also been adopted as this standard. A chemical score (amino acid score) (Block & Mitchell, 1947; Bender, 1961 and 1973) can be calculated by dividing the content of the limiting amino acid in the test protein by the content of the same amino acid in the reference protein.

The amino acid score of the limiting amino acid provides a chemical prediction of the nutritional value of the test protein and may be taken as a first approximation of the possible efficiency of its utilization by children (Table 1.1).

The procedure, however, does not take into account differences in digestibility of proteins or the biological availability of amino acids. Also, whole egg proteins are atypical in that they are very high in sulphur-containing amino acids. Historically, in 1957, a modified term "protein score" was used in place of chemical score. This used a hypothetical reference protein derived from the human requirement pattern instead of egg protein as the reference standard. However, this also led to discrepancies, and in 1965, a new pattern and method of calculation, again based on egg protein was adopted but, cumbersome calculations were involved and many workers continued to use the "protein score" method. Finally in 1973, the F.A.O./W.H.O. suggested a new pattern which is still in use, based on the more recent evaluation of human amino acid requirements for children. This returned to the use of total nitrogen rather than total essential amino acids as the basis of comparison. In Table 1.2 is shown a comparison of the whole egg pattern and the reference scoring patterns for Lys, Trp and S-amino acids. The requirements for total nitrogen and essential amino acids decline with age, therefore, the "quality" of a protein must be related to the age of the subject who consumes it.

In this work, consideration of choice of methods and procedures is limited mainly to those that could be used for plant material such as cereals and legumes, but the criteria for selection are the same irrespective of the test material.

Table 1.1

Comparison of Nutritional Quality of

Whole Egg Protein Standard and Whole Wheat Protein

	Percentage Composition	
Amino Acid	Egg	Wheat
His	2.1	2.1
Tyr	4.5	4.4
Phe	6.3	5.7
Trp	1.5	1.2
Leu	9.2	6.8
Thr	4.9	3.3
Arg	6.4	4.2
Met & Cys	6.5	4.3
Val	7.3	4.5
Ile	8.0	3.6
Lys	7.2	2.7

For wheat: Amino acid in greatest deficit is lysine

Chemical score of wheat proteins = $\frac{2.7}{7.2} \times 100 = 37\%$

Data adapted from Satterlee et al., 1979.

Table 1.2

Reference Scoring Patterns

Amino Acid	Reference Pattern		
	Whole Egg	F.A.O. 1957	F.A.O./W.H.O. 1973
Lys	440	270	340
Trp	106	90	60
Total Sulphur amino acids	355	270	220

Data adapted from Pellett, 1978.

1-3 Chemical Methods

1-3-1 Introduction

Where straightforward qualitative and quantitative results for amino acids are required, chemical methods may be adequate. Many of the methods are indirect, and they may be useful in screening procedures to identify samples of interest, although they often require fairly large samples. Although results may need to be confirmed by nutritional evaluations, chemical methods in general are quick and can often be automated to permit a rapid throughput of samples. They often lack specificity and sensitivity and the content of say, Met or Lys, determined chemically does not always correlate with the nutritional value of the component. For example, chemical methods frequently involve an acid hydrolysis step which may liberate amino acid residues not released by normal digestion in vivo. These would therefore not be absorbed by the gut and utilised. This situation may arise with samples that have become chemically modified, by heat treatment or processing, that are often not susceptible to enzymic hydrolysis (Bjarnason & Carpenter, 1969; Ford, 1975; Ford & Shorrock, 1971; Mauron, 1972). Further discussion of heating effects is found in Chapter 6. (section 6-3).

On the other hand, acid hydrolysis may destroy or modify some residues such as Trp and Thr leading to underestimates of their true nutritional value; whereas antinutritional factors in the sample may impair utilisation and in this case chemical assays overestimate nutritional values. The complementary use of two chemical methods may overcome the limitations of either method used alone, but the advantage of assay speed and simplicity is then lost.

In summary, chemical assays may be satisfactory in many cases and are widely used, but results should be interpreted with great care since such results do not provide a reliable prediction of nutritional value. Some of the commonly used procedures are discussed in the next sections.

1-3-2 Total Nitrogen

Total nitrogen values are frequently used to obtain an estimate of protein content. The Kjeldahl method in its various forms is commonly used. Acid digestion of a protein liberates ammonia which can be determined by titration or colorimetry (A.O.A.C., 1975 and Reports of P.A.G., 1975; I.A.E.A., 1977). Variations in the method are frequently being published, in a recent paper (Ngonge & Murray-Ballance, 1982), a 40% increase in sensitivity over other colorimetric procedures was claimed by optimising reagent concentrations, temperature, pH and incubation time. The Dumas combustion method gives reproducible results in agreement with those from the Kjeldahl method, although it is generally less applicable; however, it can be fully automated and hence many samples can be handled daily. Other physical methods have been described, but the need for sophisticated, expensive equipment precludes their general use. Nitrogen values, however derived, are multiplied by a standard factor to convert to protein values. This factor can vary according to the protein source (F.A.O., 1973). It is 6.38 for milk, 5.3 for some legumes, 5.7 for cereals (Bender,1978); an average value of 6.25 is often used to convert from nitrogen to protein for many mixed food proteins.

Since it is not absolutely correct to convert amino nitrogen values to protein nitrogen by using the value 6.25 and then expressing amino acids as a percentage of protein (although this is often done), a common convention is to express each amino acid in grams per 16 gram total nitrogen. This is the same as percentage assuming a 6.25 conversion factor. Some authors quote grams amino nitrogen per grams protein nitrogen (Bender, 1978). It is apparent therefore that care should be taken in the interpretation of quoted data.

1-3-3 Total Protein

Of the many methods used for total protein, none is without limitations, generally through lack of specificity or by interference from non-protein components. For many assays, preparation of the sample is important, it must be finely divided to permit access of reagents. The Biuret reagent (alkaline $CuSO_4$) reacts with peptide bonds to give a coloured complex; a simple, quick reaction, useful for protein screening (Greenway & Davis, 1975). Certain dyes (e.g., Orange G), which bind to basic groups, give an indication of the content of basic amino acids (Lys and Arg), and if these are fairly constant in different proteins, the amount of bound dye is an indication of protein content (Almog & Berns, 1981; Simpson & Sonne, 1982). A dye-binding assay (BioRad Assay) has been routinely used here to evaluate protein concentration, and is described in Chapter 2.

A new competitive dye-binding method has been developed (Best et al., 1982), but this more complex procedure increases the assay time considerably. The widely used Lowry method (Lowry et al., 1951) depends on a reaction with tyrosine residues and hence is susceptible to interference and inaccuracies caused by variable tyrosine content. The method is reviewed by Peterson (1979).

A comparison of these methods is given in Table 1.3, from which it can be seen that the absolute values for any particular protein are affected by the assay method selected.

Turbidometric measurements of protein fractions extracted by different methods gives a quick, simple assay, although generally not of high accuracy (Paulis & Wall, 1975). Fluorimetric and radioisotopic methods, both for total protein and for specific amino acids have been developed, but the techniques are fairly complex (Goodno et al., 1981). An E.S.C.A. (Electron Spectroscopy for Chemical Analysis) method (Peeling et al., 1976), has been developed but it requires sophisticated equipment and skilled operators, although it has been shown to be rapid and sensitive.

1-3-4 Amino Acids

In conventional assays to measure the amino acid content of proteins, the samples are first hydrolysed. Although the hydrolysis may be acid, alkaline or enzymic, usually a strong acid (6M HCl) is used at 110°C for up to 24 hours in sealed evacuated tubes. Certain residues, especially Trp and to a lesser extent Ser and Thr, are destroyed. Met and Cys can only be quantitated if the sample is oxidised with performic acid to produce Met O_2 and cysteic acid. To determine Trp, alkaline hydrolysis at 120°C with barium hydroxide is necessary although this destroys several other amino acids. Both acid and alkaline hydrolysis may give rise to other chemical reactions in the sample and the non-physiological nature of the process is clear. Enzymic hydrolysis in vitro with proteases and peptidases approximates better to digestive processes but for complete digestion, a mixture of enzymes is required. Automatic amino acid analysis of hydrolysed samples are generally used (Blackburn, 1968; Longenecker, 1973).

Table 1.3

Comparative Protein Analyses (10 $mg.ml^{-1}$)

	Protein (mg/ml)		
Protein	Biuret	Lowry	Bio-Rad
Alcohol dehydrogenase	5.8	5.0	7.8
α Amylase	6.8	6.0	8.3
B.S.A.	9.7	8.4	21.1
Catalase	7.6	6.3	9.7
Ovalbumin	10.2	10.1	9.4
Fibrinogen	6.2	7.3	7.8
γ globulin (rabbit)	9.4	11.8	8.0
β galactosidase	9.5	9.9	7.9
Lysozyme	10.4	12.6	9.9
Myoglobin	13.7	7.9	20.7
Pepsin	9.8	12.4	4.1
Ribonuclease	11.8	15.9	5.3
Trypsin	11.4	15.5	4.9

Data quoted from Bio-Rad Laboratories, 1979.

However, they are fairly slow and are expensive to run for routine screenings; one recent estimate quoted $100 per complete amino acid analysis for a single sample (Skogberg & Richardson, 1979)! Quantitation is usually by colorimetry after reaction with ninhydrin, although greater sensitivity can be obtained with fluorescent derivatives using fluorescamine or σ-phthaldehyde (Steinhart, 1978). Gas liquid chromatography and HPLC may be used as alternatives to ion-exchange systems (Desgres et al., 1979; Tajima et al., 1978). Comparisons have been made between autoanalyser and other methods (Evans et al., 1976).

Whichever reference scoring pattern is used, it is found that for humans, Lys is the first limiting amino acid in about half of all protein foods, whereas Met and Cys are first limiting in about one third of foods (Table 1.4). Trp, and in some circumstances Thr, Leu and Ile are also important. Chemical assays that have been described for these particular amino acids include for Lys: (Booth, 1971; Carpenter, 1960; Carpenter & Booth, 1973; Concon, 1975; Finley & Friedman, 1973; Goodono et al., 1981; Holsinger & Posati, 1975; Kakade & Liener, 1969; Skogberg & Richardson, 1979), for Met: (Concon, 1975; Ellinger & Duncan, 1976; Evans et al, 1976; Lipton & Bodwell, 1975) and for Trp: (Concon, 1975; Friedman & Finley, 1971, 1975). Met is the amino acid with which most variation has been observed (Derse, 1969; Knipfel et al, 1975), and common problems encountered with its quantitation are discussed further in Chapter 5).

Table 1.4

Comparison of Limiting Amino Acids in Foods Calculated by Using the F.A.O./W.H.O. (1973) Pattern as Reference

Food	Chemical score	First Limiting amino acid	Second Limiting amino acid
Whole egg	100	-	-
Human milk	84.1	Met, Cys	Val
Cow's milk	94.5	Met, Cys	-
Casein	91.4	Met, Cys	-
Beef	100	-	-
Pork	100	-	-
Fish	100	-	-
Maize	49.1	Lys	Trp
Millet	62.9	Lys	Thr
Oats	68.2	Lys	Thr
Wheat	56.2	Lys	Thr
Wheat gluten	26.1	Lys	Thr
Bean (Phaseolus)	54.1	Met, Cys	Val
Rice (polished)	66.5	Lys	Met, Cys
Yam	75.3	Lys	Met, Cys
Groundnut	65	Lys	Thr
Pea	57.7	Met, Cys	Trp

(from L.R. Hackler, 1977. In vivo Indices: Relationships to Estimating Protein Value for the Human).

1-4 Biological Methods

1-4-1 Introduction

Bioassays are used to measure the efficiency of utilisation of dietary proteins as sources of essential amino acids under a set of standard conditions often comparing the "unknown preparation" with a reference standard. The biological response most frequently measured is growth. These assays do not provide absolute quantitative values of a protein or its individual amino acids but rather, they provide a measure of these components in terms of their nutritional effect. The methods were designed for proteins rather than for amino acid mixtures or peptides. These measures of protein quality are a function of the nutritionally limiting amino acid which determines the overall usefulness of the protein. Biological assays do not, therefore, yield information about other non-limiting amino acids and should not be taken as full description of a protein (Bender 1958).

Animals require amino acids to synthesise new tissues when growing and also for tissue maintenance and repair in adulthood. Recommended protein allowances have been calculated although these are open to debate: recommended (high quality) protein intakes have been suggested (F.A.O. 1973), as 1.5g Kg^{-1} (infants - 6 months); 1.0g Kg^{-1} (children - 5 years) and 0.5g Kg^{-1} (adults). Certain amino acids are essential (Lys, Thr, Trp, Phe, Met, His, Leu, Ile, Val and Arg), in that they cannot be synthesised in animal tissues (Heimann, 1980) and must therefore be supplied in the diet. Cystine can replace part of the requirement for Met and Tyr part of that for Phe, therefore, these must also be considered (National Academy of Sciences, 1980).

In the absence of one of these, net protein synthesis cannot occur and others will therefore be wasted. The other protein amino acids can be synthesised by the (human) body but it is usually the case that if a diet contains adequate essential amino acids it is likely to contain sufficient non-essential amino acids.

The nutritional quality of any protein is determined primarily by the quantity, availability and proportions of the essential amino acids it contains; and also, for optimum utilisation the presence of sufficient non-essential amino acids (Wolzak et al., 1981).

A high quality protein contains all of the essential amino acids in a utilisable form in amounts that match the average composition of the body protein. In general, an animal's nutritional requirements can be met as adequately by larger amounts of poor quality protein as by smaller amounts of high quality protein, although with the former source a larger proportion of protein nitrogen will be excreted as urea and more protein-carbon will be channelled into energy. Variations in the relative utilizability of a poor protein, a mixture of its free amino acids, or small peptides of the same composition, are well known.

Although bioassays have unique advantages they cannot always be applied for one or more of the following reasons: they take a long time to complete, require a large quantity of sample, and are expensive. Being slow and imprecise precludes their use for routine screening of large numbers of samples. The validity of extrapolating results gained with, say, the rat to man is a continuous point of debate and it is frequently argued that the proper species to use in the study of nutrition of man is man himself. However, such studies pose unique difficulties and may also raise ethical questions, especially if young children are used.

In biological assays, results may be influenced by so many factors that adequate controls are difficult to incorporate and reproducibility between laboratories is not easy to achieve. Even slight differences in sample preparation level and type of energy source, age and nutritional status of test animals, presence of other nutritional factors that may spare certain amino acids, or anti-nutritional factors that may limit digestion and/or absorption may all influence test results.

The ethical problems in using humans have been mentioned, and, in addition, it has been estimated (Satterlee et al., 1979) that to determine a nitrogen balance index (Section 1-3-2) using human subjects can take 45 days and cost $18,000 per sample; clearly, on cost limitations alone, human assays cannot be used on a routine basis for protein quality testing.

In biological assays, extensive data may be collected. Methods for statistical analysis of results and for determination of intra- and inter-laboratory variation in collaborative trials have been published (Puri & Mullen, 1980; Rudemo et al., 1979).

1-4-2 Amino Acids

The conclusions from studies on the biological response to free amino acids are frequently conflicting; reports that the response to amino acid mixtures corresponds to that on proteins (Ramasarma et al., 1949; Sauberlich, 1961) conflict with other studies that have shown amino acid mixtures to be nutritionally inferior to whole proteins of identical composition (Rose et al., 1954; Ahrens et al., 1966).

Many suggestions have been made to try and explain these differences. The most likely explanation seems to involve the difference in their absorption and thus temporal presentation for protein synthesis (Rose et al., 1954; Cannon et al., 1947;

Grey et al., 1968; Meister, 1965; Gitler, 1964; Matthews & Payne, 1975b; Silk et al., 1975). Thus, different amino acids are absorbed at different rates in the intestine; Met, Leu and Ile have high affinities and are rapidly absorbed, whereas Gly, Lys, Asp and Glu are slowly absorbed. Therefore, when present in amino acid mixtures the former reach peak concentrations in the portal blood first, leading to imbalanced presentation to the liver (Meister, 1965; Christensen, 1968; Matthews & Payne, 1975b). Nutritional imbalances and antagonisms contribute to the inadequacy of many protein sources; e.g. corn and sorghum proteins contain excess Leu which depresses Ile utilisation. However, if the protein content of the diet is replaced by a correctly balanced mixture of pure amino acids this is satisfactory for both growth and maintenance (Ranhotra & Johnson, 1965; Winitz et al., 1970; Belikov & Bobayan, 1971; Winitz & Adams, 1971). A detailed account of the development and uses of chemically defined diets is given by Greenstein & Winitz (1961).

1-4-3 Peptides

It has now been firmly established that intestinal peptide absorption not only occurs, but is probably nutritionally more important than amino acid absorption (Matthews & Payne, 1975a and b, 1980; Matthews & Adibi, 1976; Matthews, 1975, 1977; Das & Radhakrishnan, 1976; Reviews in C.I.B.A. Symposia 1972, 1977).

Peptides are also absorbed more rapidly and more extensively than free amino acids, and differences in uptake of individual amino acids are considerably reduced. It is logical to believe therefore, that synthetic diets containing peptides could be better than those consisting of free amino acids (Matthews & Adibi, 1976; Bassler et al., 1976; Clegg, 1978).

1-4-4 Amino Acid Availability

Amino acid residues that are utilised nutritionally are termed 'available'.Commonly, especially for certain amino acids (e.g., Lys, Asn, Gln, Asp and Glu), the amino acid content determined biologically is less than that determined chemically in complete protein hydrolysates. The difference between the two often represents the complement of unavailable residues arising from incomplete digestion or absorption (Carpenter & Booth, 1973; Erbersdobler. 1973, 1976; Ford, 1975; Silano, 1977).

Excessive heat or other processing methods can increase the proportion of unavailable residues by chemically modifying the proteins so that they become resistant to normal digestive enzymes (Finot et al., 1978; Ford & Shorrock, 1978). However, residues may be unavailable in natural protein foods, for example, proteases may be unable to digest proteins physically enclosed within organelles or cell walls. Protease inhibitors may also occur naturally in the sample. Untoasted soya contains a high anti-tryptic activity; soya meal may contain a haemagglutinin which has a negative effect on growth. High levels of dietary carbohydrate may influence protein evaluation. Finally, non-protein amino acids or modified peptide fragments may competitively inhibit absorption of protein amino acids or normal peptides, respectively (Erbersdobler, 1973, 1976).

1-4-5 Biological Assays

For general information on biological assays see, for example (Bigwood, 1972; Bodwell, 1976; Cole et al., 1976; Friedman, 1975; Reports of I.A.E.A., 1975, 1977; and P.A.G., 1975). Collaborative trials using a variety of methods and a variety of test proteins have been reported (Boyne et al., 1967, 1975; Carpenter & Woodham, 1974).

All workers agree that: "the need for rapid (bio) assays of protein quality is obvious" (Adams, 1974). There has been much discussion on the choice and suitability of different test animals and the present concensus indicates that the rat is the most useful (Dreyer, 1973; Eggum, 1973a; Pellett, 1973), inspite of Rose's classical work (1937) that established that the requirement of the weanling rat for Lys is extremely high.

Bioassays for protein quality are constantly being revised and adapted, and data are being accumulated for various food proteins tested by various methods. At present, no bioassay method is completely satisfactory for all samples and all requirements (McLaughlan & Keith, 1975). Procedures to speed up the assays, without loss of accuracy, are constantly being investigated.

Biological methods fall into three categories: (a) growth methods; (b) nitrogen balance methods; (c) indirect methods. These will be discussed in outline only, and some of the more commonly used adaptations will be referenced.

1-4-5-1 Growth Methods

These are usually based on weight gain and for all of these methods strictly standardised conditions are essential. The most commonly used procedure is PER (Protein Efficiency Ratio); where

$$\text{PER} = \frac{\text{Mean weight gain of test group}}{\text{Weight of protein consumed}}$$

Rats are generally used and they are given the protein under test as their sole protein source (about 10% of diet) for 10 - 30 days, during which time other foods are supplied ad libitum.

Results vary, however, with the exact proportion of dietary protein and with food intake (Hegsted, 1971; Hurt et al., 1975; Jacquot & Peret, 1972; McLaughlan & Keith, 1975). Results are fairly reproducible between laboratories, especially if casein is fed as a standard. PER values range from 4.5 for highest quality proteins to zero for those that support no growth (although such proteins may have some nutritional value if they complement other dietary proteins).

To eliminate variations in PER caused by food intake and to allow for protein required for maintenance, a modified PER, known as NPR (Net Protein Ratio), may also be used (McLaughlan & Keith, 1975). In a further modification called the slope ratio method (Report of P.A.G., 1975), change in body weight of rats fed various levels of test protein or reference protein is measured, and this is said to give a better estimate of the value of poor quality proteins. Batterham (1979) compared the results obtained with slope ratio methods using pigs and rats with the results of chemical assays for Lys.

All growth methods assume that protein synthesis and body weight changes are directly related (Deltort-Laval, 1976). If this is not the case, direct growth methods cannot be accurate but nitrogen-balance procedures could be more useful.

1-4-5-2 Nitrogen-Balance Methods

Nitrogen balance is the difference between dietary nitrogen intake and total nitrogen excretion. For successful application of the approach, it is essential that all experimental conditions are standardised. The procedures have the advantage that experimental times are less than for weight-gain methods and more detailed information is provided, including protein digestibility and utilization, but they are more laborious than growth methods.

(Eggum, 1973a; McLaughlan & Campbell, 1969). To simplify the nitrogen determinations that are necessary, an indigestible marker such as titanium oxide may be added to the diet. One useful indicator, apparent digestibility (AD), varies with the protein content of the diet but it may act as a useful index and it is claimed (Njaa, 1977) that reliable results can be obtained by analysing small samples of faeces even after one day.

$$AD = \frac{N_2 \text{ intake} - \text{Faecal } N_2}{N_2 \text{ intake}} \times 100$$

A further modification, true digestibility (TD), corrects for the excretion of metabolic (endogenous) nitrogen by using a control group fed a protein-free diet, and hence has the advantage of being independent of dietary protein content or body weight of the experimental animal (Eggum, 1973a; Slump & Van Beek, 1975).

Biological value (BV) is also determined by a nitrogen-balance procedure; BV may be considered to be the fraction of absorbed nitrogen retained in the body for maintenance and growth.

$$BV = \frac{N_2 \text{ intake} - (\text{faecal N} - \text{metabolic N}) - (\text{urinary N} - \text{endogenous N})}{N_2 \text{ intake} - (\text{faecal N} - \text{metabolic N})}$$

$$\times 100 = \frac{\text{Retained N}}{\text{Absorbed N}} \%$$

The numerator represents the total nitrogen used both for growth and maintenance, and hence BV represents the percentage of digested nitrogen that is actually utilised; it therefore provides a measure of efficiency of the absorbed protein. It is a specific term and not synonomous with nutritional value. A protein of high BV that is poorly digested would have little value. One of the best indices of 'usefulness' is Net Protein Utilisation (NPU) since this makes allowance for both amino acid composition and digestibility.

$$NPU = \frac{TD \times BV}{100}$$

Individual foods have NPU values ranging from 1.0 to zero, an overall NPU of 0.5 is considered to represent an exceptionally poor diet. Both BV and NPU are influenced by levels of dietary protein; BV decreases significantly with protein uptake (Deltort-Laval, 1976; Eggum, 1973a; Hegsted & Juliano, 1974; McLaughlan & Campbell, 1969; McLaughlan & Keith, 1975). This arises partly because amounts of protein required for maintenance and growth in the rat are quite different - maintenance levels for Lys and Leu are low. Two proteins with different Lys concentrations could give similar responses near maintenance levels but could show wide variation at high intake levels. Proteins devoid of Lys may still have high NPU values (Bender, 1961; McLaughlan & Campbell, 1969). The latter authors calculated that it would be possible for a protein having an amino acid composition ideal for maintenance to have a chemical score of only 17 for growth.

Further discussion on these and other terms used in assessment of protein quality may be found in Bender (1973) and in Pellett (1978).

1-4-5-3 Indirect Methods

These involve measurements of such things as amino acids and urea in blood (Eggum, 1973b, 1976) or muscle (Pion, 1973); urinary creatine (Eggum, 1975); ribosome activity (Eggum, 1978), and the activity of particular liver enzymes (Bergner, 1978). They are not generally considered reliable and none has been generally adopted (Njaa, 1977).

1-4-6 Enzymic Methods

These are not conventional biological methods; they involve digestion *in vitro* with proteases and assay of the resulting hydrolysates. See references and reviews (Bodwell, 1977; Marable & Sanzone, 1981; Mauron, 1970a and b, 1973; McLaughlan & Campbell, 1969; Morrison & McLaughlan, 1972; Sheffner, 1967; Stahmann & Woldegiorgas, 1975). The pepsin digest residue (PDR) compares the pattern of amino acids released *in vitro* from a protein hydrolysate using pepsin with the amino acid pattern of the residual protein (Sheffner, 1967). It was found that PDR correlated fairly well with NPU for various proteins and was of value in detecting the effects of heat damage. Because of the limited digestion achieved by pepsin alone, a pepsin-pancreatin digest index (PPD) was introduced (Akeson & Stahmann, 1964). Later modified, it was compared with results from animal assays (Mauron, 1970a and b, 1973).

Individual enzymes may be useful for specific assays of free amino acids, e.g., lysine-decarboxylase, or arginase (Wall & Gherke, 1974), methionine γ-lyase (Tanaka *et al.*, 1980), lysine-α-oxidase (Kusakabe *et al.*, 1979). Many of these assays involve measuring colour released from a colorimetric substrate. Often enzymic methods are complex. Bodwell (1977) stated that: "despite the theoretical advantages of using estimates of nutritional value derived from enzyme assays relative to indices calculated from amino acid composition data, the information available does not indicate that significantly more valid information regarding actual values is consistently obtained through the use of more laborious enzymic methods"; this summarises the generally held views on these techniques.

pH-Stat Methods (Bodwell, 1977; Payne et al., 1977), that can be used to monitor the rate and extent of proteolysis in vitro are described briefly elsewhere (Section 2-8).

1-5 Microbiological Methods

1-5-1 Introduction

The underlying concept of any biological assay is to compare an unknown preparation with a reference standard (Section 1-3-1). In many cases, for example, in the assay of certain vitamins and antibiotics, a microbiological assay is the only means to assess potency (Hewitt, 1977). The methods frequently provide comparable speed, reliability, accuracy, potential for handling multiple samples and for automation as do chemical methods, whilst retaining certain of the inherent advantages of biological methods using higher organisms.

Many species, including bacteria, fungi and ciliate protozoa have been used in assays for specific amino acids, the composition of protein hydrolysates and the RNV of proteins. Methods normally rely on the principle that the test microorganism has a growth requirement for the substance being assayed, and a substrate-dependent response is measured. A parallel can be drawn between the role of microbiological assays in protein research and the development of assay techniques for vitamin research in food, feed, clinical material and metabolic studies (Adamson & Simpson, 1959). The methods are varied and it is perhaps not suprising therefore, that whereas some give results in close agreement with those from animal assays (Boyne et al., 1975; Henry & Ford, 1965) others do not (Bunyan & Price, 1960). However, all methods were able to differentiate good, high quality proteins from poor, low quality ones and this distinction may be all that is required in an initial screening programme.

This approach again poses the fundamental question of relevance of results of protein nutritional quality derived from response of a microorganism to those obtained with the rat, and indeed to human response. Other potential problems are that the response of a microorganism to a particular nutrient may not always be reliable since it may be influenced by the form of the specific nutrient (free amino acid or peptide bound); by the quality of the nutrient present and by the organism's nutritional status and growth phase. Furthermore, many of the nutritionally fastidious species used for microbiological assays have complex and often not fully defined growth requirements.

However, using carefully chosen microbial species, those assays have many intrinsic advantages, although they are not always being fully exploited in most current procedures. Many reviews have been published concerning microbiological assays, including: (Barton-Wright, 1952, 1963; Bolinder, 1972; Ford, 1960; Hutner et al., 1958; Kavanagh, 1960, 1963, 1972; Shockman, 1963).

1-5-2 Procedures For Assay

1-5-2-1 Introduction

The common methods are plate assays (agar diffusion) and tube methods. Both involve comparison of a quantitative effect of the test sample and a standard on the growth of a specific microorganism. In antibiotic assays, the measured effect is commonly inhibition; for amino acids it is growth promotion. The ideal requirements for determining growth promoting substances have been discussed elsewhere (Payne & Tuffnell, 1980). The choice of organism and method are interdependent, and by appropriate selection of microorganism and method, considerable specificity can be obtained.

1-5-2-2 Plate Assays

Plate assays cannot be used for test samples until a standard has been established (Gavin, 1957a). Although there is considerable variation in detail, a typical procedure involves preparing sterile molten agar (cooled to about 42°C), adding the test organism and uniformly dispersing it, then pouring a suitable volume (15-20 ml) into a petri dish to give a uniform layer. Solutions of test and standard substances are applied to the solid agar either into cut out reservoirs, or onto small filter paper discs, or into the centre of porous ceramic beads; this may be automated (Sykes & Evans, 1975). After about one hour in the upright position, during which the solution diffuses into the agar, the plates are incubated overnight. After incubation, a turbid growth zone occurs around the test solution, and a quantitative measure of nutrient concentration is obtained by measurement of the diameter of the zone boundary. Conditions must be standardised since a variety of factors may influence sharpness, formation and size of the zones of growth. However, in general, the method is perfectly acceptable and attempts to overcome variation in response within and between plates led to the development of large plate assays that also find some applications. Typically, these 30 cm^2 glass plates, can take 64 samples which can be applied in a randomised or quasi randomised (8 x 8) latin square design. Detailed consideration of the practice and theory of plate assays have been published, see for example; Adamson & Simpson, 1959; Bolinder, 1968a, b and c, 1969, 1970a, b, c, d, e, and f; Cooper, 1963, 1972; Gavin, 1957a; Hewitt, 1977; Lees & Toothill, 1955a, b and c. Bolinder (see above), has used various strains of Lactobacilli extensively to assay for Met, Trp, Arg, Tyr, His, Lys and Cys.

Lactic acid strains have also been used by other workers including Kojima (1965). Auxotrophs of Escherichia coli have been used in plate assays for Asn (Sköld & Wallenberg, 1969); Phe (Dickinson, 1959; Jones & Burns, 1958); Lys (Gol'dfarb et al., 1968) and Trp (Konova & Verbina, 1966).

Bacillus subtilis has been used in a screening test for detecting the incidence of phenylketonuria and for other amino acids (Guthrie & Susi, 1963; Guthrie, 1969a and b). Care must be taken in using this organism since its physiological state (vegetative or spore) profoundly influences the size and formation of growth zones.

Clearly, plate assays are useful for screening large numbers of samples, especially if the quantitation of labile amino acids is of interest, but care must be taken in interpretation of results. It has been claimed (Sotelo & Sousa, 1976), that using the disc method to assay for Lys and Met in protein hydrolysates using Leuconostoc mesenteroides, a high linear correlation ($r = 0.999$) was obtained between the disc and acidometric methods.

Diffusion assays are rapid, readily mechanised and sterile samples are not essential, thus preventing loss of activity caused by heat treatments. This makes them particularly useful to assay for labile amino acids such as Trp.

1-5-2-3 Tube Assays

Tube assays are the most common way to determine amino acids. Graded concentrations of test solution are added to a series of test tubes containing liquid growth media, after inoculation with test organism and incubation for the appropriate time the nutrient-dependent growth response can be measured, usually within 16 to 48 hours.

The method has greater sensitivity and is easier to carry out than the plate method but it is generally necessary for the sample to be sterile, and it tends to be more susceptible to interference by extraneous substances than plate assays. These various methods have been reviewed (Kavanagh, 1963; Hewitt, 1977).

The most usual ways to estimate growth is by turbidity measurements using a suitable photometer. The cell concentration can be measured by nephelometry (measurement of light scattered by the cells), or by absorptiometry (measurement of proportion of light transmitted through the suspension). The latter is the more convenient method and is more extensively used. Wavelengths from 400 - 750 nm have been used; the choice being dictated by the need to minimise interference or to enhance sensitivity - which increases as wavelength decreases (Shockman, 1963). The standard response curve has been said to be curvilinear throughout its length (Barton-Wright, 1952), but turbidity measurements are generally plotted in the form that gives the best linear relationship (Gavin, 1957b; Hewitt, 1977). Statistical procedures can be used to determine precision and accuracy (Hewitt, 1977), and these are generally superior to those obtained when titration is used as an alternative to measure growth response (Boyne et al., 1967). However, where the extract is glutinous, turbid or coloured, growth cannot be assessed photometrically and titration methods may become necessary; for example Lactobacilliaceae produce acids during growth that can be measured by titration against an alkali. However, the procedure is more time consuming and less reliable than optical growth measurements. Unfortunately, growth and acid production do not parallel each other, with maximum growth being reached before maximum acid production, and extended incubation times (about 72 hours) are needed to optimise titration results.

Ford (1960) has suggested that to increase accuracy and reproducibility lactic acid, not total acidity, should be determined, although it is the latter that is usually found.

The microbial assays described are generally capable of grading protein-rich samples in a similar order to that obtained using rat or chick bioassays. Exceptions to this may occur however. For example, when the test protein originates from certain plants which could contain toxic constituents. Also, unless a microorganism is powerfully proteolytic, it may not be able to fully utilise an intact protein. Even with powerfully proteolytic strains, additional enzymic digestion of the test sample *in vitro* is often necessary to speed the assay and improve linearity and reproducibility of the dose-response curves (Ford, 1960, 1962, 1964).

1-5-3 Other Methods

1-5-3-1 Enzyme Activity

As the extent of growth of an amino acid auxotroph of *Escherichia coli* is directly dependent on the amount of required amino acid added (Payne *et al.*, 1977), it can be speculated that the amount of synthesis of certain enzymes may also be related to the added amino acid. Under prescribed conditions, enzyme activity is linearly related to enzyme concentration and in principle, therefore, it becomes possible to determine the amount of enzyme synthesised, and thus to assay the amino acid from the enzyme activity. A preliminary account on this approach was published (Bell *et al.*, 1977), and this thesis deals with a detailed study of this method.

1-5-3-2 Radio-Active Incorporation

This is similar in principle to the enzymic method described above. The amino acid-dependent protein synthesis is measured in a double (for example Pro^{-} Lys^{-}) or multiple auxotroph of Escherichia coli. After starvation for the required amino acid, the bacteria are added to tubes containing a large excess of one amino acid (e.g., Pro), which is radioactively labelled, plus test samples containing limiting concentrations of, say, Lys. On incubation, for about thirty minutes, proteins are synthesised to an extent dependent on the amount of added Lys. The degree of protein synthesis is measured from the amount of labelled Pro incorporated. If the diverse proteins synthesised contain an overall fixed Pro:Lys ratio, Lys can be assayed by measurement of the incorporated Pro (Bell et al., 1977).

Whereas this method and the enzyme activity method described in the previous section (1-5-3-1), each, offer a novel approach to evaluation of amino acids in protein digests, the enzyme method has been investigated here because we feel that it is generally more useful. No radioactively-labelled amino acids are needed and sophisticated apparatus to measure incorporation of a labelled amino acid into the microbial cell is not required, obviating the requirement for sophisticated scintillation equipment.

1-5-4 Test Organisms

1-5-4-1 Introduction

In the preceding discussions on microbiological assays, emphasis has been placed on the comparative ease, reliability and application of the procedures. Because the correct choice of method and microbial species is essential, it is also pertinent to consider some of the assay organisms that are commonly used.

Most of the widely-used organisms are "natural" amino acid auxotrophs which require a variety of amino acids, and possibly other growth factors; Lactobacilliaceae, Streptococcaceae and Leuconostoc species have been widely used (Guirard & Snell, 1962). Several authors have discussed the nature of the organisms used for amino acid assays (Barton-Wright, 1952; Bolinder, 1972; Gavin, 1956; Shockman, 1963).

Some of the organisms that have been used traditionally may not necessarily be the most ideal. It should now be possible with increased knowledge on microbial nutrition and of transport and utilisation of amino acids, peptides and proteins, to select more suitable strains by genetic manipulation. Amongst the most desirable features that such strains should have are the following:

(a) The organism's nutritional requirements should be clearly understood.

(b) The response to a given substrate should be specific and not influenced by other components in the sample.

(c) Cultivation should be relatively simple and reliable.

(d) The organism should be non-pathogenic.

(e) The organism should have stable nutritional requirements.

(f) The organism should be sensitive to low levels of the required substrate and its response should be measurable with ease, accuracy and reliability.

(g) The response of an organism to a given sample should correlate well with results from other biological assays.

Cultures of many useful assay organisms can be obtained from the National Collection of Industrial Bacteria (N.C.I.B.), Torry Research Station, Aberdeen, and that organisation is undertaking a programme to check the suitability and sensitivity for microbiological assay of organisms held within the collection.

A brief description of some of the more frequently used assay bacteria is given below.

1-5-4-2 Lactic Acid Bacteria

Various strains of Lactobacillus, Leuconostoc, Streptococcus and Pediococcus have been used in plate and tube assays for many different amino acids. Commercial growth media are available, being specially formulated for use in amino acid assays with these organisms (see Difco Manual 9th edition 1953), although many workers have prepared their own media. The nutritional requirements of these organisms are not fully understood; they are known to require between one and sixteen amino acids, they may need one or more vitamins, bases and nucleic acid precursors and they utilise only a few simple sugars (Barton-Wright, 1963; Bridson & Brecker, 1970; Rechcigl, 1978).

As a result, suggestions are frequently made for improved media. Barton-Wright (1972) suggested the use of a more flexible basal medium modified from one of his earlier media (Barton-Wright, 1952). The cultivation of these organisms, therefore, needs great care and for most of the common assay strains these methods are well documented (Block & Weiss, 1956; Bolinder, 1972; Ford, 1960, 1962, 1964, 1965, 1977; Shorrock, 1972).

The intrinsic problem in using the lactic acid bacteria is that variations in a medium can change the growth response to a particular amino acid. This is well documented for the vitamin supplements in a medium and the topic is discussed in papers by Guirard & Snell (1962); and Matthews & Payne (1975b). A related problem, frequently not considered, is that the growth response to peptides containing an essential amino acid residue may not be the same, (it could be more, or could be less), as the response to an equivalent amount of free amino acid. (Guirard & Snell, 1962; Kihara & Snell, 1960a and b;

Law, 1978; Matthews & Payne, 1975a; Payne, 1980; Prescott et al., 1953; Rice et al., 1978. A series of papers by Bolinder describes the effects of various nutrients, including peptides, on amino acid analyses by Streptococci as measured by large plate assays (see Review in Bolinder (1972).

The presence of uncharacterised nutrients in the test sample, and continuous variation in relative concentrations in the medium during batch culture, may explain the lack of linearity between growth response and amount of test sample that is frequently observed. Interferences caused by substances related to the amino acid being assayed are not uncommon, for example, the stimulatory effect of hydroxylysine on the growth response of Leuconostoc mesenteroides P60 to Lys has been well documented (Carpenter et al., 1964; Hartley et al., 1965).

There seems to be some disagreement regarding the stability of the growth requirements of lactic acid bacteria, and this is not surprising since stability can only be established with exact certainty when growth requirements are known. Certainly growth requirements have been reported as changing on repeated subculture in the laboratory (Bolinder, 1970f, 1972; Hartley et al., 1965; Sheckleton & Haynes, 1959). However, the lactic acid bacteria have the important advantage that they are non-pathogenic, and it has been suggested therefore, that microbiological assays for Met with Streptococcus zymogenes are safer than an alternative chemical test (Ford, 1981).

Collaborative trials have been conducted to assess the applicability of chemical, microbiological and biological assays for protein quality in animal feedstuffs. Useful correlations were found between protein values for rats and chicks and those for Tetrahymena, Streptococcus zymogenes and Streptococcus faecalis and they showed the useful potential of these organisms. Subsequently, emphasis was placed on the measurement of available amino acids, in particular the assay of available Met with Streptococcus zymogenes (Ford, 1962).

The use of lactic acid bacteria has thus become fairly widespread and these microorganisms have much to recommend them (Bunyan & Woodham, 1964; Boyne et al., 1961, 1967, 1975; Carpenter & Woodham, 1974; Evans et al., 1976; Ford, 1977; Haenel & Kharatyan 1973; Henry & Ford, 1965). Tennant (1979) described a method for determination of Met in serum using a chloramphenicol-resistant strain of Pediococcus acidilacti.

1-5-4-3 Escherichia coli

As stated previously, this thesis is concerned with assays using Escherichia coli as the test organism and consideration of reasons for the choice and potential usefulness of this organism are discussed elsewhere (Sections 1-5-2-2, 1-5-3-1, 1-5-3-2 and Chapters 2 and 3).

1-5-4-4 Other Bacteria

Many species of bacteria have been used, but none of these has been widely accepted. These include Bacillus subtilis for determining Phe and Trp (Guthrie & Susi, 1963; Guthrie 1969a and b). Chromobacterium violaceum has also been used to assay for Trp by using a disc diffusion method (Sebek, 1965)

Various species of Pseudomonas have been used but they show a wide variation in response to peptides and their equivalent amino acids (Cascieri & Mallette, 1976), this makes them generally unsuitable. When using the latter organisms there is also the problem of pathogenicity. This problem also arises with the strongly proteolytic organism Clostridium welchii (perfringens); and its advantages are far outweighed by its potential danger as a pathogen frequently implicated in cases of food poisoning. Ford (1964) showed that unaccountable variation from one assay to another was common. Surprisingly, it has recently been used (Solberg, 1979), in a rapid protein quality procedure and the authors ignore possible dangers and state that the method warrants further development!

1-5-4-5 Yeasts

Although Saccharomyces fragilis was used to assay β-Ala by a large plate method (Ericson & Carlson, 1953), in what was the first report on the quantitative assay of amino acids by this method, yeasts have subsequently proved to be less useful for amino acid assays than for vitamin assays. Initial studies with yeasts are reported by Adamson & Simpson, 1959; Hutner et al., 1958; Shockman, 1963.

1-5-4-6 Other Fungi

Fungi are unlikely to be widely used in amino acid assays because they have the disadvantages of slow growth, unreliability arising from complex, poorly understood responses to nitrogen and various nutrients in the medium, and lack of sensitivity. However, they are relatively easy to cultivate and their biomass can be determined accurately.

Ryan (1946) suggested that measuring the rate of germination of conidia was preferable to biomass measurement. Regnery (1944) used a Leu-requiring mutant of Neurospora to assay Leu in protein hydrolysates. Brand et al., (1945) used a Pro-requiring mutant in the same way. It has been claimed (Hodson & Krueger, 1947), that by using this proteolytic mould, direct assays can be performed on food-stuffs. More recently, Moyhuddin et al., (1976, 1977) described assays based on biomass determination for evaluating protein quality of cereals and legumes using Aspergillus flavus and a Lys-requiring strain of Neurospora crassa. Particular emphasis on the advantages of fungal assays is presented by these authors.

1-5-4-7 Protozoa

There is considerable literature concerning the use of the ciliate protozoan Tetrahymena pyriformis in assays to measure protein nutritional quality based on the early findings that the organism is strongly proteolytic (Lawrie, 1937), readily uses intact protein as the sole source of amino acids (Rockland & Dunn, 1946), and requires a similar pattern of amino acids as the growing rat (Kidder & Dewey, a 1951). It is also non-pathogenic and has the unique advantage that particulate denatured proteins can be used directly (Orias & Rasmussen, 1979). Inspite of these advantages, Tetrahymena pyriformis is not widely used. One problem could be the reluctance of microbiologists to handle what is often an unfamiliar organism which needs complex media (Shorrock & Ford, 1973), and the procedures are more complicated than those for handling bacteria. Secondly, the incubation periods are often at least four days and hence, bacterial contamination and autodigestion can be a problem.

Finally, and perhaps most important of all, there is no simple way to assess growth of protozoan cultures especially when they contain particles of test foods in suspension. Methods that have been tried include the following:

(a) Acid production over 41 days incubation (Rockland & Dunn, 1946, 1959).

(b) Colorimetric measurement of the enzymic reduction of added triphenyltetrazolium dye (Anderson & Williams, 1951) - this measurement has been modified and used by several workers (Ford, 1960; Kaestner et al., 1976).

(c) Ammonia production (Fernell & Rosen, 1956; Rosen & Fernell, 1956).

(d) Cell count methods; these methods are tedious and slow, and the problem caused by food particles has been mentioned. However, cell count methods are widely used (Landers, 1975; Rolle, 1976; Srinivas et al., 1975) and various photographic adaptations have been described (Shorrock, 1972; Shorrock & Ford, 1973).

(e) Elutriation to separate food particles and organisms followed by the use of a Coulter-counter has been suggested (Teunisson, 1971a; Evancho et al., 1977). More recently, Satterlee et al., (1979) described a method wherein the cultures are counted electronically after 24 and 66 hours of incubation. The first value being the measure of interfering particulate material which was subtracted from the final count. However, this method would only be satisfactory if the number of food particles in the test culture remains unchanged throughout the subsequent incubation, and this seems unlikely.

(f) Gas-liquid chromatography of tetrahymanol, a terpene synthesised by the organism has been used as a growth index (Shepherd et al., 1975), as has production of 2-aminoethylphosphonic acid (Maciejewicz-Rys & Antoniewicz, 1978).

(g) Methods based on extinction measurements using test-samples clarified by predigestion with proteases in vitro have also been proposed (Frank et al., 1975; Shorrock, 1976; Shorrock & Ford, 1973). Enzymic predigestion is considered necessary prior to the assay so this method is promising if the sample under test is high in protein.

It is clear from the abundance of methods listed that there is a major problem in quantitating results from Tetrahymena assays, especially when low protein materials are tested. No method seems to have the ease, speed and precision necessary for a rapid throughput of many samples.

Other problems encountered in assays using Tetrahymena arise from lack of understanding of its nutritional responses. Its amino acid requirements are fairly well understood, although the levels needed are often influenced by the composition of the medium (Kidder & Dewey, 1951). Recent advances have been made in genetically characterising the organism, in particular in the selection of mutants (Bruns et al., 1976; Hepfer & Kaney, 1976; Orias & Bruns, 1976). Competition for uptake of structurally related amino acids is known to occur (Rasmussen, 1976). A requirement for Tyr has recently been noted (Zdanowski & Rasmussen, 1979a), and shown to occur from imbalances in the culture medium.

Other nutrients may affect the growth response to a required amino acid; for example, glucose and dextrin are more stimulatory than starch (Stott et al., 1963; Reynolds, 1970) and fatty acids have been reported to inhibit (Rosen & Fernell, 1956) or not to inhibit (Landers, 1975) the growth response.

A mutant strain of Tetrahymena thermophila that has a heat-sensitive development of its oral apparatus, such that it forms food vacuoles as normal at 28°C, but none is formed at 37°C, has been used to study the relative capacity of free amino acids, proteins and peptides to satisfy requirements for essential amino acids. Using Phe and Leu (both essential), Orias & Rasmussen (1979) showed that these do not need the oral system but are absorbed via the plasma membrane. Egg albumen however, was only taken up by cells with a functional oral apparatus. Shorrock & Ford (1973) noted that during early growth stages, free amino acids are utilised in preference to protein-bound ones; this could be caused by incomplete development of the oral apparatus. Proteolytic enzymes, therefore appear to be mainly associated with the food vacuole and those in the culture medium are unimportant (Viswantha & Liëner, 1956b). Thus, together with the finding that extracellular proteases are unable to hydrolyse egg albumin (Orias & Rasmussen, 1979), emphasise the importance of an enzymic predigestion step before protein foods are assayed.

The nutritional response to peptides has been examined. Zdanowski & Rasmussen (1979b) reported the presence of surface-bound peptidases, although 85 per cent of the measured peptidase activity is intracellular. There is evidence that peptides are cleaved prior to uptake and therefore the food vacuole is not involved. Further studies are necessary to clarify full nutritional response to amino acids, peptides and proteins.

It seems unlikely, in view of the practical problems encountered that assays with Tetrahymena will ever find extensive use, especially when alternate microbiological assays are available. The following references compare results obtained using Tetrahymena and other methods: Boyne et al., 1961, 1975: Evancho et al., 1977; Frank et al., 1975; Haenel & Kharatyan, 1973; Helms & Rolle, 1970; Landers, 1975; Rolle, 1976; Rolle & Eggum, 1971; Shorrock, 1976; Shorrock & Ford, 1973.

1-5-5 Concluding Remarks

For any microbiological assay aimed at detecting other than gross differences in nutritional quality, careful choice of the assay organism and method is necessary; no one method is ideal for every food and every situation. For the formulator of animal feedstuffs, the practical problem is to evaluate proteins as sources of individual amino acids quickly, and reproducibly, so that they can be used most economically and effectively in mixed diets. The measurement of available amino acids is therefore of prime importance, bearing in mind that the difference between an available and a non-available residue relates only to the particular set of assay conditions.

One important area that has been largely omitted from this chapter is a discussion of the preparation of the sample prior to assay. Where an enzymic pre-digestion step is used, the choice of enzyme and digestion conditions are important. For example, some cereal-based products containing protein rich in glutelin is only soluble at high pH and therefore better digested by alkaline protease. However, other considerations may be paramount, for example, the degree of grinding of the test sample needed to make it accessible to the digestive enzymes, may have a profound influence on the result.

However, microbiological assay methods have unique advantages giving them an important role in measuring the biological availability of individual amino acids in a variety of foods and feeds.

The use of one particular microorganism, the gut bacterium Escherichia coli, to assay for three essential amino acids Lys, Met and Trp is the subject of the remainder of this thesis.

CHAPTER TWO

GENERAL METHODS

2-1 Introduction

Methods which were used routinely during this work are described in this chapter.

2-2 Organisms And Growth Conditions

2-2-1 Organisms

The main strains used originated from Escherichia coli W (ATCC 9637). The lysine auxotroph M2626 and its wild type revertant M2626 Lys^+, PA0101 (Payne, 1968), were laboratory strains. Mutants were isolated from M2626 by using nitrous acid mutagenesis (Section 4-2-2), auxotrophs for Lys and Met, or Lys and Trp were designated by us PA0111 and PA0110, respectively.

A Met auxotroph of Escherichia coli W (ATCC 10799, NCIB 8134), originally isolated by Davis & Mingioli (1950), was obtained from the National Collection of Industrial Bacteria, Aberdeen; this has been used extensively for Met assays. The Escherichia coli K12 strain, 4258 Arg, Leu and His was also used (Low, 1972). See Table 2.1 for list of strains used.

2-2-2 Growth Conditions

The Lys auxotroph M2626 was grown routinely in a minimal salts A medium (Davis & Mingioli, 1950), as described by Payne (1968). This was made at ten times strength and diluted with distilled water as required. The 10X medium contained: dipotassium hydrogen phosphate (70 g l^{-1}), potassium dihydrogen phosphate (30 g l^{-1}), magnesium sulphate - hydrated (1 g l^{-1}) and ammonium sulphate (10 g l^{-1}). 0.5% w/v glucose and 0.1 mM lys were added aseptically (final concentrations), to single strength medium.

Table 2.1

Bacterial Strains

	Our Designation	Auxotrophic Requirements	Notes
M2626		Lys	From *Escherichia coli* W
M2626 Lys$^+$	PA0101	-	Revertant of above strain
	PA0111	Lys, Met	Isolated from M2626 after nitrous acid mutagenesis
	PA0110	Lys, Trp	
ATCC 10799) *	-	Met, B_{12}	Davis & Mingioli (1950) *
NCIB 8134)			
ATCC 4258	-	Arg, Leu, His	From *Escherichia coli* K12 Low (1972)

* This strain was designated 113-3 by Davis & Mingioli (1950).

Bacteria were routinely grown in 25 ml volumes contained in 100 ml conical flasks at 37°C in a water bath and aerated with shaking at 120 strokes min^{-1}.

Bacterial stocks were maintained on agar slopes made from the minimal A medium as described above, but containing 1% w/v glucose, solidified with 1.5% w/v agar and containing excess (0.5mM) of the required amino acids. Slopes were stored at 4°C and the organisms subcultured every two months.

For β -galactosidase assays, cells were grown in A medium as described but with 0.5% v/v glycerol as the carbon source.

2-2-3 Measurement Of Growth

Growth was monitored by measuring the extinction (at 660 nm) of a portion of the culture using Bosch & Lomb Spectronic 20 or Pye Unicam 1800 or Pye SP8-150 spectrophotometers, with a 1 cm diameter tube or a 1 cm path length cuvette, respectively. A linear relationship between cell number and extinction up to an E_{660} of 0.6 (measured on the Spectronic 20) was shown.

Measurements of dry weight and comparison of the spectrophotometers gave the relationship: E_{660} (Spectronic 20) 0.10 = E_{660} (SP 1800) 0.22 = 0.0457 mg dry wt. ml^{-1}. During final stages of the work, a Pye Unicam SP-150 was used, for which the relationship was E_{660} (SP 1800), 0.1 = E_{660} (SP8-150), 0.11.

2-2-4 Harvesting Organisms

(a) Membrane Filtration

Organisms for amino acid assays, for fluorescence assays of peptide uptake and for preparation of cell extracts were routinely harvested by filtration. Cells were grown routinely as described in Section 2-2-2, up to less than 10^9 cells per ml i.e. E_{660} = 0.5, (Spectronic 20), up to this density the culture was in exponential phase.

Typically, for harvesting, 10 ml of cells was passed through a membrane filter (0.4 μm pore size, 25 mm diameter, Oxoid), held in a Swinnex (millipore) filter holder. For larger volumes (up to 50 ml), a 6 cm diameter, 0.4 μm pore size Oxoid filter, attached to a water vacuum was used. The bacteria were washed on the filter with two volumes of KH_2PO_4/K_2HPO_4 buffer (0.1M; pH 7.0) equilibrated at 37°C and then resuspended in the same buffer to a concentration of 0.1 to 0.2 mg ml^{-1} dry weight, unless otherwise stated. Where required, 0.5% w/v glucose or v/v glycerol, and amino acid supplements (0.1mM final concentration) were added.

(b) Centrifugation

Where volumes of cells were large (greater than 50 ml) or where several different cultures needed to be harvested simultaneously, centrifugation was used. Cultures were transferred to sterile screw-top tubes and collected in a MSE High Speed 21 centrifuge at 18,000 r.p.m. (26,000g). The supernatant fluid was decanted, the pelleted organisms were resuspended, using a vortex mixer, in an equivalent volume of 0.1M, potassium phosphate buffer pH 7.0 at 37°C and centrifuged again to wash the cells. The supernatant solution was discarded and the cells were resuspended in fresh buffer also equilibrated to 37°C.

Where volumes were very small ($\leqslant$ 1 ml), samples were placed in capped microcentrifuge tubes and a high speed (12,000 r.p.m. 10,000g) Microhaematocrit centrifuge was used.

2-2-5 Amino Acid Starvation

To obtain amino acid starved cells, exponential-phase bacteria were harvested by filtration as described, washed with two volumes of fresh growth medium (minus the amino acid supplements), which had been equilibrated at 37°C; then resuspended from the filter into the same growth medium.

The volume used being at least the original volume of culture taken, but sometimes more medium was used to dilute the cells to the required density. The cells were then incubated at 37°C with shaking (120 strokes min^{-1} in a water bath) for 90 minutes to starve of endogenous amino acid (Section 3-3-5).

2-3 Protein Assays

2-3-1 Introduction

Throughout this work, it was often necessary to determine the protein concentration of a solution; for example, to determine the extent of cell disruption, to quantify the rate of a reaction (in terms of concentration of bacterial protein) or to determine accurately the concentration of a protein in a sample of, say, meal or rice, prior to digestion for amino acid assay.

The method of choice should be rapid, accurate and sensitive. The standard Lowry procedure (Lowry et al., 1951), is subject to interference from, for example, K^+, Mg^{++}, EDTA, tris and thiol reagents and from carbohydrates; and the microbiuret reaction (Leggett-Bailey, 1967), is subject to interference from, for example, tris, ammonia and glycerol (Bradford, 1976). Modifications have been suggested to remove interferences from certain compounds, to decrease assay time, to provide more stable reagents, to increase sensitivity and to give a linear calibration curve. These modified procedures tend to increase the time required for reagent preparation or assay, although they were used, especially during the early stages. In later work, therefore, a modified dye-binding assay was used routinely (BioRad Laboratories, California), in which the absorbance maximum of an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 - 595 nm when binding to a protein occurs. The binding of the dye to protein is rapid (approximately 2 minutes) and the protein-dye complex remains dispersed in solution for approximately one hour. Comparisons between the three methods above are given in the literature supplied by BioRad Laboratories Ltd. (Tables 1.3, 2.2).

2-3-2 Materials

BioRad protein assay solution, gamma globulin and bovine serum albumin standards were all supplied by BioRad Laboratories, California.

2-3-3 Methods

The procedures adopted essentially are the same as those described in the literature supplied by BioRad. Two different methods can be used: the standard method (sensitivity range 200 - 1400 μ g protein ml^{-1}) and a microassay method (sensitivity 1 - 20 μ g protein ml^{-1}). Bovine serum albumin and gamma globulin standards were supplied by BioRad to reconstitute at concentrations of 1.4 and 1.34 mg ml^{-1} respectively. These were stored at -20°C. For routine use, these standards were diluted as required and aliquots were stored at 4°C for a maximum of 5 days.

The dye reagent was supplied as a 5X concentrate and was used directly for the microassay procedure. For the standard assay, the concentrate was diluted with four volumes of distilled water and filtered through Whatman no.1 filter paper. This diluted reagent was found to be unstable and did not remain active for the two week period claimed by the suppliers.

For the standard procedure, 0.1 ml of standards containing 0.2 - 1.4 mg ml^{-1} protein or appropriately diluted test samples (previously centrifuged to remove any debris that might interfere with the assay), were added to clean detergent free test tubes (these had previously been acid washed, rinsed with tap water three times, and distilled water twice). 5.0 ml of diluted reagent was added, the tubes were mixed gently and E_{595} was measured versus a reagent blank, after 5 - 30 minutes.

For the micro-assay procedure, 0.8 ml of standards or test samples, appropriately diluted to contain 1 - 20 μ g protein ml^{-1} and centrifuged if necessary - were added to clean test tubes. 0.2 ml of neat reagent was added and the assay completed as above. This procedure was found to be more satisfactory, since the undiluted reagent was more stable; therefore, this was adopted routinely, the assay samples being diluted as necessary for the micro-assay.

2-3-4 Results

Fig. 2.1 shows a typical calibration curve for the gamma-globulin standard. Control tubes containing varying amounts of standard and sample together gave results in agreement with the values predicted from the sum of the two separately. Therefore, the results were taken to provide an accurate measure of protein concentration in the various samples. A comparison of protein concentrations obtained using the BioRad and the microbiuret (Leggett-Bailey, 1967), methods is given in Table 2.2.

2-3-5 Discussion

Two conditions are known particularly to interfere with the BioRad assays; these are the presence of anionic detergents in concentrations greater than 0.1% v/v and the presence of strong alkalis. Chromogenic substrates could also interfere with the assays if their colour contributed to absorption at 595 nm. However, usually these problems did not occur and the BioRad micro-assay proved to be a quick, reliable method for protein evaluation except in one situation!

In permeabilised cell preparations (prepared by the addition of 0.05% v/v Triton X-100, Section 2-5-4), interference with the assay was noted although the detergent concentration was less than the 0.1% v/v claimed by the BioRad manufacturers, to be tolerated.

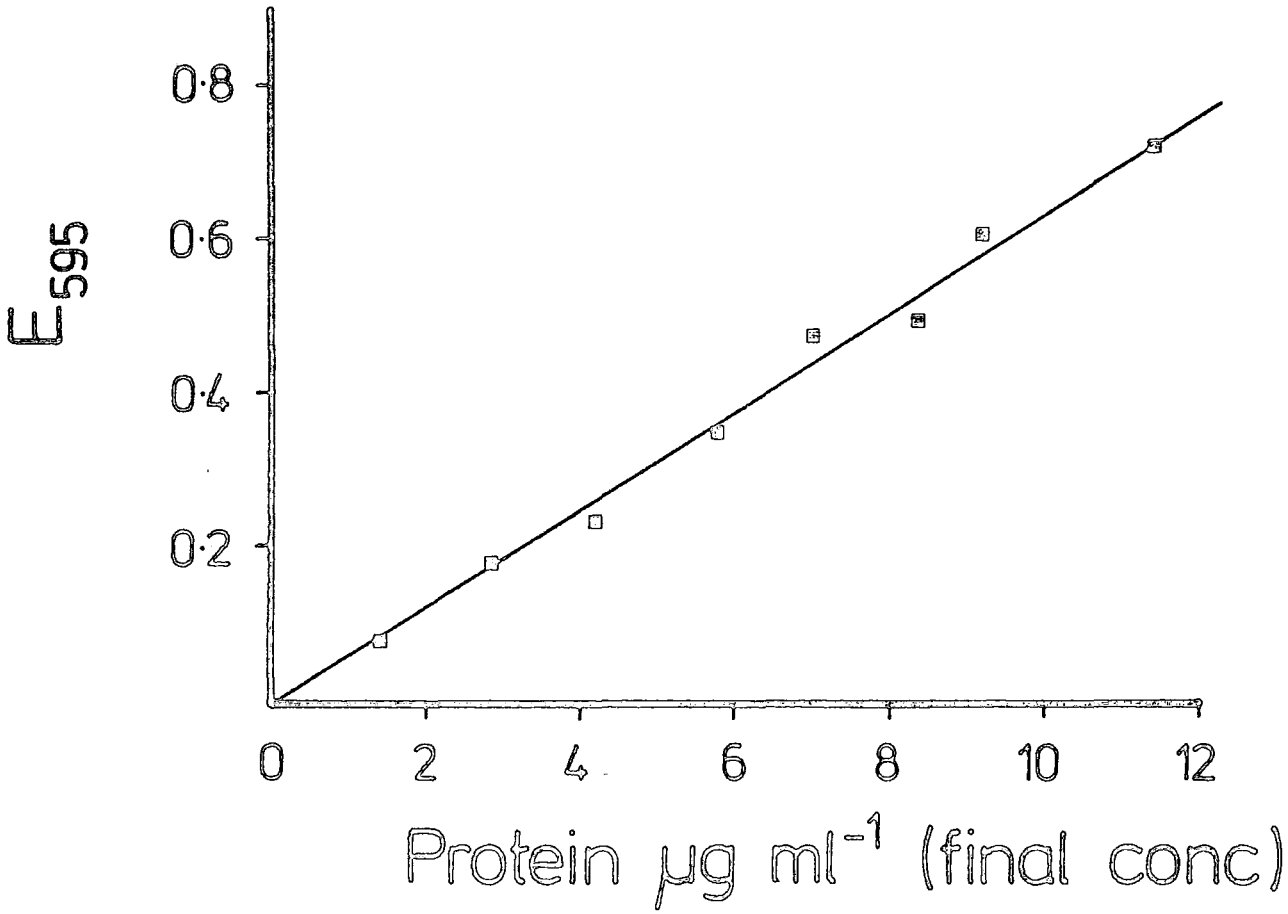


FIGURE 2.1

Protein calibration curve using standard bovine gamma globulin. BioRad micro-assay procedure as described in text.

Table 2.2

Comparison of BioRad (Micro-assay) and Modified Microbiuret Protein Assay on Sonicated Cell Extracts (Exponential Cells M2626)

Sonication Time (1 minute bursts)	BioRad μ g ml^{-1}	Microbiuret μ g ml^{-1}
2	41.3 (26.8)	44.3 (28.78)
3	91.2 (59.2)	96.3 (62.5)
4	104 (68)	117.6 (76.4)

Figures in parentheses represent percentage of soluble protein released, assuming 60% of total cellular protein is soluble. Sample was taken and after stated sonication times 2 aliquots were removed for protein assays.

For Escherichia coli E_{660} 2.19 = 1 mg protein ml^{-1}

To assay protein in these samples, the modified Biuret method was used.

Recent reports indicate that the dye-binding method can be automated for the processing of large numbers of samples (Sano et al., 1981; Simpson & Sonne, 1982), and the sensitivity can be further increased (Almog & Berns, 1981).

2-4 Dansyl-Chloride Procedure

2-4-1 Introduction

Dansyl-chloride (1-dimethylamino-naphthalene-5-sulphonyl chloride) reacts with primary and secondary amines to form fluorescent sulphonamide derivatives; nanomole quantities of amines can be detected. The reagent is routinely used in protein sequencing studies and several chromatographic techniques have been developed for the separation of dansyl-amino acids (see Seiler, 1970 for review). The reagent also reacts with the α-amino groups of small peptides. The derivatives can be separated by thin-layer chromatography and quantified by the intensity of the fluorescence compared to standards. Dansyl-chloride can therefore be used to monitor the disappearance of amino acids and small peptides from an incubation broth, or to monitor the simultaneous uptake of several amino acids or peptides and detect accumulation of peptides and changes in concentration in cells by examination of cell extracts (Payne & Bell, 1979; Nisbet, 1980)

2-4-2 Materials

Dansyl-chloride and dansyl-amino acid standards were from B.D.H. Ltd., Polyamide Sheets (15 x 15 cm) from B.D.H. or Pierce-Warriner. All solvents and reagents were of analytical grade.

2-4-3 Dansylation Procedure

Samples of standard, media or cell extract containing up to 10 nmol peptide or amino acid (usually 100 μ L) were placed in Durham tubes (6 x 30 mm) together with an ornithine, diaminopimelic acid (DAP) or other suitable standard (10 μ L, 0.5 mM). The tube contents were evaporated down *in vacuo*. Sodium bicarbonate (200 mM in deionised water, 20 μ L), was added to bring the pH to about 9.0, followed by 20 μ L of dansyl-chloride (2.5 mg ml^{-1} in acetone).

The dansyl-chloride solution was kept for up to four weeks at 4°C - stored in the dark. Tubes were sealed with silicone rubber stoppers and incubated at 40°C for 90 minutes, to allow the dansylation reaction to proceed to completion. The mixture was then evaporated to dryness and the residue redissolved in aqueous pyridine (1 : 1 v/v, 10 μ L).

In some cases, especially with extracts, an insoluble precipitate remained in the tube and this interfered with the subsequent thin-layer chromatography, therefore, this was removed by centrifugation (Haematocrit microcentrifuge, 12,000 r.p.m., 10,000 g), for 2 minutes and the supernatant solutions were transferred to clean tubes. Samples (5 μL) were spotted onto polyamide sheets and chromatographed in three solvent systems for approximately 50 - 60 minutes in each (until solvent front was about 3 cm from top of sheet). The solvents used were as in Payne & Bell, 1979.

(a) First dimension: H_2O : formic acid (98.5 : 1.5 v/v).

(b) Second dimension: acetic acid : toluene (10 : 90 v/v).

(c) Re-run in second dimension: methanol : butyl acetate : acetic acid (38 : 60 : 2 v/v).

Care was taken to dry the plates thoroughly between solvent runs. A final re-run in the first solvent sometimes helped to 'round-up' fluorescent spots. Chromatograms were viewed under long-wave U-V light, derivatives identified and where necessary, quantified by reference to standards which were either run on the back of the plates or on separate ones. Chromatograms were inspected soon after drying off the final solvent, although fluorescence does not fade over several hours in the light, or up to two weeks in the dark (T.M. Nisbet - personal communication).

A permanent record of some plates was made by photographing under U-V light using Ilfodata H.S. 23 film (Ilford), and a Wratten No.3 filter (Kodak). Plates could be reused after washing overnight in acetone : water : ammonia (50 : 60 : 4 v/v) and drying.

2-4-4 Results

Chromatography in the first dimension frequently gave poor resolution because of interferences at the origin (phosphate and glucose). The problem was minimised by using low buffer concentrations or cell extracts prepared in distilled water. However, interferences from a variety of non-protein cellular components in cell extracts persisted, especially in those samples of extracts prepared from starved cells. Some amino acids e.g. Pro/Val, Ile/Leu ran very close to each other after three solvents, but were readily distinguished by examining the plates after running in two solvents only. After three solvents, dansyl derivatives were identified from their positions on a chromatogram relative to the internal standard, to standards on the reverse of the chromatogram and to the various by-products of the reaction (dansyl-hydroxide and dansyl-ammonia), See Fig. 2.2.

Most of the derivatives flouresced green with the exeption of His (orange) and Tyr (yellow).

2-4-5 Concluding Remarks

To obtain satisfactory results, a large excess of dansyl-chloride (approximately 20 fold), was used. The pH of the reaction is important and this was checked when the bicarbonate was added, and if necessary, adjusted to pH 9.0 by further addition. The dansyl procedure allowed the assay of peptide uptake by Escherichia coli and the fate of the absorbed peptide could be followed, as could the exodus of its constituent amino acids.

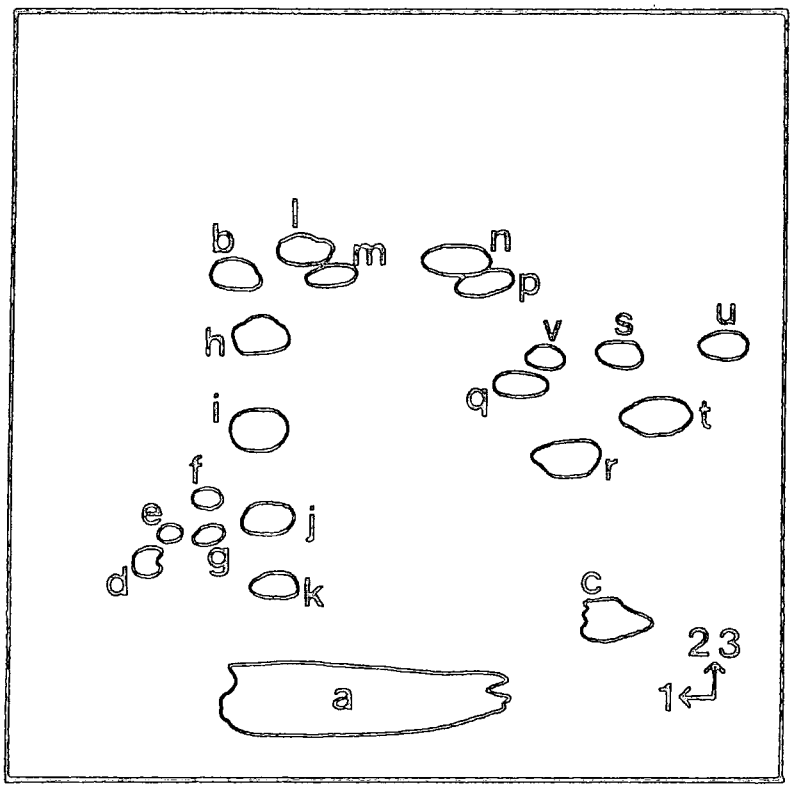


FIGURE 2.2

Positions of dansyl-amino acids after chromatography in three solvent systems.

a	dansyl hydroxide	l	Pro
b	dansyl ammonia	m	Val
c	diaminopimelic acid	n	Ile
d	Arg	p	Leu
e	Asn	q	Met
f	Thr	r	Orn
g	Ser	s	His
h	Ala	t	Lys
i	Gly	u	Tyr
j	Glu	v	Phe
k	Asp		

However, quantitation of low uptake rates was difficult as was the identification of peptides present in cell extracts prepared from starved cells.

2-5 Treatments To Cells

2-5-1 Introduction

Since the chromatographic separation of dansyl derivatives of amino acids and peptides (Section 2-4), is clear and reproducible, it allows cell extracts to be monitored for the accumulation of amino acids and peptides and for changes in amino acid levels.

2-5-2 Preparation Of Cell Extracts By Boiling

For preparation of extracts, incubations of cells were usually performed in a volume of 1 ml containing approximately 10^9 cells. The cell suspension was harvested on a membrane filter (Section 2-2-4), under a water vacuum, immediately washed with 20 ml distilled water and straightaway extracted by adding the filter to a stoppered tube containing 1 ml water, 50 μ L of toluene was added to each tube, which was boiled for 15 minutes. The resulting suspension was cooled, centrifuged (MSE Bench Centrifuge, 3,100 r.p.m., 20 minutes), to deposit insoluble debris, 100 μ L portions of the supernatant extracts were evaporated down after addition of appropriate internal standard (Section 2-4-3), and the samples were then dansylated.

2-5-3 Preparation Of Sonicated Cell Extracts

Cells for sonication were grown, collected on a filter, washed and resuspended initially in 0.1M potassium phosphate pH7.0, buffer as already described, to give an approximate concentration of 10^9 cells ml^{-1}. 5 ml volumes of such cells were placed in a McCartney bottle and subjected to 5 x 1 minute bursts of ultra-sound from a Soniprobe sonicator type 1130A tuned to optimum amplitude and power. Between each burst of ultra-sound, the samples were cooled on ice for two minutes.

The disrupted cell suspensions were centrifuged as in Section 2-5-2. To check on the efficiency of sonication, a portion of the supernatant solution from each sonicated batch of cells was subjected to a protein assay using the BioRad dye-binding method described in Section 2-3-3 and see Table 2.2.

2-5-4 Preparation Of Permeabilised Cells

In some cases it was necessary to destroy the integrity of the permeability barrier of cells, for example, to determine enzyme activities *in situ*. This was achieved by alternate freeze/thawing in the presence of the anionic detergent Triton X-100 (Mozzari *et al.*, 1978). Approximately 10^9 cells were harvested and washed on a filter as described previously (Section 2-2-4), and then resuspended in 1 ml 0.1M potassium phosphate pH 7.0 buffer in polypropylene test tubes. 2.0 µl of 2.5% v/v Triton X-100 was added to give a final concentration of 0.05% v/v detergent, and the tube contents mixed. This was reacted at room temperature for 5 minutes and the tubes were then frozen in liquid air (-160°C), for 30 minutes. The frozen cell suspensions were then thawed rapidly in a water-bath at 25°C, mixed thoroughly (Whirlimix for 10 seconds), and the freeze/thaw cycle repeated. The cell suspensions were then centrifuged for thirty minutes (3,100 r.p.m.), the supernatant solution was discarded and the cell pellet was resuspended in 1 ml of 0.1M potassium phosphate pH 7.0 buffer. For correct interpretation of experimental results, it was important that the treatment described above actually caused disruption of the cells permeability barrier. To assess the efficiency of permeabilisation with Triton X-100, a β-galactosidase assay was carried out using M2626 (See Chapter 3 for method).

For comparison, untreated cells, cells permeabilised as above and cells permeabilised using the procedure of Putnam & Koch (1975), see Section (3-3-5-3), were all assayed.

The results of this study (Fig. 2.3), showed that β -galactosidase activity was readily detectable in the freeze/thaw treated cells. However, the activities of β-galactosidase were lower than found after the normal disruption procedure of Putnam & Koch in which the disruption medium had been optimised for stabilising the liberated β -galactosidase.

2-5-5 Concluding Remarks

When intracellular amino acids and peptides were examined by dansylation, they were extracted from the cells by boiling. When cell extracts were required with enzymic activity they were prepared by sonication. In the latter case, it was found that salts from the 0.1M potassium phosphate buffer interfered with subsequent processing and assay steps, therefore the molarity of the buffer used in the extraction was reduced to 0.05M; having first demonstrated that under the conditions in which it was used, it still had adequate buffering capacity.

Before cell extracts were dansylated and examined, a portion was examined for protein content either by the BioRad dye-binding assay; or, when Triton X-100 was used in the extraction, by the modified Biuret assay. These protein assays were essential to check the efficiency of cell disruption and to calculate comparative protein concentrations used in subsequent treatments.

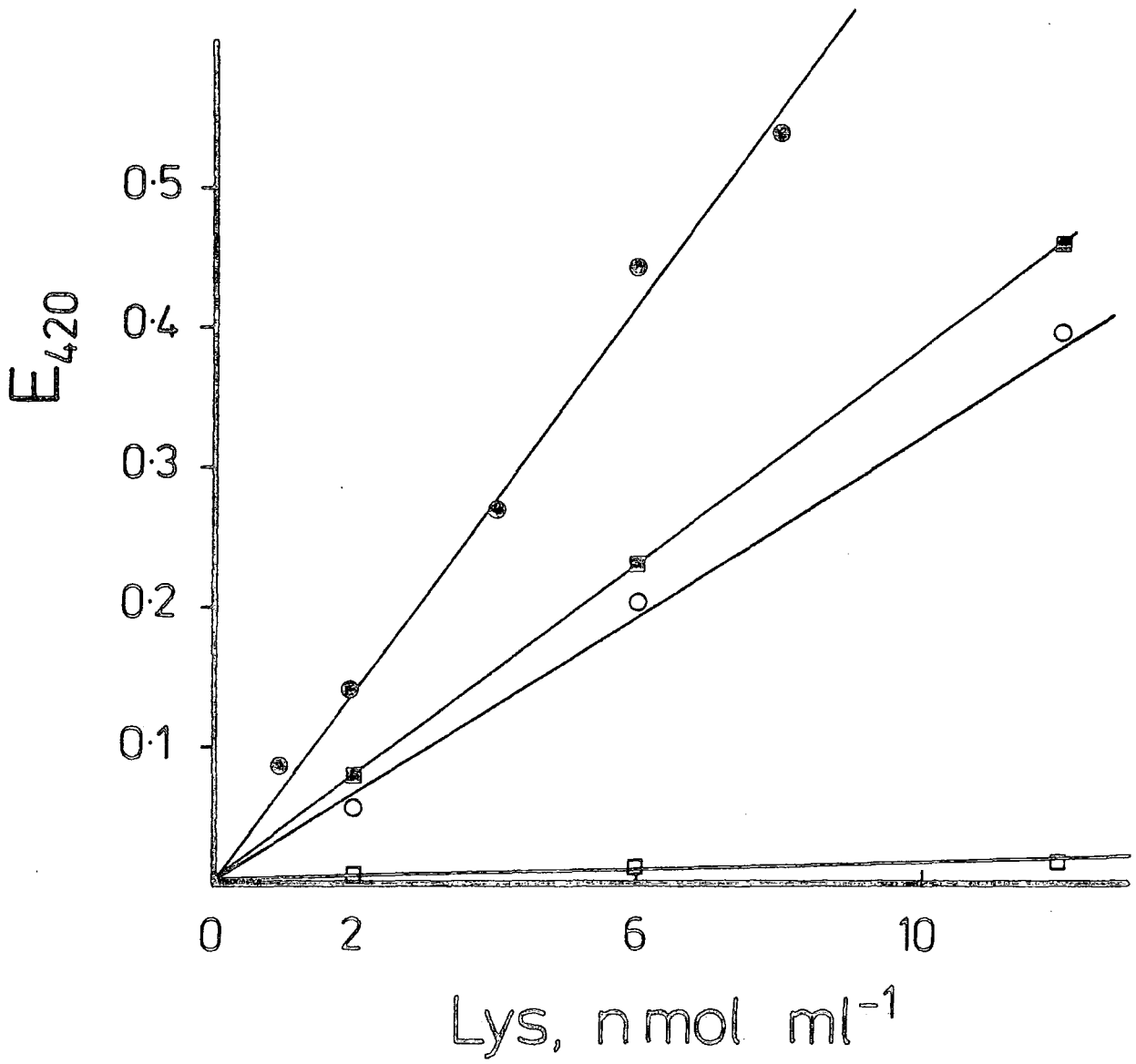


FIGURE 2.3

β -galactosidase activity in various cell preparations

- no treatment
- freeze-thaw
- freeze-thaw in the presence of 0.5% v/v Triton X-100
- normal disruption (Putnam and Koch method)

E_{420} = 1 corresponds to σ-nitrophenol (0.23μ mol/ml)

See section 2-5-4 for details of freeze-thaw treatment and section 3·3-5-3 for details of disruption by Putnam and Koch method

2-6 Fluorescamine Chromatography

2-6-1 Introduction

Fluorescamine (Fluoram®), Roche, is a non-fluorescent compound which at room temperature and alkaline pH reacts extremely rapidly (200 - 500 msec at pH 9.0; Stein et al., 1973), with amino group containing compounds, e.g., amino acids and peptides to give intensely fluorescent products; excess reagent being hydrolysed to give non-fluorescent water soluble products. The procedure has many advantages over the use of dansyl-chloride (see Section 2-6-5 for discussion); in this instance it was used with Met and its oxidised forms which were not easily identified by dansylation.

2-6-2 Materials

Fluorescamine (Fluoram®), 4-phenylspiro (furan-2(3H), 1'phthalan-3,3 dione), a gift from Roche, or purchased from Sigma (London) Ltd., was used as a freshly prepared solution (4 mg in 10 ml anhydrous acetone). Precoated silica gel 60 F_{254} thin-layer plates were purchased from Merck. Glass plates were prepared with a silica gel coating and used in initial studies, but were generally less sensitive than the commercial ones. Solvents were of the purest grade generally available.

2-6-3 Method

Although it is possible to derivatise compounds with fluorescamine at the origin of thin-layer plates (Nakamura & Pisano, 1976a), it was found here to be preferable to derivatise the amino acids and peptides first in solution and then apply them to the plates since this increased sensitivity.

(a) Preparation of sample

Samples to be derivatised, 0.1 ml of 10mM amino acid or peptide or 0.05 ml of cell extract (prepared as for dansylation - Section 2-4-3), were added to 0.5 ml, 0.1M disodium tetraborate buffer (pH 9.3). 0.2 ml fluorescamine in acetone (0.4 mg ml^{-1}) was gradually added to the reaction mixture whilst it was rapidly mixed on a whirlimix (Chen et al., 1978). 0.01 ml of the derivatised product was added to the origin of the silica gel plates in 0.001 ml aliquots. (The remainder of the solution was stored up to one week, in the dark at $4^{o}C$).

When dry, the plates were run in covered tanks, kept away from light.

(b) Running Plates

For a complete chromatographic separation of fluorescamine derivatives there is no simple, universal solvent. Since the amino acids of interest for this work were Met and its various oxidised forms these were used to test the efficiency of 12 different solvent systems, each run in a single dimension. The three solvents that gave the most promising results were selected to be tested in combination in two dimensions:

(A) Acetone : Ethyl acetate : Methanol : Water

60 : 20 : 25 : 10 (v/v)

(B) Ethanol : Hexane : Methanol : Water

60 : 20 : 25 : 10 (v/v)

(C) Isopropanol : Chloroform : Ammonia 28% : Water

70 : 30 : 18 : 7 (v/v)

When the solvent front had reached 5 cm from the top of the plate they were removed, dried and observed under long wave (366 nm) ultra-violet light.

Solvents A and C were shown to separate Met and its derivatives, solvent B gave good separation of Met-containing peptides. The application of this method would be greatly extended by development of two-dimensional solvent-systems. However, combinations of the above solvents in two dimensions were unsatisfactory and since development of new solvent-systems was outside the scope of this thesis, examination of cell-extracts was carried out in one dimension using control marker spots.

2-6-4 Results

Rf values for Met and its oxidised forms are given in Table 2.3; which shows that they can be separated by this method. However, when present in the cell extracts, components did not separate as well and a range of standards needed to be added to extracts in order to identify components therein. This was especially true for starved cell extracts. Applications of the method are discussed in Chapter 5.

2-6-5 Concluding Remarks

Detection of compounds with fluorescamine is a rapid and very sensitive procedure. As little as 10pmol of fluorescamine-derivatised compound can be detected (Nakamura & Pisano, 1976a). Fluorescamine has been used with ion-exchange columns as a detection reagent in place of ninhydrin in auto amino acid analysers (Stein et al., 1973). A solution of fluorescamine in acetone has been used as a spray for amino acids on thin-layer chromatograms (in a similar manner to ninhydrin) (Nakamura & Pisano, 1976b). A particular advantage is that the procedure can be carried out in solution without the problems that arise with fluorescent byproducts in the dansylation reaction (dansyl-amide and hydroxide). The use of fluorescent methods for isolation, characterisation and assay of peptides has been reviewed by Udenfriend (1978).

Table 2.3

Rf Values For Fluorescamine Derivatives

Of Methionine And Its Oxidised Forms

	Solvent A	Solvent B
Met	0.86	0.4
Met(O)	0.82	0.31
Met(O_2)	0.68	0.33

2-7 Automated Fluorescamine Procedure

2-7-1 Introduction

A direct method to monitor peptide uptake by microorganisms which involves the preparation of dansyl derivatives of substrates remaining in the medium after incubation with cells, separating them by thin-layer chromatography and quantitating them from their fluorescence, has been widely used (Payne & Bell, 1979). Later, fluorescamine was used in place of dansyl-chloride to prepare fluorescent derivatives with the peptides present in the medium after incubation, these derivatives being quantified directly in solution (Nisbet & Payne, 1979). This fluorescamine assay was subsequently automated and now allows continuous monitoring of peptide or amino acid transport (Nisbet, 1980; Payne & Nisbet, 1981).

2-7-2 Materials

Fluorescamine was a gift from Roche Products Ltd., or was purchased from Sigma (London) Ltd., and was used as described (Payne & Nisbet, 1981). In later stages of the work, glass fibre filters, GF/C 25 mm diameter, Whatman Ltd., were used in place of Millipore filters. These had a greater capacity and were less prone to blocking, allowing transport to be monitored for a longer period (Section 2-7-3).

2-7-3 Method

The assay uses a system based on a 3-channel pump (Pharmacia P-3), and a fluorescence spectrophotometer (Perkin Elmer model 1100), incorporating a flow cell. A suspension buffer, 0.05M potassium phosphate pH 7.2, containing 0.2mM ammonium chloride and 0.1% w/v glucose, was used to wash and to suspend the bacteria, and act as a blank for determination of peptide yields. Amino acids were added to the suspension buffer to prevent the onset of starvation in particular strains (See Table 2.1).

Thus, if strain PA0111 (Met^- Lys^-) were used to monitor the cleavage of a peptide containing an oxidised Met residue then Lys (5 μ L of 10mM), was added; but this was unnecessary if strain 8134 (Met^-), was used. For assay of peptide cleavage, a suspension of 10 ml whole or permeabilised bacteria (approximately 10^8 cells ml^{-1}), was placed in the thermostated incubation vessel and equilibrated. During this time a stable baseline level of fluorescamine was obtained by pumping the suspension buffer through the system. This buffer from Channel A was mixed in special mixing chambers, firstly with a second buffer solution (0.2M citrate-phosphate pH 5.8), and then with fluorescamine at 0.1 or 0.5 mg ml^{-1} (depending on peptide concentration and/or reactivity), in Analar Isopropanol, which was introduced through Channel C. The final reaction pH was 6.2 (pH 6.6 at flow-cell effluent which contains Isopropanol); at this value there was negligible reaction from amino acids which react optimally at about pH 8.5, hence efflux of amino acids from cleaved peptides does not contribute significantly to the fluorescence. All solutions were pumped at 0.9 ml min^{-1}. The resulting mixture was passed through a delay coil and into a flow cell allowing the fluorescence to be measured continuously. To start the assay, the pump was stopped momentarily, the filter assembly dipped into the microbial suspension, peptide (typically 10 μ L, 10mM) was added, and the pump restarted. With a suspension containing approximately 10^8 cells ml^{-1} the uptake rate could be monitored for about 7 minutes before the Millipore filter (0.4 μ pore size), started to block, or about 20 minutes with a Whatman GF filter.

In some cases, in order to monitor uptake or cleavage over a longer time, substrate was added to the incubation vessel containing cells, as usual; an initial sample was pumped through the system for approximately 2 minutes and then the filter was switched into buffer alone whilst the cells continued to take up peptide; after about 20 - 30 minutes, the incubation mixture was again monitored, allowing cleavage to be assessed over the longer time.

2-7-4 Discussion

Because of the internal volume of the system, samples took 2.8 minutes to pass from the incubation vessel to the flow cell, under standard conditions; thus, events portrayed on the chart recorder or V.D.U. actually represent the situation 2.8 minutes earlier in the incubation vessel. The rates of uptake in the first two minutes cannot readily be found directly from the output (fluorescence versus time) (Fig. 2.4). However, the subsequent linear part of the trace allows ready measurement of the steady state rate of disappearance of peptide from the solution. Uptake occuring at a steady state over a period of time indicates that the peptide is being cleaved, therefore measurement of the uptake rate for the peptide indicates the minimal rate of concurrent intracellular cleavage. Cleavage rates were expressed in nmol peptide min^{-1} mg $protein^{-1}$.

Typically, with the peptides studied here, if the rate after 2 - 3 minutes was extrapolated back to zero time, the calculated initial peptide concentration corresponded to that actually added, indicating that the average rate is constant over the first few minutes.

Work is now in progress (J.T. Gleaves & J.W. Payne) to interface a programmed micro-computer for direct determination of rates.

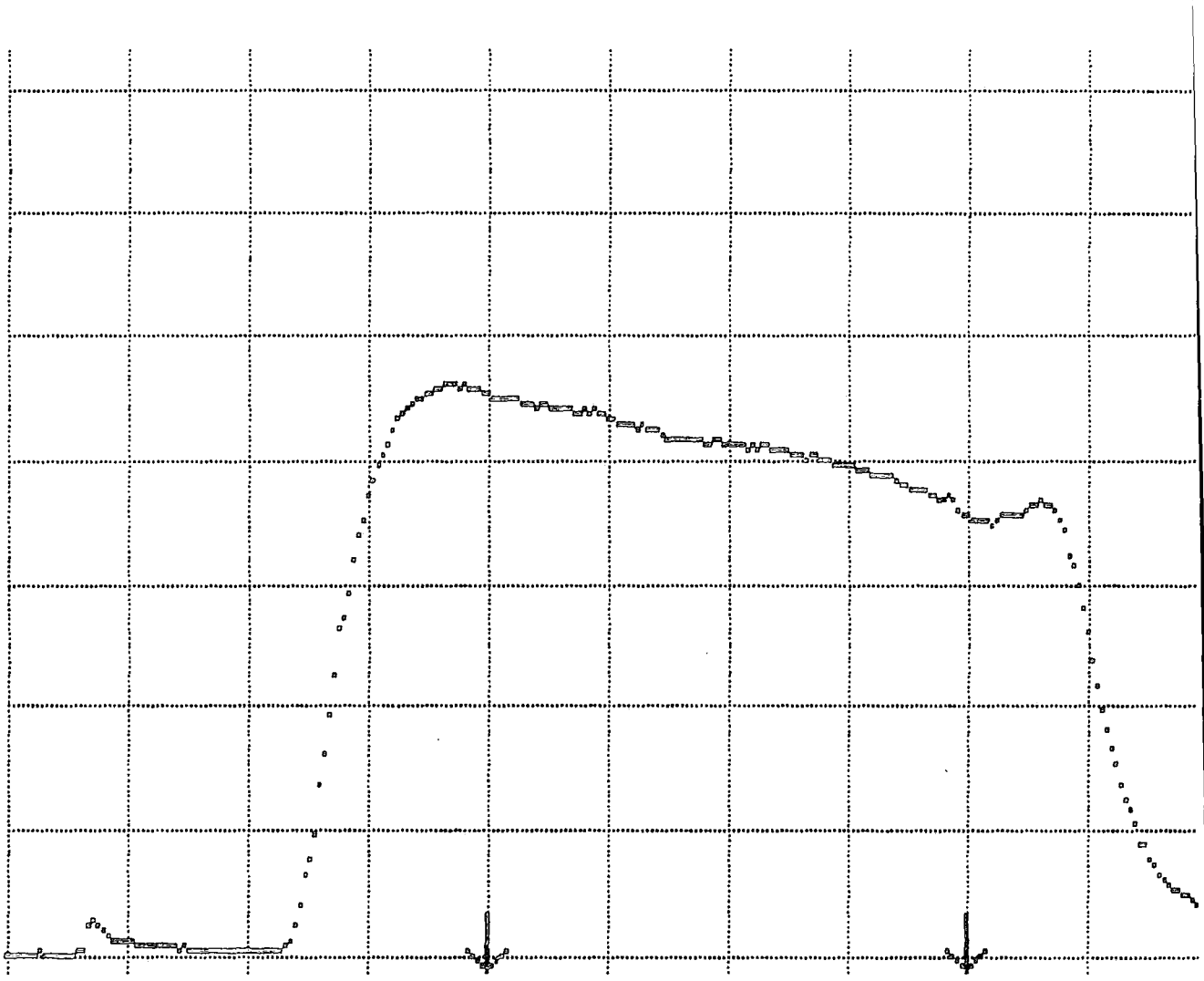


FIGURE 2.4

Uptake of Met(O_2)-Leu-Gly by cells of E.coli M2626 starved of Lys

E_{660} of starved cells = 0.18, scale expansion on fluorimeter = 5, fluorescamine conc = 0.5 mg/ml, substrate conc = 0.1 mM (0.1ml 10mM peptide in 9.9 ml reaction buffer).

Time period between arrows = 4min

Rate of uptake during this time period = 28nmol/min/mg protein

Trace taken from printer output interfaced to VDU (earlier work used a chart recorder to record output from fluorimeter)

The application of this technique for monitoring cleavage of peptides containing partially or completely S-oxidised Met residues and the results obtained are discussed in Chapter 5.

2-8 Protein Digestion (Enzymic)

2-8-1 Introduction

Escherichia coli does not secrete proteases or peptidases (Section 3-1-1), therefore, an initial enzymic digestion step *in vitro* must be included when assaying large peptide-bound substrates.

Many methods of enzymic hydrolysis have been employed in related assays. Hill & Schmidt (1962), used papain digestion followed by treatment with purified kidney peptidases, leucine amino peptidase and prolidase. Bodwell (1977), discusses the importance of digestibility in the nutritional evaluation of grains and describes several rapid multienzyme methods for its estimation.

However, for the *Escherichia coli* assay described herein, digestion to free amino acids is not essential, proteolysis need only produce fragments which are sufficiently small to pass through the outer membrane of the cell envelope and reach the cytoplasmic permeases (Nikaido *et al.*, 1977; Payne & Gilvarg, 1968). The cut-off size for peptide uptake is at a molecular weight of about 650 (i.e. approximately penta-hexapeptide size) (Payne, 1977; Payne & Gilvarg, 1968). Successful digestion procedures therefore, must be capable of cleaving to this level (i.e. on average a 20% cleavage).

2-8-2 Materials

A pH stat automatic titrator Model TTT60, was supplied by Radiometer, Copenhagen. Protease P, Protease type 14 (pronase), and Peptidase (porcine intestine), were supplied by Sigma (London) Ltd., as were the purified protein samples: α -chymotrypsinogen A, type II from bovine pancreas; whale myoglobin type II - skeletal muscle, and lyzozyme, grade I from chicken egg white. Various food protein samples were kindly supplied by Dr. A. Williams (N.I.R.D.) and Dr. B. Juliano (I.R.R.I.).

2-8-3 Methods

Enzymes were stored at -20°C. For use in digests, enzyme solutions (0.5 mg ml^{-1}), were freshly made in sterile 0.2M potassium phosphate, pH 7.0 buffer to minimise possible bacterial interference during digestion.

Initially, an automatic titrator was used to monitor the rate and extent of proteolysis (Fig. 2.5). A mixture of pronase and peptidase gave about 44% hydrolysis (calculated from the equivalents of base added after allowance for titres of control samples containing substrate or enzyme alone), after four hours. This is sufficient to give mostly dipeptides as cleavage products. However, pH-stat titration was not used routinely to monitor the digestion of samples but was used to establish the activity of each fresh batch of enzyme on a standard substrate prior to its use with test samples.

To determine moisture contents, protein samples were weighed and placed in an evacuated desiccator over calcium chloride *in vacuo* overnight at 80°C. The following morning, the samples were re-weighed, returned to the 80°C oven, and after one hour they were again re-weighed to show that they had reached constant weight. The percent moisture in each sample was calculated. Samples were kept in an evacuated desiccator at room temperature until required.

Samples of pure proteins were subjected to various enzymic combinations and digestion times, and from the data obtained (See Results, Section 6-5-4), a standard method of digestion was developed. Since initial experiments were aimed at establishing optimum digestion conditions, some test procedures did not give complete digestion. This proved to be a problem with lysozyme which can lyse *Escherichia coli* and hence such studies were confined to myoglobin and chymotrypsinogen.

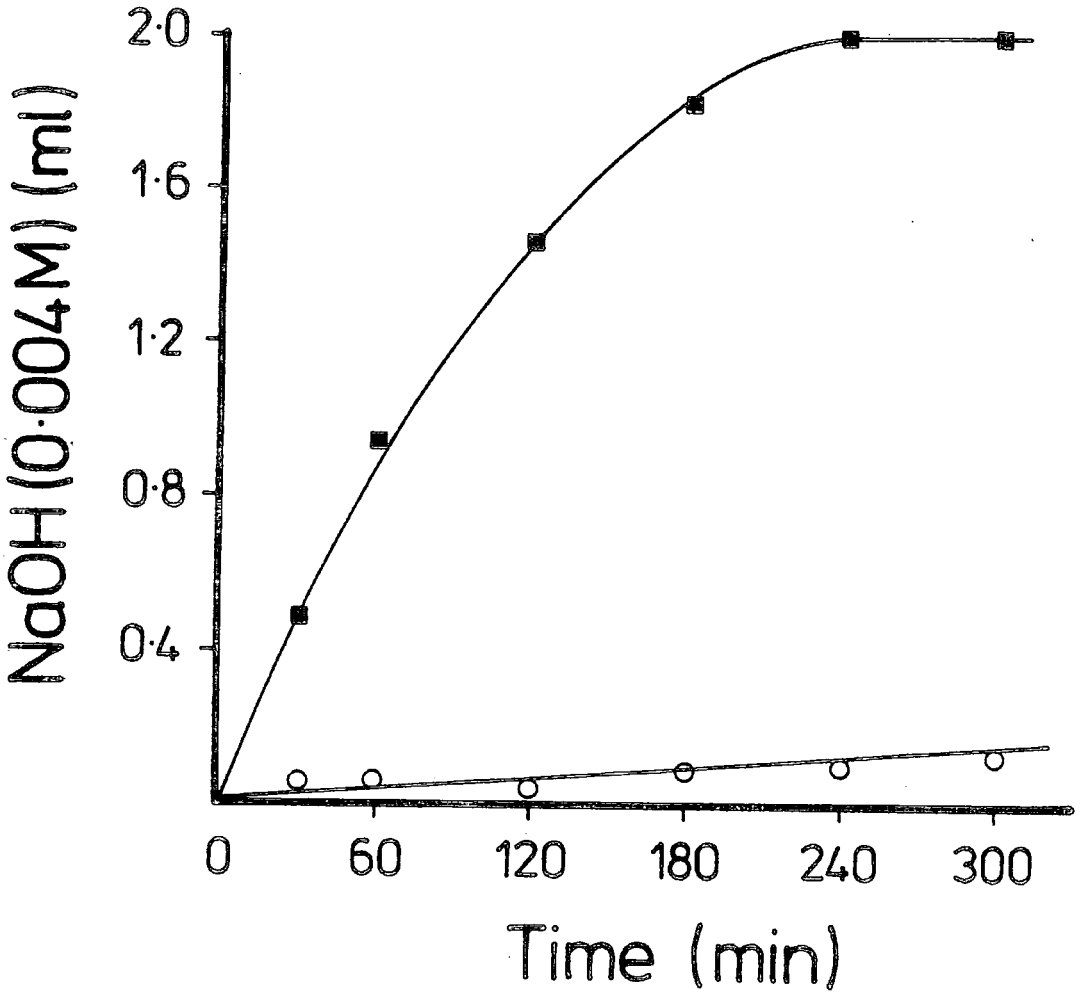


FIGURE 2.5

Proteolytic digestion of Chymotrypsinogen A by pH-stat method

Proteolytic digestion of Chymotrypsinogen A, 5mg/ml, 5ml, with pronase, 0.25mg/ml, 1 ml. ■——■

Control ○——○, pronase and peptidase - no substrate

Temperature 37°C, end-point pH 8.0.

The digestion procedure finally adopted for the pure proteins was as follows:

10 ml samples were prepared in boiling tubes, these contained 6 ml sterile 0.2M potassium phosphate buffer, pH 7.0. 2 ml protein solution (5 mg dry wt. ml^{-1}), and 1 ml of each enzyme solution (freshly prepared solutions of pronase and peptidase containing 0.5 mg ml^{-1} of each enzyme in sterile buffer - as above), giving final concentrations of 1 mg ml^{-1} protein and 0.05 mg ml^{-1} of each enzyme.

Samples were covered with Parafilm to prevent evaporation and incubated, with gentle shaking (60 strokes min^{-1}), at 37°C for 16 hours. Autolysis controls, containing only 0.05 mg ml^{-1} of each enzyme were treated in the same way. After digestion, samples were diluted with 0.05M buffer, pH 7.0 prior to assay for Lys, Met or Trp by β-galactosidase assay. In each case, controls containing samples of the digested proteins plus graded amounts of the particular amino acid under test were included to check for the possible presence of inhibitors in the test sample.

The digestion of the meals, rice etc., was carried out in a similar manner to that of the pure proteins. Substrates were used directly without defatting, after first being ground finely and sieved (Baird & Tatlock test sieve M69, 355 micron). Sieved samples were added to 0.2M potassium phosphate buffer, pH 7.0 (sterile), to give about 10 mg meal ml^{-1} (subsequently corrected for percent protein and moisture in sample). The suspensions were dispersed by 30 sec. treatment with a polytron mascerator and if necessary, any residue was washed from the blades with more buffer solution, (measured volume). The suspensions were covered with Parafilm and incubated at 45°C for 30 minutes with gentle shaking (60 strokes per minute).

A dye-binding protein assay (Section 2-3-3), was carried out on aliquots of the suspension as an independent check of concentration in some cases.

Pronase and peptidase were added and the digestion carried out as described above for the pure proteins. After digestion for 15 - 18 hours, samples were centrifuged (10 min, 3100 r.p.m., bench centrifuge), and the supernatant extracts were diluted as necessary with 0.05M pH 7.0 buffer, prior to assay for Lys, Met and Trp. Controls, as described for the pure proteins were included in all assays.

2-8-4 Concluding Remarks

In a time course for digestion, the amino acids in the proteins were made completely available after fifteen hours, using the digestion procedure described above. However, prolonging the time (up to 22 hours), had no adverse effect, therefore, routinely digestion was allowed to proceed overnight (16 - 18 hours). The results obtained from analyses on pure protein and food protein digests are described in Sections 6-5-4 and 6-6-3, respectively.

2-9 Automatic Amino Acid Analyses

2-9-1 Introduction

It was often necessary to analyse the amino acid constituent of samples, for example, to check the purity of peptides, to corroborate results from dansyl or fluorescamine chromatography or to separate and identify Met and its oxidation products (Chapter 5). For this work, a Locarte amino acid analyser equipped with an automatic peak area integrator was used.

2-9-2 Method

(a) Hydrolysis Procedure.

Samples containing approximately 0.5 μmol of any amino acid residue were added to Durham tubes, together with 10 μl of 2.5mM norleucine internal standard and evaporated to dryness. 50 μL of constant boiling (5.7M) hydrochloric acid was added, the tubes were evacuated, sealed and hydrolysis was performed for 18 hours at 105^{o}C. The tubes were opened, the contents evaporated to dryness over sodium hydroxide, and the samples were redissolved in 500 μL citrate buffer pH 2.2, for loading onto the analyser.

(b) Calculation of Results.

The concentration of each amino acid in the hydrolysate was calculated from the following formula:

$$\text{Concentration (mM)} = \frac{X \times 0.025}{CF \times Y}$$

where X = peak area of amino acid

Y = peak area Norleucine (Nle=internal standard)

0.025 = concentration of Nle (mM)

CF = colour factor (determined for each batch of ninhydrin)

$$CF = \frac{\text{Peak area of amino acid (std)}}{\text{Peak area of Nle (std)}}$$

2-9-3 Concluding Remarks

Using the acid hydrolysis described, Trp is destroyed and cannot be estimated. One advantage of the bacterial assay described in this thesis is that it permits determination of Trp in biological materials.

Methionine sulphoxide is a product of mild oxidation of Met, whereas strong oxidising agents e.g. performic acid, are needed to produce methionine sulphone. It was found that, providing care was taken to exclude oxygen from the tubes during hydrolysis, i.e. hydrolysis under N_2, Met could be recovered from acid hydrolysates of methionine-containing peptides with a yield of at least 97%.

Commercial samples of methionine sulphoxide Met(O) and methionine sulphone Met(O_2), or samples derived from the hydrolysis of peptides containing either of these compounds could be separated using the analyser. Met, Met(O_2) and Met(O) elute at 80, 36 and 32 minutes, respectively. Threonine elutes at 37 minutes and aspartic acid at 33 minutes; therefore, if there were any possibility that these amino acids could be present in hydrolysates containing Met(O_2) or Met(O), samples were also examined using dansyl (Section 2-4), or fluorescamine (Section 2-6) chromatography, which allow these various amino acids to be distinguished.

The colour-yield of methionine sulphoxide was found to be 99% that of aspartic acid, in agreement with that quoted by Spackman *et al.*, (1958). The sulphone gave a consistently high colour-yield, 15% higher than that of Met.

CHAPTER THREE

BETA-GALACTOSIDASE ASSAY FOR AVAILABLE LYSINE

3-1 Introduction

Microbiological assays for amino acid availability offer many advantages over conventional chemical or biological methods. However, most commonly they use fastidious microorganisms and are, therefore, inherently susceptible to "often unrecognised and essentially unquantifiable errors" (Bell *et al.*, 1977); some aspects of these errors were discussed earlier (Section 1-5-1).

3-1-1 Advantages of Escherichia coli for Microbiological Assays

Many of the disadvantages of using 'natural' mutants could in principle be overcome by the use of 'artificial' mutants of well characterised organisms such as *Escherichia coli*. This species can be cultivated in a simple defined medium (see, for example, Section 2-2-2). It grows easily and has a short generation time; the size of the inoculum is essentially unimportant; identical growth yields can be obtained from cells taken from the exponential or stationary phase, independently of whether the inoculum was prepared in minimal or enriched media and the cells can be stored for several weeks in liquid culture at $4^{o}C$.

Escherichia coli is a gut bacterium, which has well characterised transport systems for amino acids and peptides, that in many respects parallel the absorption systems found in the mammalian intestine (Matthews & Payne, 1975; Payne, 1975b, 1976, 1977). Therefore, *Escherichia coli* would seem to be a relevant and useful tool with which to assess the nutritional value of proteins. It has been shown for the amino acids tested that the same growth responses occur to free amino acids and to peptides.

Similarly, mixtures of these amino acids, their peptides, protein digests and other nutrients gave growth yields determined only by their content of required amino acids. In addition, growth yields on meal digests were linearly related to the amount of digest in the growth medium (Bell *et al.*, 1977).

The genetics of *Escherichia coli* are well characterised and much of the linkage map has been determined (Bachmann & Brooks-Low, 1980), the cells are amenable to genetic manipulation by simple, well documented techniques (Section 4-2-2). The *Escherichia coli* lysine auxotroph (M2626), which was used in early work, has a mutation in the gene coding for the last enzyme of the lysine biosynthetic pathway (diaminopimelate decarboxylase), and thus, no biosynthetic precursor of Lys can meet the need for this amino acid. Double amino acid requiring mutants can be selected for desirable amino acid assays, e.g. Lys-Met, Lys-Trp (Chapter 4), and this facility offers advantages over the use of 'natural mutants', whose complex growth requirements and genomes are not fully characterised.

Escherichia coli does not normally secrete free proteases or peptidases but its natural environment is the peptide rich lumen of the gut and it has efficient transport systems for absorbing these products as well as amino acids (Matthews, 1975). This means that when it is used to assay amino acids, an enzymic digestion of test proteins *in vitro* is needed. This proteolysis must produce fragments which are sufficiently small to pass through the outer cell envelope and reach the cytoplasmic peptide permeases (Payne & Gilvarg, 1968). Conditions for such proteolysis have been developed and described earlier (Section 2-8).

In fact, even when proteolytic organisms such as Streptococcus zymogenes are used; because they are only weakly proteolytic under assay conditions, prior enzymic digestion similar to that for Escherichia coli is also necessary. In Streptococcus zymogenes the level of exogenous proteolysis is variable and can be influenced by culture conditions, growth media and possibly other factors; there is also the possibility that secreted proteases may be hydrolysed by added proteases and vice versa; thus it is necessary to run enzyme blanks. These are perhaps less essential with Escherichia coli where conditions for proteolysis in vitro can be better controlled.

3-2 Bacterial Growth Assay For Available Lysine

3-2-1 Method

The Escherichia coli W lysine auxotroph M2626 was grown in test tubes containing 10 ml minimal medium A (Section 2-2-2), supplemented with sources of Lys. After autoclaving (15 psi, 15 min), glucose (0.25 ml, 20% w/v) was added aseptically to give a final concentration of 0.5% w/v, followed by an inoculum (0.1 ml, ca 10^6 cells). The tubes were incubated at $37^{o}C$ with shaking (100 strokes min^{-1}), in a water bath for 16 hours, and the absorbances were read at 660 nm using a Bausch & Lomb Spectronic 20, the zero time absorbances were subtracted from the final values. Using other mutants, (Section 4-2), Met and Trp were assayed in a similar way.

Previous studies confirmed early results (Gilvarg & Katchalski, 1965), that there is a linear relationship between growth yield (determined by E_{660}), and concentration of free Lys (in the range 0 - 0.15 μ mol Lys ml^{-1}). The same growth yield is obtained with equal amounts of free, or of peptide-bound, Lys in a variety of defined peptides and biological digests and the values were not influenced by the presence of additional nutrients (see Bell et al., 1977). Furthermore, when Met and Trp mutants were examined, their responses to these amino acids were comparable to that of M2626 to Lys. In conclusion, therefore, the use of Escherichia coli has advantages over some of the more widely used organisms.

3-3 β-Galactosidase Assay For Available Lysine

3-3-1 Introduction

A new approcah to the determination of available Lys using M2626 was developed by Bell et al., 1977; in which, rather than measuring cell proliferation specific enzyme synthesis was measured. In this way, a more rapid, sensitive and potentially automated colorimetric assay could be performed. Enzyme synthesis can be made to reach completion in less than the time required for a cell to divide and yet still be detected accurately and reproducibly, reducing the total assay period to less than 2 hours. Less than one percent of the Lys needed in the usual growth test is sufficient for the enzyme method. The amount of enzyme synthesis can be measured accurately using substrates that give coloured or fluorescent products.

3-3-2 Enzymes

Escherichia coli synthesises various well-characterised, inducible or derepressible enzymes that could be assayed by this method, and in preliminary studies β-galactosidase and alkaline phosphatase were evaluated (J.W. Payne - unpublished). However, here studies have been confined to the inducible enzyme β-galactosidase (E.C. 3.2.1.23.), which has over the years become the enzyme of choice in a variety of studies. For this work it has the particular advantage that the number of residues of Lys, Met and Trp are similar(29, 24, and 35 respectively / ¼ molecule) Table 4.1; thus making the sensitivity comparable for each amino acid.

3-3-2-1 Properties of β-Galactosidase

β-galactosidase is an intracellular enzyme that hydrolyses β-galactosides such as the milk sugar lactose, to give glucose and galactose.

When Escherichia coli is grown with glucose as the main carbon source, only a few molecules of the enzyme are present in the cell i.e. constitutive synthesis occurs, but when cells are grown with a β -galactoside as main C source, β -galactosidase synthesis is greatly increased, i.e. induced synthesis occurs.

The inducer need not be a substrate for β -galactosidase e.g., isopropyl- β -D-thio-galactopyranoside (IPTG), is a commonly used gratuitous inducer. The synthesis of β -galactosidase in Escherichia coli is regulated not only by induction but also by catabolite repression. Certain'preferred' carbon sources e.g., glucose or gluconate repress synthesis of the enzyme even in the presence of an inducer; succinate, acetate and glycerol are poorer catabolite repressors than glucose (Section 3-3-5-2).

The colourless compound σ -nitrophenol- β -D-galactopyranoside (ONPG) (Fig.3.1), is the substrate commonly used to assay β -galactosidase. The enzyme cleaves it to release σ -nitrophenol that ionises to the σ -nitrophenolate anion with an absorption maximum at 420 nm.

3-3-3 Initial Studies

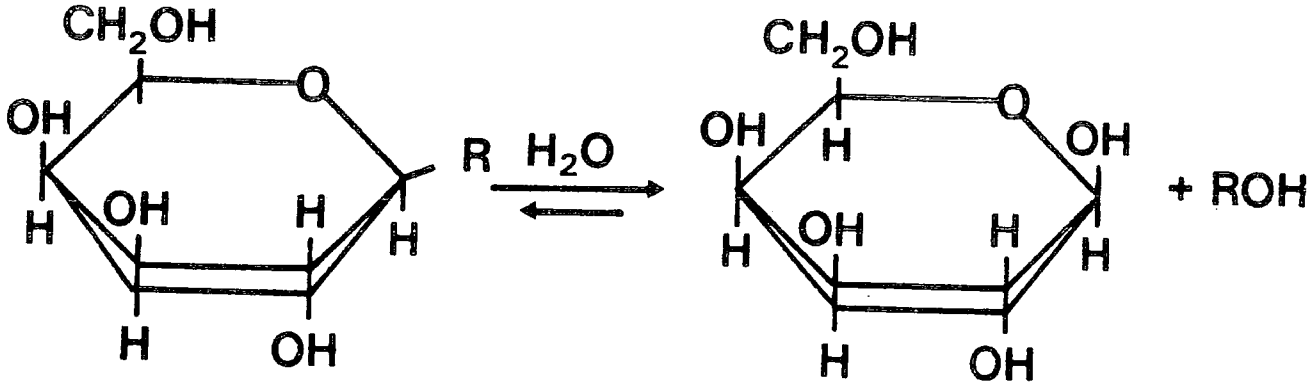
The sequence of operations necessary for the assay of available Lys using the Escherichia coli β -galactosidase method were described by Bell et al., 1977. Using this method, a linear response was obtained in the range 0 - 25 nmol Lys ml^{-1} final solution. Briefly, the sequence of events is as follows:

(a) Lys-starved cells of the Lys auxotroph were prepared by growing in a medium containing limiting Lys (0.05mM), which caused growth to stop at E_{660} of approximately 0.5 (i.e. 5×10^8 cells ml^{-1}). After this, the cells were incubated for a further 1 - 2 hours to deplete any residual Lys.

FIGURE 3.1

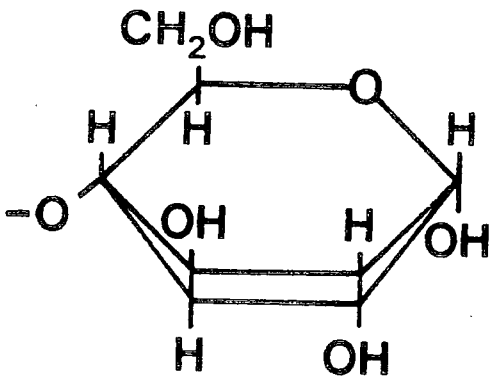
Reactions Catalysed by β-galactosidase (E.C. 3.2.1.23)

General Reaction



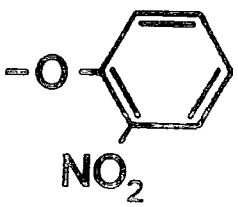
Specific Reactions

When R =



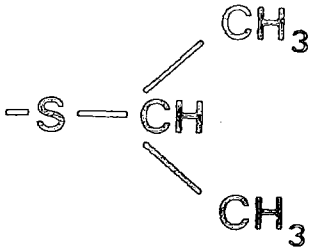
The substrate is lactose

When R =



The substrate is o-nitrophenyl-β-galactoside (ONPG)

When R =



The substrate is isopropylthio-β-galactoside (IPTG), an inducer but not a substrate.

(b) The starved bacteria were collected by centrifugation (8000 g, 20 minutes), and resuspended at a density of approximately 2×10^8 cells ml^{-1} in a complete growth medium, equilibrated at 37°C and containing 1mM IPTG. A medium was used with 0.6% w/v lactate as the C source (prepared by neutralisation of lactic acid with NaOH).

(c) After 10 minutes incubation at 37°C, 0.8 ml of induced cells were added to tubes containing 0.2 ml of a source of Lys (free, peptide-bound or biological digest), and incubated for 2 hours to allow synthesis of that quantity of enzyme which could be formed from the available Lys in the digest.

(d) 50 μL of toluene was added, the suspension was mixed on a vortex mixer and incubated for 20 minutes at 37°C to disrupt the bacterial permeability barrier. 0.2 ml 5mM ONPG, equilibrated at 37°C was added and the enzymic reaction allowed to proceed at 37°C for 20 - 60 minutes as convenient.

(e) Enzyme action was stopped by the addition of 0.2 ml 1M sodium carbonate solution and enzyme activity was measured (420 nm, 1 cm pathlength cuvette), from the colour yield (amount of σ-nitrophenol released from the substrate).

In the above system, enzyme activity is linearly related to enzyme concentration, thus a direct measure of enzyme synthesis can be obtained from the σ-nitrophenol released. The number of active enzyme molecules that can be synthesised by the auxotroph depends on the amount of available Lys and this was determined using a calibration curve of β-galactosidase activity (E_{420}) versus Lys concentration. Where $E_{420} = 1$ corresponds to 0.23 μ mol ml^{-1} of σ-nitrophenol (Fig. 3.2).

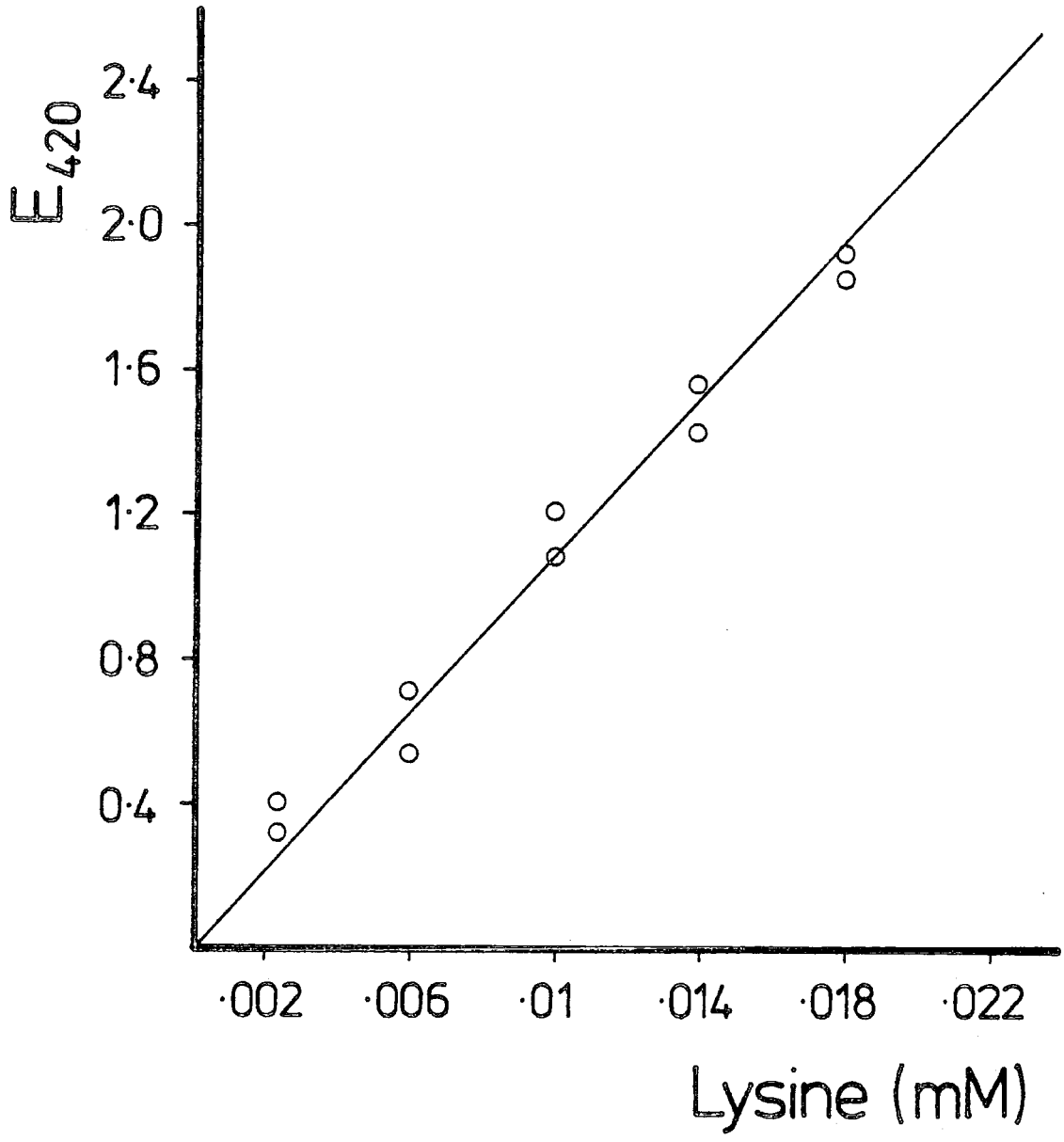


FIGURE 3.2

Lysine Calibration Curve

Available Lys versus amount of β-galactosidase synthesis by E.Coli.
Incubation of substrate for thirty minutes with toluene-treated bacteria.

E_{420} = 1.0 corresponds to o-nitrophenol (0.23μ mol/ml).

Subsequent work described herein was carried out with the objective of increasing the sensitivity of the assay and reducing the assay time.

3-3-4 Development of β-Galactosidase Assay

3-3-4-1 Effect of Cell Concentration

With a fixed concentration of Lys (4 nmol ml^{-1}), the rate of enzyme synthesis was directly proportional to the number of bacteria used (Fig. 3.3). The final yield of enzyme was equal in each case, being determined by the amount of added Lys and independent of the concentration of bacteria within the range $1 \times 10^8 - 5 \times 10^8$ cells ml^{-1}.

3-3-4-2 Kinetic Studies

When a fixed concentration of bacteria (ca 3×10^8 cells), was used and the concentration of Lys was varied (0-10 nmol ml^{-1}), the rate of enzyme synthesis increased with increasing Lys concentration up to a maximum rate; although the final yield of β-galactosidase showed the expected variation. Maximum enzyme synthesis was achieved by 60 minutes and this was taken as a suitable incubation time (Fig. 3.4).

The E_{420} values measured are not simply dependent on the amount of β-galactosidase but also upon the incubation time of the enzyme with the substrate. Thus, if this time is increased, it offers an "amplification factor" and low enzyme synthesis could be measured more sensitively by extending this period.

3-3-5 Preparation of Cells

It is necessary that the cells are starved of the amino acid to be assayed and any residual pool of that amino acid depleted.

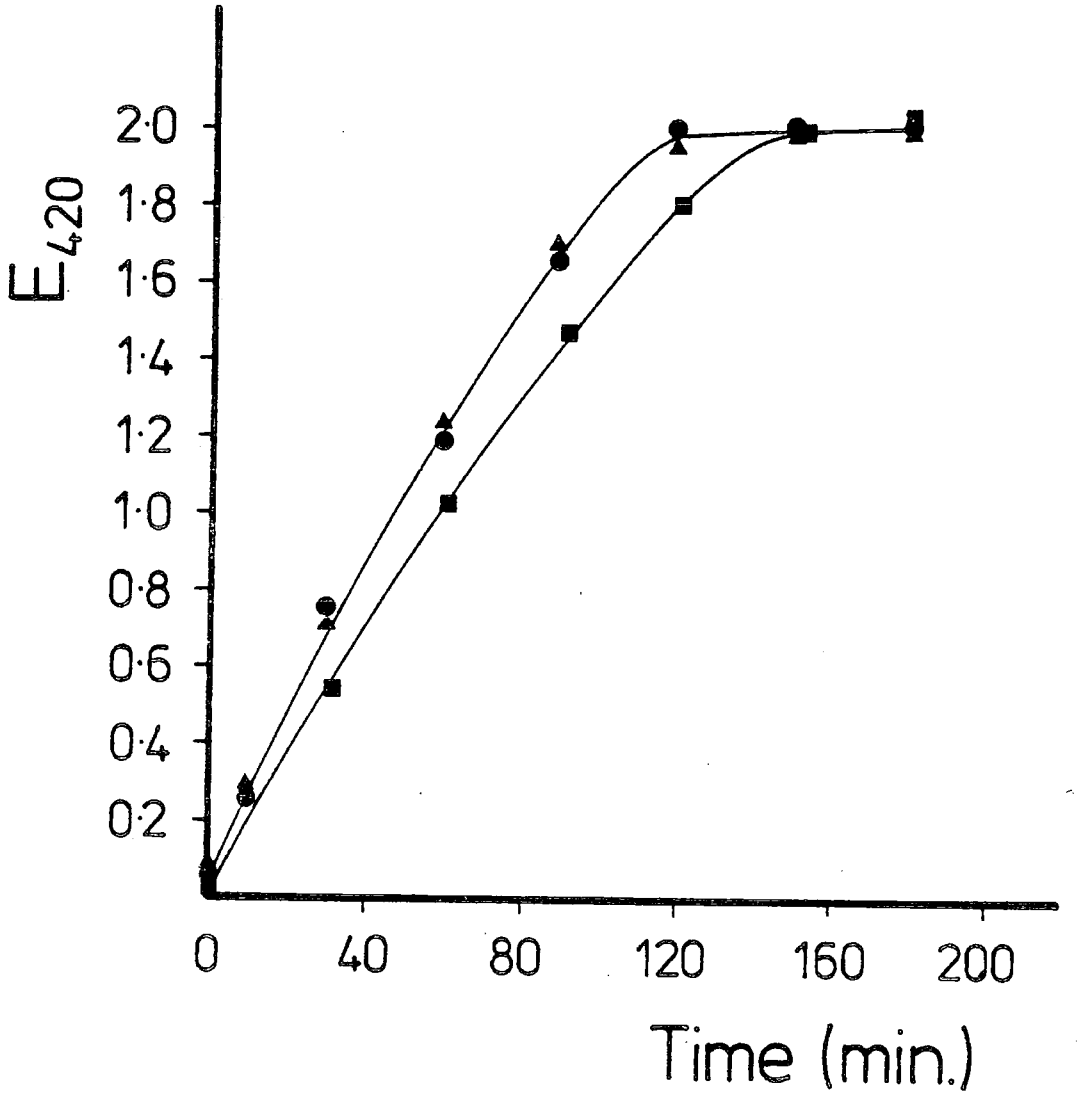


FIGURE 3.3

Rate of Beta-galactosidase synthesis by the E.coli Lys auxotroph M2626 as a function of bacterial concentration.

Lys (20nmol/ml) added at zero time. Approximate cell concentrations:

△——△ $5x10^8$ cells/ml; ●——● $2.5x10^8$ cells/ml;

■——■ $1x10^8$ cells /ml. Enzyme assayed by method described in section3-3-3

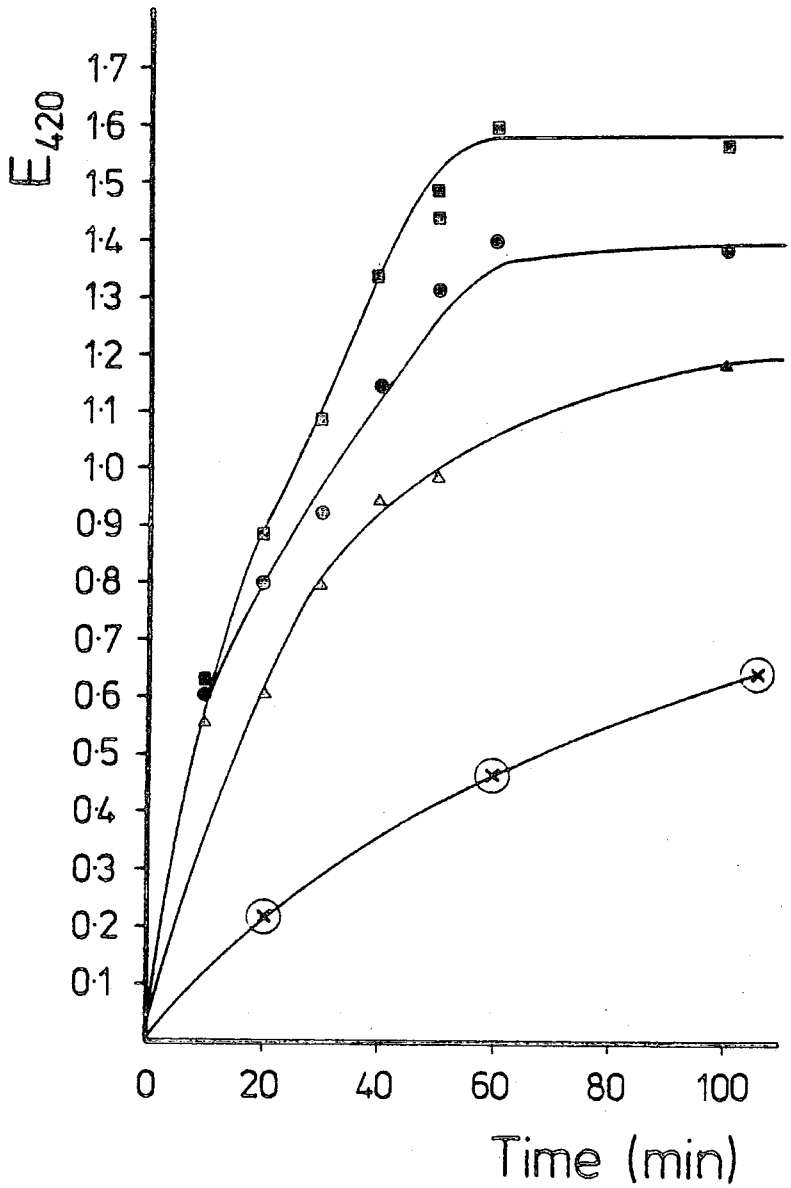


FIGURE 3.4 Kinetics Of β -galactosidase Synthesis By E.coli M2626. Cell concentration = 3×10^8 ml^{-1}. Cells were incubated with Lys concentrations for times shown and β-galactosidase was assayed by procedure as described in Section 3-3-3. Starvation periods were reduced hence blank values ⊗——⊗ were high.

■——■ 10 nmol ml^{-1} Lys

⊙——⊙ 7.5 nmol ml^{-1} Lys

△——△ 5 nmol ml^{-1} Lys

However, the practice of growing cells to stationary phase in limiting amino acid (Section 3-3-3), and harvesting by centrifugation is not very convenient, as timing of subsequent steps is important and centrifugation and washing is time consuming. Attempts were therefore made to improve this procedure: M2626 was grown as previously described, but with 0.2mM (excess) Lys with either 0.5% w/v lactate or 0.5% v/v glycerol as C source. About 20 ml of exponentially-growing cells (generally approximately 5×10^8 cells ml^{-1}), were collected on a membrane filter (Section 2-2-4). The cells were washed with twice the original volume of A medium containing 0.5% w/v of the required carbon source, and equilibrated to 37°C. They were then resuspended in the original volume of A medium plus lactate or glycerol, but minus Lys, and incubated at 37°C with shaking, for various times. To determine the rate at which residual Lys in the cells became depleted, 2 ml samples were removed from the incubation, induced, and β-galactosidase was assayed by the published method in which starvation was achieved by depletion of limiting Lys (Section 3-3-3). The results (Fig. 3.5), show that the intracellular Lys pool was essentially depleted within 30 minutes; therefore, to allow an extra safety margin a 60 minute starvation period, prior to induction, was adopted as standard. There was a low 'background' level of enzyme synthesis that continued after the adopted starvation period and we presume that this arises mainly from intracellular protein turnover. However, this level was relatively low, even with ca 5×10^8 cells, and was compensated for in all assays by appropriate blanks

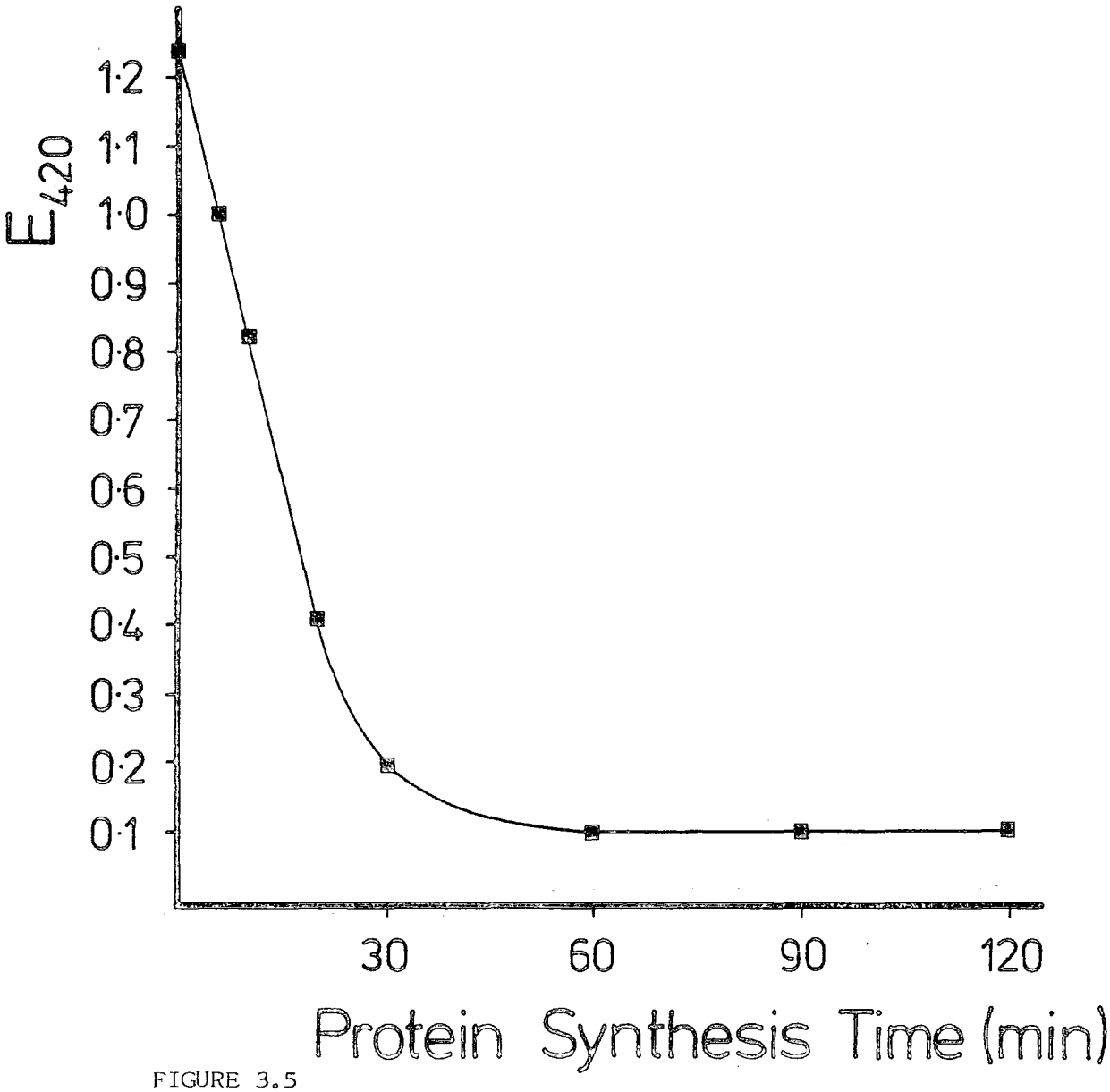


FIGURE 3.5

Beta-galactosidase synthesis by M2626 using endogenous Lysine.

Cells were grown to exponential phase, washed and starved of Lys as described in text. At stated times 2ml aliquots were induced and β-galactosidase assayed. Cell concentration: approx $2x10^8$ cells/ml.

In the final version of the routine β -galactosidase assay (Section 3-4), which utilises about 10^7 - 10^8 cells ml^{-1}, the quantity of amino acid liberated by protein turnover of starved cells is an insignificant source of error. Less than 1 nmol Lys could be detected in cell extracts prepared from 2.5×10^9 cells starved of Lys for 60 minutes.

3-3-5-1 Effects of Starvation After Induction

For β -galactosidase to be a useful enzyme in this context, it is important that its activity is stable once synthesised. Thus, an attempt was made to determine whether there is any significant loss of activity of the enzyme in amino acid or carbon-starved cells. 100 ml of bacteria were grown overnight in limiting Lys, as already described with sodium lactate (0.5% v/v), as carbon source. The E_{660} was found and the cells were induced at zero time and 0.02 mM Lys was added to allow enzyme synthesis. β -galactosidase levels were measured after intervals up to 90 minutes. The cells were collected on a filter (Section 2-2-4), washed to remove inducer using equilibrated 1 x A medium and the E_{660} adjusted to the individual level. Aliquots of these cells were added to 5 boiling tubes with combinations of Lys, lactate or glucose or to controls minus the amino acid or carbon source and the tubes were incubated at 37°C. The level of β -galactosidase present at different times thereafter under these starvation conditions were then measured. Fig. 3.6 shows that there was no systematic variation in the enzyme present in the cells whilst they were kept under starvation and thus, this is unlikely to be a serious source of error if prolonged incubation periods are necessary for a particular assay.

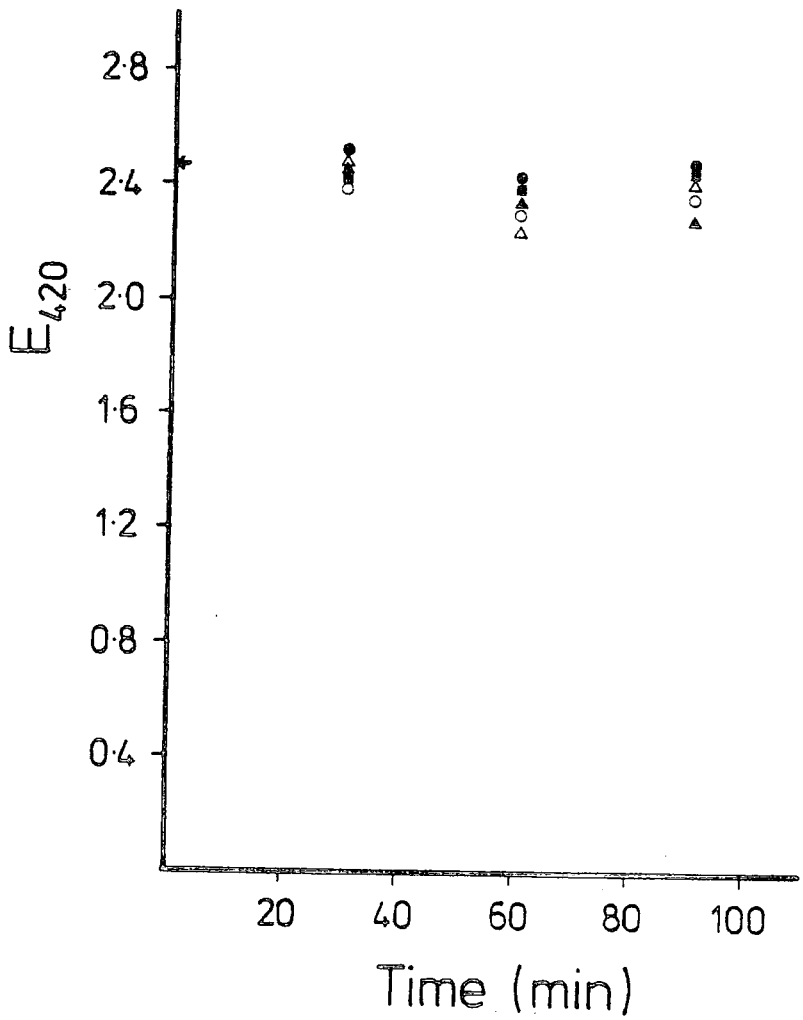


FIGURE 3.6 Stability Of β -galactosidase Under Various Conditions

After synthesis of enzyme cells were washed (to remove inducer and Lys) and 20 ml cells (ca. 10^8 ml^{-1}) were added to flasks at zero time, the incubations continued and β -galactosidase assayed at stated times. At T=0, E_{420} = 2.42: minus Lys + Glucose 0.5% w/v o——o ; minus Lys + Lactate 0.5% w/v Δ——Δ ; + Lys (0.01 mM) + Glucose 0.5% w/v ⊙——⊙ ;+ Lys (0.01 mM) + Lactate 0.5% w/v Δ——Δ ;Lys present but cells starved of main C source ⊡——□ .

Arrow on ordinate indicates initial concentration.

3-3-5-2 Carbon Source For Growth and Assay

Commonly, when growing Escherichia coli, glucose is used as carbon source. However, in the β -galactosidase assay, as originally described (Bell et al., 1977), sodium lactate was used since this avoids problems of catabolite repression (Section 3-3-2-1). The use of glycerol (0.5% v/v), and succinate (0.5% w/v), as alternate C-sources was examined. Fig. 3.7 shows that glycerol is a satisfactory C-source for the assay. However, in this experiment, all flasks were aerated and this could be an inconvenience when seeking a simple assay procedure. Incubations were therefore carried out in duplicate with lactate (0.5% w/v), and glycerol (0.5% v/v), in each case, one flask was shaken (100 strokes min^{-1}), to aerate, and the other was static. The results (Fig. 3.8), show that aeration is necessary for enzyme synthesis with lactate as C-source but is unnecessary with glycerol. The weak catabolite repression (Section 3-3-2-1), caused by glycerol is insignificant compared to that with glucose, and since the use of glycerol negated the requirement for aeration during protein synthesis, assays were routinely carried out in the presence of 0.5% v/v glycerol.

When cells (M2626), growing in A medium with 0.5% w/v glucose as C-source, were transferred to an identical medium containing 0.5% v/v glycerol, there was a lag varying from typically 2, up to 8 hours. However, once growing exponentially, M2626 had a mean generation time of 45 minutes on glycerol compared with 35 minutes on glucose (flasks being well aerated, 100 strokes min^{-1}, in each case).

In a separate growth test, where cells were grown in limiting (0.1mM) Lys, the same E_{660} was reached with both carbon sources, with growth stopping at E_{660} = 0.51 for glucose grown and 0.50 for glycerol grown cells.

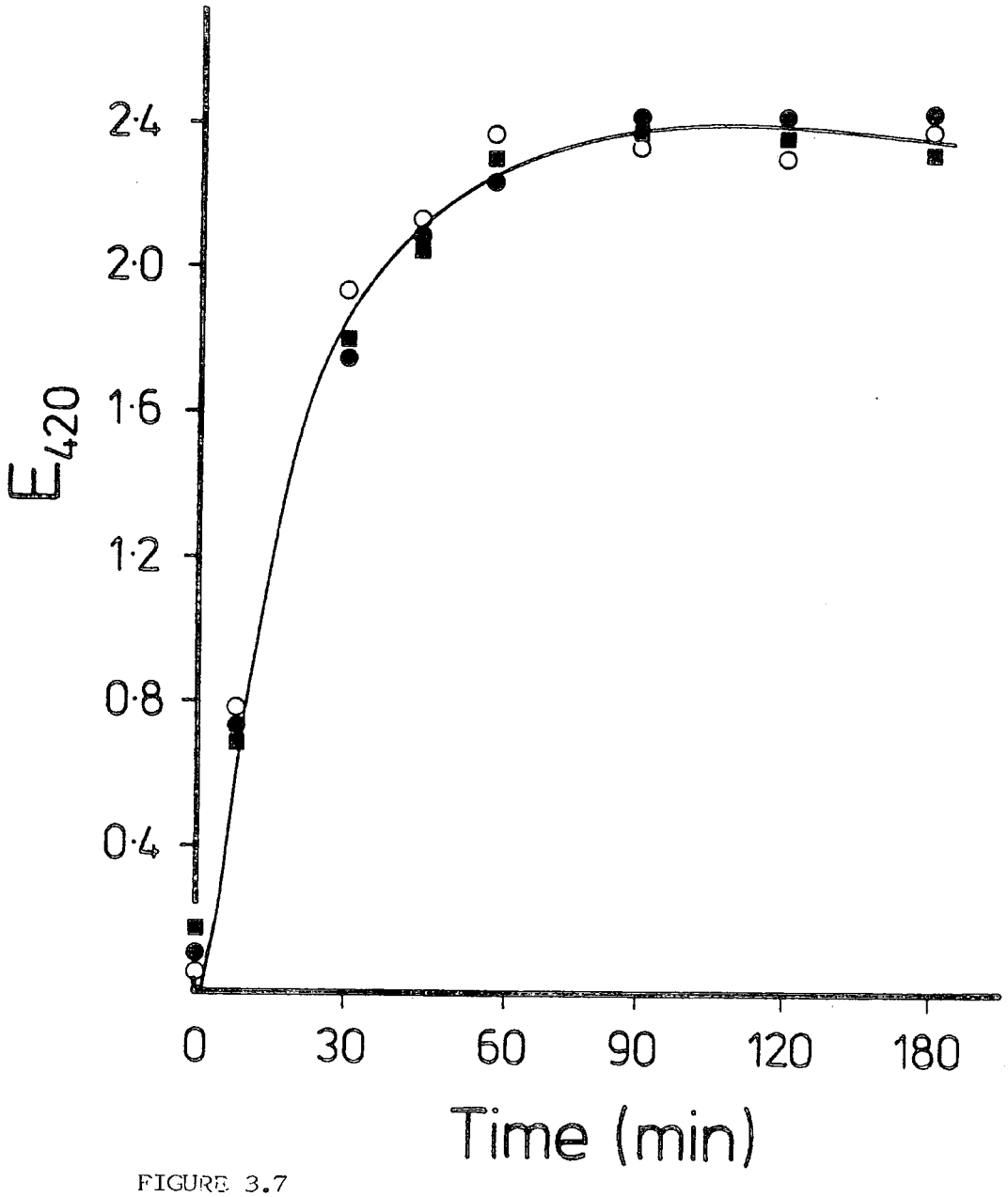


FIGURE 3.7

β -galactosidase synthesis in the presence of various C sources

■——■ Glycerol 0.5%v/v
●——● Lactate 0.5%w/v
○——○ Succinate 0.5%w/v

Experimental method as in Figure 3.6. All flasks containing ca. 10^8 cells /ml, shaken for aeration (100 strokes / min, shaking water-bath). β -galactosidase assayed by original procedure (section 3-3-3). All cells pre-grown in C source used for the experiment.

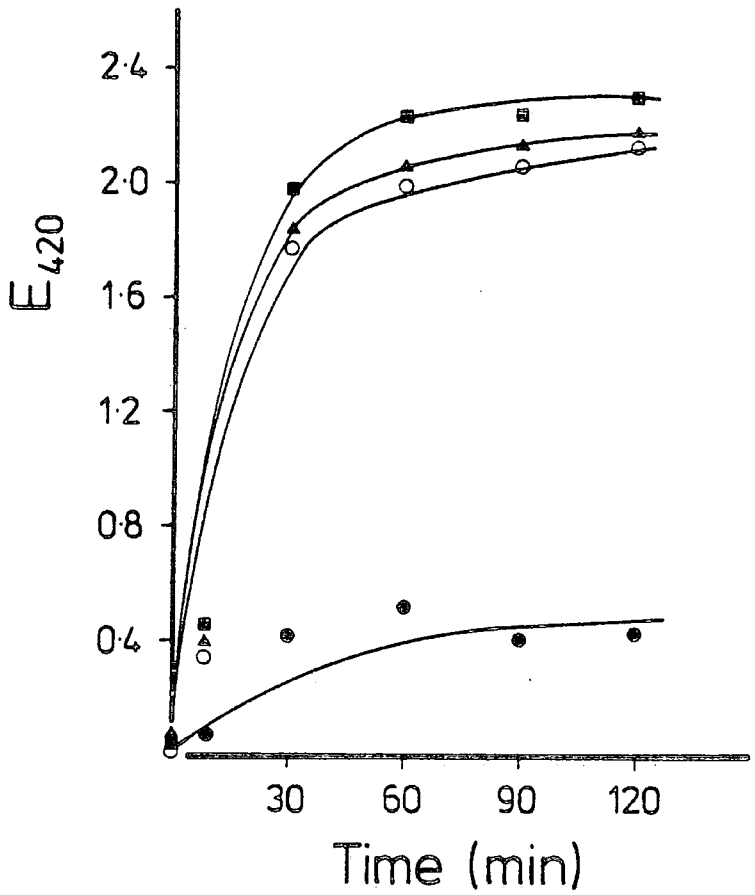


FIGURE 3.8 Effect Of Aeration On Synthesis Of β-galactosidase By E.coli M2626 With Lactate Or Glycerol As Carbon Source.

M2626 grown on appropriate C source to exponential-phase. Cells were collected and starved in normal way. Equal volumes of induced lactate- and glycerol-grown cells were added to flasks containing 0.5% w/v or v/v appropriate C source and 0.2 mM Lys. Flasks were incubated at 37°C in static or shaking (100 strokes min^{-1}), water baths. At stated times 2 ml aliquots were removed from each flask and β-galactosidase assayed.

O——O Lactate + aeration ●——● Lactate no aeration

□——□ Glycerol + aeration △——△ Glycerol no aeration

3-3-5-3 Cell Disruption

The synthetic chromogenic substrate (ONPG) used for the assay of β-galactosidase will not enter Escherichia coli cells unless the permeability barrier is broken. Hence, cell disruption is an essential step in the assay. Initially, (Bell et al., 1977), this was accomplished using toluene (20 u L ml^{-1} final concentration, added to tubes containing ca 10^8 cells ml^{-1}), the toluene being mixed thoroughly with the cell suspension and incubated at 37°C for 20 minutes prior to the addition of substrate. Toluene is used commonly to make bacterial cells permeable to exogenous substrates with molecular weights up to about 50,000. It is not clear exactly what damage to the cell envelope occurs; several reviews give detailed accounts of the effects of toluene on cells of Escherichia coli (Jackson & Demoss, 1965; Woldringh, 1973).

One fact that these authors make clear, however, is that the extent of damage depends on the methods of treatment (toluene concentration and temperature). Any attempt to simplify the assay in such a way that it could be more readily automated by disrupting cells prior to induction cannot be successful because this abolishes any amino acid dependent synthesis of β-galactosidase (Fig. 3.9). It was found that toluene concentrations could be reduced slightly, but when the reduction was by two fold or more, enzyme yields were significantly reduced (Fig. 3.10). This Figure also shows that for good permeabilisation incubation periods of at least 30 minutes are required.

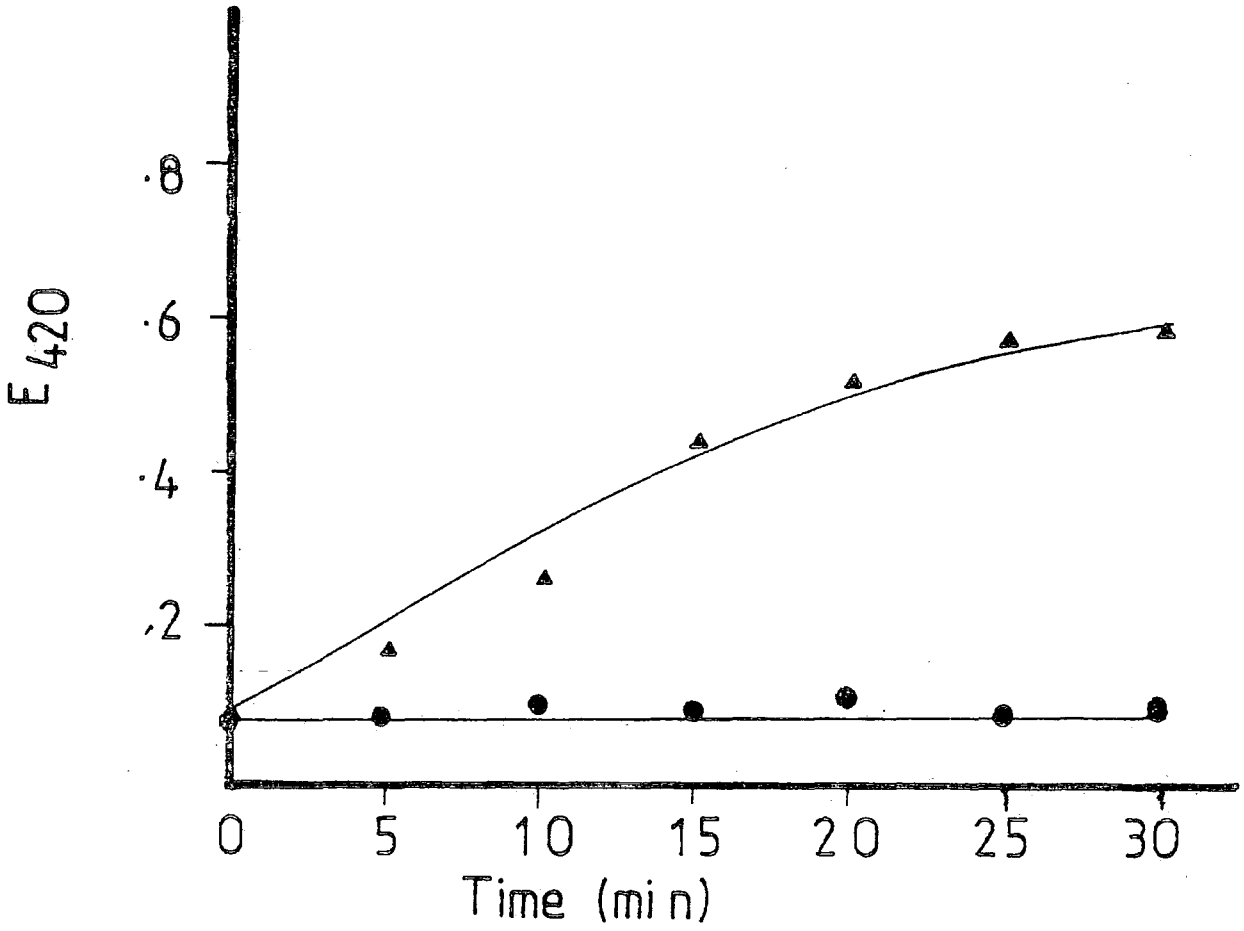


FIGURE 3.9 Disruption of E.coli M2626 By Toluene

5×10^8 cells ml^{-1} disrupted with 20 µl ml^{-1} toluene before ●——●, or after ▲——▲ induction. Cells incubated with toluene for 20 min at 37°C for disruption. Enzyme assayed by method described in Section 3-3-3

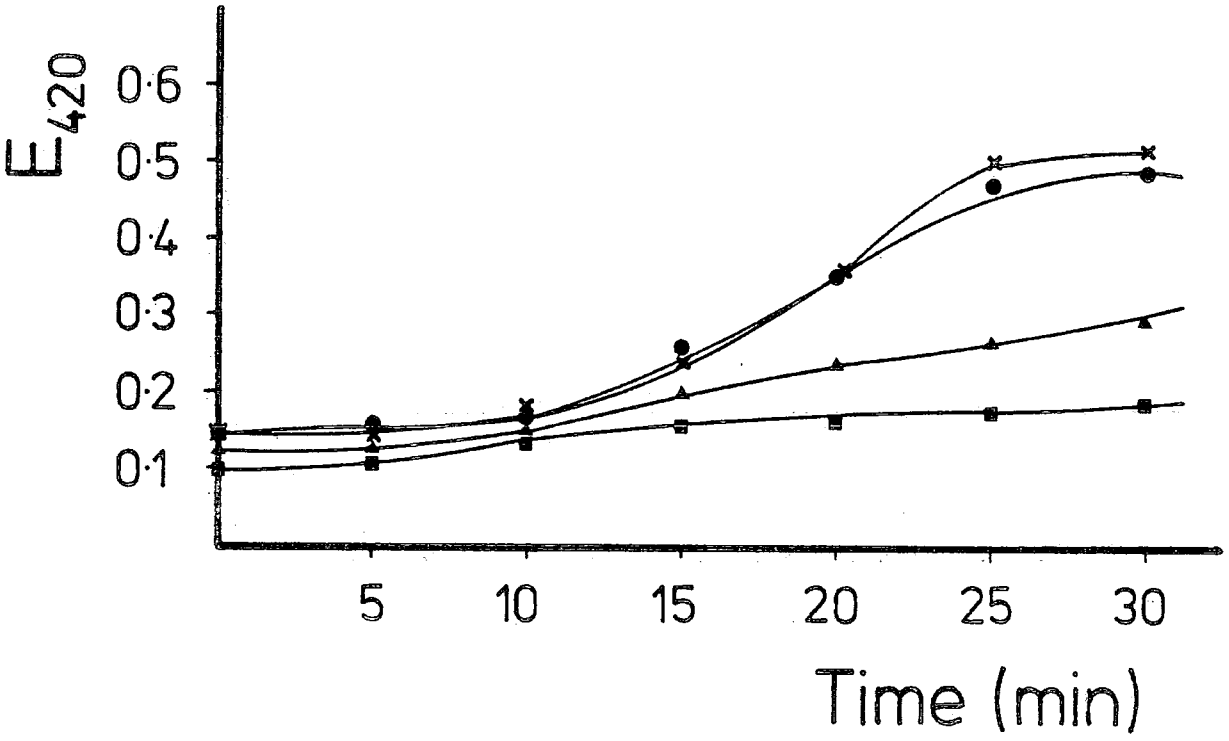


FIGURE 3.10

Effect of Toluene Concentration and Time of Disruption on β -galactosidase activity in E. coli M2626.

Cell concentration ca. $5x10^7$ / ml; x axis shows time of incubation of cells in toluene at 37°C, tubes thoroughly mixed (whirlimix) every 10 min.

×——× 20µl toluene / ml cells

●——● 10µl toluene / ml cells

△——△ 5µl toluene / ml cells

▣——▣ 2.5µl toluene / ml cells

An alternative method for the disruption of the permeability barrier of bacterial cells of particular use when assaying β -galactosidase, has been described by Putnam & Koch, (1975). This employs the following disruption medium: 1 part 10% w/v sodium dodecyl sulphate (SDS); 1 part 0.02M manganous sulphate; 1 part toluene; 5 parts 2-mercaptoethanol. The mixture forms a single phase with incipient cloudiness in the presence of water. The toluene and SDS both serve to break down the permeability barrier. The manganous cation acts as a co-factor to stabilise β -galactosidase (Rickenberg, 1959), and also to enhance the effect of the mercaptoethanol which keeps the enzyme in a more stable, reduced state (Craven et al., 1965). The solution can be stored in a glass bottle (ensure that a rubber insert is not used), at room temperature for up to six months - after which time it begins to darken and should be discarded. To compare the efficiency of the various disruption procedures and to establish the function of the various components, M2626 was used in the following tests:

(a) Disrupted with 10 μ 1 ml^{-1} toluene at 20 - 22°C for 30 minutes.

(b) Disrupted with 10 μ 1 ml^{-1} toluene incubated at 37°C for 30 minutes.

(c) Disrupted with 10 μ 1 ml^{-1} medium of Putnam & Koch at 20 - 22°C (room temperature for 10 minutes).

(d) As (c) above but with 10 μ 1 ml^{-1} disruption medium made up minus mercaptoethanol.

The results of kinetic assays (Fig. 3.11), demonstrate that disruption using the Putnam & Koch method was more effective than using toluene alone ~~but~~ and that the mercaptoethanol was essential to act as a reducing agent and maintain the stability of the enzyme.

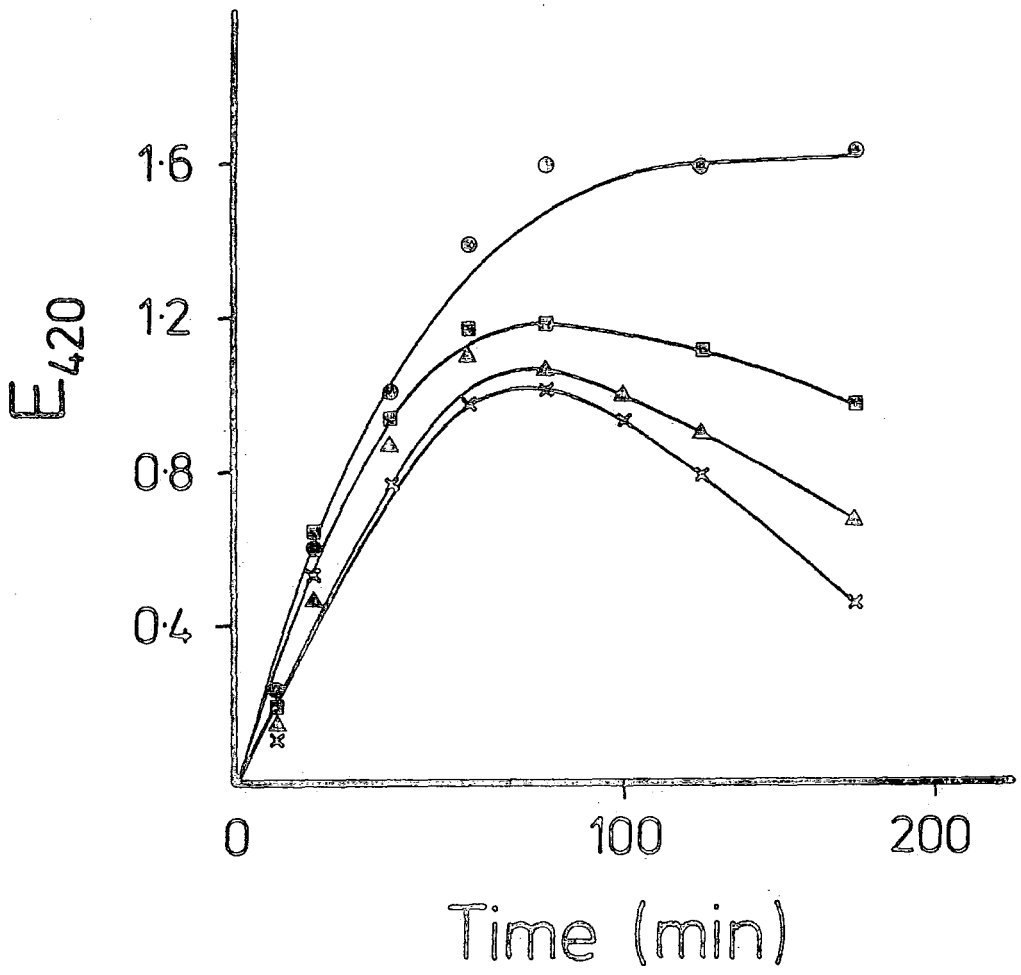


FIGURE 3.11

Examination of Disruption Methods

M2626 grown in medium containing 0.5% v/v glycerol and excess Lys.
Exponential cells collected, washed and starved as described in text
Resuspended in medium containing 0.1mM Lys ca $2x10^8$ cells / ml.
At time intervals duplicate 2 ml samples removed and treated:

○——○ Putnam and Koch medium 10 µL 10 min, room temp.

△——△ as above but less mercaptoethanol.

▣——▣ Toluene 10µ L / ml, 37°C , 30 min.

⨯——⨯ Toluene 10µ L / ml, room temp, 30 min.

(These results could explain why, in some previous kinetic assays, where incubation times exceeded 90 minutes, enzyme activity began to fall).

In the previous experiment, disruption of the cells, using the method of Putnam & Koch, was carried out as they recommended, at room temperature (20 - 22°C). Subsequent experiments (Fig. 3.12), established that higher values of enzyme activity were indeed obtained when disruption was carried out at 20 - 22°C rather than 37°C.

The mercaptoethanol could be replaced by an equivalent concentration (5 parts, 0.02M), of dithiothreitol (Cleland's reagent), with no effect on enzyme activity (Fig. 3.13). However, this was not used routinely because it is much less stable in solution.

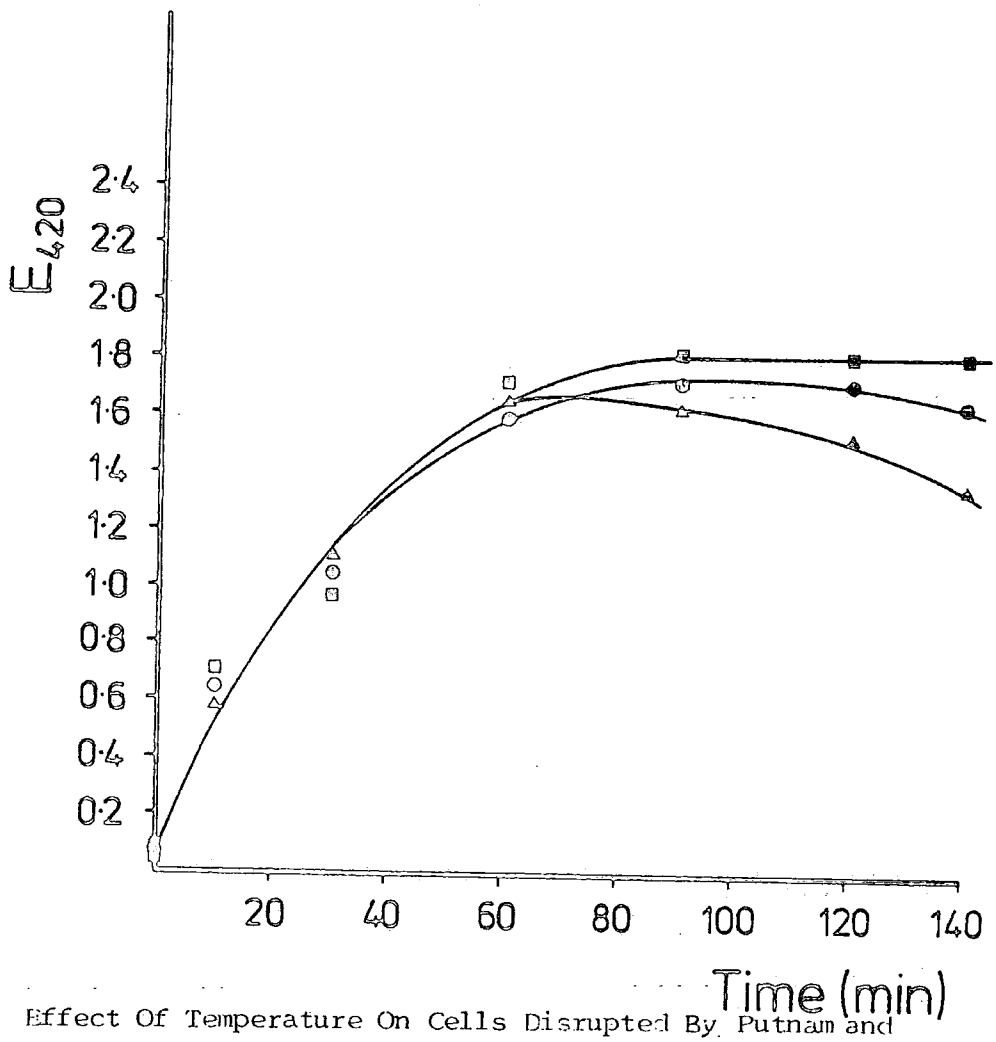


FIGURE 3.12 Effect Of Temperature On Cells Disrupted By Putnam and Koch Method : Effect On β-galactosidase Activity.

M2626 grown , collected in exponential-phase, washed and starved of endogenous Lys (as described in text). Resuspended in A medium + 0.5%v/v glycerol + 0.1 mM Lys at ca. 2×10^8 cells ml^{-1}.

□——□ Putnam and Koch Medium $10\mu l\ ml^{-1}$. 20 - 22°C

○——○ as above incubated at 37°C

△——△ Putnam and Koch Medium minus mercaptoethanol, 20 - 22°C

All incubations with disruption medium were for 10 min. Enzyme assayed by method described in Section 3-3-3.

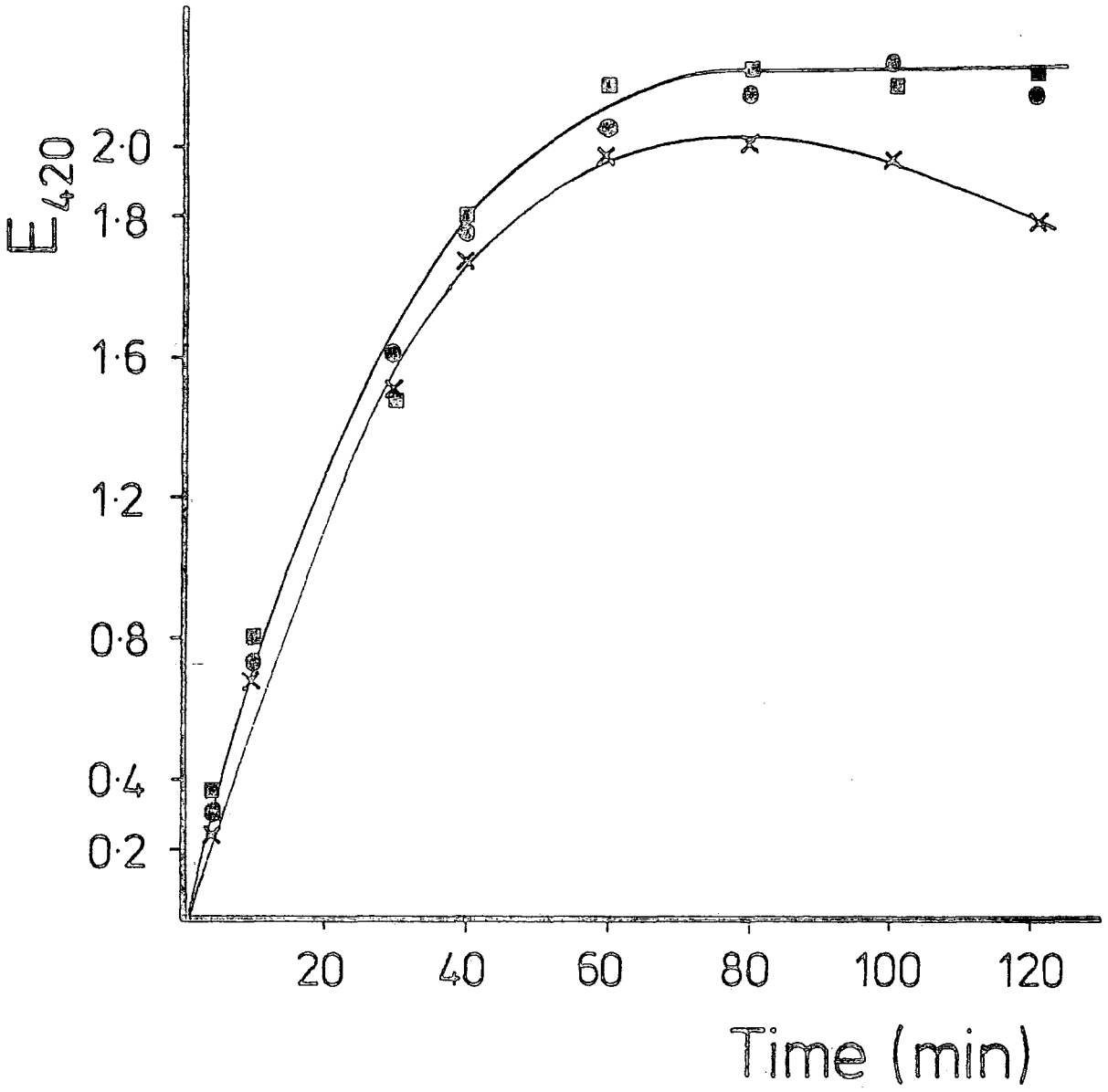


FIGURE 3.13 Use Of Dithiothreitol As Reducing Agent In Disruption Medium.

M2626 exponential cells collected, washed and starved in normal manner; resuspended in medium containing 0.1 mM Lysine. Cell density ca. 5×10^8 ml^{-1}. At stated time intervals 2 ml samples removed and treated:-

⊡—⊡ Putnam and Koch disruption medium, 10_μ 1 ml^{-1}, 20 - 22°C.

○—○ Medium as above but minus mercaptoethanol, with the addition of 1 part 0.02M dithiothreitol, 20 - 22°C.

✕—✕ As above but with neither reducing agent present.

Incubation times for disruptions ≤ 10 min

3-4 Recommended Protocol For Final Assay Procedure

The summary below gives the sequence of operations for the modified method which was used routinely in subsequent assays.

1. For running 40 assay tubes (20 duplicates), grow 20 ml Escherichia coli W auxotroph M2626 in a 100 ml conical flask at 37°C with shaking at 100 - 120 strokes min^{-1}, using A medium containing 0.5% (v/v) glycerol as C source and 0.2mM Lys.
2. Collect exponentially growing cells on a Millipore filter (6 cm diameter, 0.45 pore size), and wash filter with twice original volume pre-equilibrated (37°C) A medium to remove excess Lys.
3. Suspend cells in 20 ml fresh, equilibrated A medium containing glycerol, as before, but without Lys. Incubate at 37°C with shaking for 60 minutes to exhaust endogenous Lys.
4. Adjust cell density to give approximately 10^8 cells ml^{-1} (OD = 0.05 Bausch & Lomb Spectronic), and add inducer IPTG 0.01M, 0.2 ml, to give a final concentration of 0.1mM.
5. Incubate cells with inducer for 15 minutes at 37°C. Remove 0.5 ml samples and add to numbered tubes containing the material to be assayed (to give a final volume in the assay tube of 2 ml), pre-equilibrated at 37°C. Test samples should contain Lys in the range 0 - 1.0 μM.
6. Incubate tubes at 37°C for 75 - 90 minutes with gentle shaking; during which time the bacteria synthesise a quantity of β -galactosidase dependent on the Lys available in the assay sample.
7. In a fume cupboard, add 20 μl of Putnam & Koch disruption medium (Section 3-3-5-3), to give 10 μl ml^{-1} to each tube. Mix each vigorously on a whirlimix (5 seconds at maximum speed).

Leave at room temperature ($20^{o}C$), for 10 minutes, giving a second mix after 5 minutes.

8. Add 0.2 ml of 10mM substrate, ONPG, to each tube, mix thoroughly but gently and incubate in a fume cupboard at room temperature for a period appropriate for activity (usually about 30 minutes).

9. Add 0.1 ml 1M sodium carbonate to raise the pH to 11.0 and to stop the reaction. Read the yellow (E_{420}) of the liberated o-nitrophenol and plot values of absorbance against time (corrected as necessary).

10. Read the available Lys concentration for test samples against a calibration curve relating β-galactosidase activity to standard amounts of Lys, covering the range 0.1 - 1.0 μM Lys.

Results of assays for proteins of known composition, high protein meals, and rice samples are discussed in Sections 6-6, 6-7 and 6-8 where detailed methods of calculation are described.

3-5 Assay Results

3-5-1 Stability of Liberated σ -nitrophenol

In order to determine the stability of the σ-nitrophenol in assay tubes one set of 20 tubes was retained after the E_{420} had been read at the end of the assay. The value of the E_{420} was read (Gilford Spectrophotometer), and recorded at fifteen minute intervals for a six hour period, during which time the tubes were kept at room temperature. The results (not shown), demonstrated that the σ -nitrophenol in solution with the test sample, disrupted cells and disruption medium, was stable for up to two hours. After which a gradual decrease occurred (average decrease 13.1% over 6 hours). This demonstrated the importance of reading all results within two hours of completing the experiment. In practice, tubes were read immediately at the end of the incubation period.

3-5-2 Computation of Results

As already described (Section 3-4), the enzyme activity present in the assay tubes is determined by measuring the absorbance at 420 nm. The optimum range for E_{420} readings should be between 0.3 - 2 (SP1800).

The E_{420} reading is a combination of the absorbance of the σ -nitrophenol and the light-scattering from cell debris. This latter component can be corrected for by measuring the extinction at 550 nm, at which there is only light scattering and no absorbance of the σ -nitrophenol (Fig. 3.14).

For Escherichia coli (light scattering) $E_{420} = E_{550} \times 1.75$ (Fig 3.15). Using this correction factor the true absorbance of σ -nitrophenol was computed for each sample Actual (corrected) $E = E_{420} - (E_{550} \times 1.75)$. This was used routinely at first.

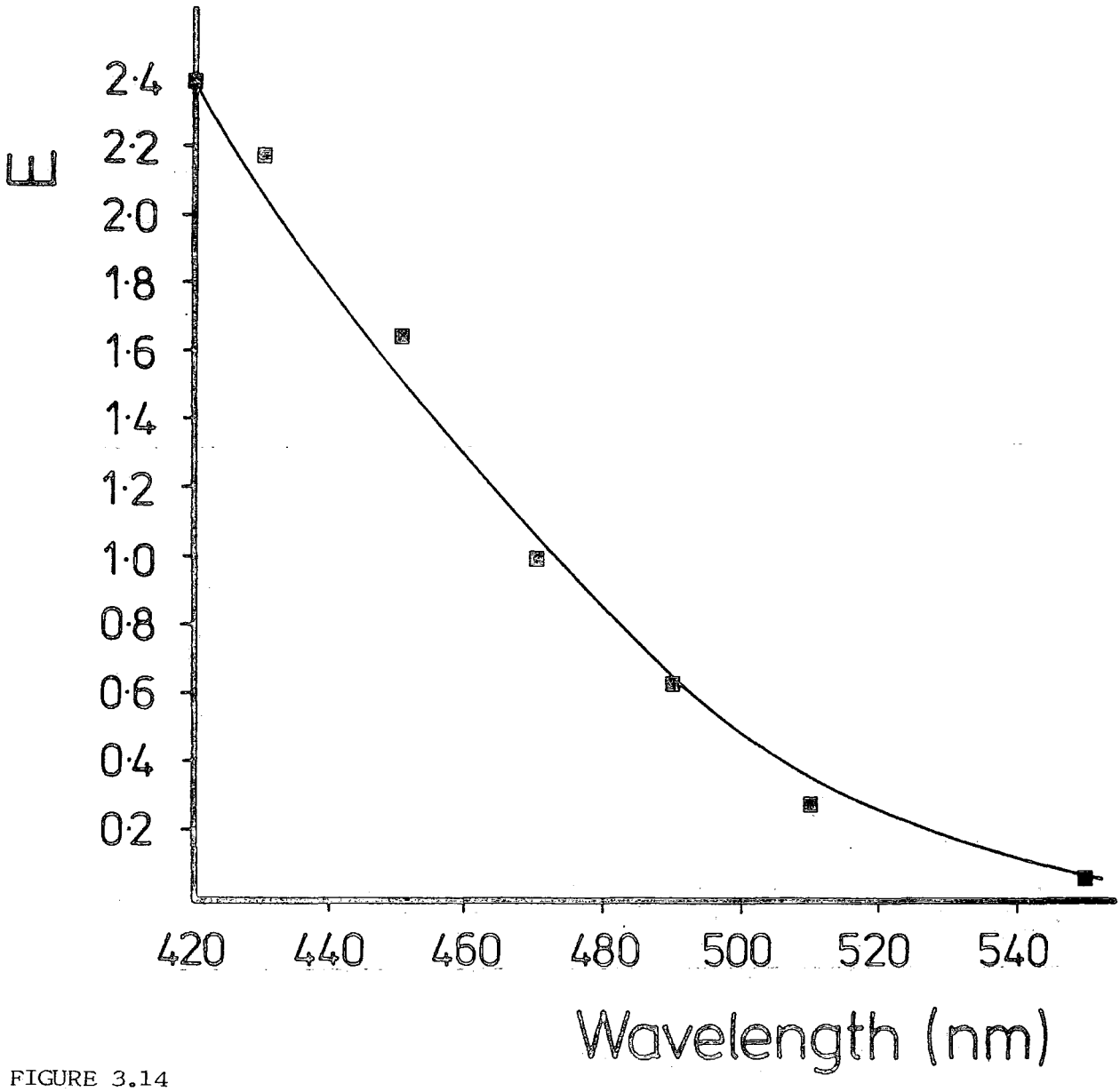


FIGURE 3.14

Relationship between wavelength and extinction for solution of

σ-nitrophenol (0.55mM)

E_{420} corresponds to σ-nitrophenol =0.23 μmol /ml.

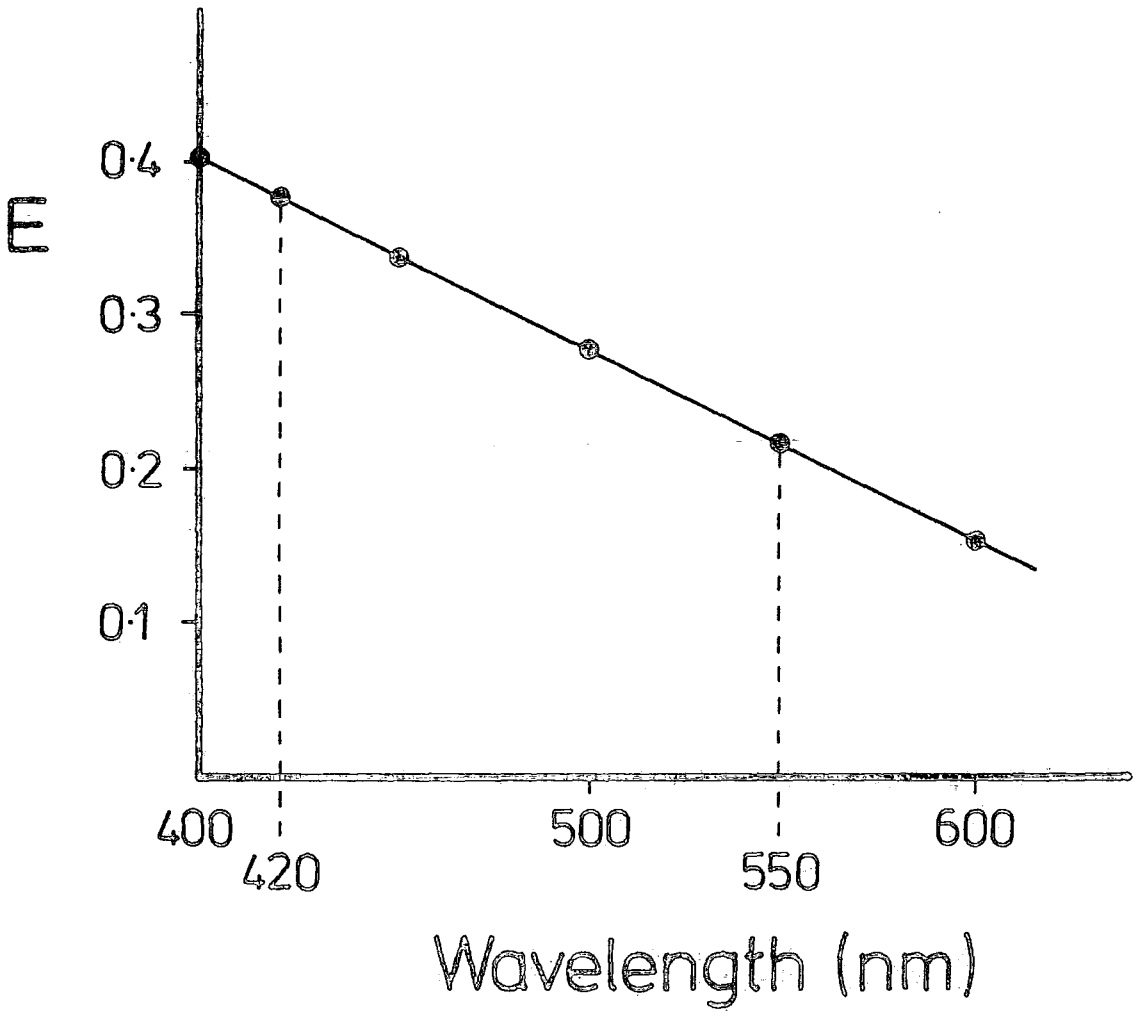


FIGURE 3.15

Relationship between Wavelength and Extinction for Cell Suspension (ca. 10^8 cells / ml)

E_{660} 1.0 = 2 x10^9 cells

However, when the development work had been completed, and optimum assay conditions established, cell densities could be reduced from ca 5 x 10^8 ml^{-1} to less than 10^8 ml^{-1} (E_{660} = 0.05). At this level the light scattering caused by cell debris was shown to be insignificant, E_{550} less than 0.01 compared with absorbance from the o -nitrophenol, and thus, could be ignored.

After the corrections had been made to the E_{420} reading (where necessary), the value of the control blanks (tubes to which no test sample was added), was subtracted from all readings. To speed the calculation, a programmed calculator was used. In all Figures (except where otherwise stated), where E_{420} values are plotted, the data have been corrected as necessary.

In some earlier experiments, instead of correcting the E_{550} contribution of the cell debris, the sample tubes were spun on a bench centrifuge (3100 r.p.m., 20 minutes). Although this procedure was satisfactory, it was not used routinely because it was more time-consuming.

A typical Lys calibration curve, showing the levels of Lys which can be detected using the final modified protocol (Section 3-4), is given in Figure 3.16. Results obtained using this modified protocol show approximately 20-fold greater sensitivity than in the original procedure (Fig. 3.2). A linear response being obtained in the range 0 - 1.0 μ M (final concentration) Lys.

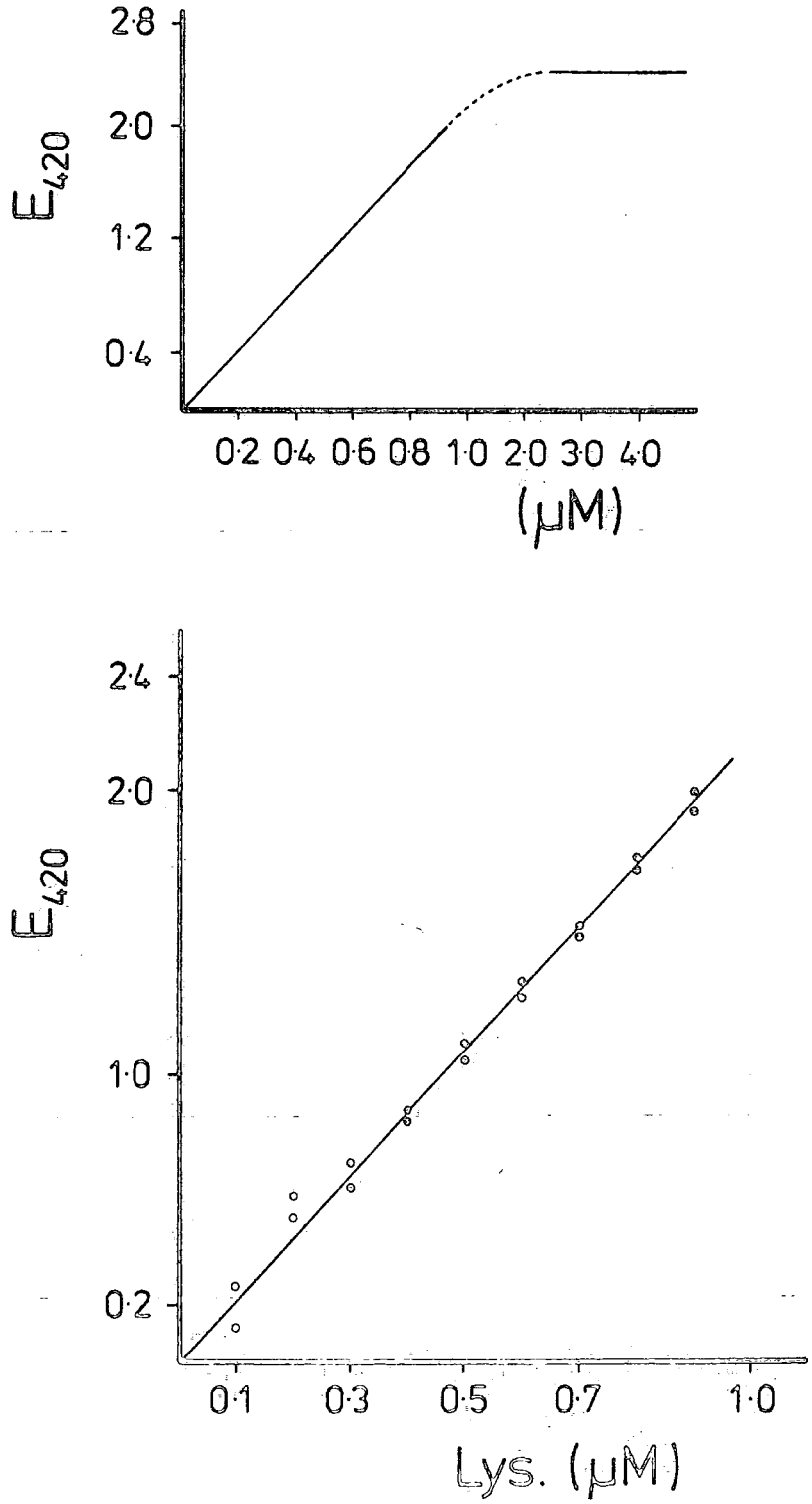


FIGURE 3.16

Lysine Calibration Curves

Available Lys versus amount of β-galactosidase synthesis by M2626 using modified protocol (section 3.4); increased sensitivity over original method (fig 3.2) of twenty-fold.

3-6 General Conclusions

The assay described can be completed in $2\frac{1}{2}$ hours, with up to 30 samples being handled. The sensitivity can be further increased by extending the enzyme-substrate incubation times; but using the standard modified procedure, as described, 0.1 μM Lys can be determined. Results demonstrating the application of the method to measure peptide-bound Lys and Lys present in a variety of digests are described in Chapter 6.

CHAPTER FOUR

SELECTION AND CHARACTERISATION OF MUTANT STRAINS FOR USE IN ASSAYS FOR AVAILABLE METHIONINE AND TRYPTOPHAN

4-1 Introduction

Whereas Lys is probably the most frequently deficient amino acid in the European diet at present because wheat proteins are deficient in Lys; Met is another essential amino acid that is often deficient, especially when the staple consists of legumes (Woodham, 1978). It has been claimed that Met is the most important S-containing amino acid (Heimann, 1980), through its action as a methyl donor. The human requirement for Met may be satisfied by homocysteine, or by certain methionine hydroxy analogues, provided methylating agents (such as choline and vitamin B_{12}), are present in the diet. Cysteine however, will only partially replace Met (Meister, 1965). Cysteine is readily converted to Met chemically and analyses generally quote Cys plus Met.

Trp is an essential aromatic amino acid that is rarely first limiting in food protein sources but which does present a particular problem because it is rapidly destroyed by acid hydrolysis and hence cannot be determined by using conventional chemical analyses. Therefore, reliable data of Trp contents are rarely encountered. Cyst(e)ine is also destroyed by acid hydrolysis (Williams et al., 1979), and is usually determined after conversion to stable cysteic acid using performic acid oxidation. After prolonged acid hydrolysis Met is destroyed (Chapter 5).

For these reasons, it was decided to isolate double auxotrophic mutants to permit the assay of Lys, Met and Trp. Because the β -galactosidase molecule contains approximately equal numbers of each of these residues (See Section 3-3-2 and Table 4.1), sensitivity to each could be expected to be of the same order (Section 4-3-1).

Table 4.1

Amino Acid Composition Of β -galactosidase

Residues per ¼ Molecule		Ratio
Lys	29	1.0
Met	24	0.8
Trp	35	1.2

Total molecular weight = 135,000

Data from Craven et al (1965), Steers & Cuatrecasas (1974)

4-2 Isolation of Double Mutants

4-2-1 Introduction

The auxotrophic mutants required for the enzymic assay of amino acids should ideally be deletions since these would be stable and reversion would be avoided. To try and achieve this simply, nitrous acid was used as a mutagenic agent. It acts directly on nucleic acids by oxidatively deaminating their bases. Thus, adenine is deaminated to hypoxanthine which pairs with cytosine instead of thymine and cytosine is deaminated to uracil which pairs with adenine. Guanine is changed to xanthine which continues to pair with cytosine but with only two instead of three H bonds. Thymine has no amino group, hence it is unaffected by nitrous acid. Thus, nitrous acid causes two way transitions A - T $\Longleftrightarrow$ G - C and can lead to part of the chromosome being deleted (Hayes, 1968).

4-2-2 Isolation Techniques

Nitrous acid was prepared by using the method of Schwartz & Beckwith (1969), by the addition of 0.1725 g $NaNO_2$ to 25 ml sodium acetate buffer (0.1M, pH 4.6), at room temperature (approximately 20°C). This gives 0.1M HNO_2; 0.05M when diluted in the reaction tubes. Because it is unstable, it is used immediately. The 0.1M acetate buffer was prepared using sodium acetate and acetic acid and its pH was adjusted to 4.6 before autoclaving (15 p.s.i., 15 minutes).

To isolate mutants with nitrous acid, the treatment should give a survival rate of 0.1 - 0.01% (Miller, 1972a); therefore, a preliminary experiment to determine the necessary conditions for this was performed.

Approximately 5 x 10^8 cells of M2626 were added to each of 6 sterile 15 ml centrifuge tubes; collected in a bench centrifuge at 3,100 r.p.m., washed once with 10 ml acetate buffer at 37°C then resuspended in 1.0 ml of the same buffer. 0.1 ml samples were removed and viable cell counts performed (initial cell numbers). Freshly prepared nitrous acid was added to duplicate tubes to give final concentrations of 0.07, 0.05 and 0.03M.

After 10 minutes at 37°C, 10 ml of excess of potassium phosphate buffer, pH 7.0, 0.2M was added to one of each duplicate pair to stop the reaction, and after a further five minutes the reaction in the remaining three tubes was stopped in the same way. Viable counts were performed to determine the percentage survival in each tube (Table 4.2). 1.0 ml of each of the mutagenised samples in buffer were also added to six flasks containing 24 ml of A medium enriched with 2mM Lys, 2mM Met or Trp, 0.5% w/v glucose and 0.1% yeast extract and incubated for 12 hours at 37°C, for growth of survivors. These flasks were then stored at 4°C for 24 hours awaiting the results of the viable cell counts.

Optimum killing conditions were found to be fifteen minutes with 0.07M nitrous acid; therefore, the flasks that had been subjected to other times and concentrations were discarded. The remainder were used for the next stage: Penicillin enrichment.

Because the mutants required are auxotrophs distinguished by their lack of growth on media that support growth of the wild type, replica plating must be used for their selection.

Table 4.2

Nitrous Acid Treatment - Percentage Survivors

	Percentage Survivors		
Contact Time (min)	0.07M[a]	0.05M[a]	0.03M[a]
10	0.02	1.0	20
15	0.01*	0.1	4

* Because 0.1 - 0.01% survival is required, cells treated in this way were used subsequently.

[a] Molarity of nitrous acid.

However, a penicillin enrichment step was first used to 'concentrate' the desired mutants. Escherichia coli, like other Gram negative bacteria, is fairly resistant to penicillin, therefore, the N-substituted penicillin, Ampicillin, was used (Molholt, 1967; Christensen, 1981). A solution of 0.2 mg ml^{-1} (aq), of Ampicillin, sterilized by filtration, retained its activity against Escherichia coli for six months (data not shown). A solution of 0.05M Mg^{++} in sucrose (100% w/v), was prepared and filter sterilized.

For the enrichment procedure, approximately 5 x 10^9 cells from the mutagenised cultures were grown up at 37°C in medium containing 1mM Lys and either 1mM Met or Trp for 3 hours. Cells were then collected, washed and resuspended in the same media but minus the Met or Trp and again incubated for 3 hours, for the cells to use up the endogenous pool of these amino acids. After this time, the E_{660} was taken, and if the cell density exceeded 2 x 10^8 ml^{-1}, the culture was diluted with A medium (Molholt, 1967). Sucrose, Mg^{++} and Ampicillin were added to each flask to give final concentrations of 20% w/v 0.01M and 0.02 mg ml^{-1} respectively and incubated for 90 minutes at 37°C. 2.5 ml of cell suspension was removed, the cells were collected in a bench centrifuge (3,000 r.p.m., 20 minutes), and resuspended in A medium equilibrated to 37°C and washed to remove the antibiotic. Washed cells were resuspended in A medium enriched with Lys plus Met or Trp as before.

The enrichment procedure was repeated once more only (any further cycles would increase the proportion of required mutants but would be likely to select for Ampicillin-resistant strains).

Dilutions were then made and 0.1 ml of diluted suspension from each tube (to contain 100 - 150 viable cells), was spread on an agar plate (enriched with Lys and Met or Lys and Trp). Six plates of each dilution were prepared for each auxotroph sought. After incubation at 37^{o}C for 24 hours to give suitable colonies, the colonies were replicated onto minimal + Lys plates in the normal way (Lederberg & Lederberg, 1952; Miller, 1972b).

Twelve presumptive Met, Lys and thirty-seven presumptive Met, Trp auxotrophs were initially isolated. Each colony was checked by streaking onto duplicate gridded plates containing Lys and plus or minus Met or Trp. In this way colonies of four Lys, Met and three Lys, Trp auxotrophs were selected.

4-3 Characterisation Of Mutants

4-3-1 Growth Response Curves

The generation times on glucose and glycerol were determined for each double auxotroph and no significant differences were found between the parent and derivatives. For complete growth in A medium, the mutant strains required approximately 0.15mM Trp and 0.1mM Met.

Figure 4.1 shows growth yields for two double mutants versus amino acid concentration for each of their required amino acids. On agar plates, 0.3mM of Met or Trp were required to give large colonies, with 0.2mM being the minimum concentration to support visible colony growth in each case.

4-3-2 Relative Sensitivity to Lys, Met and Trp

In addition to the growth tests, the more sensitive β-galactosidase assay was performed. Each of the isolates was tested in a combined assay for Lys and Met or for Lys and Trp. In each case one amino acid was supplied in excess (0.2mM), and graded doses (0.0002 - 0.02mM), of the other were supplied. Thus, the amount of enzyme synthesised in response to equimolar amounts of each of the required amino acids could be compared. Results (Fig. 4.2), showed that the sensitivity was of the same order in each case, with only slight variation in enzyme yield for each amino acid as measured from the ratios of the slopes. These observed values are in close agreement with the calculated ratios for these three amino acids in β-galactosidase (Tables 4.1 and 4.3). Deviations from these theoretical ratios might arise from differential catabolism of the amino acids but seemingly, this is of minor consequence. See discussion (Section 7-3).

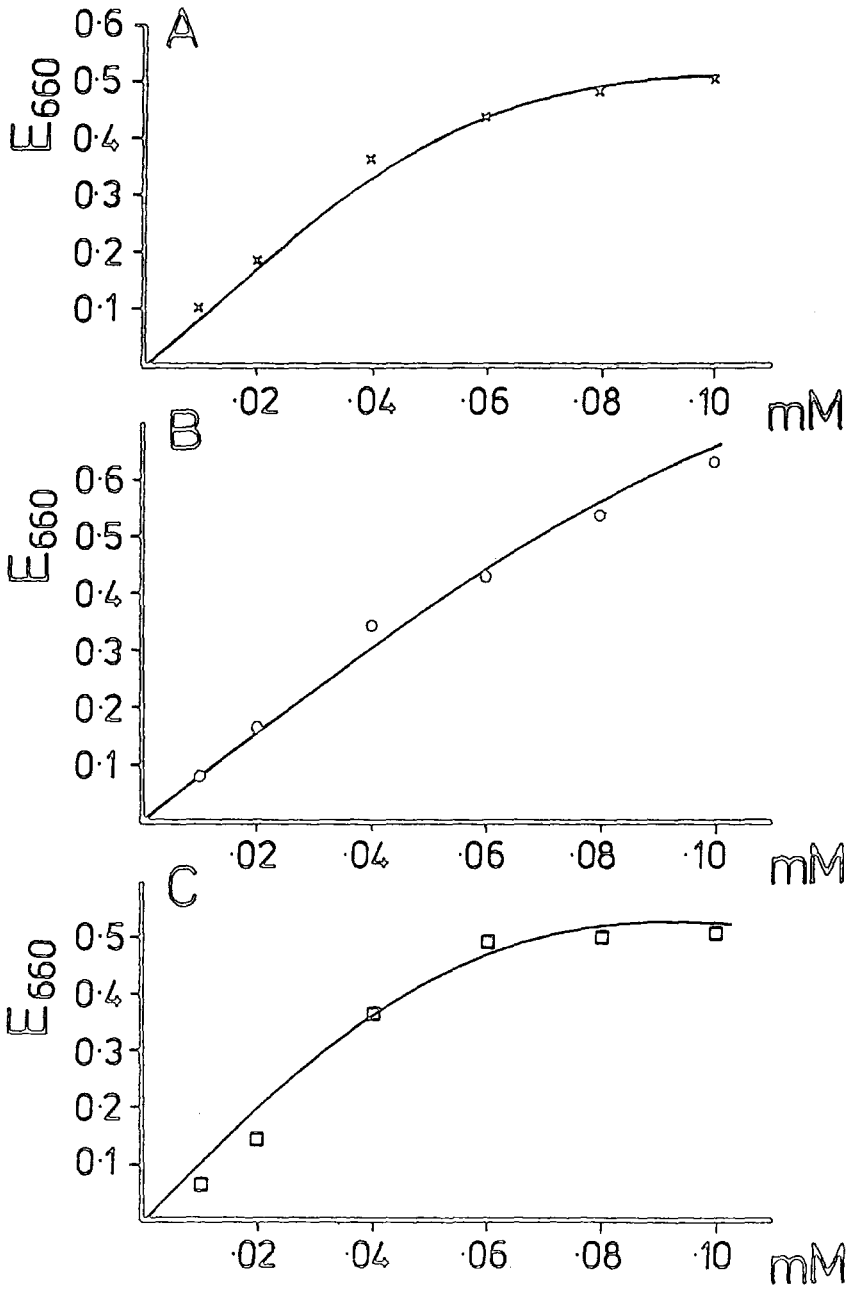


FIGURE 4.1 Growth Yields Of Double Auxotrophs

Cells grown in presence of stated amino acids for 18h.

A M2626 grown in A medium + 0.5% w/v glucose and 0.01 - 0.1 mM Lys.

B Strain POA111 (Lys^- Met^-) grown in A medium + 0.5% w/v glucose, 0.01 - 0.1 mM Met and 1.0 mM Lys.

C Strain PAO 110 (Lys^- Trp^-) grown in medium as above (1.0 mM Lys) but containing 0.01 - 0.1 mM Trp.

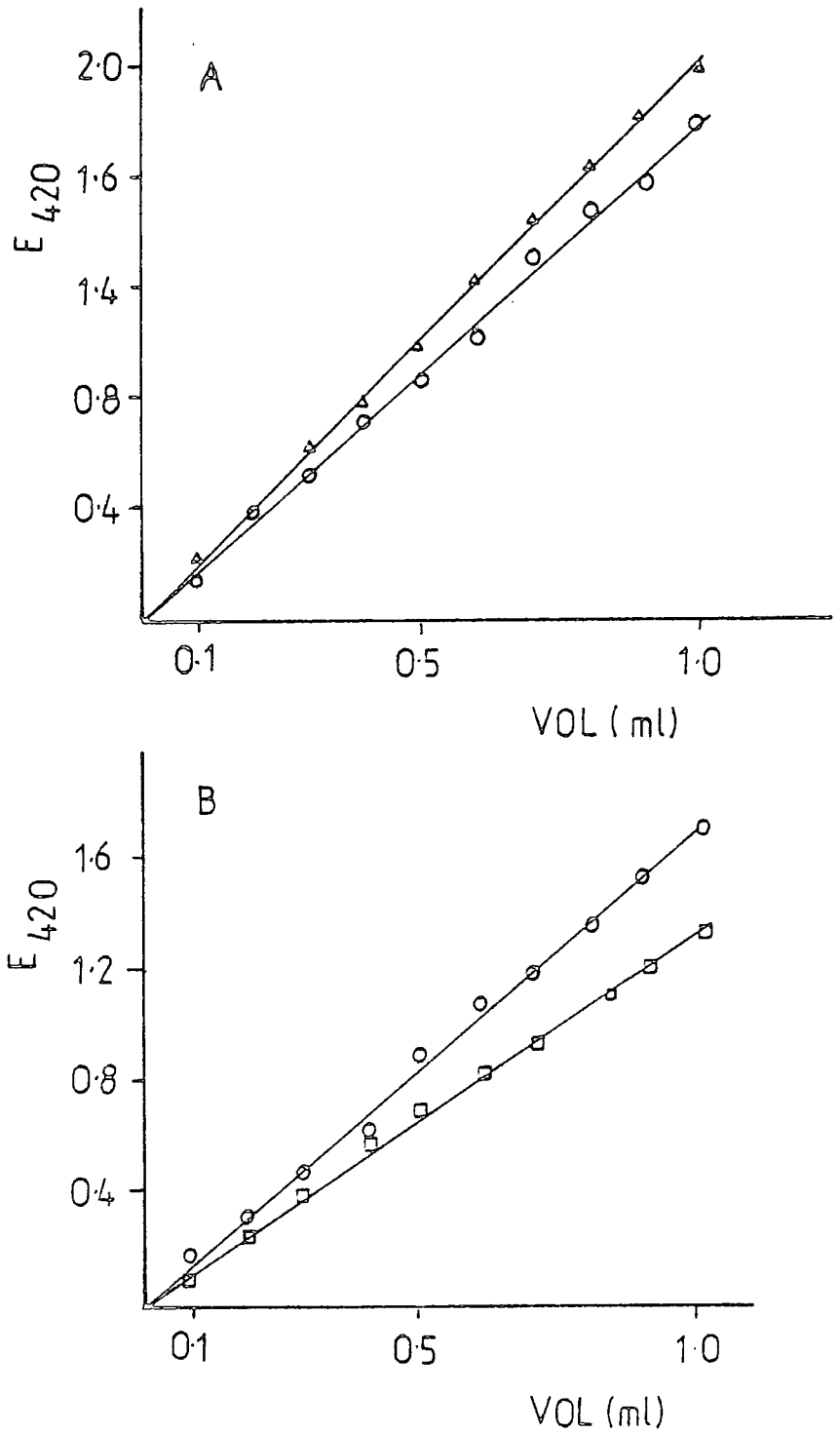


FIGURE 4.2 β-galactosidase Assay For Lysine, Methionine and Tryptophan.

Amino acids being assayed are supplied at 0.04mM volumes stated added to give total volumes of 2.0 ml. In each case second amino acid required by strain supplied at 0.2 mM. A = strain PAO 1Φ1, Lys⁻Met⁻ ; B = strain PAO 1Φ, Lys⁻Trp⁻. β-galactosidase assayed by modified method described in section 3-4. ○——○ , Lys; △——△ ,Met ; □——□ ,Trp.

Table 4.3

Comparison Of Observed Relative Sensitivities To Lys, Met And Trp And Their Calculated Molar Ratios In β-galactosidase

	Slope of response lines[a]	Ratio of slopes	Calculated Molar ratio in β-galactosidase[b]
Met	2.4		
Lys	1.8	1.33	1.25
Lys	1.75		
Trp	1.37	1.30	1.28

[a] Calculated from Figure 4.3

b Data taken from Table 4.1

See also discussion - Section 7-3.

These results also imply that this standard β -galactosidase procedure should be applicable to a variety of other amino acids provided appropriate auxotrophs are available.

4-3-3 Reversion Rates of Auxotrophs

4-3-3-1 Introduction

As a simple way of testing whether the mutants PA0111 or PA0110 arose by deletion, spontaneous reversion rates to loss of auxotrophy were determined. Point mutations have typical reversion rates around 1 in 5 x 10^7.

4-3-3-2 Method and Results

Strain M2626, the Met^- strain (ATCC 10799, NCIB 8134, isolated by Davis & Mingioli, 1950 now to be refered to as 8134), the Lys^- Met^- strain PA0111 and the Lys^- Trp^- strain PA0110 were grown up in A medium to mid-exponential phase in the presence of 0.5% w/v glucose, supplemented with 0.2mM of their required amino acids. The bacteria were harvested on a filter, washed twice with A medium, equilibrated to 37°C (Section 2-2-4). The cells were then resuspended in A medium to give approximately 10^9 cells ml^{-1} and serially diluted from 10^{-1} to 10^{-9} in A medium. Duplicate 0.1 ml aliquots from each dilution were plated out onto suitable agar plates containing all the required amino acids (to determine total cell counts); also onto plates lacking the amino acid for which reversion was being tested. Plates were incubated at 37°C and observed after 24 and 48 hours.

The presence of fast growing colonies on the plates lacking e.g. Met was taken as indicating spontaneous Met^+ revertants. In both strains 8134 and PA0111 there was less than 1 revertant in 10^8 cells (Table 4.4). However, with both strains there were minute transparent colonies on the minus Met plates.

Table 4.4

Reversion Rates

	Lys	Met	Trp
M2626	1 in 10^8	-	-
PA0111	1 in 7 X 10^7	1 in 10^8	-
PA0110	1 in 5 x 10^7	-	1 in 10^8
8134	-	1 in 10^8	-

These were not visible by eye after 24 hours and they grew much more slowly than the colonies on the plates containing Met, although the numbers were essentially equal on both plates. These colonies were not revertants and it seems likely that the mutants were "leaky" in that limited, very slow, growth occurred in the absence of Met; either, because the mutations were not complete or, possibly by synthesis of Met through an alternate pathway. This slow growth would not be a problem in the use of either the strain 8134 Met^- or for the double auxotroph PA0111, Lys^- Met^-, for Met assays; especially by the β -galactosidase method. Care should be taken, however, not to subject the cells of either strain to excessive starvation periods prior to their use in assays for Met.

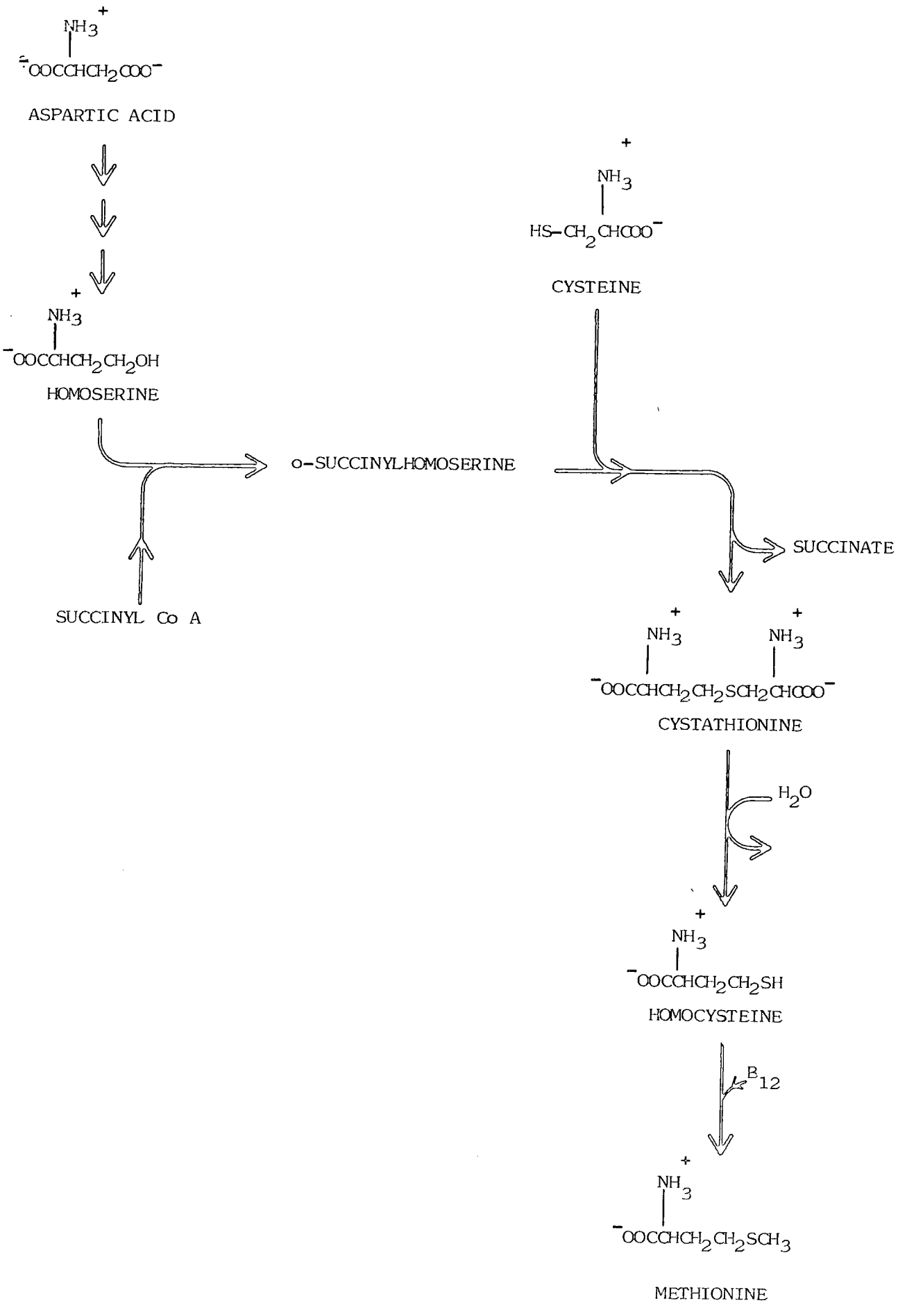
The frequency of reversion of the Trp-requiring strain to loss of Trp auxotrophy was of the order of 1 in 2×10^7, and for both double auxotrophs the frequency of reversion for loss of Lys auxotrophy was approximately 1 in 10^8 (Table 4.4).

4-3-4 Characterisation of Strain PA0111 Lys^- Met^-

4-3-4-1 Growth Response to Met Precursors - Introduction

The strain M2626 used to assay Lys has a mutational lesion in the gene coding for the enzyme of the last step in the Lys biosynthetic pathway and hence, no precursors of Lys substitute for the amino acid when the strain is used for assay (Section 3-1-1). In the same way, it is important to determine the position of the mutation conferring Met auxotrophy. A scheme for the biosynthesis of Met is given in Figure 4.3

FIGURE 4.3 BIOSYNTHESIS OF METHIONINE



4-3-4-2 Method

The following Met precursors were tested: Cystathionine, Homocysteine, Homoserine and Cysteine. (All were obtained from Sigma, London Ltd., except Homocysteine which was obtained from Koch-Light Ltd.). Solutions of each were made up at 10mM in distilled water and were filter sterilised. Strains 8134 (Met^-), and PA0111 ($Lys^- Met^-$), were grown up to mid-exponential phase in the presence of 0.5mM Met. About 10 ml of the culture was harvested, washed on a Millipore filter (Section 2-2-4), and resuspended in A medium to give ca 10^8 cells ml^{-1}. 0.1 ml Aliquots (ca 10^7 cells), were added to a series of sterile tubes containing A medium, 0.5% w/v glucose and one of the precursors or Met. Each of the precursors was tested at concentrations of 0.2mM, 1.0mM and 2.0mM; except cystathionine for which only the two lowest concentrations were used. Met was tested at either 0.2mM or 2.0mM. Two sets of tubes were prepared, one (A) being inoculated with strain 8134, the other (B) being inoculated with PA0111; in this case, 0.2mM Lys was also present in each tube. For each set, a control tube (minus Met or the precursors), was included.

Tubes were incubated in a water-bath at 37°C with shaking (120 strokes min^{-1}), and E_{660} values were monitored at intervals up to 24 hours. E_{660} values for the control tubes were subtracted from the remaining tubes.

4-3-4-3 Results

For both strains there was some slight growth in the tubes without Met or precursors for about two hours, cells were growing on endogenous Met; after this no further growth occurred.

No growth occurred on cysteine or homoserine at any concentration tested (up to a ten-fold excess of the Met concentration that supports good growth, Fig. 4.4). Both strains behaved in an identical manner in that they were able to grow to a limited extent on homocysteine (Fig. 4.5), and cystathionine (Fig. 4.6). In both cases, growth at 0.2mM was less than would be obtained with this concentration of Met. The initial growth response was much slower than was found with Met; but after 8 hours both precursors were able to support good growth of these strains. Growth on these two precursors at 2.0mM being equivalent to growth on Met at 0.2mM.

4-3-4-4 Discussion

These results imply that the mutation in these Met-requiring strains is located in a gene coding for an enzyme before cystathionine synthesis. Compounds in the pathway up to this point could not act as substrates for growth. It is unlikely, however, that cystathionine or homocysteine would occur significantly in digests of seed meals, biological fluids etc. Therefore, suitability of the strain to assay for Met is confirmed.

In the paper describing the isolation of the Met^- strain 8134, Davis & Mingioli (1950), demonstrated its response to Met and B_{12}. They also tested for growth on Homocysteine and reported no growth after 72 hours in the presence of 40 μg ml^{-1} (0.14mM), of this compound, they did not examine response to cystathionine. The findings reported here show a limited growth response to cystathionine and homocysteine at 0.2mM., but there was a higher growth yield in the presence of these two compounds at 1.0 or 2.0mM. The apparent discrepancy in these results and those reported by Davis & Mingioli could be explained by the variation in concentrations used.

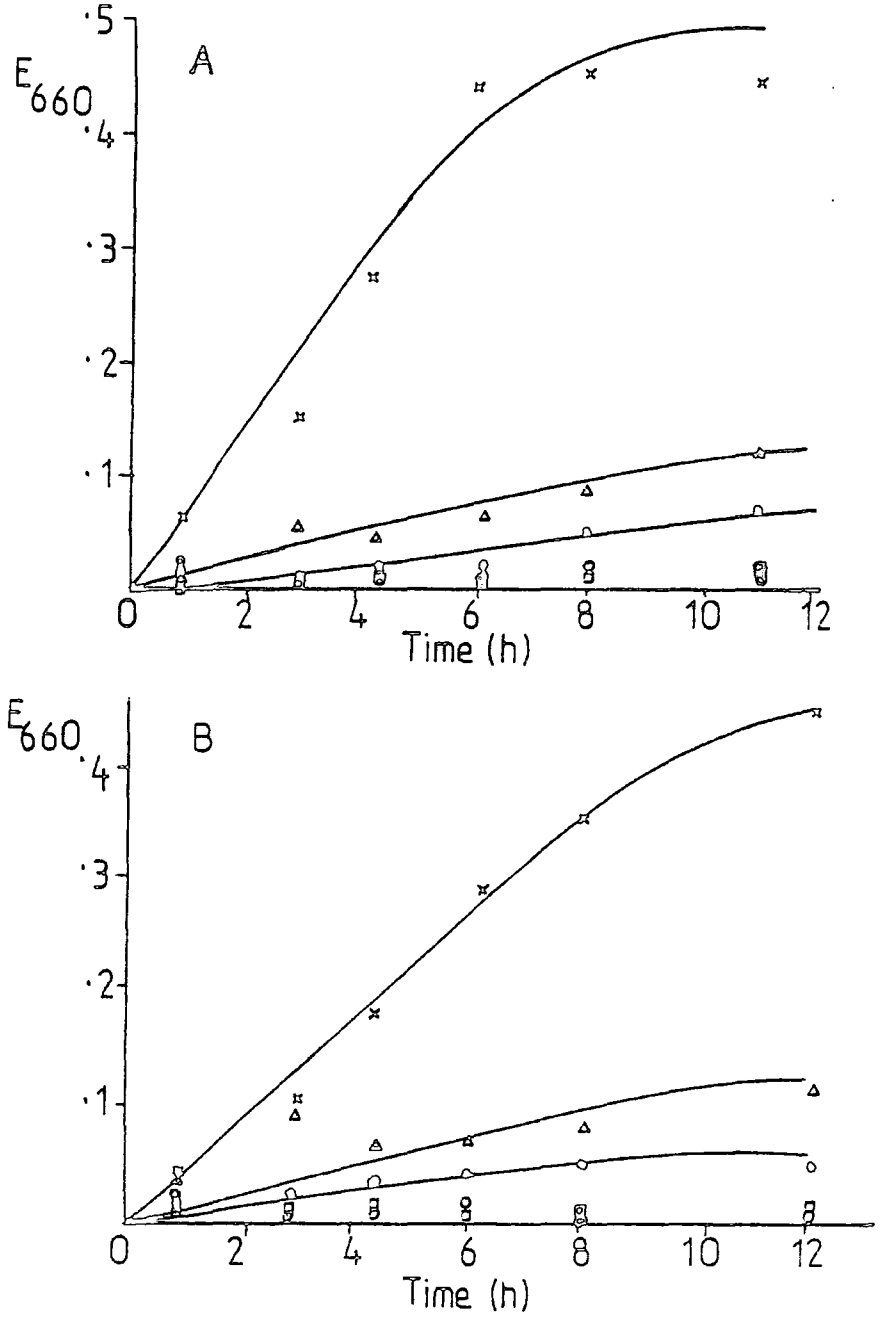


FIGURE 4.4 Growth Response Of Met-Requiring Strains To Met Precursors

Growth of strain 8134, Met^- (A) and PAO 111, Lys^-Met^- (B) in the presence of Met or precursors, all at 0.2 mM. ⊠—⊠, Met; △—△, Cystathionine; ○—○, Homocysteine; ◎—◎, Homoserine; □—□, Cysteine. Control tubes (minus Met or precursors) were included and values subtracted from results. Tubes were incubated at 37°C in a shaking water-bath (100 - 120 strokes min^{-1}), and E_{660} read at stated times.

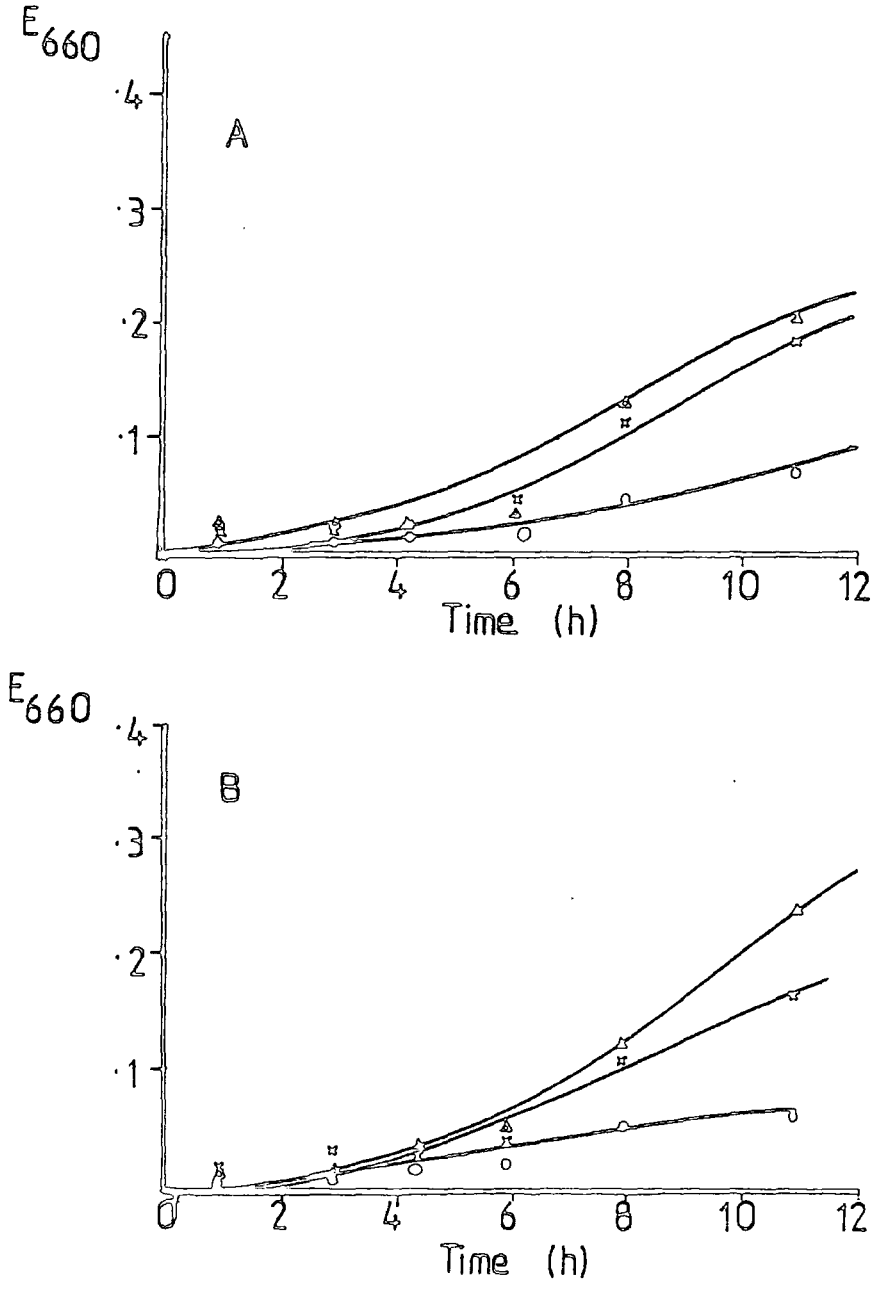


FIGURE 4.5 Growth Response Of Met-Requiring Strains To Homocysteine.

Growth of Met⁻requiring strain 8134, Met^-, (A) and PAO111, Lys^-Met^- (B) in the presence of homocysteine at 2.0 mM △——△ ; 1.0 mM ×——× ; and 0.2 mM ○——○ . Experimental details as described in the text (section 4-3-4-2) and for figure 4.4.

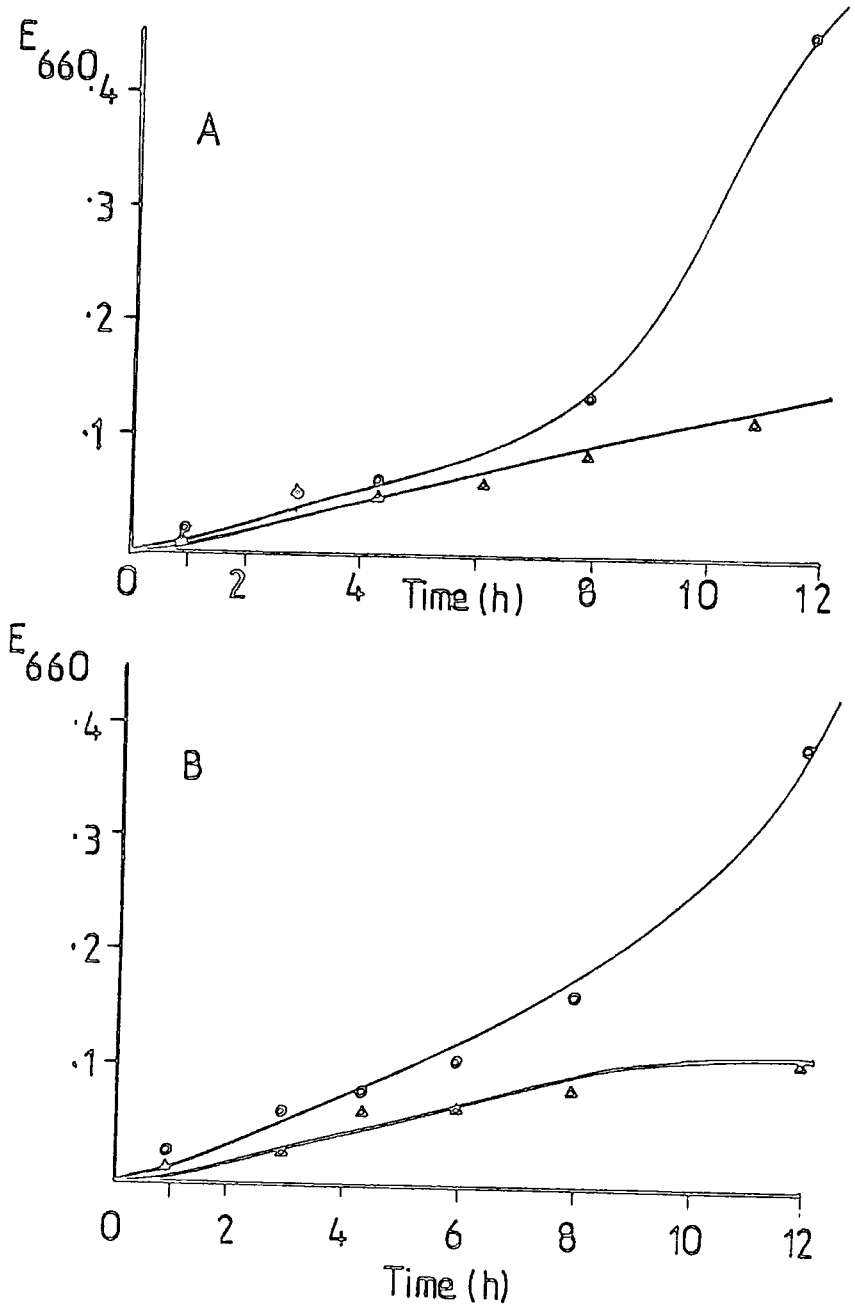


FIGURE 4.6 Growth Response Of Met-Requiring Strains To Cystathionine

Growth of strain 8134, Met^-, (A) ; and strain PAO111, Lys^-Met^- (B) in the presence of 0.2 mM △——△ and 1.0 mM ○——○ cystathionine. Experimental details as described in text (section 4-3-4-2) and for Fig 4.4.

The higher concentration used here being 14.3 times that used by Davis & Mingioli.

4-3-4-5 β-Galactosidase Assay - Response to Met Precursors - Method

A β-galactosidase assay was carried out using the standard procedure (Section 3-4). Cells of strains 8134 Met^-, and PA0111 Lys^- Met^- were grown up in the presence of 0.2mM Met to mid-exponential phase. After the standard washing, starvation of Met and induction; aliquots of cells were added to prepared tubes containing either Met or one of the precursors and protein synthesis was allowed to continue for 90 minutes. Cysteine and homoserine were assayed at final concentrations of 0.01 and 0.018mM, Homocysteine and Cystathionine were each assayed at 0.01, 0.018, 0.001, and 0.0018 mM and Met was assayed at 0.001 and 0.0018 mM (all final concentrations). 0.01mM Lys was present in all assay tubes used with strain PA0111. All concentrations were assayed in duplicate. Zero blank values (minus Met or precursor), were subtracted from the E_{420} yield for each strain and results were plotted in graphical form (Figs. 4.7 and 4.8).

4-3-4-6 Results

The results of the β-galactosidase enzyme assay were in agreement with conclusions obtained in growth tests. There was some response to cystathionine and homocysteine but this was less than the response to an equivalent concentration of Met (Fig. 4.8). When the concentration of these precursors was raised ten-fold then a higher yield was obtained; the response to cystathionine and homocysteine at 0.02mM being about equal to that obtained from Met at 0.002mM.

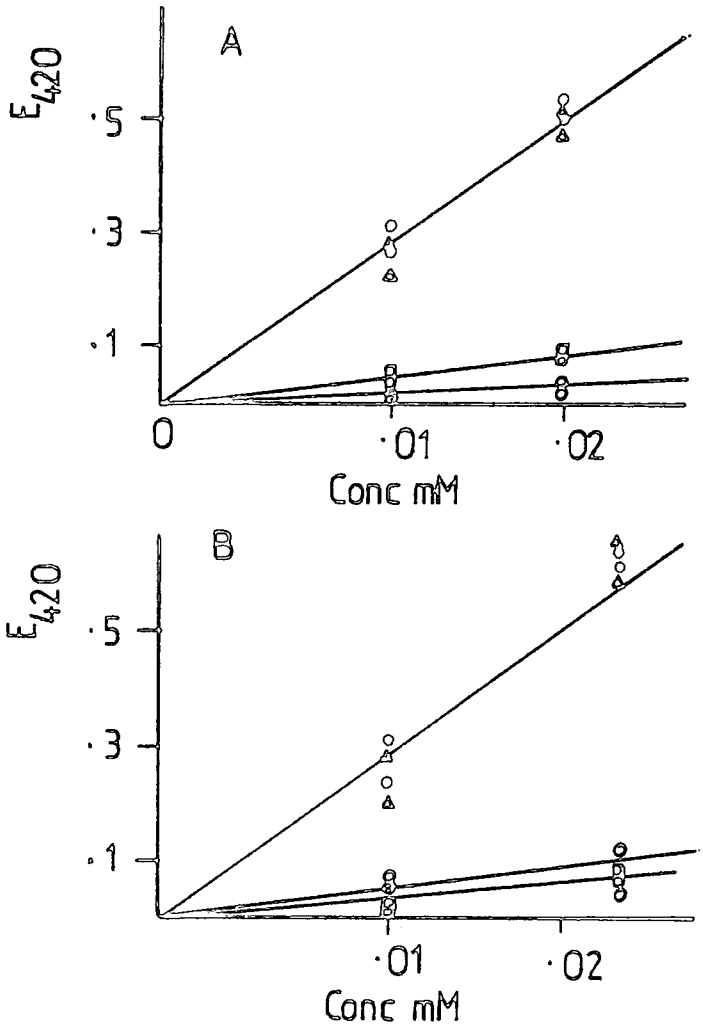


FIGURE 4.7 Response Of Met Biosynthetic Precursors In β -galactosidase Assay.

Response of Met-requiring strain 8134, Met^- (A) and PA0111, $Lys^- Met^-$ (B) to cystathionine △——△ ; homocysteine ○——○ ;homoserine ⊙——⊙ ; and cysteine □——□ . Values shown were obtained after subtracting control (minus Met or precursor). Enzyme was assayed by method described (section 3-4). For further experimental details see text section 4-3-4-5.

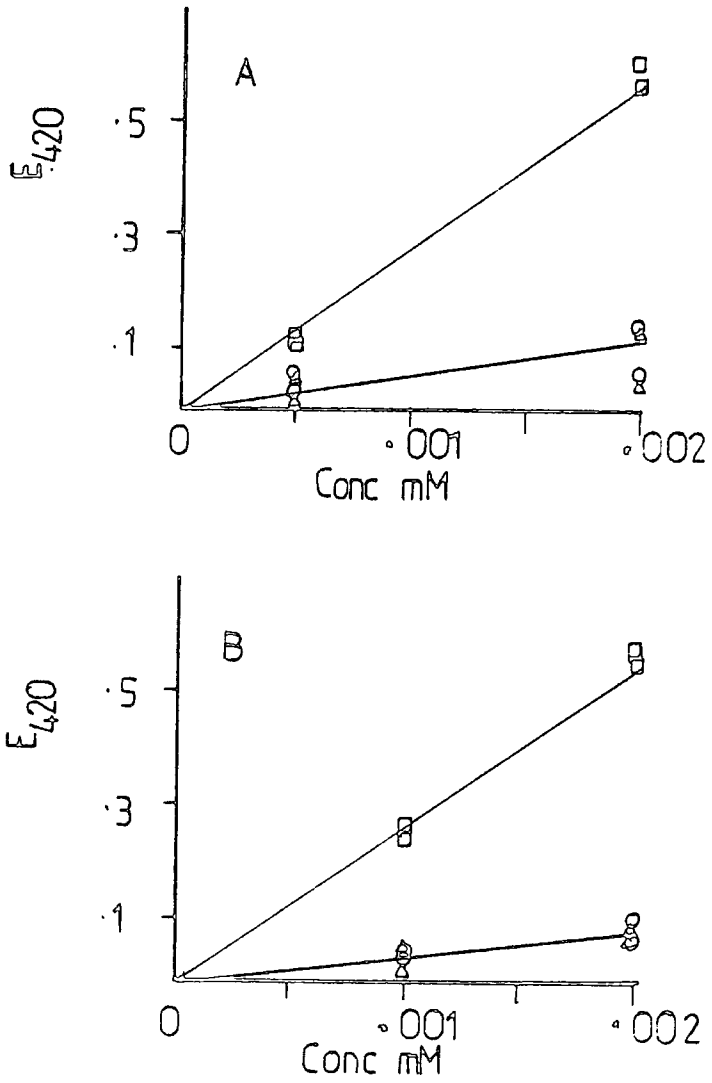


FIGURE 4.8 β -galactosidase Assay Response To Methionine and Its Biosynthetic Precursors.

Assay was carried out as described in previous figure, using strain 8134 Met^- (A) and strain PAO111 Lys^-Met^-(B). Responses to Met□——□ ; Cystathionine △——△ ; and to Homocysteine ○——○ ; are shown after subtracting value from control (minus Met or precursors). β -galactosidase was assayed by method described in section 3-4

No response was obtained to Homoserine or cysteine up to 0.01mM but there was some detectable production of enzyme at 0.018mM.

4-3-5 Characterisation of Strain PA0110 Lys$^-$ Trp$^-$

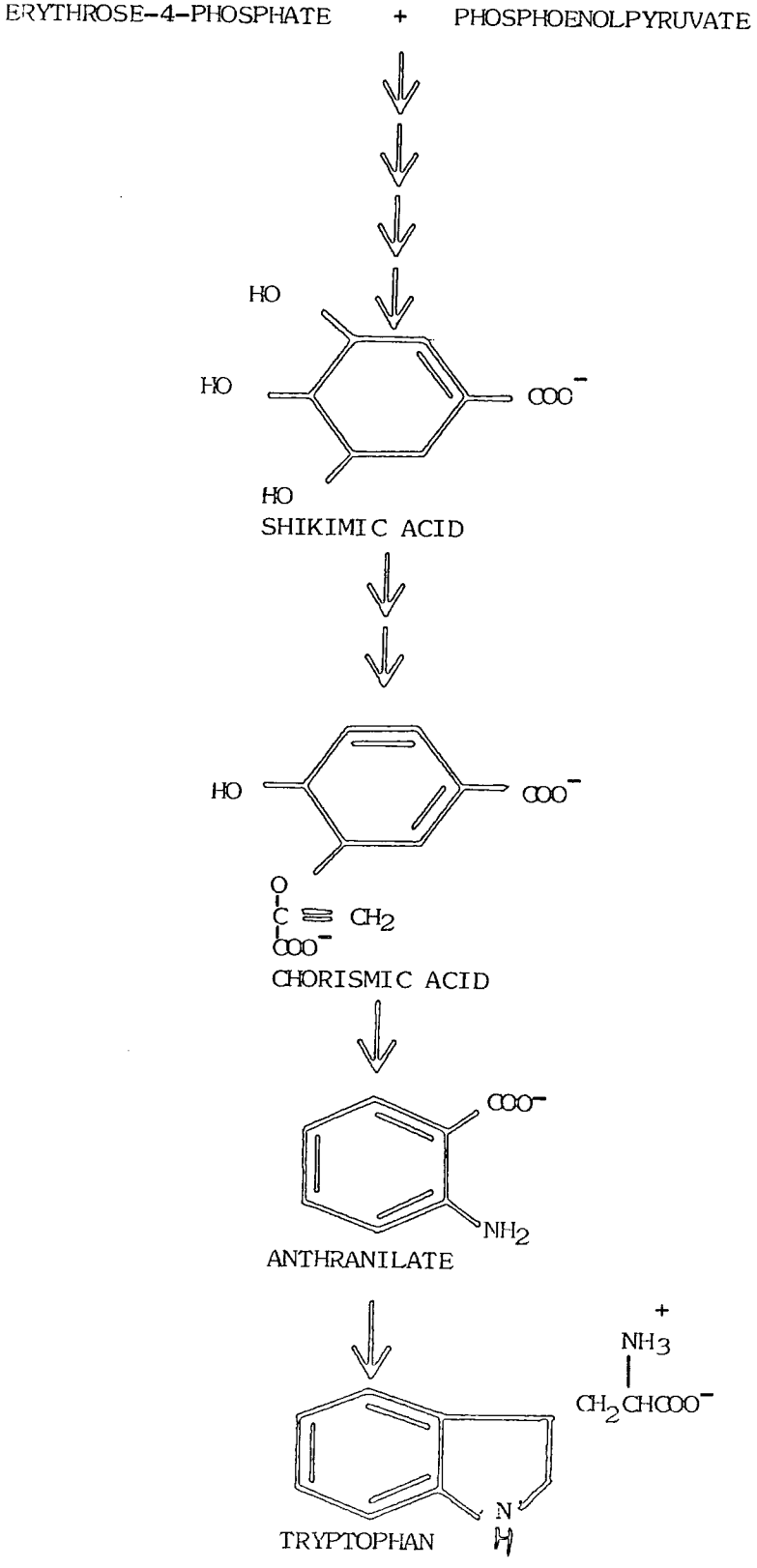
4-3-5-1 Growth on Precursor

The biosynthetic pathway for Trp is given in Fig. 4.9. The only intermediate compound of Trp biosynthesis examined was anthranilic acid. Growth tube tests and β-galactosidase assays were carried out using analogous methods described for the Met precursors (see Section 4-3-4). Although anthranilic acid can be transported and utilised by Escherichia coli (Miller, 1961), results (not shown), indicated that it could not replace the growth requirement of strain PA0110 for Trp, nor could it substitute in β-galactosidase synthesis (Fig. 4.10). Therefore, the mutation would appear to be in a gene controlling the last step of the biosynthetic pathway, between anthranilic acid and Trp. Hence, any metabolic precursors of Trp which may be present in cell extracts will not substitute for Trp.

4-3-5-2 Examination of Part of Genome of Trp$^-$ Isolates

The oligopeptide permease (opp) locus maps at 27 min in Escherichia coli and this is close to various genes involved with Trp biosynthesis/transport (Trp A, At, C, Cp, D, E, Ea, Ee, Eo and Ep), (Bachmann & Brooks-Low, 1980). Therefore, it was important to establish whether the opp locus was intact in the 3 Trp-requiring strains isolated, in order that they could be used to assay for peptide bound Trp. The Trp$^-$ isolates were shown to have the ability to use the tetrapeptide Trp-Met-Asp-Phe-amide as the sole Trp source for growth and β-galactosidase synthesis (results not shown).

FIGURE 4.9 BIOSYNTHESIS OF TRYPTOPHAN



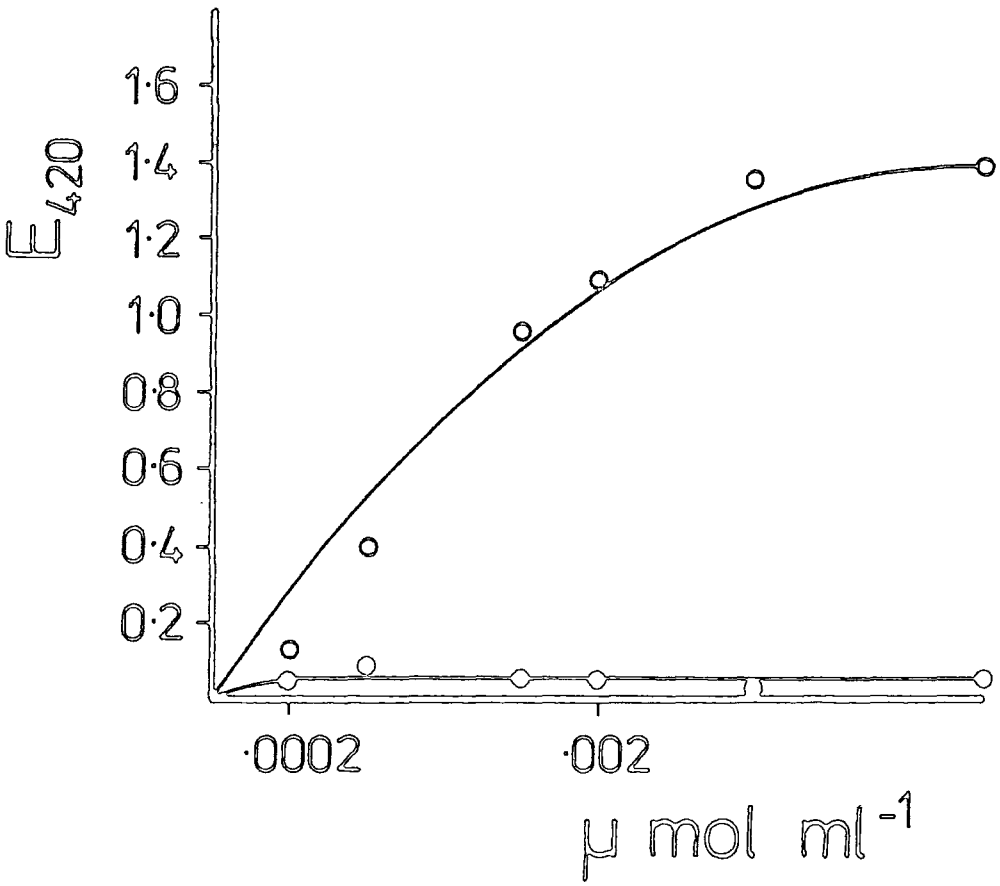


FIGURE 4.10 β -galactosidase Assay Response To Anthranilic Acid and Tryptophan.

Response of strain PAO 110 to Trp (open circles) and to Anthranilic acid (closed circles). Cells prepared and enzyme assayed as described in Section 3-4. 0.2 mM Lys present in incubation mixture in both cases.
Cell density ca. 2 x 10^8 ml^{-1}. For full experimental details see Section 4-3-5-1.

However, it was still possible that this peptide might have entered through the third peptide permease (map position 86 min), and therefore alternative methods were tried in which resistance to the toxic tripeptide, triornithine (which exclusively uses opp), was determined. All 3 Trp auxotrophs were grown in the presence of Lys and Trp (0.2mM), approximately 10^8 cells were collected, washed and resuspended in A medium containing supplements as shown in Table 4.5. Tubes were incubated at 37^oC for 24 hours after which time growth was noted. The results (Table 4.5), indicate that triornithine (Orn_3) inhibited growth of the Trp auxotroph (later designated PA0110), and therefore the opp locus was intact. Each of the other auxotrophs showed an identical response (results not shown).

To check these results, a more sensitive test was used. This was based on the fact that the toxic tripeptide, Orn_3, inhibits protein synthesis, and thus, would prevent β -galactosidase synthesis in all but opp^- strains (where it is not taken up). Therefore, cells of each strain were grown up in the usual way, induced and incubated in the presence of 0.04mM Orn_3 for 20 minutes. Aliquots of induced cells or induced cells plus Orn_3 were added to tubes containing Trp and Lys and allowed to synthesise protein (including the induced β -galactosidase) for 90 minutes. The enzyme was then assayed in the normal way (Section 3-4), and β -galactosidase production was scored. Results (Table 4.6), showed that the strain, later to be designated PA0110, produced β -galactosidase in the presence of Lys and Trp, but no enzyme was produced either in the absence of Trp, or in the presence of Orn_3, indicating that the cells were sensitive to Orn_3 and their opp locus was intact.

Table 4.5

Effects Of Triornithine On Growth Of *Escherichia Coli* Trp Auxotrophs

Strain	Growth Supplements *	Growth Response
M2626	0.2mM Lys	+++
M2626	0.2mM Lys, 0.2mM Orn_3	-
** PA0110	0.2mM Lys, 0.2mM Trp	+++
PA0110	0.2mM Lys, 0.2mM Trp, 0.2mM Orn_3	-
PA0110	0.2mM Lys	-
M2626 opp^-	0.2mM Lys	+++
M2626 opp^-	0.2mM Lys, 0.2mM Orn_3	+++

* All tubes contained A medium and 0.5% w/v glucose

** Results for other 2 Trp auxotrophs were identical

+++ Growth

\- No growth

Table 4.6

Response Of Two Trp⁻ Isolates And Parent Strain To Triornithine

Strain	Lys	Trp	Lys Trp	Lys Trp Orn_3
T_1 *	0	0	+	0
T_{19}	0	0	+	0
M2626	+	0	+	0

Amino acids and Orn_3 supplied at 0.4mM. Aliquots of induced cells plus supplements, as above, were allowed to synthesise β-galactosidase for 90 minutes. The enzyme was then assayed by using the normal procedure and tubes were scored on a simple + (enzyme synthesised) or ⊖ (no β-galactosidase produced) basis. Each test was carried out in triplicate.

* Later designated PA0110

Two of the isolates gave identical results in all tests; they were both derived from the same original flask of mutagenised cells therefore one was chosen for all subsequent work and designated PA0110.

4-3-6 Examination of Cell Extracts of PA0111 and PA0110 for Accumulation of Precursors

In order to gather more information regarding the nature of their mutations, cell extracts of strain PA0111 and PA0110 were examined after dansylation. The cells were grown up in A medium in the normal way in the presence of their required supplements at 0.5mM. Samples, approximately 10^9 cells, were either washed to remove excess buffer salts and extracts were prepared immediately (Section 2-5-2), or they were washed, resuspended in the original growth medium minus one of the required amino acids and incubated at $37^{o}C$ for 90 minutes with gentle shaking (80 strokes min^{-1}). During this progressive starvation, samples of cells were removed and extracts were prepared, dansylated, spotted with marker amino acids and run as described (Section 2-4). Lys, Met and Trp precursors were also dansylated in the same way after first checking that subjecting them to the extraction procedure caused no chemical modification.

Results showed that as cells were starved of a required amino acid (Lys, Met or Trp), it disappeared from the intracellular pool. As expected, a build up of the Lys precursor, diaminopimetic acid (DAP) was seen when M2626 was starved of Lys (Section 3-1-1 and 3-3-5-1).

None of the Met precursors tested (cysteine, homocysteine or cystathionine), was detected even when cell extracts were later added at a five-fold excess concentration but at this concentration the extracts did not run well and there was a great deal of interference on the plates at the positions where cystathionine and homocysteine would be expected to run. Although in the light of subsequent studies that showed some capacity of cystathionine and homocysteine to substitute for Met (Section 4-3-4), failure to detect a build up in their concentrations is hardly surprising. With hindsight, attention should perhaps have been focussed on cysteine and earlier precursors.

As cells of strain PAO110 were starved of Trp this amino acid disappeared from the intracellular pool. Anthranilic acid (which runs close to His on dansyl plates), was seen to accumulate in Trp-starved cells and no other spot increased in intensity. These results are compatible with the conclusion that the Lys^- Trp^- strain had a mutation affecting the last stage of the Trp biosynthetic pathway (tryptophan synthetase); and are in complete agreement with the conclusions from the growth response to the precursors (Section 4-3-5-1). Hence, any possible precursors present in a digest are unable to substitute for Trp; the mutational locus therefore is ideally situated for a test organism.

CHAPTER FIVE

BETA-GALACTOSIDASE ASSAY RESPONSE TO OXIDISED FORMS OF METHIONINE: INFLUENCE OF PHYSIOLOGICAL STATUS ON OXIDATION/ REDUCTION OF METHIONINE SULPHOXIDE AND CLEAVAGE OF MET-OXIDISED PEPTIDES

5-1 Introduction

Diets in many parts of the world are poor in protein quality and quantity and the limiting amino acid of such diets (if they are not cereal based), is frequently Met (Cuq et al., 1973; Miller & Samuel, 1970).

In recent years, there has been much attention paid to the reduction of nutritive value of foods resulting from food processing which may render many amino acids biologically unavailable (Section 6-3-1), (Slump & Schrender, 1973). The chemical interpretation of these events is often unexplained or confused.

Met may be partially S-oxidised to form methionine sulphoxide (Met(O)). This has been shown to occur in food proteins as a result of the action of oxidising lipids and hydrogen peroxide which is widely used in the food industry (Cuq et al., 1973). The latter has been shown to convert protein-bound Met to Met(O), (Ellinger & Palmer, 1969; Slump & Schreuder, 1973; Snow et al., 1976). It is logical to expect that in the absence of a protective enzyme Met(O) would be found to occur naturally in food proteins through air-oxidation of Met.

Nutritionally, Met(O) has been shown to have a lower availability than Met. The nutritional response to Met(O) is often reported as being variable, typically 60 - 100% that of free Met; young animals often showing a greater response than old ones, (Aymard et al., 1979; Anderson et al., 1976; Cuq et al., 1978; Miller & Samuel, 1970; Njaa, 1962; Tuffnell & Payne, 1981). Since Met is the limiting amino acid of many diets, the nutritional implications of this are important.

Many examples of the loss of biological activity resulting from oxidation of Met residues are now known. Such oxidation may arise *in vivo* from the action of oxidising agents such as peroxides, hydroxy radicals, and superoxides (Varma *et al.*, 1977; Rowley & Halliwell, 1982). In the human lens, for example, one of the main differences between normal and cataractous lens proteins is the relatively large proportion (up to 60%), of methionine sulphoxide residues in the latter (Garner & Spector, 1980). Significantly perhaps, development of cataracts and nutritional status appear to be linked (Bhat, 1982; Brot & Weissbach, 1982). It has also been shown that the oxidation of Met in proteins may significantly affect enzymic activity. For example, RNA-ase, and subtilisin from commercial sources, loose 20 - 30% of their activity following oxidation of specific Met residues (Reiss & Gershon, 1978). Met oxidation in the cardiotoxin from snake venom has been shown to abolish its lethal effect (Louw & Carlsson, 1978), and the same chemical modification causes the inactivation of human α_1-proteinase inhibitor (Johnson, 1980; Carp *et al.*, 1982). Oxidation of Met is also implicated as one of the mechanisms that human polymorphonuclear leucocytes use to damage microorganisms (Tsan & Chen, 1980).

For use in protein synthesis, Met(O) must first be reduced to Met: Met(O) reductase activity has been reported in various animal tissues such as HeLa cells, *Tetrahymena pyriformis* (Brot *et al.*, 1981), rat tissue extracts (Aymard *et al.*, 1979; Reiss & Gershon, 1978), surf clams (Kikuchi & Tamiya, 1981); in plants such as spinach and *Euglena gracilis* (Brot *et al.*, 1981), and bacteria (Ejiri *et al.*, 1979, 1980; Payne & Tuffnell, 1981).

In most cases the reductase has only been shown to act on free Met(O), although some evidence for the reduction of protein-bound Met(O) has been presented (Reiss & Gershon, 1978; Ejiri et al., 1980; Brot et al., 1982).

Recently, it has been reported that a Met auxotroph of Escherichia coli could utilise Met(O) in place of Met; this occurred through the action of a reductase, the activity of which was maximal in exponential-phase bacteria and which, declined in lag-phase cells (Ejiri et al., 1979, 1980). These reports appeared at essentially the same time that we were examining the response of the Met auxotroph PA0111 to oxidised Met residues using both growth tests and β -galactosidase assay.

Met may also be completely S-oxidised to methionine sulphone ($Met(O_2)$). It is well documented that this compound has no biological activity and cannot satisfy the nutritional requirement for Met (Miller & Samuel, 1970; Anderson et al., 1976). There have been virtually no reports of the natural occurrence of $Met(O_2)$ in proteins; in fact, the normal method for determination of the amino acid composition of proteins includes an oxidation step with performic acid so that all Met residues are determined as $Met(O_2)$. As a result any Met(O) or $Met(O_2)$ residues present in the native protein would not be detected as such, but would be presumed to have been Met.

5-2 Oxidation Of Methionyl Peptides

5-2-1 Preparation of Methionine Sulphoxide Derivatives (partial oxidation)

For all studies here, peptides and amino acids were purchased from Sigma (London) Ltd., B.D.H. (Poole), Phase Separations Ltd., or UniScience (Cambridge).

Methionine sulphoxide derivatives were prepared by a modification of the method of Neumann (1972).

1 ml of 10mM D-Met or a Met-containing peptide was equilibrated in a water bath at 30°C and 1M HCl was added to give a pH of 4.0 - 4.5. 30 μL of 30% hydrogen peroxide was added and the solution was incubated for 30 minutes at 30°C with gentle agitation. The reaction was then stopped by the addition of 1M NaOH or 0.88 ammonia to neutralise.

To remove excess H_2O_2, the solution was placed in a desiccator attached to a water-pump to allow gentle degassing. Known volumes of the oxidised product were evaporated to dryness. The Met(O) which will be a mixture of two diastereoisomers was always contaminated with about 25% Met(O_2). It is not possible by this approach to prepare pure Met(O), (Fujii *et al.*, 1978); more gentle treatment left some Met residues unoxidised. Therefore, a subsequent purification step was carried out using descending paper chromatography: 1 ml of the sample was streaked onto the origin of Whatman 3MM paper. After running for 18 - 20 hours (descending), in a solvent of butanol : acetic acid : water : pyridine (75 : 15 : 60 : 50 by vol), the paper was dried, the positions of the spots were identified by comparison with known standards, the strips were excised and eluted with 20% v/v aqueous pyridine solution.

Purified peptides were stored at -20°C. It was found that 75 - 80% of the peptide was eluted from the sheets and their concentration could therefore be approximately estimated. Exact concentrations were found following hydrolysis and use of the amino acid analyser (Sections 5-4-4 and 5-7-1a).

5-2-2 Preparation of Methionine - Sulphone Derivatives (total oxidation)

Various methods have been described for the preparation of free or protein-bound methionine sulphone (Neumann et al., 1961; Njaa, 1962; Toennes & Kolb, 1941); they frequently involve oxidation with H_2O_2 at low pH. Many of these conditions are inapplicable here since they could hydrolyse the peptides. The following method was adopted: A solution of performic acid was prepared by the addition of 1 ml 30% H_2O_2 to 9 ml 90% (aq) formic acid, and the solutions were allowed to react at room temperature for one hour. The performic acid solution was diluted 1 in 10 with distilled water and 70 µL were added per ml of peptide (10mM). This was allowed to react at pH 1.5 4°C for 18 hours. The solution was neutralised with 1M NaOH and purified by using paper chromatography as for the sulphoxide (Section 5-2-1).

5-3 Organisms And Culture Conditions

5-3-1 Organisms

The following strains of Escherichia coli W (ATCC 9637), were used:

The Lys auxotroph M2626 Lys^- and its wild type revertant M2626 Lys^+ (Payne & Bell, 1979), together with the derived Met^- Lys^- auxotroph PA0111. The Met^- auxotroph, NCIB 8134, isolated by Davis & Mingioli (1950), and extensively used for Met assays. The Escherichia coli K12 strain 4258 Arg, Leu, His, obtained from B. Bachman, Yale University, was also used.

5-3-2 Media and Growth Conditions

Growth media and incubation conditions were as described in Section 2-2-2, except where extracts were being prepared when the molarity of the medium was decreased to 0.05M to minimise buffer salts interfering with the running of the chromatograms. In such cases, the pH of each incubation was checked to ensure the buffering capacity of the medium was adequate.

Lys- or Met-starved cells were prepared as described in Section 2-2-5. Carbon-starved cells were prepared by harvesting exponential-phase bacteria on a filter, resuspending the cells in normal growth medium minus carbon and then incubating at 37°C for 90 minutes. Because the Lys can act as a poor C source these experiments were carried out using strain M2626 Lys^+ so Lys could be omitted.

Stationary-phase cells were also used in certain studies, these being used after three successive 30 minute absorbance (E_{660}) readings were identical.

5-3-3 Chloramphenicol Treatment

An exponential-phase culture was incubated for 60 minutes with 250 $\mu g\ ml^{-1}$ chloramphenicol to inhibit protein synthesis. At this concentration the chloramphenicol did not affect the fluorescent yield of the peptide during peptidase assays (Section 5-5).

5-3-4 Preparation of Cell Extracts

a) Permeabilised Bacteria:

Permeabilised bacteria were prepared by freezing and thawing in the presence of Triton X-100 (Sections 2-5-4 and 5-7-1f).

b) Broken Cells:

Broken cells were prepared using a Soniprobe (Type 1130A), sonicator (Section 2-5-3). Protein content of cell extracts was determined with the Coomassie Blue Protein Assay (Section 2-3-3).

5-4 Chromatography

5-4-1 Dansyl Derivatives

Dansyl derivatives of test compounds in incubation media and cell extracts were prepared and run as described (Section 2-4), using ornithine and dansyl amide (ammonium), as internal standards.

5-4-2 Fluorescamine Derivatives

A rapid qualitative measure of peptidase activity was obtained using thin-layer chromatography on cellulose plates (Section 2-6).

5-4-3 Cellulose Thin Layers

20 x 20 cm glass plates were coated with cellulose powder (MN500, Machery & Nagel, Duren, Germany), or pre-coated plates were purchased from Merck. They were run in n-butanol : acetic acid : water : pyridine (75 : 15 : 60 : 50, by vol). After drying, the plates were developed by spraying with ninhydrin : cadmuim acetate reagent. This method allowed the identification of Met, Met(O_2) and Met(O) from cleaved peptides or present in cell extracts or incubation media; they had Rf values of 0.6, 0.46 and 0.38, respectively.

5-4-4 Automated Amino Acid Analyses

Automated amino acid analyses of incubation media were performed as described (Sections 2-9 and 5-6-3), using a Locarte auto analyser on a standard long-column (6 hour) run.

5-4-5 Discussion

The methods described above, which enable the separation of Met from its oxidised forms and the separation of Met-oxidised methionyl peptides, were used to study these compounds in media incubated with either whole cells or cell extracts of varied physiological status.

In this way it was possible to study their uptake and hydrolysis and to observe the appearance in the medium of cleaved amino acid residues as a result of exodus.

5-5 Peptidase Assays

To measure peptidase activity, a computer-linked, automated fluorescamine assay was used to continuously measure peptide concentration in the media (Section 2-7), using a reaction pH of 6.2 (6.8 measured at the waste outlet). With whole cells this is strictly a measure of peptide transport, for this is the rate limiting step in bringing substrate in contact with intracellular peptidases. However, for net uptake to continue rather than reaching an equilibrium, peptide must continuously be cleaved within the cell. Thus, a convenient measure of the minimum peptidase activity in vivo is obtained. This is particularly useful with oligopeptide substrates, for their intermediate peptide cleavage products do not appear in the incubation medium where they would contribute to the fluorescence and lead to underestimates of cleavage rates.

Controls were run to determine the fluorescence yield from the peptide, its component amino acids, the cells, buffer or any other substance present in the reaction mixture (e.g. chloramphenicol). Sometimes, as an alternative to continuous monitoring, samples were also removed periodically from incubations with whole cells or cell extracts and examined by using thin-layer chromatography. In this way, the cleavage products could be identified and semiquantitative measures of hydrolysis rates obtained (Section 5-4-3).

5-6 Results And Discussion

5-6-1 Growth Assays

The Met-requiring strains (8134 and PA0111), both showed equal growth rates and growth yields for L-Met and L-Met(0), (Fig. 5.1); D-L Met(0) gave half the growth yield of the L-isomer. No growth was observed without Met or with Met(0_2); and the presence of Met(0_2) did not affect the response to the utilisable substrates (results not shown). These results support those of Ejiri et al., 1979, who used an Escherichia coli K12 Met auxotroph (2276).

Strain PA0111 also utilised Leu-Met(0), Met(0)-Ala and Ala-Met(0) as Met sources to give equal growth with that on free Met(0) (Fig.5.2). No growth was obtained with Leu-Met(0_2), Met(0_2)-Ala or Ala-Met(0_2) and neither were they inhibitory to growth on Met. These observations extend those of Ejiri et al., (1979), and indicate that Escherichia coli must contain peptidases able to cleave peptide-bound Met(0) residues (Fig. 5.1). The overall results of the growth tests indicate that Met(0) can substitute equally for Met and that reductase(s) active on free Met(0), and/or peptide-bound Met(0), (Table 5.1) are present in Escherichia coli.

Based on the results of these growth assays, it therefore appeared a simple and routine matter to apply the β-galactosidase procedure to the oxidised Met substrates to confirm their relative nutritional values. In fact, such studies showed this not to be so, the situation to be complex and the findings novel.

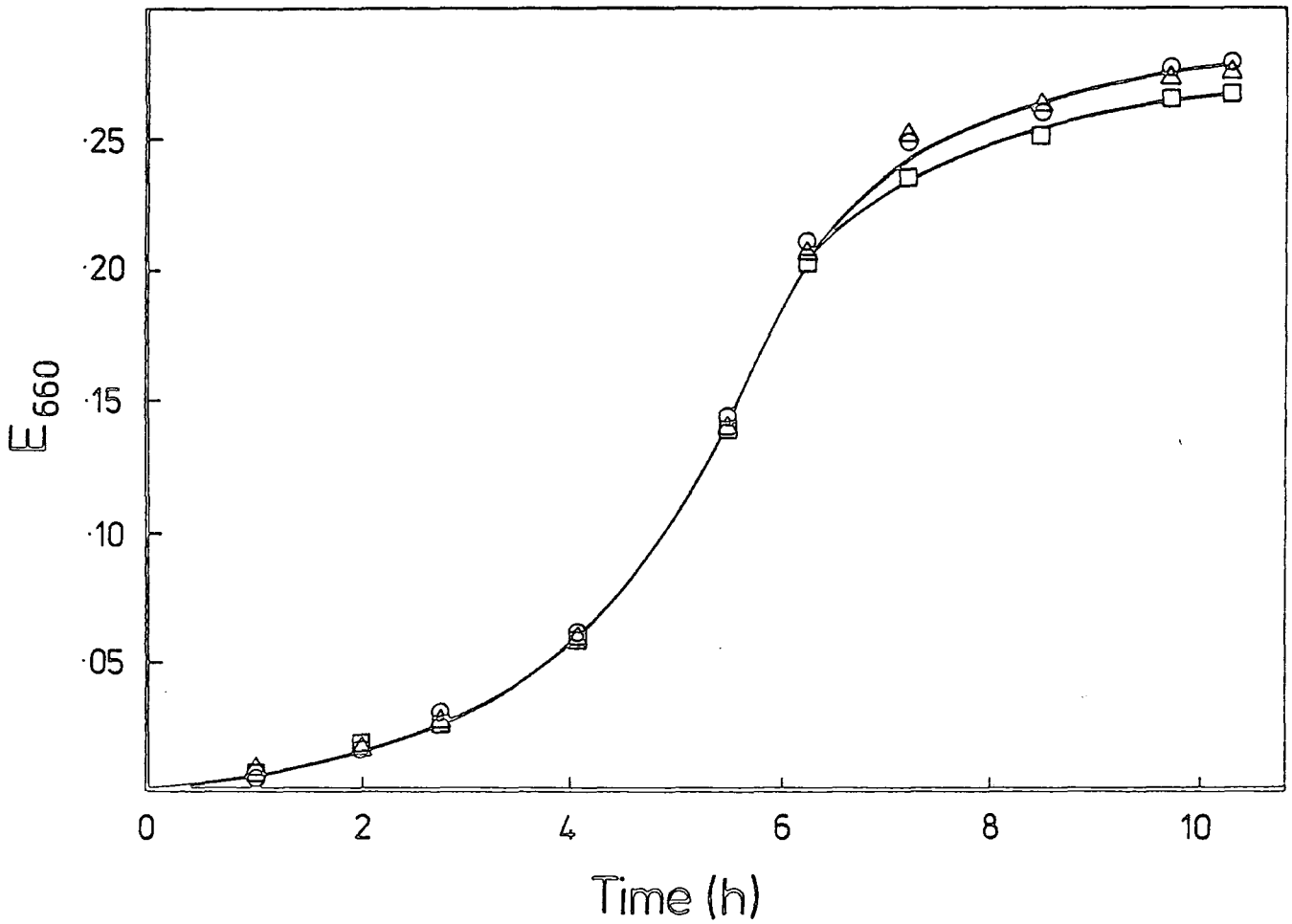


FIGURE 5.1 Utilization of Methionine Sulphoxide For Growth By Strain PAO111.

Growth response to 0.04 mM L-methionine △—△; 0.04 mM L-methionine sulphoxide □—□ and 0.08 mM D,L-methionine sulphoxide ○—○ . Bacteria were grown in minimal medium A supplemented with 0.1 mM Lys and containing 0.5%v/v glucose; incubated at 37°C with shaking.

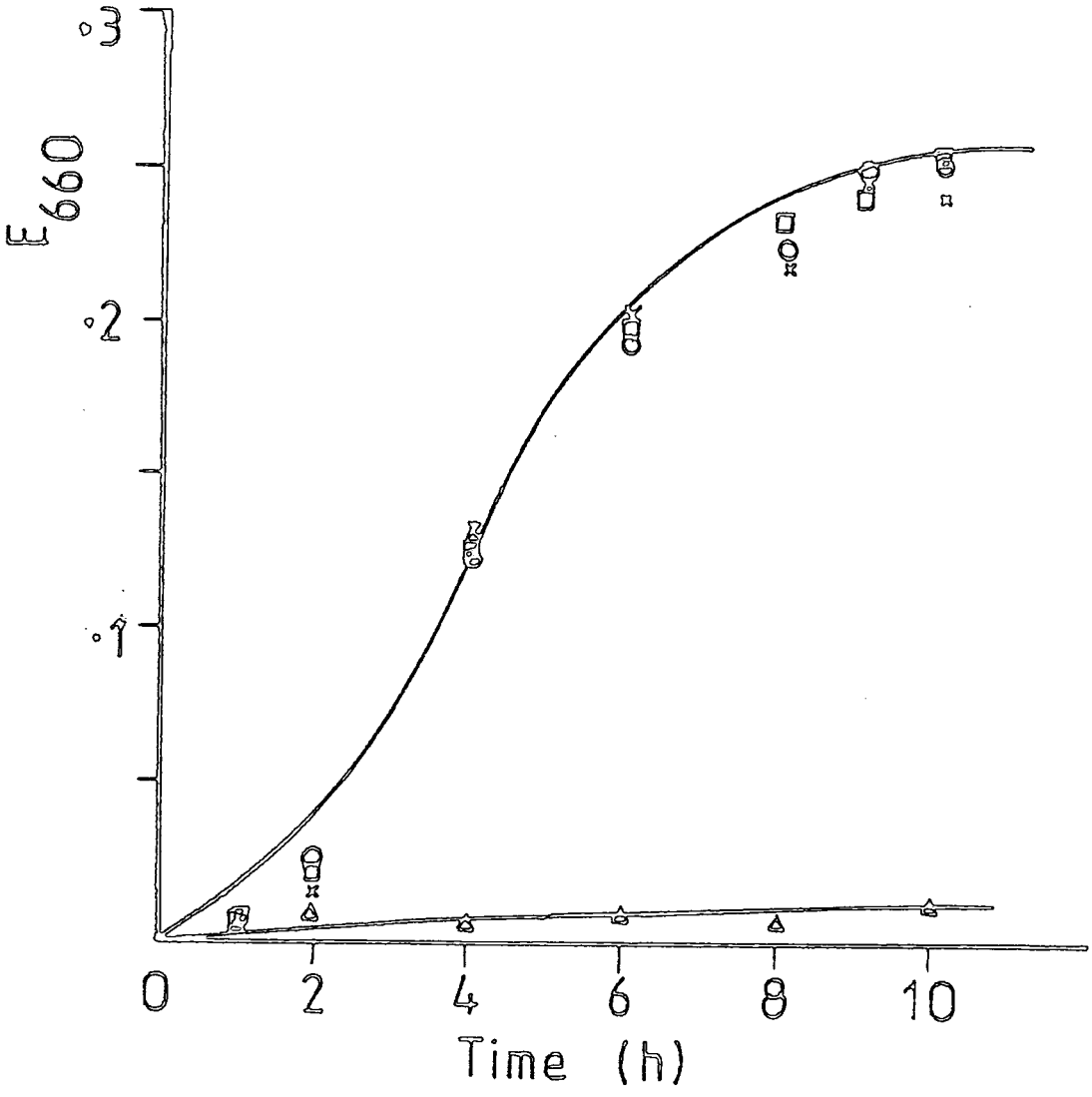


FIGURE 5.2 Utilization Of Methionine-Sulphone and Methionine-Sulphoxide-Containing peptides For Growth By E.coli PAO 111

All peptides were at 0.04 mM, Met(O_2) was at 0.08 mM.

Met(O_2)

Leu-Met(O)

Met(O)-Ala

Ala-Met(O)

TABLE 5.1

Rate Of Conversion Of Met(O) To Met.

Exponential Cells

Time Of Incubation With Met(O). (0.1 mM)	Rate Of Reduction Of Met(O) to Met. (n mol min^{-1} $mg\ protein^{-1}$)
5 min	2.146
15 min	1.007
30 min	0.775
60 min	0.416

5-6-2 β -Galactosidase Assays

When the routine β -galactosidase procedure (Section 3-4), in which bacteria are depleted of their reserves of essential amino acid by 90 minutes starvation, before enzyme induction, was applied, Met(O) was found to support only about 60% of the enzyme synthesis obtained with an equivalent amount of Met (Fig. 5.3). Similar results were obtained with Met(O)-Ala and Ala-Met(O). Met (O_2) and peptides containing Met(O_2) were without activity in the enzyme assay, in agreement with the conclusions from the growth tests. To try and understand further the basis for the decreased enzyme yield (60%) from Met(O) kinetic assays of enzyme synthesis were performed (Fig. 5.4). These showed initial rates to be the same for both Met and Met(O) and prolonging the protein synthesis time failed to give a relatively higher yield of enzyme with Met(O). It appeared, therefore, that the decreased enzyme yield in the standard assay did not arise because of a slower rate of synthesis perhaps arising from a slow reduction step.

We next studied the effect of varying the starvation period on the response to Met(O). Figure 5.5A shows that as starvation proceeds, a decreased amount of enzyme is synthesised from endogenous Met sources; and after 75 minutes, these are virtually exhausted. When constant Met was added at different times throughout a 90 minute starvation a steady net yield of β-galactosidase was obtained, being the sum of the residual endogenous level and the added Met increment (Fig. 5.5B). In contrast, the same procedure with Met(O) gave a decreasing net yield of enzyme when the starvation period was varied which reached about 60% that of Met after 90 minutes starvation (Fig. 5.5B).

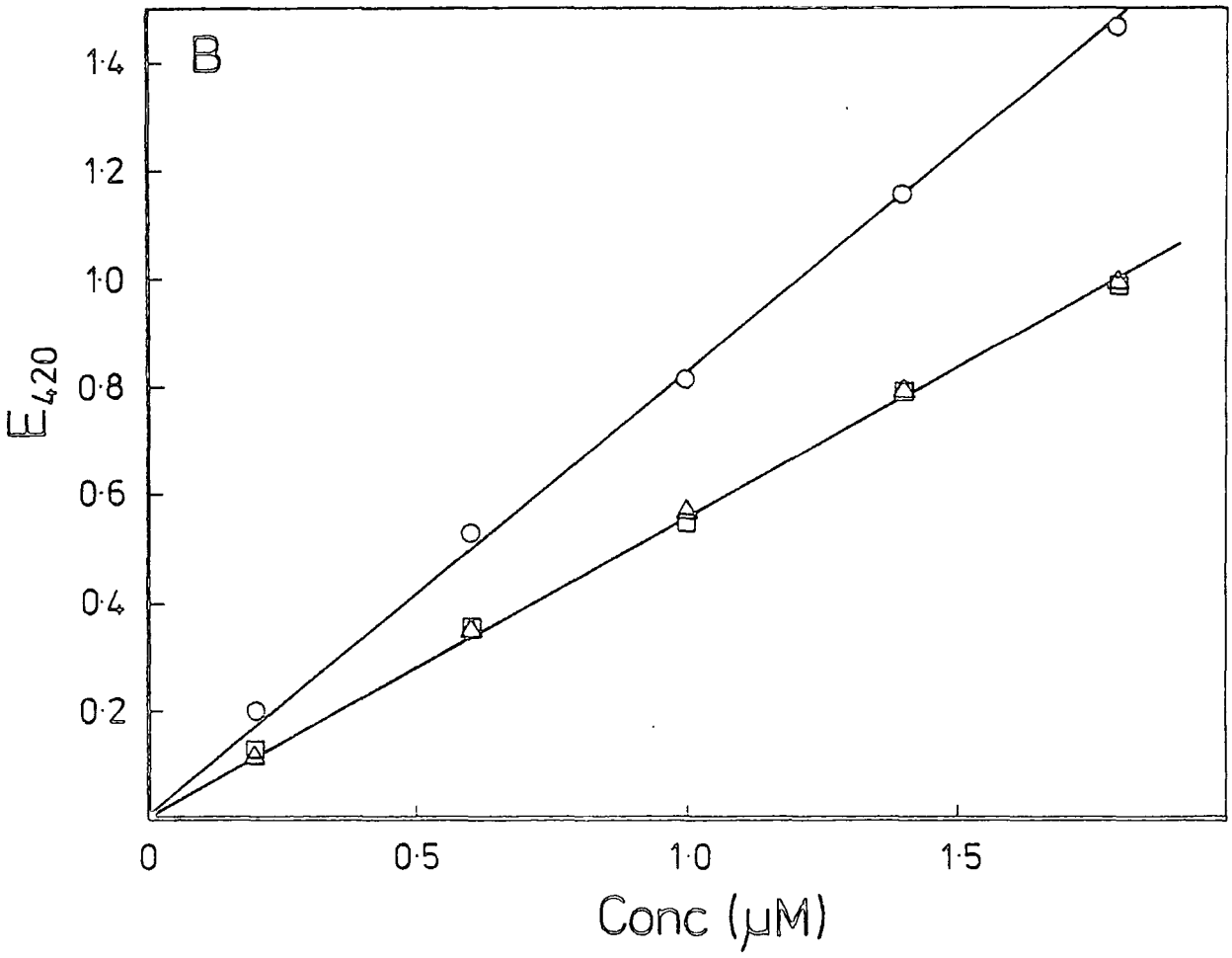


FIGURE 5.3 β -galactosidase Synthesis in Response to Methionine and Methionine Sulphoxide.

Various concentrations of L-methionine ○——○ ; L-methionine sulphoxide □——□ , and (at twice the concentration) D,L - methionine sulphoxide △——△ . Enzyme was assayed by normal procedure (section 3.4) in the presence of 0.5% w/v glycerol. 2×10^7 cells were used for each assay, enzyme synthesis was for 90 min; 0.1mM o-nitrophenol gave $E_{420} = 0.45$.

Enzyme incubation was for 30 min.

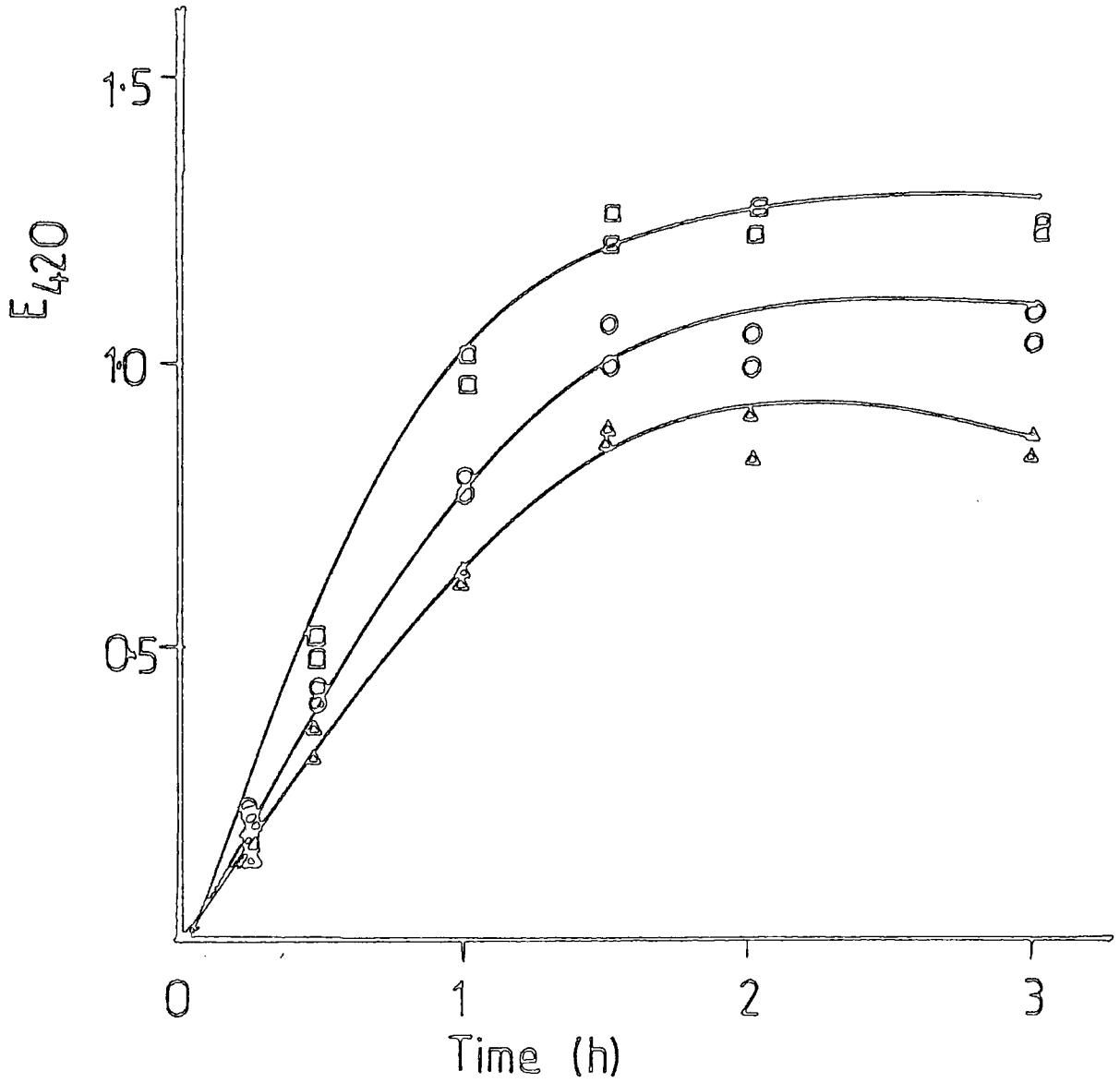


FIGURE 5.4 Kinetic Assay Of β-galactosidase Synthesis In Response To Methionine and Methionine Sulphoxide.

15 ml of Met-starved cells (8134) were added to tubes containing 5 ml 0.004mM Met □——□ , 5 ml 0.008mM D,L-Met(O) O——O , or to 2.5 ml of each of the above solutions Δ——Δ . Duplicate samples were removed at stated times and β-galactosidase assayed in the normal way (Section 3-4)

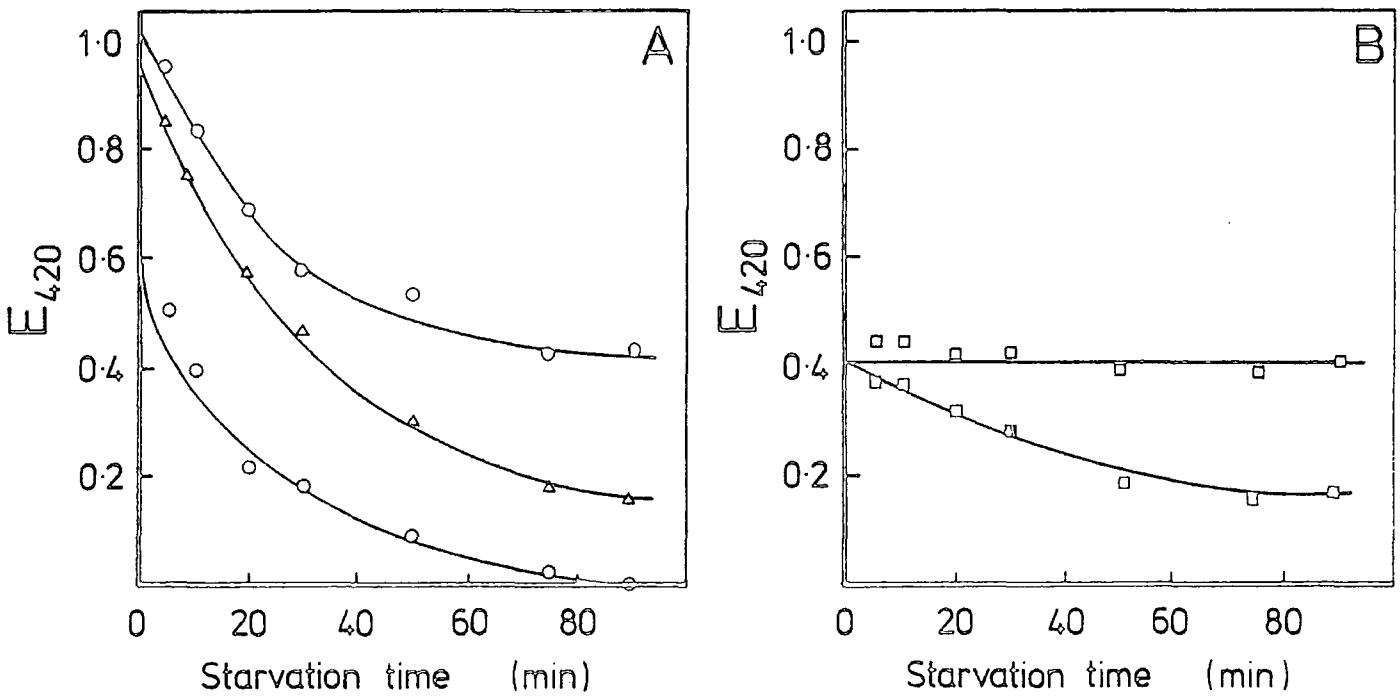


FIGURE 5.5 Effect of Starvation Period On Efficiency Of Methionine Sulphoxide Utilisation For Enzyme Synthesis In E. coli PA0111.

A Bacteria were starved of Met for various periods; 0.16 nmol of either Met○——○ or L-met(O) △——△ was added and the yield of β-galactosidase was measured; ◎——◎ is the unsupplemented control.

B Net enzyme synthesis on supplementation with Met□——□ or with L-met(O) □——□ ; (data derived from A).

That is, as cells became progressively starved their relative utilization of Met(O) decreased from 100% that of Met in unstarved cells to about 60% after 90 minutes starvation. One possible cause for decreasing yield of Met(O)-dependent enzyme synthesis during starvation could be that starving Escherichia coli for an amino acid produces conditions whereby Met(O) is partially modified into a derivative that cannot be converted to Met. If this is the case, the derivative could possibly be detected within the cells or the incubation medium.

5-6-3 Incubations of Whole Cells With Met(O)

Exponential-phase cells of PAO111 and 8134 (approximately 10^9), and cells in different stages of starvation were incubated in A medium containing 0.5% w/v glucose at 37°C with gentle shaking with 0.1mM (final concentration), Met(O) and at 15 minute intervals 2 ml samples were removed and cell extracts were prepared by boiling (Section 2-5-2). These were dansylated and examined on polyamide sheets (Section 2-4). In every case a derivative appeared in the boiled cell extracts that corresponded to dansyl-Met and no other significant new derivative was detected. When the medium from these same incubations was examined, continuous uptake of Met(O) was demonstrated, from exponential and starved cells. As intracellular reduction of Met(O) occurred, excretion of Met took place followed later (20 minutes), by its gradual reabsorption. To demonstrate the effect of incubation of starved cells with Met(O) approximately 10^{11} cells of the Met auxotroph 8134 were first washed in A medium then resuspended in 9 ml fresh A medium, at $^1/_{10}$ dilution, containing 0.075% w/v glucose for two hours at 37°C to starve of Met prior to the addition of 1 ml 1mM Met(O) (0.1mM final concentration).

5-6-4 Incubation of Sonicated Cell Extracts With Met(O)

Exponential phase or Lys starved cells of M2626 were suspended in 0.01M potassium phosphate, pH 7.0 buffer, after harvesting on a filter (Section 2-2-4), to give an E_{660} of 2.2 (1 mg ml^{-1} protein).

Cells were sonicated (Section 2-5-3), and 10mM Met(O) was added to the sonicated cell extract to give a final concentration of 2mM. The mixture was incubated at 37°C for 90 minutes; at zero time, 30, 60 and 90 minutes 1 ml aliquots were removed. 1 ml 10% TCA was added to each sample and after chilling on ice for 15 minutes, centrifugation and lyophilisation (with NaOH in desiccator), extracts were resuspended in half original volume and examined on cellulose thin layers as described in Section 5-4-3.

Met was produced by both preparations being more extensive from exponential-phase cell extracts, whereas Met(O_2) was only demonstrated in the incubation with starved bacterial extracts. Thus, endorsing the results obtained with whole cells (Fig. 5.6).

5-6-5 General Discussion

These results endorse the conclusions of Ejiri et al., (1979, 1980), that Met(O)-reductase activity is greater in exponential-phase bacteria and decreases in starved/stationary-phase bacteria. However, our demonstration of a concomitant oxidation of Met(O) to Met(O_2) could make measurement of reductase activity, by the procedure of Ejiri, misleading. The nature of the oxidation of Met(O) to Met(O_2) described here implies that it is enzymically mediated, since it shows a stereospecific preference for L-Met(O). Furthermore, all incubations were carried out at pH 7, whereas to obtain chemical oxidation of Met(O) to Met(O_2) a low pH (approximately 2.0), and strong oxidising agents (e.g. performic acid), are required.

FIGURE 5.6

Reduction and Oxidation Of Methionine Sulphoxide By Sonicated and Exponential-Phase Bacteria and Lysine Starved Cells (M2626).

A Sonicated bacteria (1mg protein ml^{-1}) in 10mM K phosphate buffer pH 7.0 were incubated with 2 mM L-Met(O) at 37°C. At the indicated times (min) samples were removed, treated with 10% w/v trichloracetic acid and the supernatant solutions lyophilised prior to examination on cellulose thin-layers.

B Sonicated, lysine-starved bacteria were incubated with L-, or D-Met(O) as described above. Rf values were 0.60, 0.46 and 0.38 for Methionine, Met(O_2) and Met(O) respectively.

When the medium from these incubations was examined at intervals up to about 90 minutes, using dansylation as described for exponential cells, there was a reduced appearance of Met in the medium and an additional component appeared (being approximately 10% that of Met), this chromatographed to the position of dansyl-Met(O_2), (Fig. 5.6A). When D-Met(O) was used as the substrate (other experimental conditions being as described for L-Met(O)), there was a marked decrease in production of the compound that chromatographed with Met(O_2), but less change in the degree of conversion to Met.

To gain support for this provisional identification of dansyl Met(O_2), further incubations were carried out so that the media could be examined using an automatic amino acid analyser (Section 2-9). To obtain these samples for the analyser, 9 ml of 2 x 10^9 cells ml^{-1} of strain 8134 (either exponential-phase or starved of Met for 2 hours and resuspended in $^1/_{10}$ A medium plus 0.075% w/v glucose, as before), were incubated at 37°C with shaking (120 strokes min^{-1}), in the presence of 0.1mM (final concentration) Met(O). Duplicate 1 ml samples were taken at zero, 5, 10, 15, 30 and 90 minutes. 400 µ L aliquots of each sample were evaporated down for the amino acid analyser. These were resuspended in 500 µ L pH 2.3 buffer and 10 µ L nor Leu (2.5 µ mol ml^{-1}) standard was added (Section 2-9-2). Met, Met(O) and Met(O_2) were identified and quantified - these three compounds elute at 77-78, 32-33 and 37-38 minutes, respectively. A typical analyser trace is shown in Figure 5.7. In certain cases other amino acids present are seen to be undergoing exodus but during a 90 minute incubation Met(O_2) was shown to be produced at an average rate of 15 n mol mg dry wt^{-1} h^{-1} by Met-starved 8134 (Fig. 5.8).

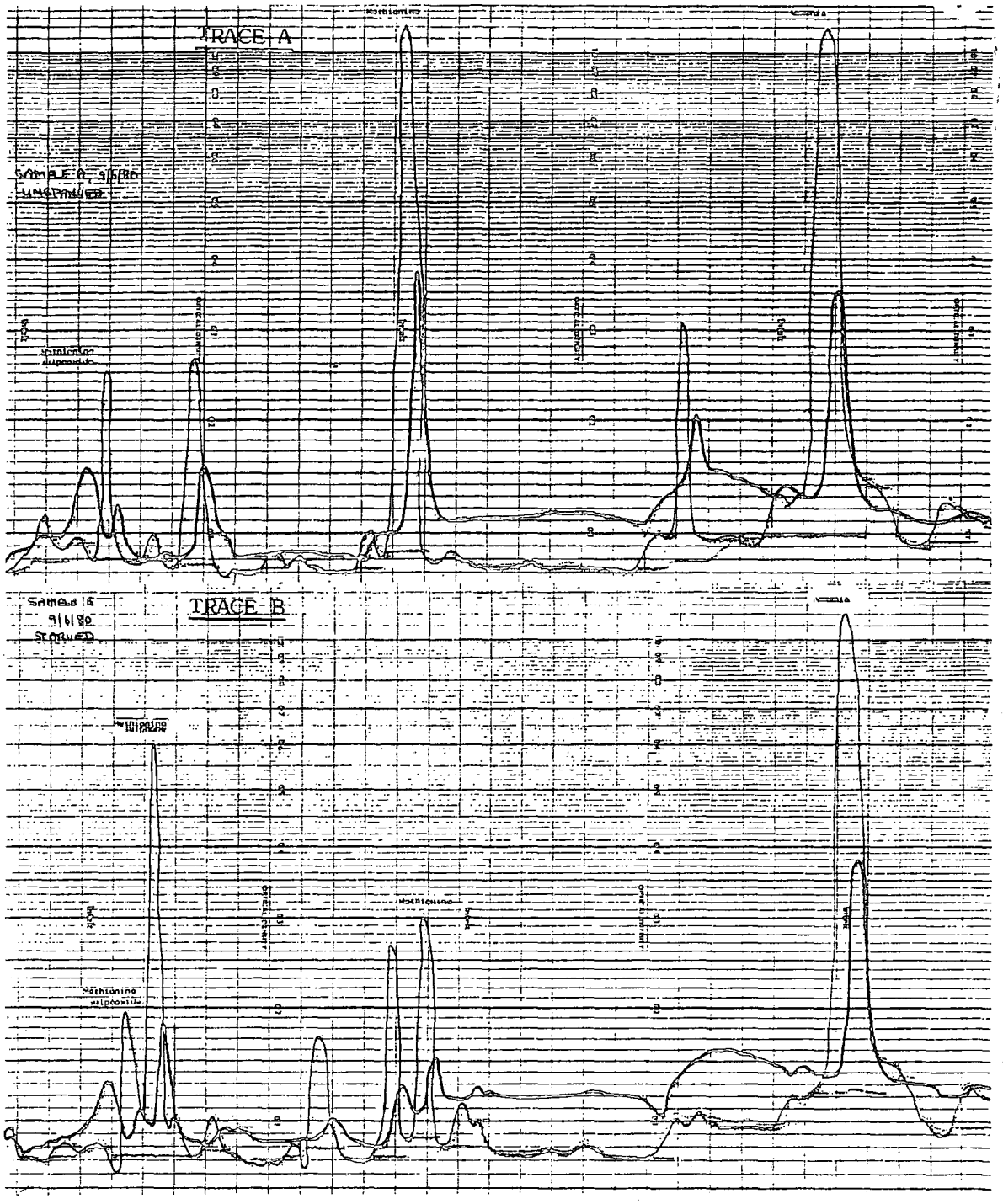


FIGURE 5.7 Amino Acid Analyser Traces

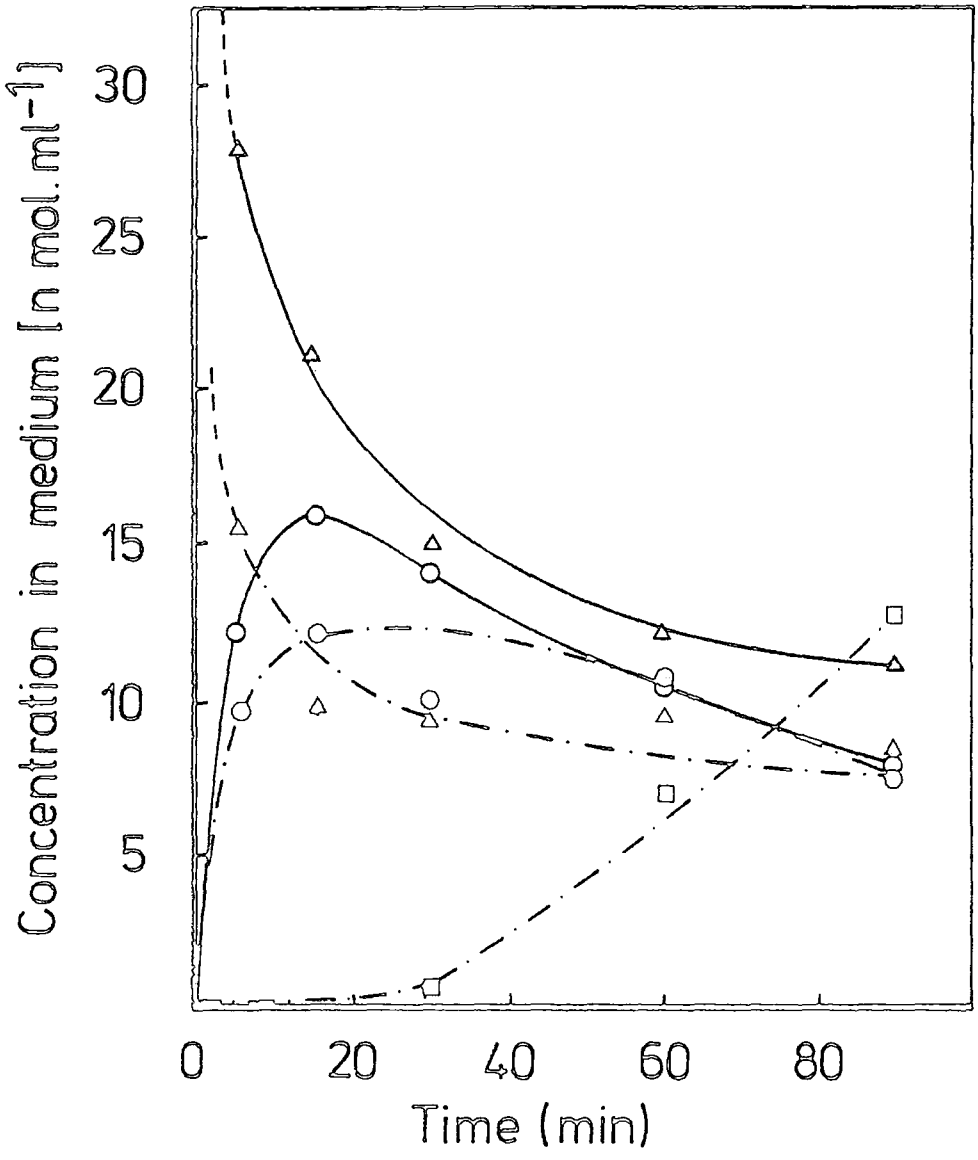
9 ml of 2 x 10^9 cells ml^{-1} were incubated in the presence of 0.1mM Met(O) and samples were taken and run on analyser (long run);

Trace A = exponential cells incubated with Met(O) for 90 min

Trace B = Met-starved cells treated in same way.

Elution times: Met 77-78, Met(O) 32-33 and Met(O_2) 37-38 mins.

See section 2-9 for details of analyser runs and section 5-6-3 for further details of this experiment.



FIGUER 5.8 Reduction and Oxidation of L-Met(O) by Exponential-Phase and Met-Starved E. coli 10799 (8134).

Met(O) was added to exponentially-growing bacteria (full lines) and to cells starved of Met for 90 min (broken lines) and samples of the incubation media were removed at the indicated times and examined on an automatic amino acid analyser: △ , △ , L-Met(O); ○ , ○ , Met; □ , $Met(O_2)$.

Although peroxides and superoxides are believed to accumulate in ageing and starved cells and these could be implicated in these oxidations, on balance the evidence seems to be against their significant involvement.

5-7 Cleavage Of Peptide-Bound Met(O) And Met(O_2)

It has been shown (above results and Ejiri *et al.*, 1979, 1980), that *Escherichia coli* produces a Met(O) reductase, active on free Met(O) and that the activity of the enzyme(s) decreases in starved/stationary-phase bacteria; in starved cells we also demonstrated that there is in addition, a marked production of Met(O_2) from Met(O) *in vivo*.

Strain PAO111 grew on Leu-Met(O), Met(O) Ala and Ala-Met(O); as readily as on Met (Fig. 5.2), indicating that the cells must either hydrolyse the peptides and then reduce the cleaved Met(O) and/or first reduce the Met(O) and then cleave the peptides. There was, at this time, some evidence in the literature for reduction of protein-bound Met(O) (Brot *et al.*, 1981). However, the search for enzymes able to cleave peptide-bound Met(O), (and Met(O_2)) residues had not been reported, and we examined *Escherichia coli* therefore, for the presence of such activities.

5-7-1 Methods

a) Quantitation of Peptide Substrates

Various Met-containing peptides with the designation X-Met, X-Met(O), X-Met(O_2), Met-X, Met(O)-X and Met(O_2)-X - where X is an amino acid; were prepared as described (Sections 5-2-1 and 5-2-2). These were purified using paper chromatography and run on cellulose thin-layers to confirm their purity and to find their approximate concentrations from the intensity of their ninhydrin derivatives. Samples (containing approximately 500 n mol) were hydrolysed and 50 n mol portions were examined on the automatic amino acid analyser to determine their exact concentration (Hydrolysis procedure and controls were as described in Section 2-9-2).

Because the hydrolysis procedure converts some Met(O) to Met(O_2), it was not possible to find the concentration of Met(O) containing peptides from the Met(O) component - the calculations to determine concentration were therefore based on the other amino acid residue. Valine (5 μL, 20mM) was added to the tubes prior to hydrolysis to provide a measure of overall recovery. During these analyses the peptides were stored at -20°C, once their exact concentrations were known they were evaporated and reconstituted in sterile distilled water to give a known concentration (10mM or 1mM depending on quantity present).

b) Cell Strains

Cells used were: M2626, 8134 and PA0111, either exponential-phase, or starved of Lys (M2626) or Met (8134, PA0111). In other experiments, cells were treated with chloramphenicol (250 μ g ml^{-1}, 90 minutes), (Section 5-3-3), which it was demonstrated, inhibited growth (protein synthesis); controls showed that this concentration of antibiotic does not affect the fluorescence of peptide substrates. Additionally, stationary-phase cells, resulting from acidification of the growth medium, were used. Nitrogen-starved cells were prepared using the revertant M2626 Lys^+.

c) Preparation of Cells for Assays

For transport assays using the fluorescamine procedure, the suspension buffer (0.05M potassium phosphate, pH 7.2 containing 0.05% w/v glucose and 0.5mM NH_4Cl), was used, both to wash the cells and to resuspend the cells for assay. This same buffer was used as the blank to determine fluorescent yields of the peptides or blank yields (cells or extracts minus peptide).

Exponential-phase cells were grown up in A medium plus 0.5% glucose plus 0.1mM required amino acid(s), and collected, washed in the suspension buffer plus supplements, as above and used immediately. Where amino acid starved cells were required they were washed as above, resuspended in A medium minus their required amino acid supplement plus 0.5%w/v glucose for 2 hours incubation at 37°C prior to collection, washing and resuspension in the potassium phosphate buffer as described for exponential cells. Lys, at 0.05mM, was always present for Met starved PA0111. Where nitrogen-starved cells were required, M2626 Lys^+ was used and the resuspension buffer did not contain ammonium chloride. Stationary-phase cells were grown in A medium plus 0.5% w/v glucose plus 0.1mM required amino acid(s), and were used after three sucessive 30 minute E_{660} readings were identical. Chloramphenicol-treated cells were prepared as described (Section 5-3-3).

d) Conditions for Fluorescamine Assay With Cell Suspensions

Fluorescamine assays were as described in Section 2-7. 0.05M suspension buffer pH 7.2 and 0.15M phosphate citrate reaction buffer, pH 5.9 were used, giving a reaction pH of 6.2 (6.8 as measured at effluent). Fluorescamine was present at 0.5 or 0.1 mg ml^{-1} (in isopropanol), the concentration depending on peptide concentration or reactivity. Typically, 50 μL of 10mM peptide was added to 9.95 ml cells (ca 10^8 ml^{-1}) in suspension buffer or equivalent concentration of permeabilised or sonicated cell extracts (see part (f) - this Section).

In order to measure cleavage (transport) over a prolonged time (up to 30 minutes), the incubation medium was initially assayed for about 2 minutes, the incubation was continued but not monitored for 15 - 20 minutes, after which the medium concentration was again measured. It was not usually feasible to assay continuously over this period since under the conditions described the 4 μm filters blocked in ca 7 minutes, or 20 minutes if glass fibre (Whatman GF) filters were used.

e) Cellulose Thin-Layer Chromatography

Cleavage of peptide-bound Met(O) and Met(O_2) was confirmed using cellulose thin-layer plates (prepared and run as described in Section 5-4-3). Exponential-phase, and Lysine-starved M2626 were incubated at a concentration of ca 10^9 ml^{-1} in 0.5 ml 50mM potassium phosphate buffer, pH 7.0, containing 100 n mol peptide. At intervals, samples were removed and supernatant solutions (0.05 ml) were examined, after centrifugation, (Haemotocrit microcentrifuge 12,000 r.p.m., 10,000 g) and lyophilisation on cellulose thin layers using butanol : acetic acid : water : pyridine (75 : 15 : 60 : 50 v/v) solvent and developed as described in Section 5-4-2.

f) Permeabilised Cells

5 ml of ca 5 x 10^8 cells ml^{-1} were washed and resuspended in phosphate buffer (5 ml, 0.1M, pH 7.2) 20 μL of 2.5% Triton X-100 was added. The mixture was then frozen in liquid air for 5 minutes, stored at -20°C for about 30 minutes, and placed in a water bath at 37°C for 10 minutes to thaw; this procedure was then repeated. The efficiency of permeabilisation was checked initially by using treated cells in a β-galactosidase assay in which the normal disruption step (using the method of Putnam & Koch, Section 3-3-5-3) was omitted.

Permeabilised cells were shown to produce markedly more β-galactosidase than untreated control cells, therefore the substrate must have entered the cells demonstrating that the treatment was effective. Therefore, permeabilised cells were routinely prepared in this way using 0.5% v/v Triton in cell suspensions of ca 5 x 10^8 cells ml^{-1}. After treatment, permeabilised cells were collected by centrifugation. For peptidase assays the pellet was resuspended in 0.4 ml 0.05M phosphate buffer, pH 7.2, 100 μ L peptide (10mM) was added. At 30 minute intervals, 2 x 0.05 ml samples were removed and examined directly, initially by using paper chromatography using butanol : acetic acid : water : pyridine 75 : 15 : 60 : 50 as a solvent, and in subsequent experiments using cellulose tlc after treatment with 10% TCA, followed by centrifugation (Haematocrit microcentrifuge; 12,000 r.p.m., 10,000 g) and lyophilisation (Section 5-4-3).

5-7-2 Results - Hydrolysis of Met(O) Peptides

Rates of transport for the peptides listed were measured and expressed in n mol min^{-1} mg protein (Table 5.2). In whole cells peptides are actively transported to reach the intracellular peptidases and uptake is the rate limiting step (Payne, 1980b), since assays were continued for 8 - 10 minutes, or sometimes transport was measured at the begining and end of a 20 - 25 minute period; and transport continued throughout, the transported peptide must be metabolised. In agreement with this, no intracellular accumulation of intact peptide could be observed using the dansyl-labelling procedure and, as expected, rapid exodus of the cleaved amino acid residues occurred.

Table 5.2

Influence Of Physiological Status On Methionine Peptide Cleavage By Escherichia Coli

Peptide	Exponential phase	Stationary phase	Lysine-starved	Chloramphenicol-treated	Methionine-starved
MetAla	26	-	25(17)	-	-
Met(O)Ala	27(21)	-	19(22)	-	-
$Met(O_2)Ala$	3(2)	18	21(13)	30	27
MetLeuGly	25	16	28	-	24
Met(O)LeuGly	16	16	15	14	-
$Met(O_2)LeuGly$	2	21	28, 36	23	-
AlaMet	15	-	13	-	11
AlaMet(O)	16	-	12	-	-
$AlaMet(O_2)$	4	-	5	-	6
LeuMet	27	-	-	-	28
LeuMet(O)	15	-	11	-	11
$LeuMet(O_2)$	2	-	2	-	2

Rates are expressed in nmol/min/mg protein.

Values in parentheses are for permeabilised cells.

- Not determined.

The rates of cleavage of all the Met(O) peptides tested were high and comparable with the analagous Met peptides. There was no significant difference between exponential, stationary or starved cells. These rates of hydrolysis imply either a rapid reduction of peptide-bound Met(O) followed by cleavage by normal peptidases or the presence of peptidase(s) active towards Met(O) residues. To try and distinguish between these possibilities the cleavage products were examined by using thin-layer chromatography. To minimise possible reduction of Met(O_2), cell extracts were made of Lys-starved M2626 (no requirement for Met, sonicated cells), and they were incubated with Met(O) Ala. The incubation medium was sampled periodically and examined and Ala, Met(O) and Met were detected. This confirmed the presence of peptidase activity towards Met(O) residues.

5-7-3 Cleavage of Met(O_2)-Containing Peptides

Since Met(O_2) is not utilised by Escherichia coli, no information on possible cleavage of Met(O_2) peptides had been obtained from growth tests using Met auxotrophs (Section 5-6-1). However, direct peptidase assays using whole cells were possible with peptides containing Met(O_2), (Table 5.2). They showed that exponential-phase bacteria have low activities towards peptides containing Met(O_2) but hydrolysis of Met(O_2)-X peptides increased about ten-fold in stationary phase bacteria, in auxotrophs starved for Lys or Met and in chloramphenicol-treated cells. A sample trace showing cleavage of Met (O_2)-Leu-Gly by cells starved of Lys is given (Fig. 5.9).

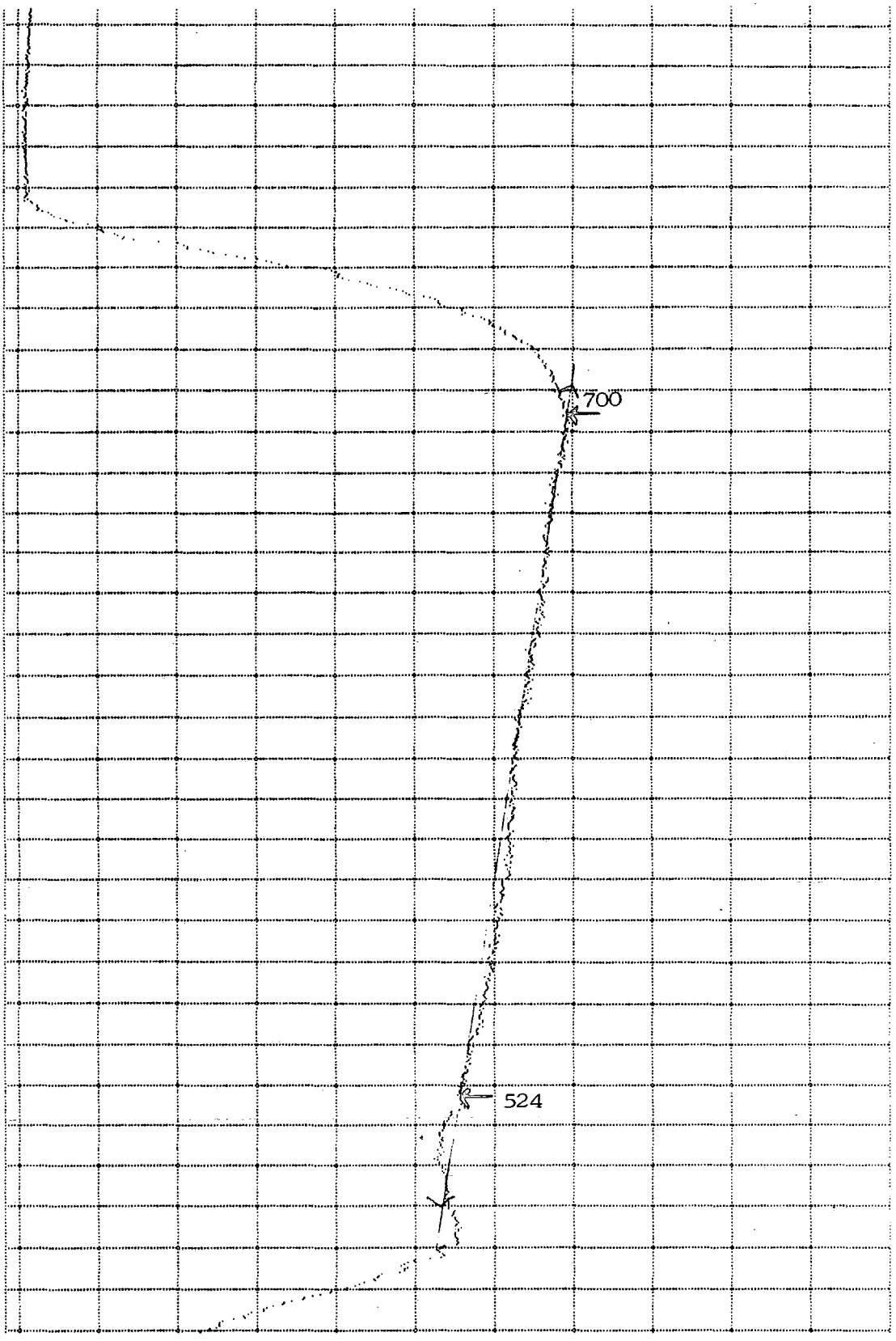


FIGURE 5.9 Sample Trace Showing Met(O_2)-Leu-Gly Cleavage By Lys-Starved Cells of M2626.

Fluorescamine conc = 0.5 mg ml^{-1}. E_{660}cells = 0.18. Time between arrows = 5.33 min. Numbers on trace represent fluorimeter reading. Calculated rate in this case = 36.09 nmol min^{-1} mg $protein^{-1}$.

Another trace of uptake of this same peptide is given in Chapter 2, Figure 2.4 (page 70).

This general conclusion was confirmed using thin-layer chromatography to examine cleavage of Met(O_2)-Leu-Gly by broken cell extracts (Fig. 5.10). The photograph shows that Met(O_2) is released from Met(O_2)-Leu-Gly, together with Leu-Gly, Leu and Gly, on incubation with a broken cell extract of Lys-starved bacteria but cleavage is markedly less with exponential-phase bacteria. Following this finding, a growth test was set up using Escherichia coli K12 strain 4258 Arg, Leu, His, and response to free Leu and to Met(O_2)-Leu-Gly as Leu sources was measured. Both supported growth although the rate was faster on the amino acid (Fig. 5.11). The ability of the strain to grow on Met(O_2)-Leu-Gly as a Leu source confirms the presence in vivo of Met(O_2)-X peptidase activity. This conclusion has subsequently been confirmed using other Met(O_2)-containing peptides and other auxotrophs (J.W. Payne, personal communication).

FIGURE 5.10

Cleavage of Met(O_2)LeuGly By Permeabilised Exponential-Phase and Lysine-Starved Cells Of E.coli M2626.

Bacteria at $5x10^8$ ml^{-1} were permeabilised, resuspended at $5x10^9$ ml^{-1} in 0.5 ml of 50mM K phosphate buffer pH 7.0 containing 100 nmol peptide and incubated at 37°C for the indicated times. After centrifugation the supernatant solutions were lyophilised and examined on cellulose thin-layers. Rf values were 0.68, 0.60, 0.56 and 0.45 and 0.35 for Leu, Met(O_2)LeuGly, LeuGly, Met(O_2) and Gly respectively.

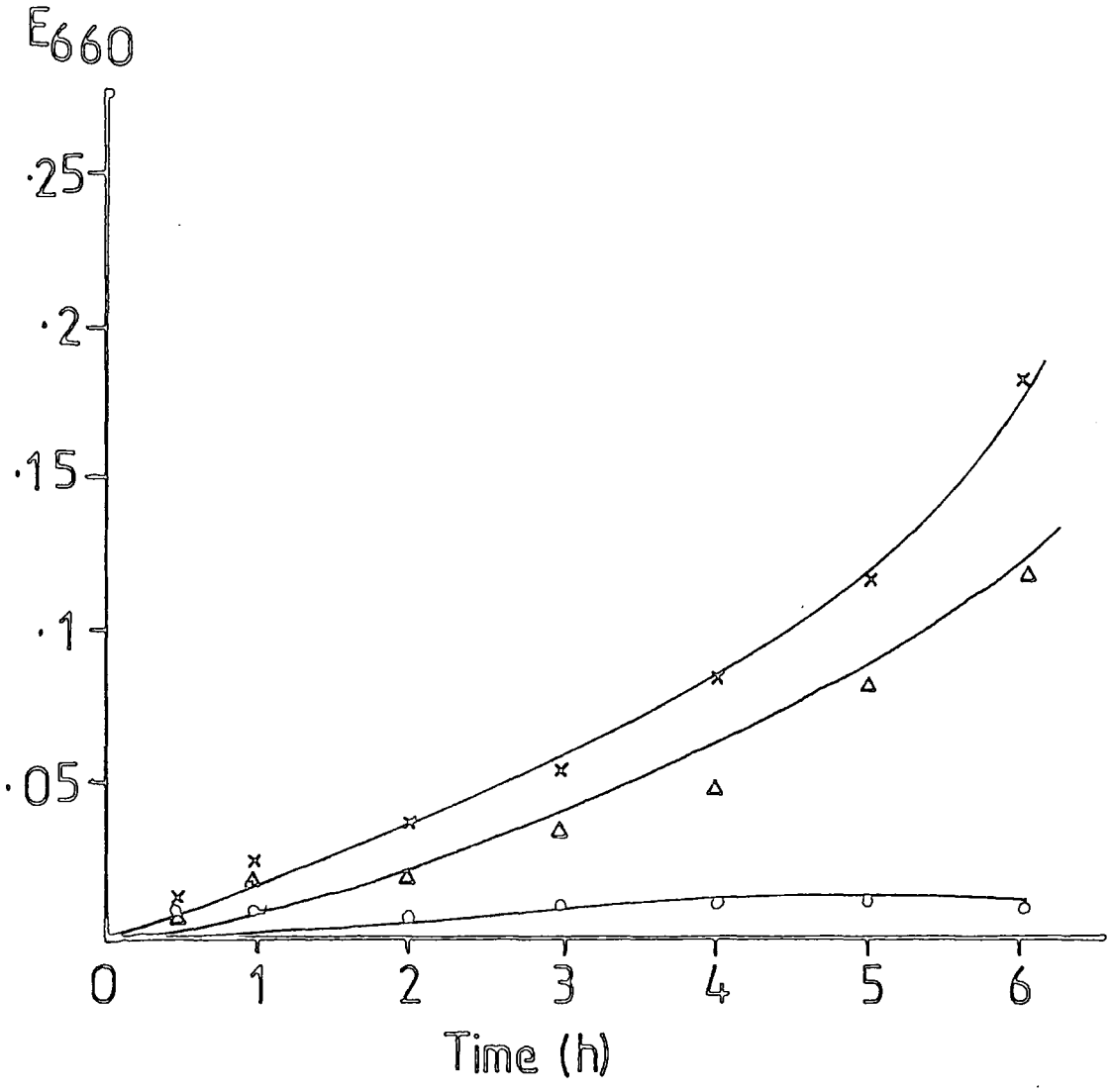


FIGURE 5.11 Comparison Of Strain 4258 Arg⁻ Leu⁻ His⁻ On Leu Or Met(O_2)-Leu-Gly As Sole Source Of Leu For Growth.

Cells were grown (A medium, 0.5% glucose, 0.5mM amino acids to exponential-phase. Collected and washed and ca. 10^8 cells were added to tubes containing 0.1 mM peptide Δ——Δ , 0.1 mM Leu ✕——✕ or no Leu source ○——○. In all cases His and Arg were added at 0.1 mM.

Tubes were incubated at 37°C with shaking (100 strokes per min) and each was aerated for 30 sec every hour since this strain grows poorly on defined media.

5-8 Concluding Discussion

It is interesting that exponential-phase and starved Escherichia coli exhibit different utilisation and oxidation/reduction patterns of Met(O); this could perhaps be analogous to the differential response of young and old animals to Met(O) as a dietary source of Met (Aymard et al., 1979; Miller & Samuel, 1970; Anderson et al., 1976; Cuq et al., 1978; Njaa, 1962).

There is a well documented increase in oxidation of protein-bound Met that accompanies ageing (Reiss & Gershon, 1978; Garner & Spector, 1980); this could be an indirect effect resulting from a decrease in activity of a methionine-reductase and/or an increase in an oxidising species.

The ability of starved cells of Escherichia coli to convert Met(O) to Met(O_2) seems on the face of it to be disadvantageous since Met(O_2) cannot be used as a Met source for growth by Escherichia coli (it has been reported (Tonzetich, 1976) that oral plaque microflora can metabolise Met(O_2) to methyl mercaptan, dimethyl sulphide and dimethyl disulphide but at a slower rate than Met(O)). However, the Met(O_2)-X cleaving activity that has been demonstrated to occur when Escherichia coli is under this same physiological stress could be a means of recovery of amino acids that are present in peptides/proteins containing Met(O_2) residues. It is known that these same stress conditions trigger enhanced and specific intracellular proteolysis but the actual mechanism of the stimulation is unclear (Pine, 1980).

Clearly, it is an interesting speculation arising from the work described that certain Met(O_2) residues produced in specific surface positions on proteins or at specific places within the primary sequence may provide cleavage sites to initiate enhanced proteolysis; subsequent hydrolysis would involve the cells known complement of proteases and peptidases (Hermsdørf & Simmonds, 1980). Alternatively, cleavage at a Met(O_2) residue could provide a regulatory mechanism to modify the activities of existing enzymes associated with proteolysis *in vivo*. Further discussion of this topic is given in Payne & Tuffnell, 1981, and Tuffnell & Payne, 1981, but clearly further work is needed to determine whether protein-bound Met(O_2) and Met(O) residues can be cleaved.

Recently, increasing evidence on the effect of oxidative changes on susceptibility to proteolysis have appeared. Thus, oxidative inactivation of glutamine synthetase of *Escherichia coli* has been shown to precede proteolysis and catalase has been shown to block this oxidative modification step (Levine *et al.*, 1980; Goldberg & Boches, 1982).

The β-galactosidase Met assay, using PA0111 or 8134, employs cells starved of Met for a 90 minute period. The response to any Met(O) residues present in the test samples would therefore be decreased to about 60% of the equivalent Met value. In virtually all cases this should not be a significant problem because Met(O) is not normally a significant constituent in proteins (Aymard *et al.*, 1979; Reiss & Gershon, 1978; Garner & Spector, 1980). However, in certain assays of processed foodstuffs, a significant proportion of Met might possibly be present as Met(O) or even Met(O_2).

Because of its metabolic inertness there is no microbiological or growth assay for Met(O_2); if it were felt necessary the β-galactosidase assay could be modified for determining Met(O) by using cells that were not Met-starved, but merely washed to remove exogenous Met from the growth medium. This would give rise to higher blank values and the range of concentrations over which a response could be measured would thus be decreased.

Another approach, that has not been examined here would be the chemical reduction of protein-bound Met(O) to Met. Snow et al (1976), reported that bisulphite solution could be used to convert free Met(O) to Met in aqueous solution over a pH range of 1.0 - 9.0 but whether the reduction of protein-bound Met(O) groups occurs in the same way was not studied. The chemical reduction of Met(O) in peptides and proteins has been reported by Houghten & Hao Li (1979).

An alternate approach is to assay the Met(O) present separately and to correct the total Met values obtained accordingly. Njaa (1980), reported a chemical method for determination of unoxidised and total Met in protein concentrates using an iodoplatinate reagent (Met(O) does not react under the chosen conditions), then repeating the determination after reduction of the sample with titanium chloride; Met(O) values can then be obtained by difference. This is faster than the normal methods which involve chromatography of digests before and after reduction (Cohen et al., 1979). These same authors describe a non-destructive means of analysis of Met(O) in protein by ^{13}C NMR techniques.

Another technique uses the property that Met(O) peptide bonds are not cleaved by the cyanogen bromide reaction that converts Met residues to [illegible](Savige & Fontana, 1977). Further details of enzymic assays for Met in food proteins, digests and biological fluids are described in Chapter 6.

CHAPTER SIX

RESULTS OF ASSAYS FOR AVAILABLE LYSINE, METHIONINE AND TRYPTOPHAN

6-1 Effect of Enrichment on β-Galactosidase Lysine Assay

6-1-1 Introduction

In order to examine the possible effects of the presence of additional nutrients on the assay for Lys, Lysine assay medium (Difco, Detroit, code number B422), was used. This is a rich, defined medium containing various nutrients, vitamins and co-factors but excluding Lys (see Table 6.1 for composition). In later work, methionine or isoleucine assay media were used (code numbers B423 and B437). These have the same composition as Lys assay media but exclude Met or Ile respectively and include a final concentration of 0.5 g l^{-1} L-lysine hydrochloride when reconstituted as recommended.

6-1-2 Methods and Results

The assay media were used at dilutions such that the final amino acid concentration would be of the same order as that likely to be present in a seed protein mixture. The recommended concentration of the media for the cultivation of Leuconostoc spp. is 10.1 g 100 ml $water^{-1}$ and the medium was usually used at a fifty or hundred-fold dilution of this value. Where Lys was added it was at a final concentration of 0.02 or 0.06mM (low and high Lys respectively). In later experiments where Met or Ile assay media were used they were diluted so that the Lys they contained (0.5 g l^{-1} when reconstituted as above), was present at a final concentration of 0.02mM.

The Lys in the Met and Ile assay media was determined by using M2626 and the standard β-galactosidase assay with various modifications.

Table 6.1

Composition Of Difco Lysine Assay Medium Formulation Per Litre - At Recommended Concentration

(Data taken from Difco Handbook 9th edition, 1953)

Bactodextrose	50g	Adenine sulphate	0.02g
Sodium acetate	40g	Guanine HCl	0.02g
Ammonium chloride	6g	Uracil	0.02g
DL-Ala	0.4g	Xanthine	0.02g
L-Arg HCl	0.484g	Thiamine HCl	0.001g
Bacto-Asn	0.8g	Pyridoxine HCl	0.002g
L-Asp	0.2g	Pyridoxamine HCl	0.006g
L-Cys	0.1g	Pyridoxal HCl	0.006g
L-Glu	0.6g	Ca panthothenate	0.001g
Gly	0.2g	Riboflavin	0.001g
L-His HCl	0.124g	Nicotinic acid	0.002g
DL-Phe	0.2g	p-aminobenzoic acid	0.002g
L-Pro	0.2g	Biotin	0.000002g
DL-Ser	0.1g	Folic acid	0.00002g
DL-Thr	0.4g	Monopotassium phosphate	1.2g
DL-Trp	0.08g	Dipotassium phosphate	1.2g
L-Tyr	0.5g	$MgSO_4$	0.4g
DL-Met	0.5g	Ferrous sulphate	0.02g
DL-Ile	0.5g	Manganous sulphate	0.02g
DL-Leu	0.5g	Sodium chloride	0.02g

In one experiment the possible effects of catabolite repression by the glucose in the assay media was studied; exponential phase cells were harvested, starved and induced (as standard method), and either, added immediately to tubes containing glycerol, Lys (0.02mM and 0.06mM), and various volumes of 2.02 mg ml^{-1} assay media or preincubated with glycerol for 30 minutes and then added to the assay media.

In both cases samples were removed periodically and β -galactosidase was assayed. Fig. 6.1 shows that where there was simultaneous addition of inducer and assay medium (which contains Bacto-Dextrose), lower levels of β-galactosidase were produced and the response of the added Lys was masked. However, when cells were induced for 30 minutes in glycerol prior to addition to the incubation mix, the effects of added Lys were apparent. This difference is compatible with catabolite repression mediated by the glucose in the assay medium (Fig. 6.2), see also (Section 3-3-2-1).

To examine the possibility that other substances in addition to glucose in the assay media might also be repressing the synthesis of β-galactosidase, experiments were performed in which instead of adding induced cells to assay media both immediately and after 30 minutes induction, uninduced cells were added at these times to minimal media containing glucose at the concentration present in the diluted assay media (1 mg ml^{-1}). Glycerol (0.5% v/v), was present and duplicate assays were carried out at high (0.06mM), and low (0.02mM), Lys concentrations (Fig. 6.3 A & B). The results were essentially similar to those obtained previously (Fig. 6.2), supporting the idea that the low enzyme synthesis seen when cells were added to enriched medium immediately after induction did indeed arise from the presence of glucose in the assay media.

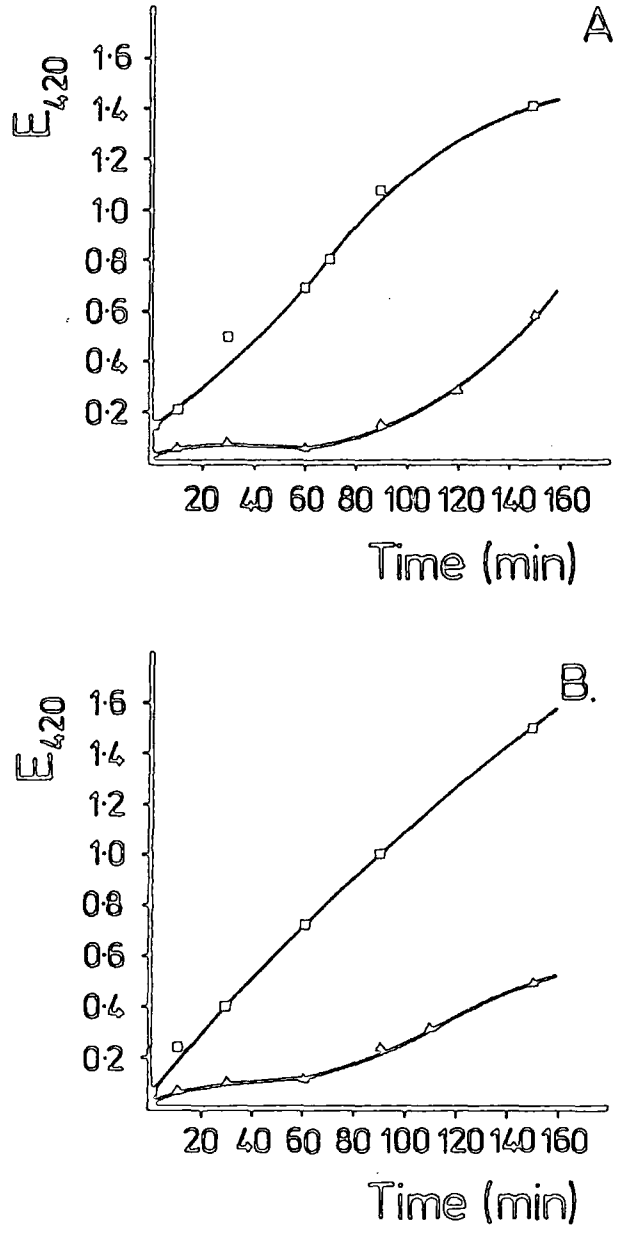


FIGURE 6.1 Effect Of Glucose In Assay Medium On Response To Lysine

2×10^8 cells (M2626) were collected in exponential-phase, starved and induced immediately. Lys at either 0.06mM(A) or 0.02mM(B) was added together with either minimal medium □—□, or Lys assay medium △—△, (15 ml, 2.02 mg ml^{-1} in 30 ml total vol) and at indicated times 2 ml samples were removed and β -galactosidase was assayed using the method in section 3-4.

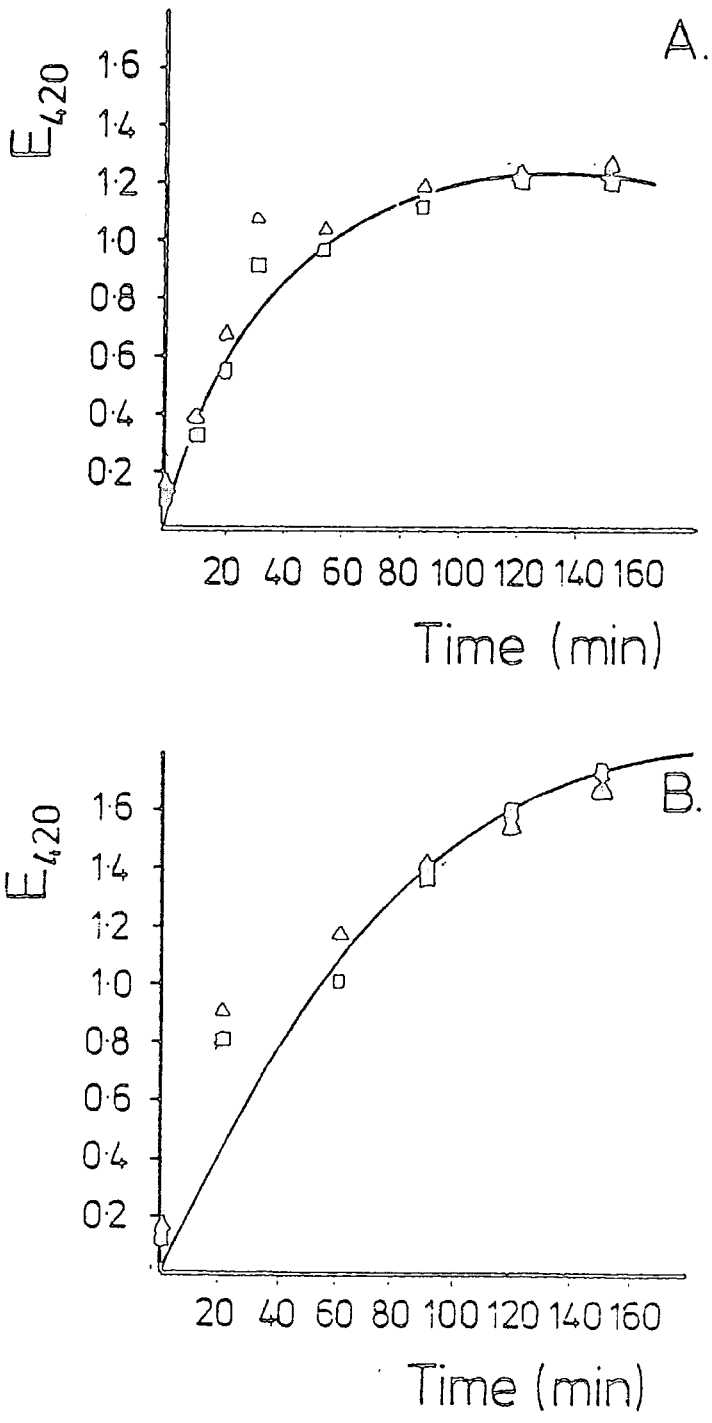


FIGURE 6.2 Effect Of Enrichment On Induced Cells

Approximately 2 x 10^8 cells were grown, starved of Lys and induced for 20 min as in normal procedure. The induced cells (M2626) were then added to either minimal medium□——□, or to 2.02 mg ml^{-1} Lys assay medium△——△, plus either 0.0[illegible] mM (A) or 0.0[illegible] mM (B) Lys. At stated times samples were assayed using the standard method as in section 3-4.

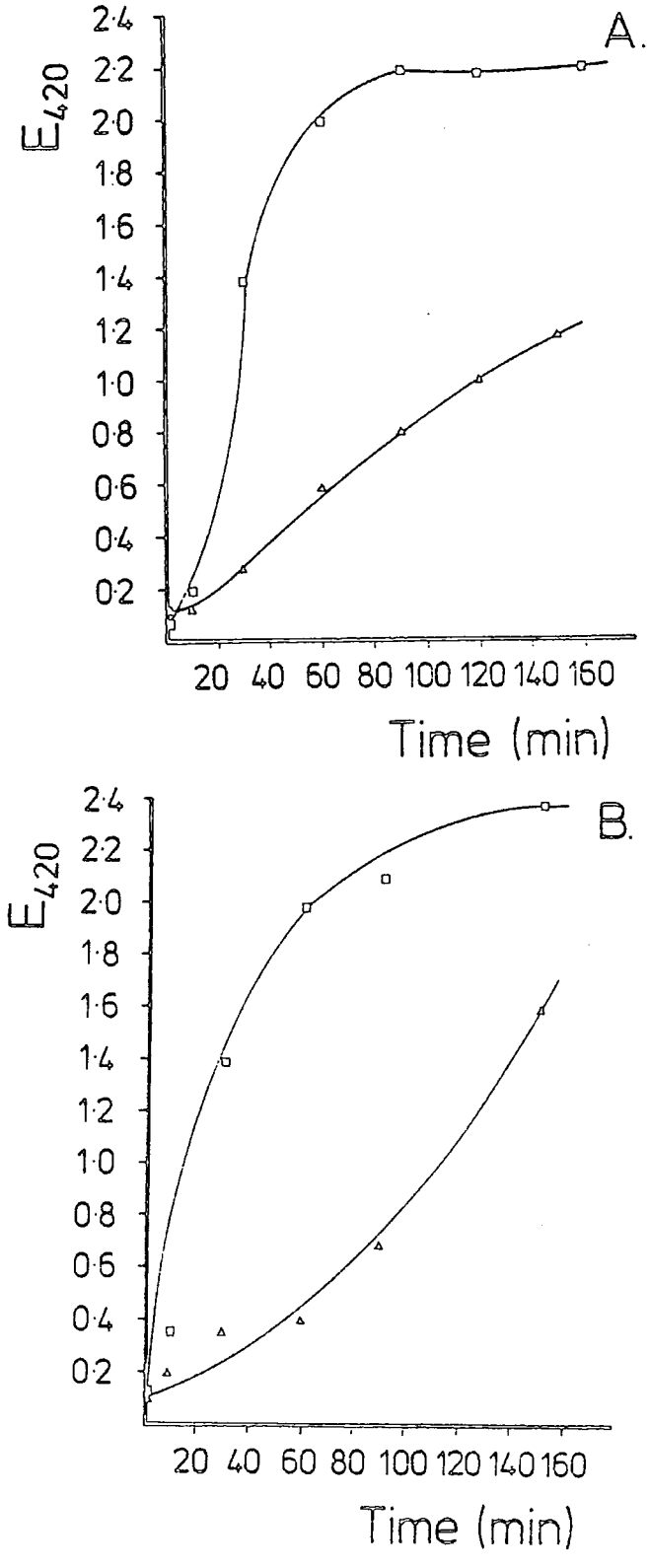


FIGURE 6.3 Effect of Glucose On β-galactosidase Synthesis.

Approximately 5 x 10^8 cells of M2626 were induced and immediately added to Lys at 0.02mM (A) or 0.06mM Lys (B) in the presence of 0.05% v/v glycerol □—□ , or 0.5% v/v glycerol + 0.015% w/v glucose △---△ At stated times samples were removed and the enzyme was assayed by the normal method; section 3-4.

To avoid possible problems of catabolite repression caused by any glucose that might be present in digests it is necessary to induce the cells for about 20 minutes (Fig. 6.4), prior to addition to the digest sample. Since 20 minutes induction prior to addition of glucose removed any catabolite repression, longer induction periods were not examined. As a further check for the possible presence of inhibitors in the assay media a recovery experiment was carried out in which Lys was assayed at various concentrations (0.02 - 0.0002mM), either alone (i.e. minimal medium), or supplemented with 5 ml 2.02 mg ml^{-1} Lys assay media in a final volume of 25 ml. It can be seen, (Fig. 6.5), that the presence of enrichments in the assay media does not interfere with the Lys assay, and it may be concluded that no inhibitors are present in the assay medium.

6-1-3 General Conclusions

The results show that provided cells are fully induced prior to their addition to test nutrients, the synthesis of β-galactosidase by M2626 is solely dependent on the Lys present; other nutrients present at concentrations that might occur in test digests do not significantly alter the response.

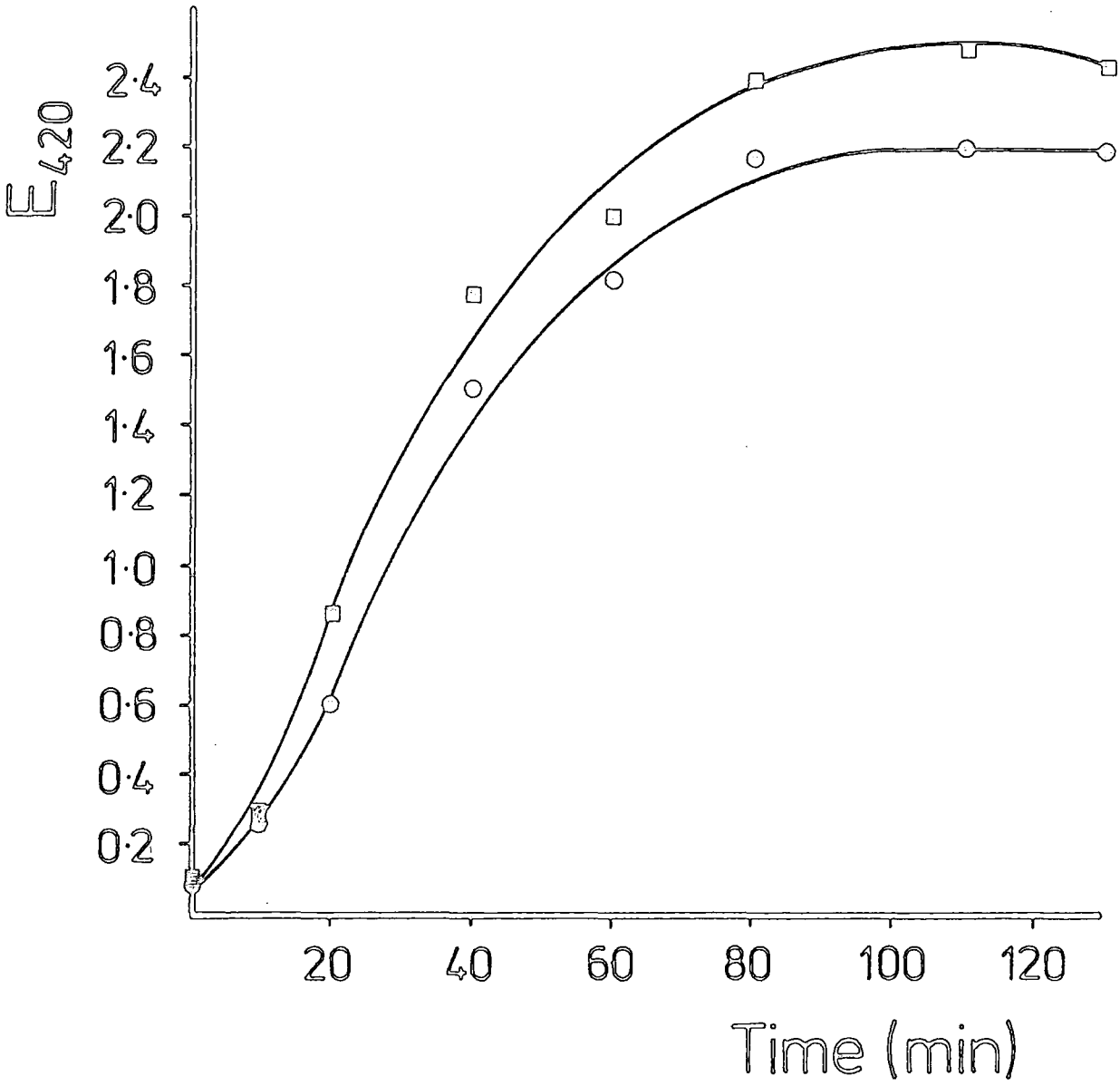


FIGURE 6.4 Effect Of Induction Time On β-galactosidase Synthesis.

30 ml of ca. 5 x 10^8 cells ml^{-1} (M2626) were grown in Lys and starved by the normal procedure. 15 ml samples were then induced for 15 ○—○ or 20 □—□ min before the addition of Lys and at stated times samples were removed and β-galactosidase was assayed by the normal method.

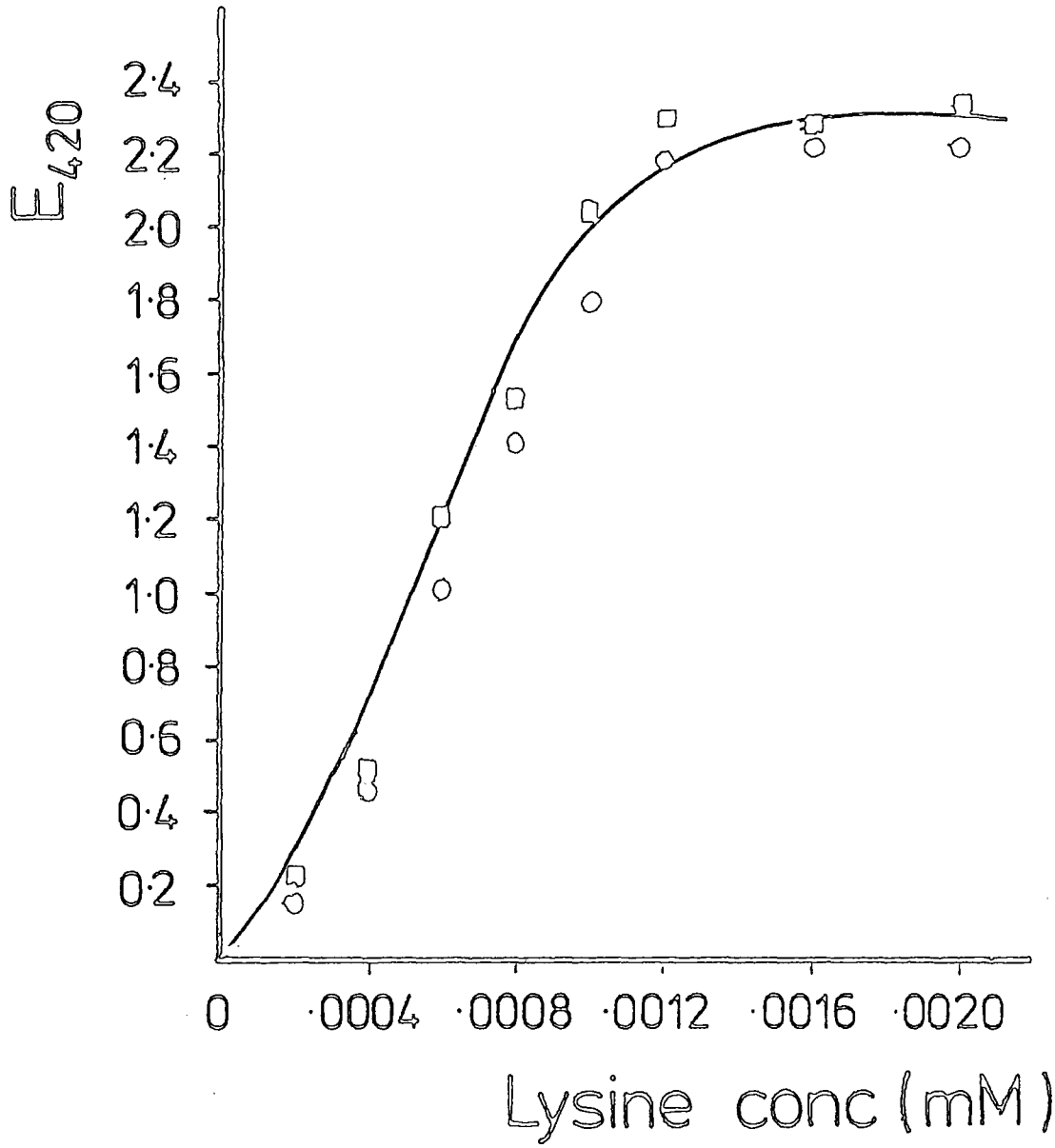


FIGURE 6.5 Effect Of Enrichments On Lysine Assay

Concentrations of Lys were assayed by normal method (section 3-4) using M2626, either alone □—□ or in the presence of Lys assay media (final conc = 0.404 mg ml^{-1}), ○—○. Cells were grown in normal way, starved of Lys and induced for 20 min prior to their addition to the two test media.

6-2 Effect of Hydroxylysine on β -Galactosidase Assay For Available Lysine

6-2-1 Introduction

The enzymic defect in M2626 precludes other biosynthetic precursors from substituting for Lys but it was important to determine whether other Lys derivatives might satisfy the cells requirement for this amino acid; the only potentially important derivative is hydroxylysine.

It has been reported (Carpenter et al., 1964; Hartley et al., 1965), that the presence of δ -hydroxylysine caused overestimates in the assay of Lys with Leuconostoc mesenteroides P60. Hydroxylysine is found in gelatine and collagen, being formed by oxidation at the δ-carbon of lysyl residues in these proteins. It was important to determine whether hydroxylysine could be used as a source of free Lys for the synthesis of β-galactosidase.

6-2-2 Methods and Results

A β-galactosidase assay was carried out as already described (Section 3-4), with either free Lys (0.002mM - 0.1mM final concentration), or δ -hydroxylysine (0.004 - 0.02mM), or mixtures of both compounds up to a maximum of a ten-fold excess of hydroxylysine. Because the δ -hydroxylysine was a mixture of D,L and DL-allo isomers it was used at two-fold equivalent concentrations of L-Lys. These studies showed that hydroxylysine could not satisfy the requirement for Lys (data not shown), and furthermore, at the ratios tested (up to a ten-fold excess), it did not have any inhibitory effect or a sparing effect for Lys (Fig. 6.6).

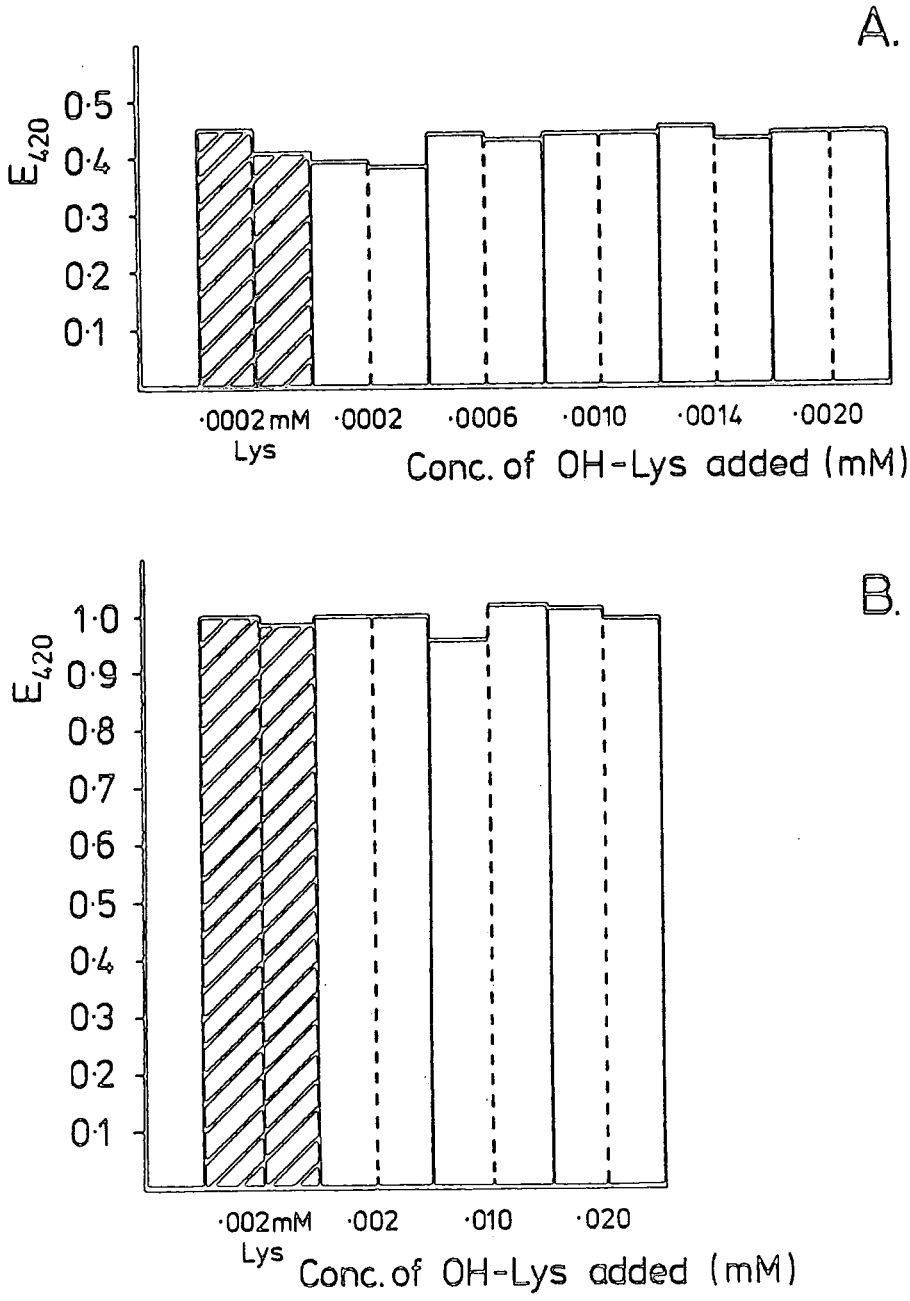


FIGURE 6.6 Effect Of Addition Of δ-Hydroxylysine On Response To Lysine.

To duplicate samples containing Lys at either 0.0002mM (A) or 0.002mM (B) was added varied concentrations of hydroxy-lysine and resultant β-galactosidase was assayed by using the standard method.

Therefore, the presence of hydroxylysine will not interfere with the determination of Lys by the β-galactosidase assay. This conclusion is similar to the finding of Lindstedt (1953), who showed that hydroxylysine was unable to promote growth in rats. The evaluation of various N-substituted lysines as nutritional sources was considered to be outside the scope of the present study. For details of the utilization of α-N and ε-N substituted lysine derivatives in a rat growth assay see Finot et al., 1978.

6-3 Assessment of Heat Damage to Available Lysine

6-3-1 Introduction

The Mailliard reactions in foods in which sugar aldehyde groups react with free amino groups causing non-enzymic browning are well known (see reviews by Bender, 1978; Carpenter and Booth; 1973). The reactions usually cause undesirable effects such as changes in colour, solubility or reduced digestibility and hence, decreased biological availability. It has been suggested (Bender, 1971; Adrian, 1974), that certain Mailliard compounds may be toxic.

The reactions leading to the formation of Mailliard products are well documented (Feeney et al., 1975; Finot, 1973), although certain aspects still remain unclear. Carpenter & Booth (1973), studied the rate of loss of reactive amino groups at different temperatures and presented the data in the form of an Arrhenius' plot. The activation energy (E) for Mailliard reactions has been quoted as 31.2 Kcal $gmol^{-1}$ (Bailey & Ollis, 1977). (For comparison, the values are 50 and 100 Kcal $gmol^{-1}$ for cell or spore destruction respectively). Other factors such as the presence of metal ions, pH, moisture content etc., also affect Mailliard reactions (Kata et al., 1981).

Much of the research into Mailliard browning has been carried out with the aim of trying to control the process. The most common type of damage is that which occurs when Lys reacts with reducing sugars, and although there may be concomittant damage to other amino acids such as Met (Ford & Salter, 1966), and Trp (Finot et al., 1982), damage to Lys can be used as an index of the severity of processing.

Because Lys is limiting in most cereals and the sulphur amino acids (Met + Cys), are limiting in most other foods, it is clearly important to prevent damage to these particular amino acids.

The effect is not limited to foods containing a large proportion of carbohydrate. Damage occurs if high protein meals or fish muscle are heated excessively (Bjarnson & Carpenter, 1970; Ford & Shorrock, 1971; Miller et al., 1965; Shorrock & Ford, 1978), although both are essentially carbohydrate free. The damage is accompanied by a decrease in the ε-amino groups of Lys. Model systems e.g. egg albumin/glucose (Tanaka et al., 1977), or casein/glucose (Osner & Johnson, 1974, 1975), have often been used for these studies but although commercial casein, for example, contains several distinct proteins, foodstuffs are more complex. Dümmer, 1971 showed a heated casein-glucose mixture lost Arg, Thr, His, Ile in that order, as well as Lys but when milk was subjected to the same treatment only Lys was lost.

Bjarnson & Carpenter (1969, 1970), have studied possible ways of preventing Lys losses during heating, drying and storage of food proteins, for example, by prior acylation. In particular, formylation was studied (Hurrell & Carpenter, 1978). It is common to add sulphite or SO_2 to foods to retard non-enzymic browning. However, there are objections to the use of these compounds and recent evidence (Robbins & Baker, 1980), indicates that although Lysine-sulphite may be resistant to Mailliard reactions it nevertheless is nutritionally unavailable. A further aspect of the problem is that not only are the complexes themselves unavailable but they may also interfere with the absorption of other nutritional compounds.

For example, reduction of protein quality by Mailliard-type reactions can occur to a certain extent even at room temperature, e.g., during food storage, but there is a marked acceleration of damage by heat (Lea & Hannan, 1949). There is disparity in figures quoting fall in digestability after various heat treatments; for example, some workers have suggested that milk suffers no damage on heating whereas others (Dümmer, 1971), have reported losses in Lys. However, milk proteins are limited by S-amino acids and it may be that biological assays might well not reveal small Lys losses unless they were carried out under conditions where Lys was made limiting, e.g., by supplementing the diet with other essential amino acids.

Chong-Min Lee et al (1977), reported competitive inhibition of the absorption, in vitro and in vivo of Trp by fructose-tryptophan. Horn et al (1968), reported that fructose-methionine promoted growth of Leuconostoc mesenteroides P60 with 80% of the efficiency of pure Met, although the same compound was totally unavailable to the rat. Both of these are Amadori compounds formed by reactions of sugar and amino acid residues. Although amino acids will be liberated from these complexes by acid hydrolysis they are not expected to be cleaved by digestive enzymes; hence, the bound amino acids will be nutritionally unavailable. The effects of Mailliard complexes on amino acid availability have been discussed (Finot et al., 1982; Erbersdobler, 1976, 1977).

Foods undergo a variety of treatments during manufacture to preserve, to improve taste and to add variety, and because heating is often involved, a reduction in amino acid availability is likely.

It was important therefore, to determine if the β -galactosidase assay responded to changes in amino acid content after typical heat treatments. In carrying out these studies we focussed specifically on Lys.

6-3-2 Materials

Met and Ile assay media were described in Section 6-1-1. Casein hydrolysate Type I and Casein enzymic hydrolysate (both from milk), were obtained from Sigma.

6-3-3 Methods

a) Casein hydrolysate

In a previous study (Payne et al., 1977), the Lys content of an acid hydrolysate of casein was assayed using a growth test with M2626. This same substrate was assayed here using the modified β -galactosidase procedure (Section 3-4); because casein hydrolysate is essentially free of any carbohydrate, it was chosen to investigate the effect of heat on proteins. Samples containing 5 to 0.5 μ g ml^{-1} of acid - or enzymically-digested casein were assayed as normal (Section 3-4), and the concentrations of Lys in each sample was calculated. Controls in which additional Lys was added to the digest were included in the assay to check for the possible presence of inhibitors; no interference was detected.

To study the effects of heat treatment, 10 ml of each casein digest, 10 μ g ml^{-1} was added to 25 ml screw-capped medical flats and autoclaved (15 p.s.i., 15 minutes). These heated samples were then assayed for Lys and compared with the results of the untreated controls. Results are given in Table 6.2. The enzyme and acid hydrolysates contained 0.064 and 0.065 g Lys g $digest^{-1}$ respectively. After heating there was a mean decrease of 4.7% in the available Lys in the enzymic digest and a 6.2% decrease in the acid digest.

Table 6.2

Assay Of Lys In Casein Hydrolysates

Sample	Conc. Test Solution (µg ml)	Lys Content µg ml^{-1}	g Lys g $Digest^{-1}$	Mean
Enzymic hydrolysate	5.0	0.30	0.06	
	2.5	0.153	0.061	0.064
	1.0	0.07	0.07	
Acid hydrolysate	5.0	0.29	0.058	
	2.5	0.182	0.073	0.065
	1.0	0.063	0.063	
Enzymic hydrolysate heated 15 p.s.i., 15 minutes	5.0	0.29	0.058	
	2.5	0.15	0.060	0.061
	1.0	0.065	0.064	
Acid hydrolysate heated 15 p.s.i., 15 minutes	5.0	0.29	0.058	
	2.5	0.153	0.061	0.061
	1.0	0.065	0.064	

Each value is the mean of 4 determinations

b) Assay Media

Met and Ile assay media both contain defined quantities of Lys and carbohydrate; when reconstituted as recommended (10.1 g 100 ml^{-1}), these are 0.5 g Lysine hydrochloride l^{-1} and 50 g dextrose l^{-1}. They are used here to study the effect of heating when Lys was present together with carbohydrate. Samples of assay media at various concentrations were subjected to several different moist heat treatments including

(i) Heating stock solutions (10.1 g l^{-1}) at 15 p.s.i. 15 min.

(ii) Heating solutions, 1.01 g l^{-1} and 0.505 g l^{-1}, at 15 p.s.i. for 15, 30 and 40 min.

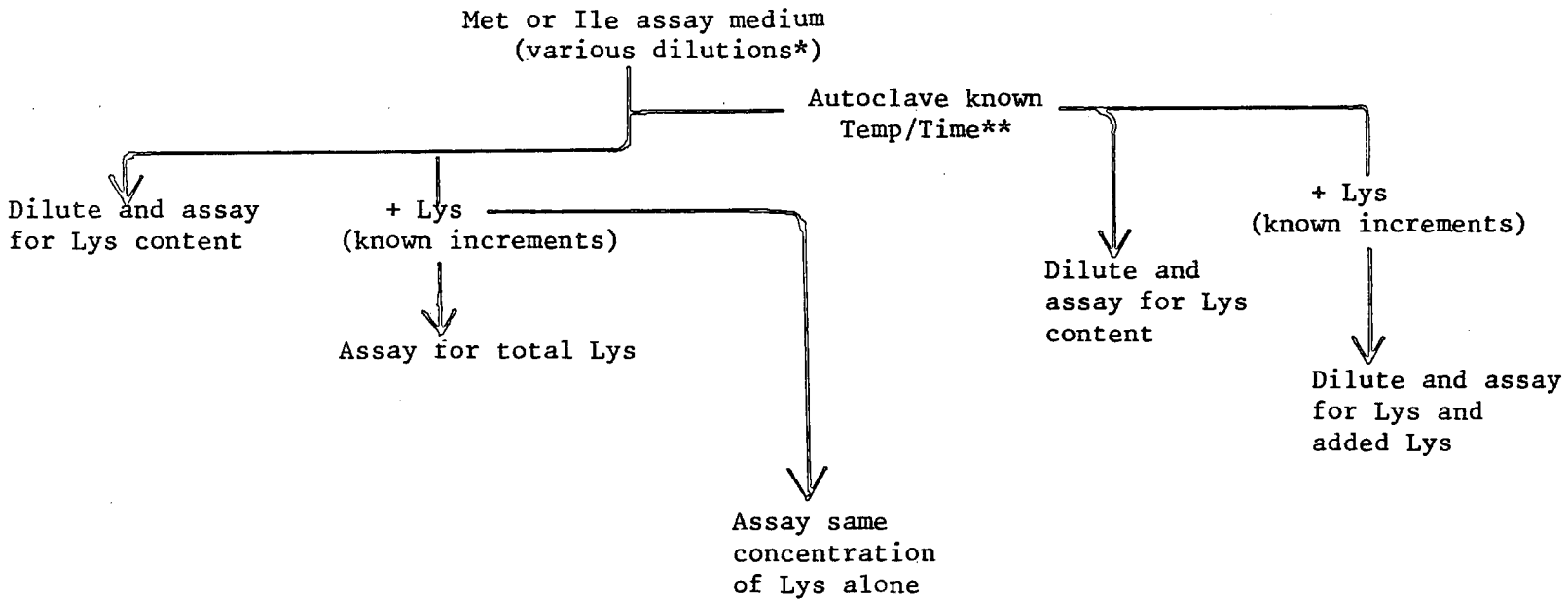
(iii) Heating 1.01 and 0.505 g l^{-1} solutions of assay media at 10 p.s.i. for 10 min. on two successive days.

Each treatment was carried out in duplicate and controls were included in which known amounts of Lys were added to heat-treated samples to check for the possible formation of products that might interfere with the assay. In other controls, various amounts of Lys were also added to the assay media before heat treatment (15 p.s.i., 15 min.).

In all cases the pH of the test solutions were checked before and after autoclaving and were found to be unaffected by the heat treatment. Samples were diluted with 0.1M, pH 7.0 buffer, as necessary prior to assay for Lys by the β-galactosidase method (Section 3-4). The protocol of a typical experiment is given in Fig. 6.7.

Figure 6.7

Typical Scheme For Study Of Heating Effects



* Variables (see Section 6-3-3(b) for details)

**

Samples assayed by β -galactosidase (Section 3-4)

6-3-4 Results and Conclusions

Results of the untreated controls were in agreement with those obtained using Lys assay medium (Section 6-1), and confirmed that the presence of carbohydrates, amino acids, vitamins and various co-factors in the medium did not alter the response to a given amount of Lys. When a 10.1 g l^{-1} dilution of assay media was heated at 15 p.s.i. for 30 minutes it became straw coloured, although when diluted as required for use in the β -galactosidase assay (usually a further $^{1}/_{20}$), it had negligible absorbance at 420 nm and did not, therefore, interfere with the measurement of σ -nitrophenol.

Duplicate samples (with and without various heat treatments), were assayed at several (at least 5), Lys concentrations. Graphs were plotted of volume of sample v E_{420} and by using least squares analyses the gradient (m) and intercept (c) were calculated (See Appendix 1 for worked example). From these the percentage decrease in Lys caused by the various heat treatments could be calculated. A considerable decrease in the Lys-dependent β-galactosidase synthesis occurred as a consequence of these heat treatments. Fig. 6.8 shows that when samples of Met and Ile assay media (both at 10.1 g l^{-1}), were autoclaved at 15 p.s.i. for 15 minutes they suffered a 54% and 53% decrease in available Lys, respectively. The concentration of the mixture affected the degree of Lys loss (Fig. 6.9), thus with 10.1, 1.01 and 0.505 g Met assay medium l^{-1}, autoclaved at 15 p.s.i. for 15 min. the percentage Lys loss was 56, 50 and 37, respectively.

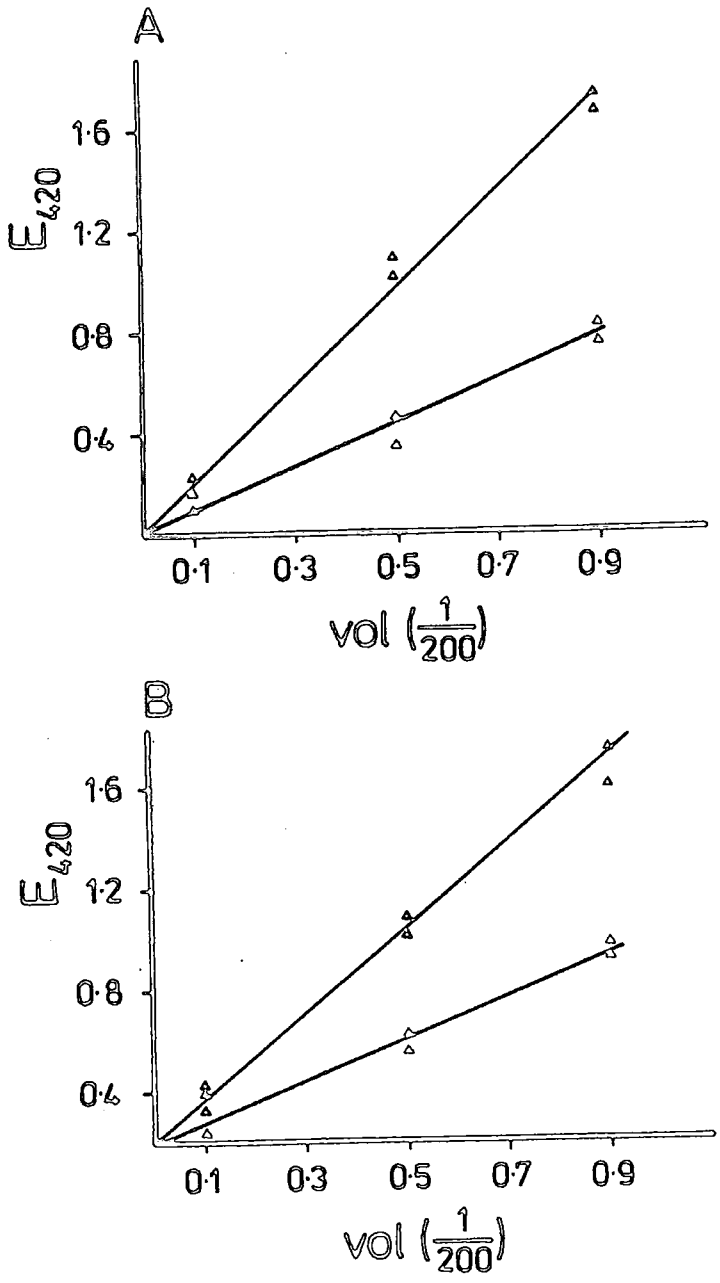
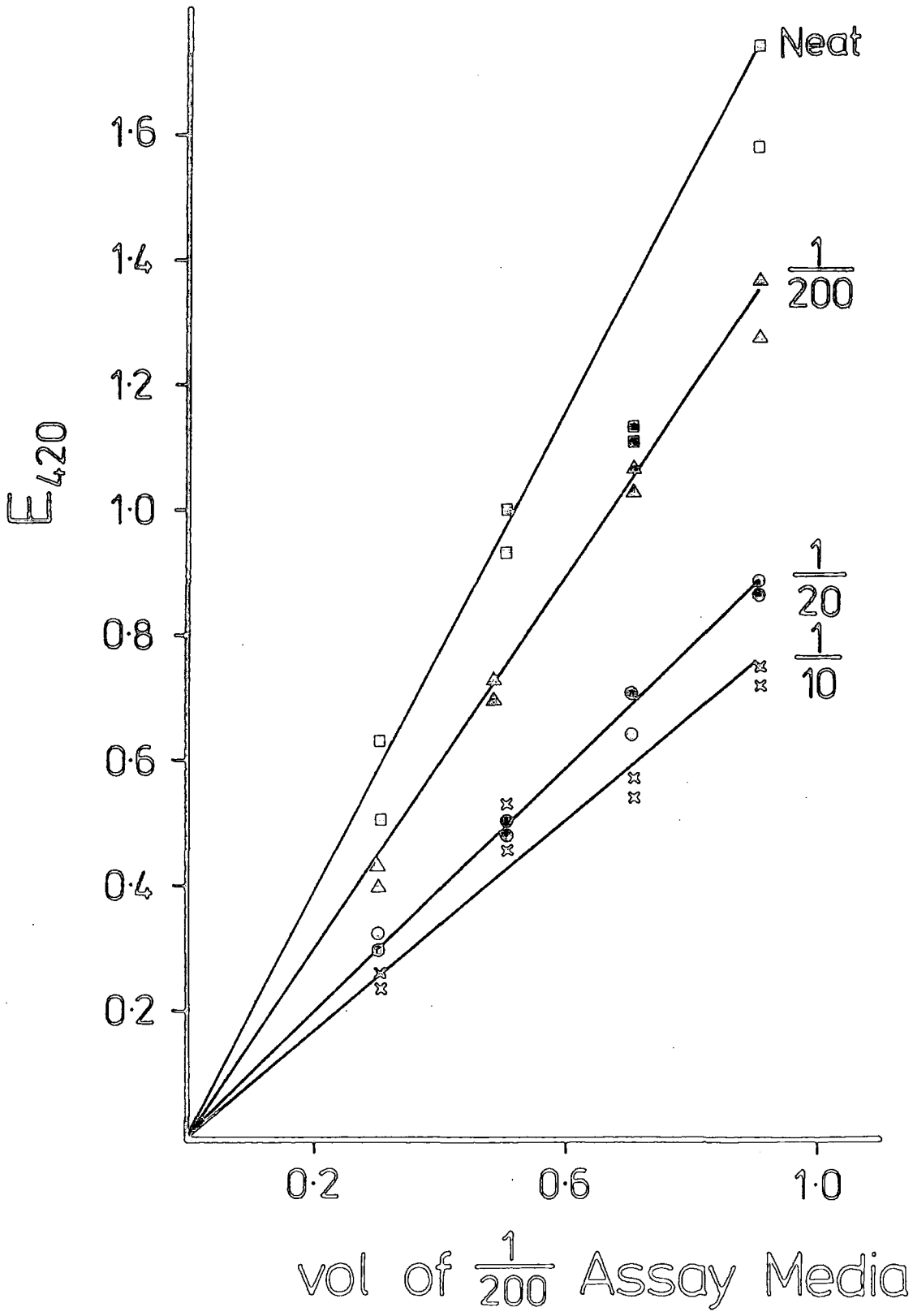


FIGURE 6.8 Effect Of Autoclaving On Lysine In Assay Media.

Met (A) and Ile (B) assay media were assayed at 1/200 dilution (0.0505 g 100 ml^{-1}) before heat treatment △——△. 1/10 dilutions were heated 15 min at 15 psi, cooled, diluted to 1/200 and Lys again assayed△. Graphs show 54% decrease (A) and 53% decrease in Lys (B). Corresponding losses when samples were heated at 1/20 dilution were 51% (Met assay medium) and 49% (Ile assay medium) - data not shown.



FIDURE 6.9 Effect Of Autoclaving At Different Dilutions

Samples of Ile assay media were autoclaved at stated dilutions and diluted to 1/200 for β-galactosidase assay. Lys content of control = 100%, Lys losses : 1/200 = 37%, 1/20 = 50% and 1/10 = 56%. Controls not shown; results are based on mean values from 8 individual assays. For carbohydrate content at each dilution see text.

Finally, it was demonstrated that the degree of damage to Lys depends on the severity of heat treatment. Table 6.3 shows the effect of heating identical samples at 15 p.s.i. for 10 minutes for one or two cycles was a 5.7% and 7.3% drop in Lys respectively; whereas, the percentage Lys decrease after heating at 15 p.s.i. for 30 or 40 minutes was 25.9 and 26.6 respectively.

It appears therefore, that Escherichia coli behaves like other biological systems in that reactions resulting from heating can affect Lys availability. This conclusion is significant in relation to the handling of samples prior to β-galactosidase assay. For example, assay material should not be sterilised by heat. However, one of the advantages of the method described is that it can be completed in a short time and therefore samples do not need to be sterilised prior to assay to prevent bacterial degradation of the material.

Table 6.3

Summary - Effect Of Heat Treatments On Available Lysine Content In Assay Media

Treatment	Percent decrease in available Lys[a]		
	10.1[b]	1.01[b]	0.505[b]
Standard 15 min., 15 p.s.i.	54	51	
	53[c]	49[c]	
	56	50	37
10 min., 10 p.s.i. x 1		5.7	
10 min., 10 p.s.i. x 2		7.3	
30 min., 15 p.s.i. x 1		25.9	
40 min., 15 p.s.i. x 1		26.6	

[a] Each value is the mean of at least four determinations

[b] Concentration of assay medium (g l^{-1}) in heated sample

[c] Ile assay medium all others are Met assay medium

6-4 Response of Auxotrophic Strains to Peptides

6-4-1 Introduction

Before attempting to analyse the enriched materials for Met and Trp it was important to determine the response of the mutants to peptides containing these residues. Therefore, assays were carried out to determine whether peptide-bound residues gave the same enzyme yields as equivalent amounts of the free amino acids and to determine if the assays were similar kinetically.

6-4-2 Purity of Peptides

50 n mol of each of the peptides listed below were dansylated and run on polyamide sheets. This grossly overloaded the plates but since less than 1 n mol can be detected it could be demonstrated that in each case there was less than 2% of any free amino acid contaminant. Peptides tested were Met_2, Met-Ala-Ser, Met-Leu, Met-Leu-Gly, Trp-Leu, Leu-Trp and Ala-Trp. Also Ala_2 and Ala_3 were used in competition studies.

6-4-3 Methionyl Peptides

a) Assay Response

β -galactosidase assay was carried out using the strain PA0111 (Lys^- Met^-), with Met, Met_2, Met-Leu-Gly, Met-Ala-Ser and various concentrations of Met and Met_2 together as substrates. The kinetics of β -galactosidase production for various Met and Met_2 concentrations were also determined. The results show that equivalent enzyme yields are obtained from equivalent concentrations of Met, Met_2 and other methionyl peptides (Figs. 6.10 and 6.11).

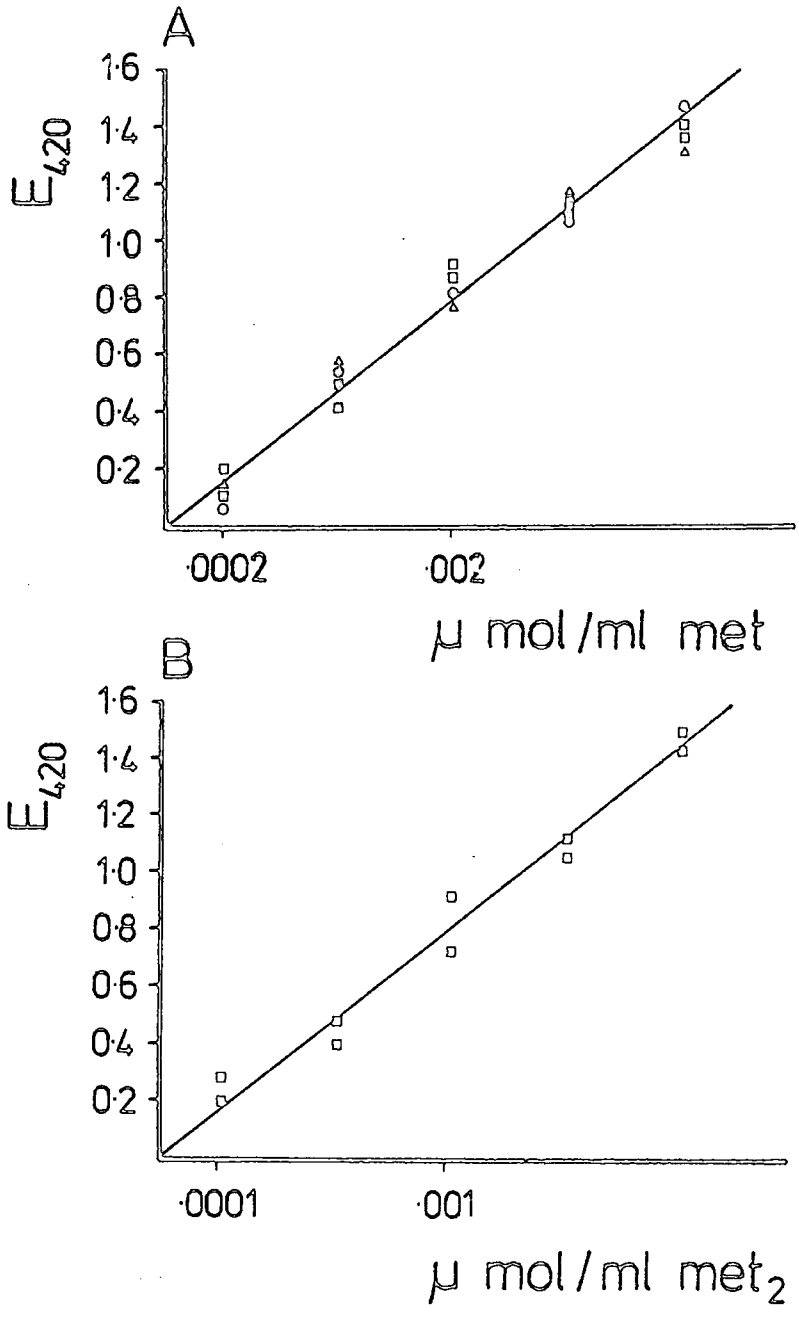


FIGURE 6.10 Assay Response To Methionine and Methionine Peptides.

Response of strain PA0111 ($Lys^{-}Met^{-}$) to :

A Met ○—□ , Met-Leu-Gly △—△ , Met-Leu ○—○ .

B Met_2 ○—□ . Cells were grown to exponential-phase, washed, starved of Met for 90 min and the enzyme was assayed by the normal procedure (section3-4).

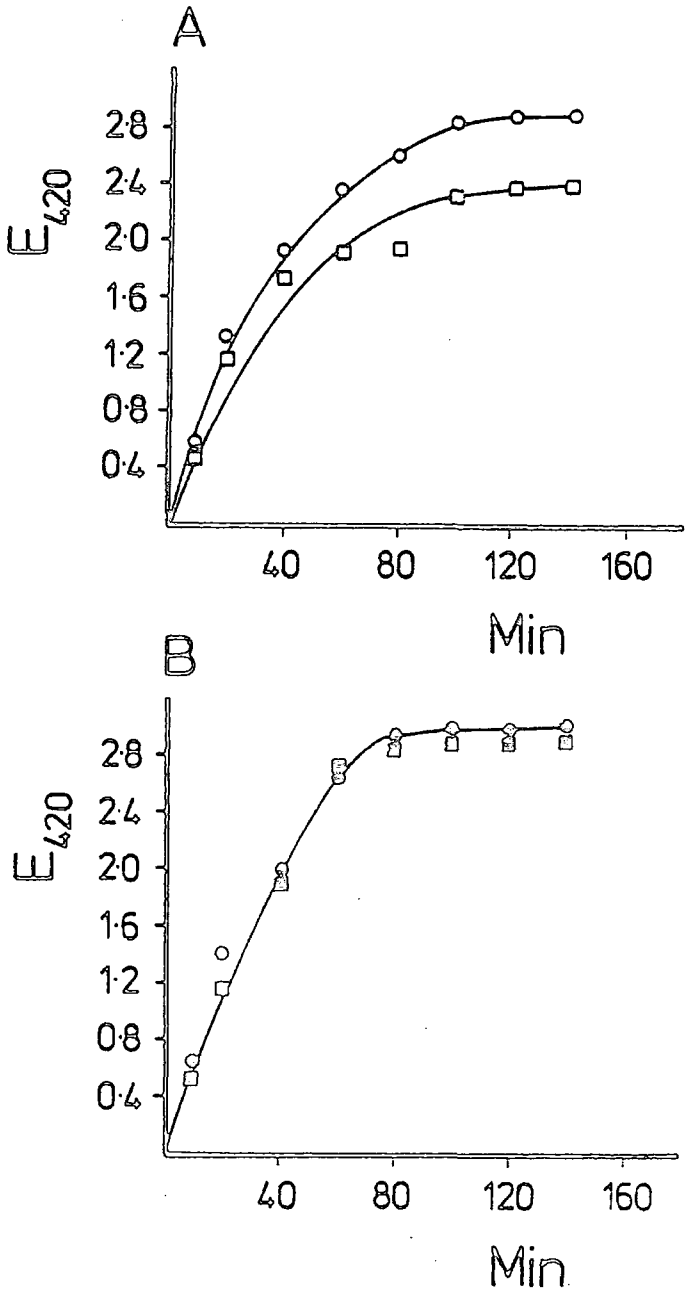


FIGURE 6.11

Kinetics Of β-galactosidase Synthesis By Strain PAO111 With Met and Met Peptides.

E.coli PAO111 was grown, washed, starved for Met as in previous figure. Cells were then incubated in the presence of A : 0.008 mM Met ◎—◎ , 0.08 mM Met-Ala-Ser ▣—▣ ,(this peptide was subsequently found to be contaminated). In figure B ; 0.004 mM Met + 0.002 mM Met_2 □—□ , 0.002 mM Met + 0.003 mM Met_2, ○—○

b) Competition for Uptake (Methionyl-peptides)

In digests, biological fluids, etc, there will be many substances present including peptides and free amino acids. In principle, therefore, it is possible that competition for uptake by peptides lacking the amino acid being assayed could slow down the rate of uptake of peptides containing the essential amino acid and hence increase the time needed for a complete response to say a Met-containing peptide. To establish the relative levels at which this effect might become significant, cells of PA0111 Lys^- Met^- were grown up, starved and induced in the normal way (Section 3-4). Samples were then added to tubes containing a Met source alone or plus excess of a high-affinity peptide (Ala_2 or Ala_3). At various time intervals, duplicate 2 ml samples were removed and β-galactosidase was assayed. Initially, Ala_2 and Ala_3 were each present at a five-fold excess, and neither affected either the rate of enzyme synthesis, or the final yield of enzyme (Fig. 6.12). Subsequently, 25, 50 or 100-fold excesses of either Ala_3 (Fig. 6.13), or Ala_2 (Fig. 6.14 A and B), were used with Met or Met peptides. In both cases a 100-fold excess of the Alanine peptide was necessary to markedly affect enzyme yields.

6-4-4 Tryptophanyl-Peptides Competition for Uptake

Experiments described in the above section with Met peptides were repeated with the Trp-containing peptides Leu-Trp-Ala-Lys, Trp-Leu and Ala-Trp, and the strain PA0110. Calibration curves of enzyme yields obtained with Trp and equal concentrations of these peptides were identical (results not shown). The effect of Ala_2 on the response to Trp-containing dipeptides is shown in Fig. 6.15.

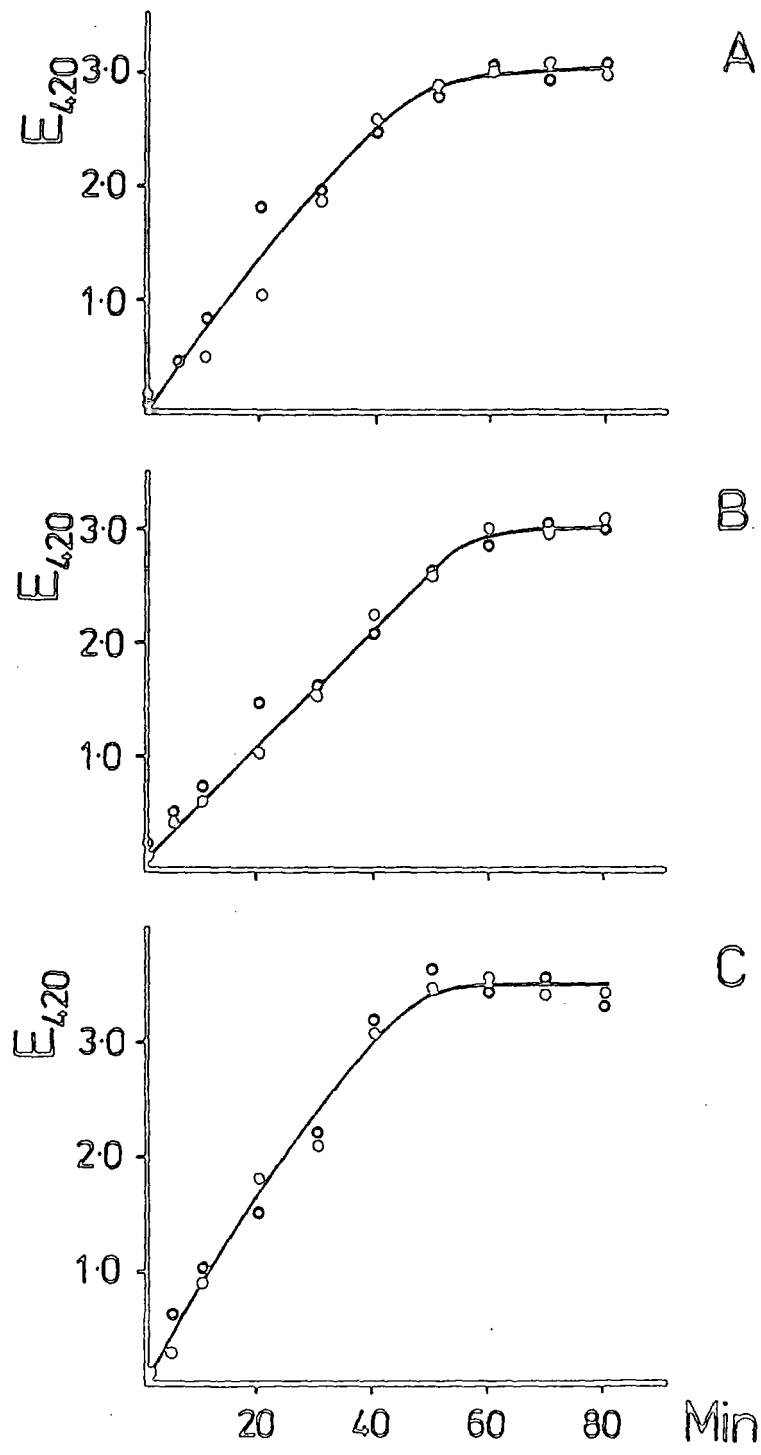


FIGURE 6.12 Competition Of Ala_2 and Ala_3 On Uptake Of Met and Met_2.

A Response of strain PAO111 to Met ○—○ and Met_2 ◎—◎ .

B Response of same concentration of cells to equal concentrations of Met but in the presence of five-fold excesses of Ala_2 ○—○ , or Ala_3 ◎—◎ .

C As B above but with Met_2 and five-fold excess of Ala_2 and Ala_3.

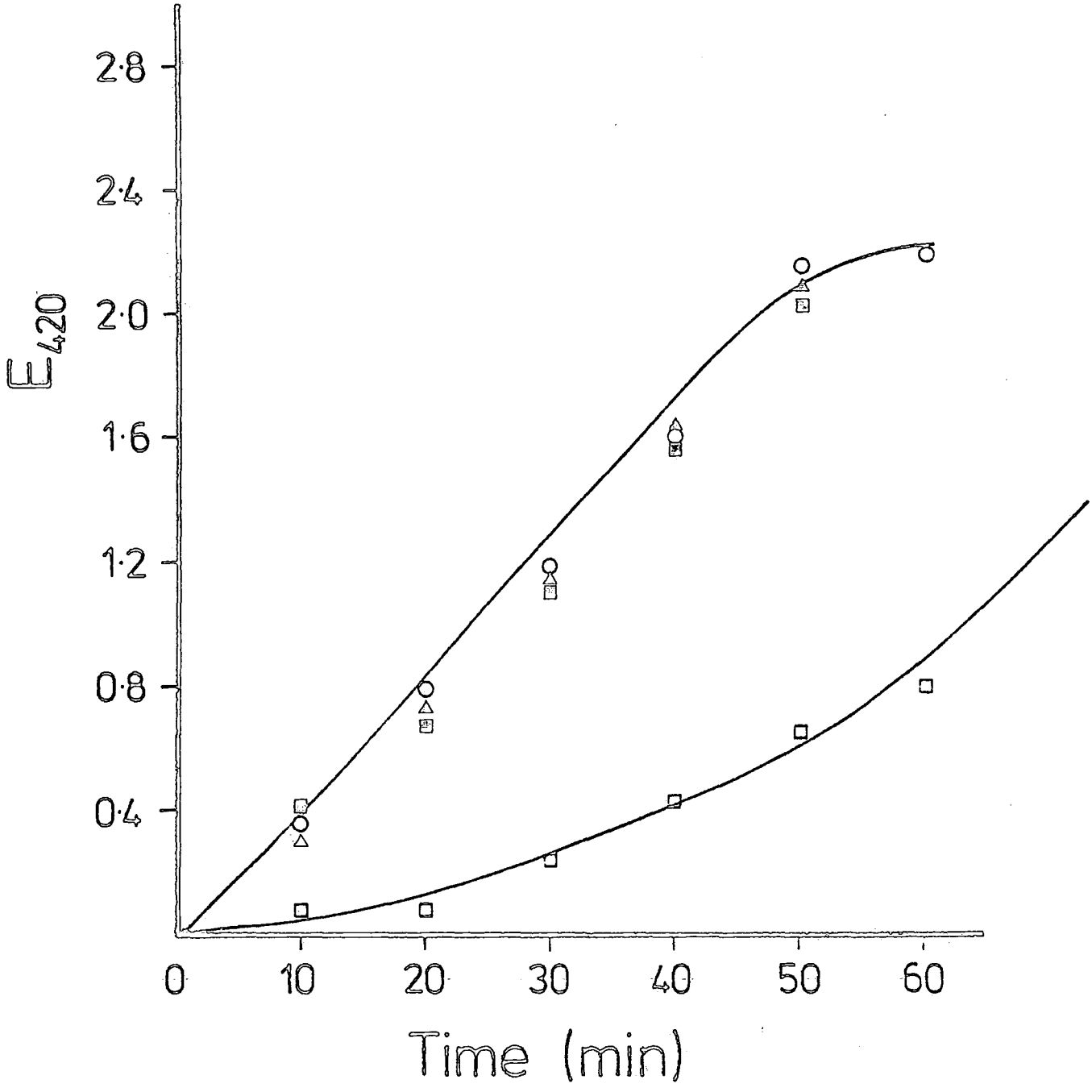


FIGURE 6.13 Effect Of Ala_3 On Assay Of Met In Met-Ala-Ser Using Strain PAO111, Lys^-Met^-.

Met or peptides were added to induced cells (ca. 10^7 ml^{-1}), 25 ml total volume, and incubated at 37°C with shaking (100 strokes min^{-1}). At stated times samples were removed and β-galactosidase assayed by standard procedure (section 3-4). Met ○—○, Met-Ala-Ser △—△, Met-Ala-Ser + 25-fold excess Ala_3 □—□, Met-Ala-Ser + 100-fold excess Ala_3 □—□.

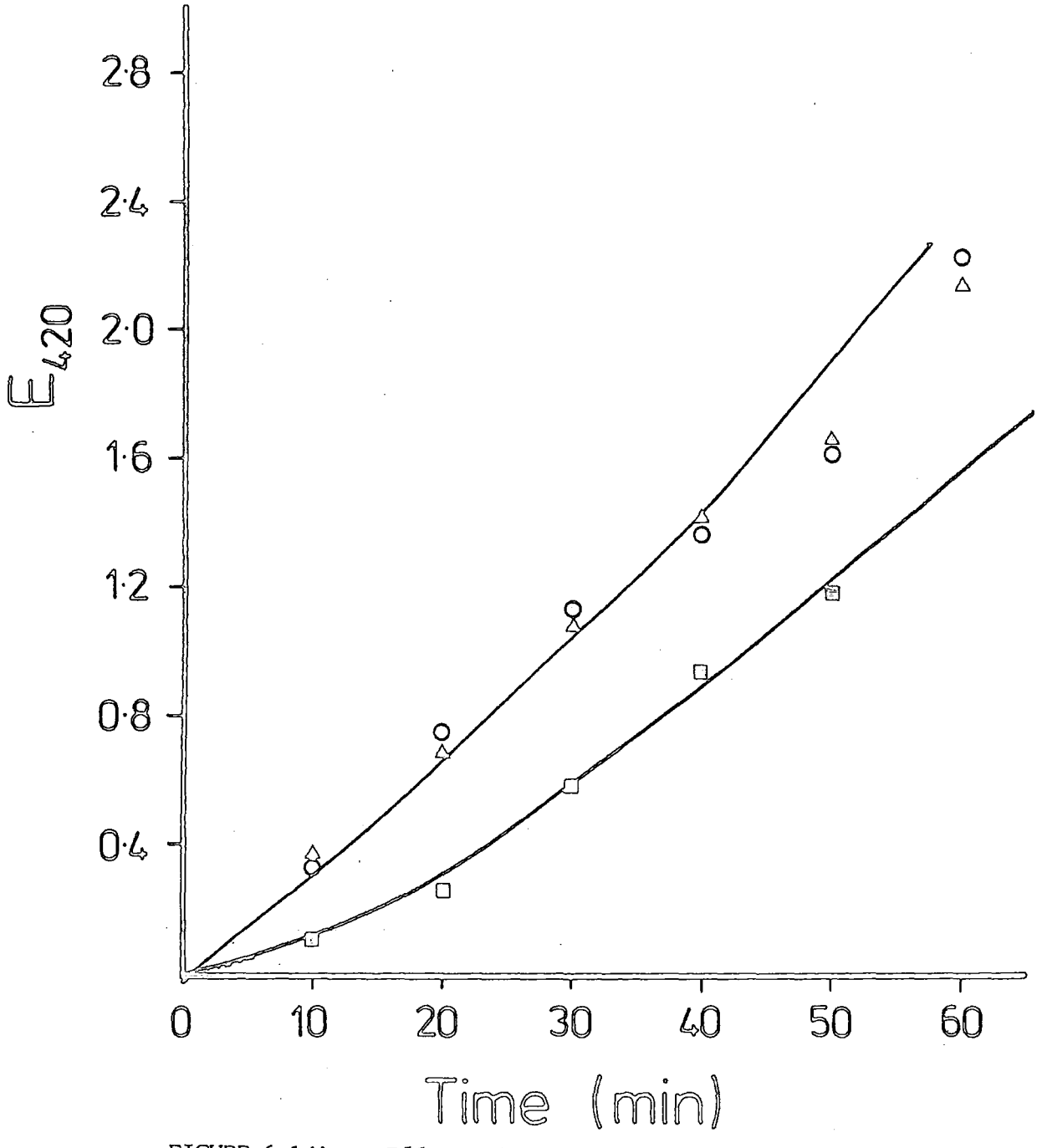


FIGURE 6.14A Effect Of Ala_2 On Assay For Met In Met_2 Using Strain PA0111, Lys^-Met^-.

Met_2 alone or together with Ala_2 was added to induced cells, ca. 10^7 ml^{-1}, 25 ml total volume and incubated at 37°C with gentle shaking. At stated times samples were removed and β-galactosidase was assayed by the normal method (section 3-4). Met_2 ○—○ , Met_2 + 10 -fold, excess Ala_2 △—△ , Met_2 + 100-fold excess Ala_2 □—□ .

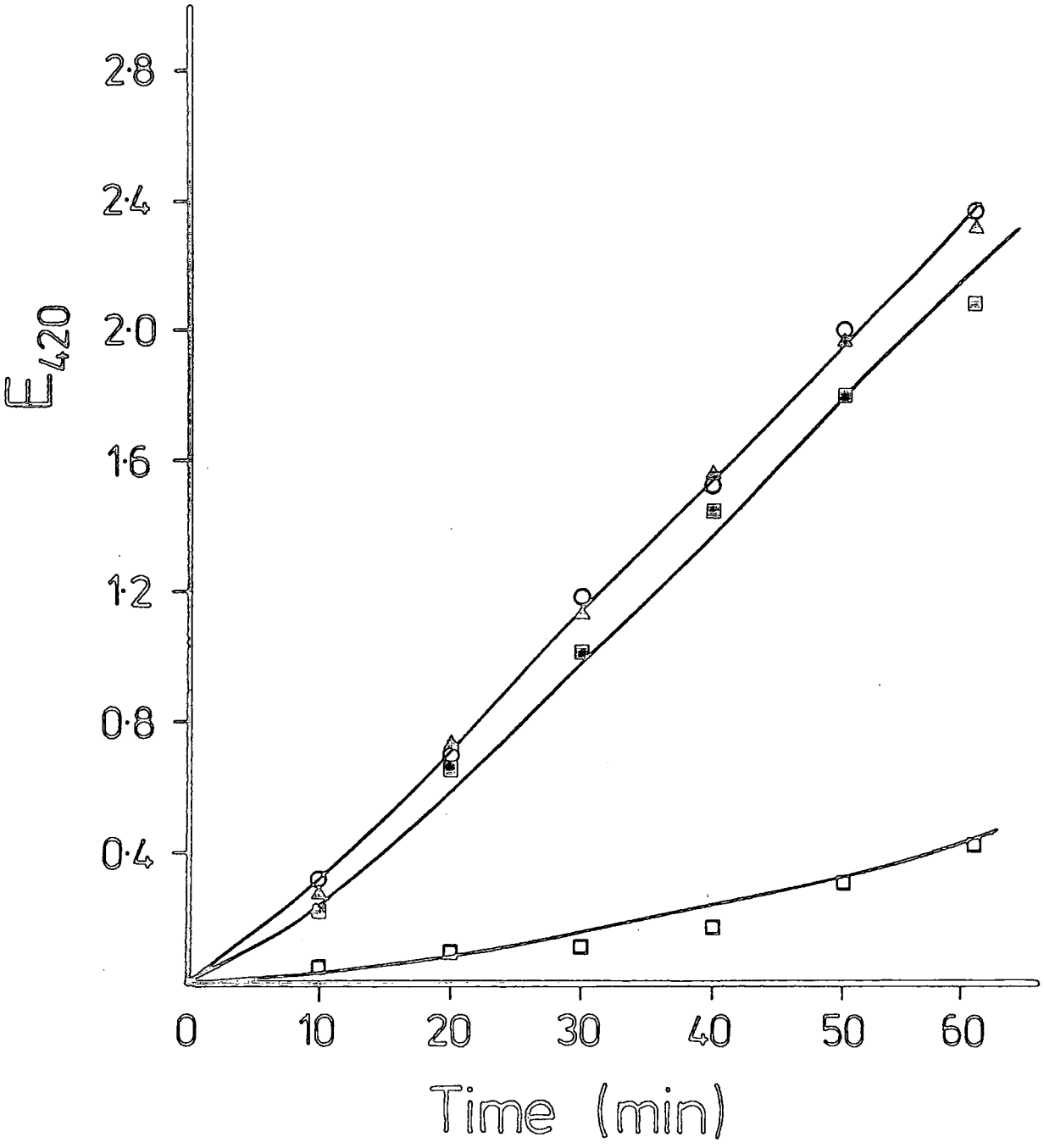


FIGURE 3.14B Effect Of Ala_2 On Assay For Met In Met-Gly Using Strain PAO111 Lys^-Met^-

Peptides were added to flasks containing induced cells (ca. 10^7 ml^{-1}), 25 ml total volume and incubated at 37°C with gentle shaking. At stated times samples were removed and assayed for β-galactosidase by standard method (section3-4). Met-Gly ○—○ , Met-Gly + 10-fold excess Ala_2 △—△ , Met-Gly + 50-fold excess Ala_2 □—□ , Met-Gly + 100-fold excess Ala_2, ▣—▣.

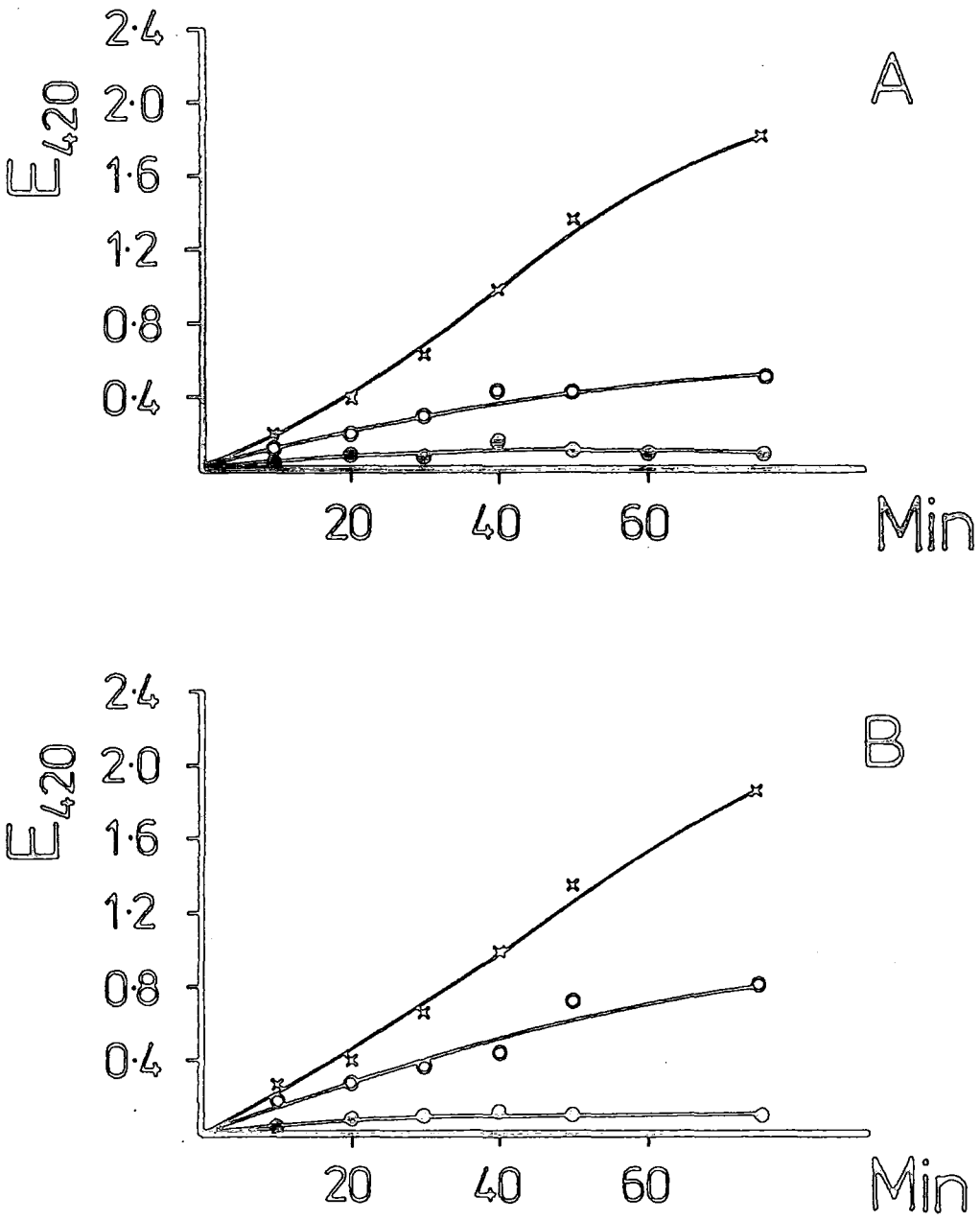


FIGURE 6.15 Effect Of Ala_2On Assay For Trp In Trp-Leu and Ala-Trp Using Strain PA0110, Lys^-Trp^-.

Peptides were added to flasks containing induced cells (ca.10^7 ml^{-1}), 25 ml total volume and incubated at 37°C with gentle shaking. At stated times samples were removed and assayed for β-galactosidase by standard method (section 3-4). A Trp-Leu ✕—✕ , Trp-Leu + 100-fold excess Ala_2 ○—○ , Trp-Leu + 500-fold excess Ala_2 ○—○ .
B Ala-Trp ✕—✕ , Ala-Trp + 100-fold excess Ala_2 ○—○ , Ala-Trp + 500-fold excess Ala_2 ○—○ .

Although there was only slight competition between Met_2 and a 100-fold excess of Ala_2 this concentration showed a greater inhibition with Trp dipeptides reflecting the varied affinities of the peptides in question.

6-4-5 General Conclusions

These results show that an excess of at least 25-fold, and typically 100-fold, of high affinity peptides is required to cause competition for uptake and hence any detectable effect on the yield of β-galactosidase. Comparable situations are unlikely to occur in digests and significant underestimates arising from competition are therefore thought not likely to be a problem. In this regard it should be noted that one amino acid will statistically occur in 10% of all dipeptides, 14% of all tripeptides and 17% of all tetrapeptides.

6-5 β-Galactosidase Assay For Available Lysine Methionine And Tryptophan In Proteins Of Known Composition

6-5-1 Introduction

As shown earlier, several auxotrophic mutants were isolated and their response to their required amino acids (Lys, Met or Trp), in both free form and peptide-bound was determined for growth and enzyme synthesis. In this section, their responses to these protein-bound residues are examined and they are used to measure the availability of these amino acids in digests of pure proteins of known compositions.

6-5-2 Materials

The proteins and digestive enzymes supplied by Sigma (London) Ltd., were as described in Section 2-8-2.

6-5-3 Methods

The proteins were digested as described in Section 2-8-3. Their moisture contents were determined prior to digestion and samples were weighed to contain exactly 1 mg ml^{-1} (dry weight), of protein. In preliminary experiments either a single enzyme (0.1 mg ml^{-1} final concentration), was used or the digestion time was varied (Figs. 6.16 and 6.17).

Having established the optimum digestion period of 15 hours, three separate digests were made for each protein using the standard digestion procedure (Section 2-8-3), and diluted as appropriate (10 or 100 times), into 0.05M potassium phosphate buffer, pH 7.0. Each of these was assayed immediately for available Lys using the standard method (Section 3-4), over a range of five concentrations.

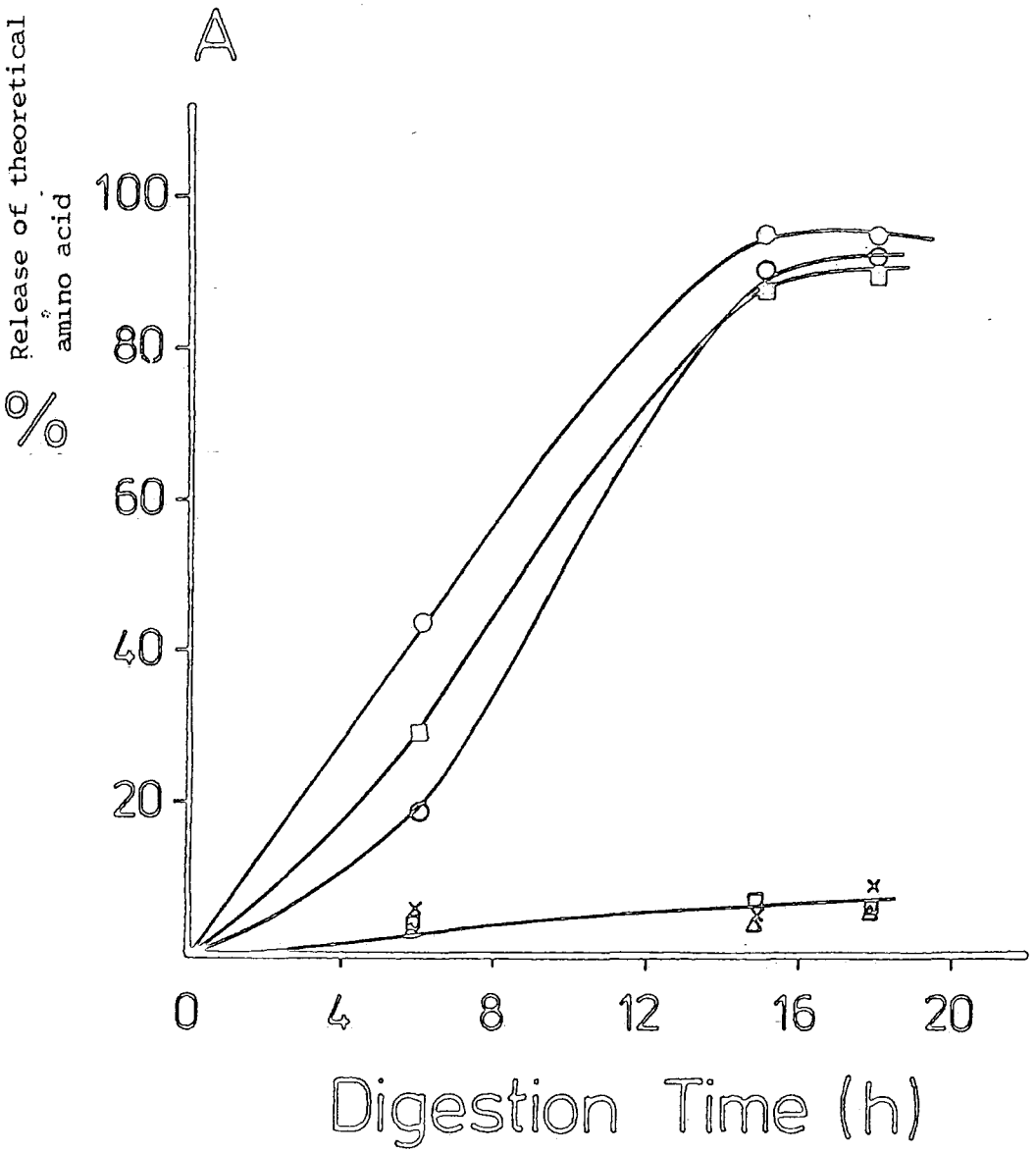


FIGURE 6.16 Progress Of Digestion Of α-chymotrypsinogen A Type II (Sigma).

Percentage of each amino acid determined from known composition after various digestion periods. 1.0 mg ml^{-1} protein, 0.05 mg ml^{-1} pronase and 0.05 mg ml^{-1} peptidase. See appendix 4 for method of calculation of results. These digests are nos. 1 & 4 in Table 6.5. Digestion procedure as described in section 2-8-3. Lys ○—○ , Met □—□ , Trp ◎—◎ . 100% = 14 residues Lys, 2 residues Met and 8 residues Trp (all expressed in terms of residues / molecule. Autolysis: Lys ☆—☆ , Met ▯—▯ Trp △—△ .

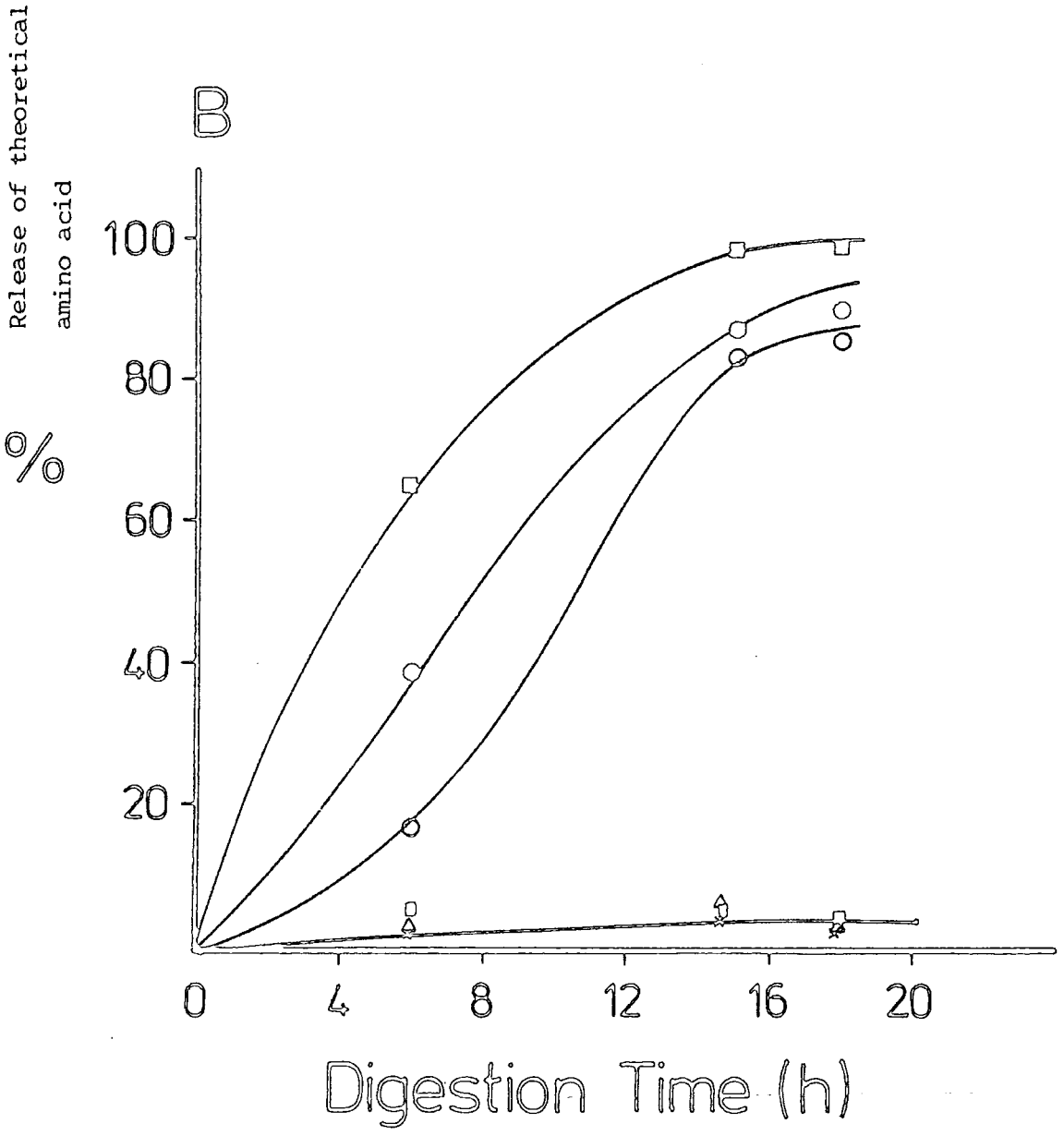


FIGURE 6.17 Progress Of Digestion Of Whale Myoglobin Type II

Percent of each amino acid determined from known composition after various digestion periods. See appendix 4 for calculation of results. Protein and enzyme concentrations and method of digestion as described in Fig 6.16 These values are digests A & D in Table 6.5. 100% = 19 Lys residues, 2 Met residues and 2 Trp residues (all / molecule). Lys, Lys-autolysis ; Met , Met-autolysis ; Trp , Trp - autolysis .

Controls, as described in Section 2-8-3, were included with every assay. Residual digests were stored at -20°C for up to 7 days until separately diluted for Met/Trp assays. Autolysis blanks gave a low response ($<$1% final total value), up to the standard 15 hours digestion time and were excluded from the calculations (Section 6-5-5). E_{420} values were plotted against weight of protein and statistically evaluated to establish the best line and to determine the correlation coefficient between the points. If the correlation (for 5 pairs of points), was less than 0.9 the data were rejected (in a total of 42 assays on pure proteins one set of data was rejected on this basis), this was an assay for Trp with a 6 hour digestion period.

Two examples showing detailed working for two sets of data are given in Appendix 3 and 4. These examples are indicated by * and ** on Table 6.5. A graphical representation of these data is also given. Table 6.4 gives the amino acid composition, from sequence data, of the proteins used.

6-5-4 Results

In the summary of results (Table 6.5), the figures are percentage amino acid determined. Both proteins were used at a concentration of 1 mg ml^{-1} and digested with 0.05 mg ml^{-1} of each enzyme (or 0.1 mg ml^{-1} where pronase alone was used). Data for other enzyme concentrations or other digestion times which were tried before the standard method was established are not included. In a preliminary experiment (Figs.6.16 and 6.17), it was shown that overnight digestion (15 - 18 hours), is satisfactory.

Table 6.4

Protein Structure Data

Protein	Amino Acid residues	Mol. wt.	Lys	Met	Trp
[a] Chymotrypsinogen A (Bovine)	245	25,105	14	2	8
[b] Myoglobin (Whale)	153	17,816	19	2	2

a Data from Walsh & Neurath (1964); Croft (1980)

b Data from Fasman (1976)

Table 6.5

Amino Acid Composition Of Protein Digests -

(Percentage of Theoretical Amino Acid Content Determined By

Beta-galactosidase Assay

Protein	Digestion	Digest No	Lys	Met	Trp
CHYMOTRYPSINOGEN	Pronase and peptidase 6 hour	1	28.6	44.8	19.3
		2	39.2	42.9	17.6
	Pronase 15 hours	3	29.4	59.1	37.5
	Pronase and Peptidase 15 hours	4	88.1	95.0	90.1
		5	88.8	87.7	90.1
			89.8	90.7	92.0
		6	86.0	86.9	78.5
	Mean of 4,5 &6		88.18	90.08	86.03
MYOGLOBIN	Pronase and Peptidase 6 hours	A	65.2	39.9	17.8
		B	42.9	20.7	--
	Pronase 15 hour	C	78.1	63.1	43.1
	Pronase and Peptidase 15 hours	D	98.6	87.5	85.2
		E	94.7*	78.3**	74.3
			96.9	82.3	75.3
		F	93.1	82.3	78.5
	Mean of D,E & F		95.8	82.6	78.3

Results expressed as % of calculated value based on amino acid composition

*, ** :see appendix 3,4,for calculation of these results. Graphical data shown in Fig 6.18 and 6.19.

-- Data not used ($r \leqslant 0.9$).

Digests 1 and 4 are two digests incubated 6 and 15h used to plot data in Fig 6.16 and A and D are same digests used to plot Fig 6.17.

6-5-5 Discussion and Analysis of Results

a) Autolysis Blanks

Although autolysis controls were assayed for Lys, Met and Trp the results were low up to the standard 15 hours incubation but they contributed more to the final enzyme yield when incubation periods were prolonged to 22 hours (data not shown). However, it is probable that in these controls, in which the enzymes were incubated alone, the contribution to test digestions from autolysis may actually be overestimated because autolysis is likely to be greater for enzyme blanks than when the enzyme is incubated together with the protein substrate. In theory, in the protein digests, where enzyme:substrate ratios were 1 : 10 if autolysis and substrate digestion occur at the same rate the initial enzyme autolysis in the enzyme mix could only be a maximum of 10% of the substrate digestion.

If it is assumed the average molecular weight of an amino acid in solution is 120

$$0.1 \text{ mg ml enzyme} = \frac{0.1}{120} \times 10^3 \ \mu \text{ mol ml}^{-1}$$

$$= 0.83 \ \mu \text{ mol ml}^{-1} \text{ each amino acid}$$

If each enzyme contains 5% Lys, 5% Met and 5% Trp then their concentration will each be

$$0.83 \times \frac{5}{100} = 0.042 \ \mu \text{ mol ml}^{-1}$$

Clearly 0.042 μ mol ml^{-1} enzyme-derived amino acid which would be released from complete autolysis would be insignificant compared to the 1 mg ml^{-1} initial protein concentration of the digest.

b) Protein Digests

The results (Table 6.5), show the percentages of Lys, Met and Trp determined when Chymotrypsinagen A and Myoglobin are digested at 1.0 mg ml^{-1} with various combinations of pronase and peptidase for different times, at 37°C. Taking the 15 hour digest with both enzymes the mean percent determinations for Chymotrypsinogen A were 88.18, 90.08 and 86.03 and for whale myoglobin 95.8, 82.6 and 78.3 for Lys, Met and Trp respectively.

Now from Table 6.4, 1 Mole (25,105 g), of Chymotrypsinogen A will contain 14 Moles Lys, 2 Moles Met and 8 Moles Trp. Therefore, using the percent determinations above the number of residues that would be detected would be:

$$\frac{88.18}{100} \times 14 = 12.34 \text{ for Lys} \quad (14)$$

$$\frac{90.08}{100} \times 2 = 1.80 \text{ for Met} \quad (2)$$

and $$\frac{86.03}{100} \times 8 = 6.88 \text{ for Trp} \quad (8)$$

Where number in brackets represents actual number of residues.

Similarly, in 1 Mole Myoglobin there are 19 Moles Lys and 2 Moles each of Met and Trp; from the percentage determination the number of each of these residues that would be determined would be 18.3, 1.65 and 1.57 respectively.

Possible sources of error could include amino acids that were not available after the digestion. The contribution due to autolysis has been ignored but this has already been discussed and the assumption that it is negligible has been defended.

Reduction in amino acid content in the test sample due to degradation by contaminants during digestion has also been ignored; the enzymes themselves could also be affected by this. Finally, the calculations assume that the proteins are 100% pure w/w. They could be contaminated by salts or other compounds. It is perhaps significant in this respect that all of the assay values determined were low.

Graphical representation of the assay of Lys in a myoglobin digest is given in Figure 6.18 and the assay for Met in the same digest is shown in Figure 6.19.

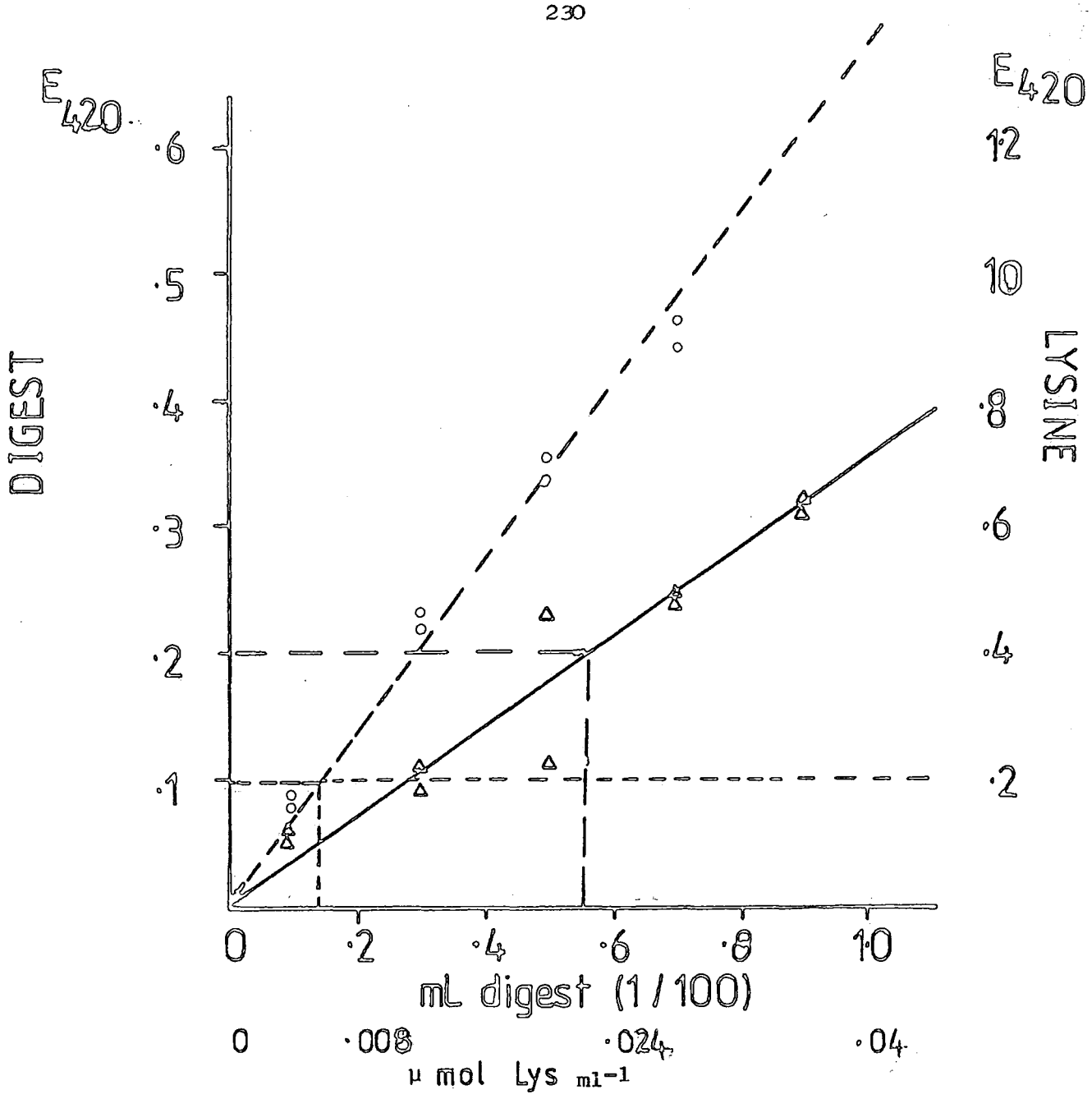


FIGURE 6.18 Assay of Lysine In Myoglobin Digest

Result of assay of Lys in myoglobin after digestion as described in text. On x axis weight of protein taken = 2, 4, 6, 8 and 10 μg. For digest △——△ , (y axis on l.h.s)

Intercept = 0.0213
Slope = 0.3294
r_2 = 0.9412
R^2 = 88.595
where x = 1.0, y=0.3507

For Lys cal. curve (y axis on r.h.s) ○- - -○

Intercept = 0.0025
Slpoe = 1.485
r_2 = 0.985
R^2 =97.023
where x = 1.0, y=1.48

Digest E in Table 6.5

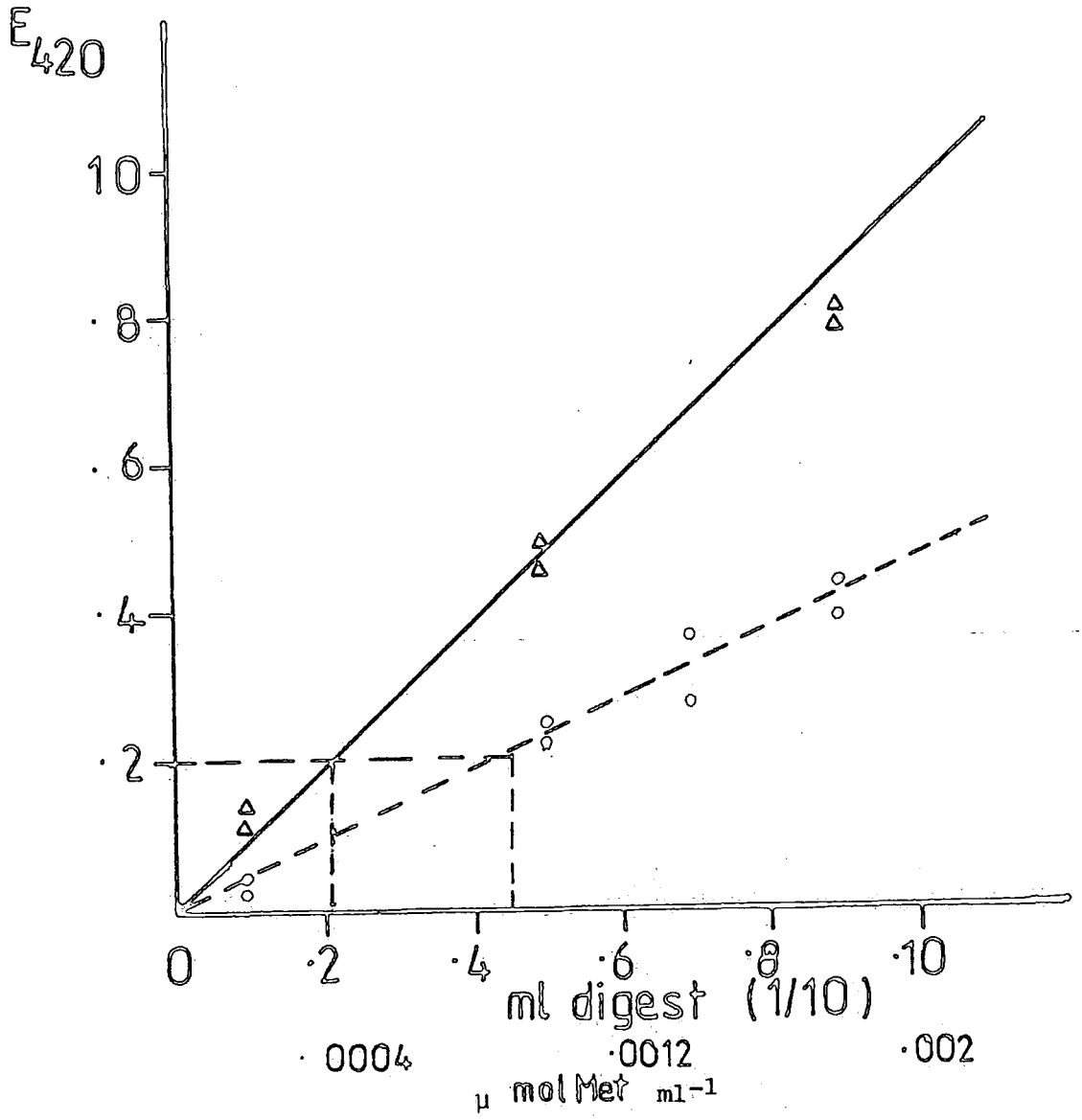


FIGURE 6.19 Assay Of Methionine In Myoglobin Digest

Volumes on x axis represent 20, 40, 60, 80 and 100μ g protein assayed. Experimental procedure as in Fig 6.18. Enzyme assayed by method described in section3-4 using strain PAO111, Lys^-, Met^-.

For digest △——△ , Intercept = -0.1116
Slope = 0.9256
r_2 = 0.9865
R^2 =97.25
where x = 1.0, y = 0.9145

For Met Cal Curve ○- -○ , Intercept = -0.045
Slope = 0.5493
r_2 = 0.9770
R^2 = 95.5
where x = 1.0, y = 0.45

Digest E in Table 6.5

6-6 Analysis of High Protein Feed Meals

6-6-1 Introduction

In 1955 the ARC formed a Protein Quality Group to undertake collaborative work on the development of laboratory procedures for the assessment of protein quality in animal feeding stuffs. By 1959 it was apparent that of the methods chosen, microbiological methods had certain unique advantages (Section 1-5). A Microbiological Panel was formed in 1959 and part of their programme was to assess the procedures on a collaborative basis. The first publication of the ARC Protein Evaluation Group was in 1961 and much data has been published since then.

Three panels: Biological, Microbiological and General Analytical were set up. The microbiological panel concentrating its efforts on measurement of availability of amino acids such as Lys, Met and Trp. Initial results showed significant correlations with previously noted values between microbiological responses to protein quality and published values for Lys and Met obtained with chick and rat bioassay (Boyne et al., 1967). Particular problems, however, were reported with results from Trp assays.

Three of the meals that have been extensively analysed in ARC collaborative trials were used here. These were GN101 (ground nut meal, solvent - extracted in the U.K.), SB101 (soya bean meal - grown in China, extracted in the U.K.) and FM101 (a white fish meal of U.K. origin), and were kindly supplied by Dr. A. Williams (NIRD).

In assessment of nutritional value three problems are paramount:

(i) Reproducibility of results - when same sample is assayed on different occasions

(ii) Reproducibility of results when same sample is assayed by different laboratories

(iii) Relevance of experimental results obtained to actual values of the samples, in practice.

The results obtained using the Escherichia coli assay described herein for Lys, Met and Trp, was evaluated with the aim of establishing the validity of (i) and (iii). To achieve this, the results obtained are compared with published data; the main source of which was Carpenter & Woodham, 1974.

6-6-2 Methods

The procedure for digesting the meals has been described (Section 2-8), and the assay has been detailed in Section 3-4.

For each of the protein-rich meals three separate digests were made using fresh enzyme solutions for each. After digestion, one sample was immediately taken, diluted as necessary for assay (usually $^1/_{10}$ or $^1/_{20}$ and the remaining digest was divided into aliquots, frozen at -20°C for subsequent assay as required. Each meal was assayed for each of the three amino acids of interest several times. For assaying Lys the assay organisms were varied in some cases for example digest 1 was assayed with M2626, digest 2 with PA0111 Lys^- Met^- and 3 with PA0110 Lys^- Trp^-. However, Met was assayed with the Met auxotroph PA0111 and Trp with the Trp auxotroph, PA0110 throughout.

For each single determination at least 3 duplicate concentrations of the sample were assayed; An amino acid calibration curve, and controls for autolysis and inhibition were also included. Autolysis controls at no time contributed to more than 1% of the total response. The addition of known amounts of test amino acid to the digest indicated that in no instance were inhibitors present.

For each assay E_{420} values were measured and corrected for blanks (endogenous amino acid), (Section 3-5-2). Data ~~was~~ were then dealt with in two ways.

a) Values obtained from amino acid calibration curves and for digests (E_{420} v. conc), were plotted and the best lines between the various sets of duplicate points were drawn by eye. Hence, from the lines obtained, the amino acid concentration in a given volume of digest could be calculated (see Appendix 5 for typical graphs and calculation).

b) The same data used in (a) ~~was~~ were taken and plotted and a least squares regression analysis used to determine the best fit of the line joining the points (see Appendix 6 for program used and results obtained with a representative set of data).

Final results were expressed as g / Kg crude protein to facilitate comparison with the values of protein content quoted by Carpenter & Woodham, 1974. These values assume 900 g / Kg dry matter (i.e. 10% moisture), they were therefore corrected for the true percentage moisture determined experimentally. (Section 2-8-3).

6-6-3 Results and Discussion

a) Lysine

Values of Lys in each of the three meals as determined by using the Escherichia coli assay are given in Table 6.6. Values for GN101 were between those obtained with the rat and the chick, for SB101 and FM101 lower values were obtained than quoted for chick bioassay. There is a tendency (Carpenter & Woodham, 1974), for samples of lower potency (e.g. SB101), to give relatively higher values by chemical tests than with chicks.

Table 6.6

Results For Lys Assay Of High-Protein Feed Meals

Sample	* Crude Protein N x 6.25	Crude Protein Dry Weight	% H_2O (a)	* Chem	* Available T	R	C	Digest Results g Kg^{-1} 1	2	3
GN101	503	558	5.99	34	-	39	25	28.60 28.19	28.19 29.02	33.92 33.75 ** 31.67
									σ = 2.84 m = 30.48 v = 8.08	
SB101	475	528	482	59	-	-	54	-	49.86	53.32
									σ = 2.45 m = 51.59 v = 5.99	
FM101	708	786	3.8	62	54	71	60	-	49.76 49.69	51.28
									σ = 0.898 m = 50.24 v = 0.807	

Comparison of Lys contents g Kg^{-1} - calculated using method (a), (points joined by eye), and literature values.

(a) Determined experimentally (As for rice - Sections 2-8-3 and 6-7-3).

** This is given as worked example in Appendix 5.

Table 6.6 (cont.)

- Not determined
T = Tetrahymena Assay
R = Rat Bioassay
C = Chick Bioassay
* Data from Carpenter & Woodham, 1974
Chem = Chemical value - acid hydrolysis and autoanalyser
σ = Standard Deviation
m = Mean
v = Variance

Since the published data and the experimentally derived data were based on different moisture contents, a comparison of chemical total Lys values at 10% water and the measured moisture content are given in Table 6.7. It should be noted that the 10% values for moisture content are assumed rather than measured.

Two methods were used to calculate results from the test data and a direct comparison of these is given in Table 6.8. For GN101 and SB101 the best fit line gives rise to lower final values than joining the points by eye. One problem with determining the best fit line is that it may not necessarily pass through the origin - although clearly there should be no response if there is no amino acid present and after endogenous values have been corrected. Therefore, when the best fit line did not pass through the origin, a line was drawn parallel to it such that this required condition was fulfilled. Since the values of the intercept of the best fit line were small (but always positive), moving the line could have caused this decrease in response.

Table 6.9 shows the percentage of Lys available in each of the meals. The values are calculated in relation to the total Lys which was determined after acid hydrolysis (100%). It is of interest here that the percentage availability values are very similar for the Lys in all three meals when calculated from the best-fit lines but there is a greater variation when the lines are drawn by eye.

However, with the limited data for available Lys (only chick results known for all three meals), it is difficult to draw any firm conclusions. A graphical comparison is given in Fig. 6.20.

Table 6.7

Comparison Of Chemical Assay For Lys And *Escherichia coli* Assay Values On Same % Moisture Basis

Sample	Chemical Total Lys[a]	Total Lys[b]	Mean Value From *Escherichia coli* Assay (Table 6.6)
GN101	34	35.52	30.48
SB101	59	62.40	51.59
FM101	62	66.27	50.24

[a] Determined by acid hydrolysis - assumes 10% moisture content

[b] Based on measured moisture content

Table 6.8

Lysine Contents - Comparison Of Results Derived From Lines Joined By Eye[a] And By Least Squares Analysis[b]

	Digest 1		2		3	
Sample	a	b	a	b	a	b
GN101	28.60	27.95	28.19	28.15	33.92	32.68
	28.19	27.46	29.02	28.51	33.75	33.70
					31.67*	31.96*
SB101	-	-	49.86	47.78	53.32	51.15
FM101	-	-	49.76	49.81	51.28	52.36
			49.69	53.54		

Mean Values g Kg^{-1}

	Method (a)	Method (b)
GN101	30.48 (90.5, 123.1)	30.05 (88.4, 120.2)
SB101	51.59 (87.4, 95.54)	49.47 (83.8, 91.61)
FM101	50.24 (81.03, 119.4)	53.58 (86.41, 99.22)

First figures in parentheses represent percentage availability based on published results from chemical analysis; second figures give comparison with published chick bioassay results.

* Worked example see Appendix 5.

Table 6.9

Percentage Availability Calculated From Lys Determined By Escherichia Coli Assay* Related To Chemical Values

Sample	Chemical Score	Digest 1 a	Digest 1 b	Digest 2 a	Digest 2 b	Digest 3 a	Digest 3 b
GN101	34	84.1	82.2	82.9	82.79	99.76	96.12
		82.9	80.76	85.35	83.58	99.26	96.18
						*93.15	*94.0
SB101	59	-	-	84.50	80.47	90.34	86.69
FM101	62	-	-	80.25	80.34	82.71	84.45
				80.15	86.35		

Mean Values (% Availability)

	Method (a)	Method (b)
GN101	89.63	88.4
SB101	87.42	83.58
FM101	81.04	83.71

* Standard Procedure Section 3-4

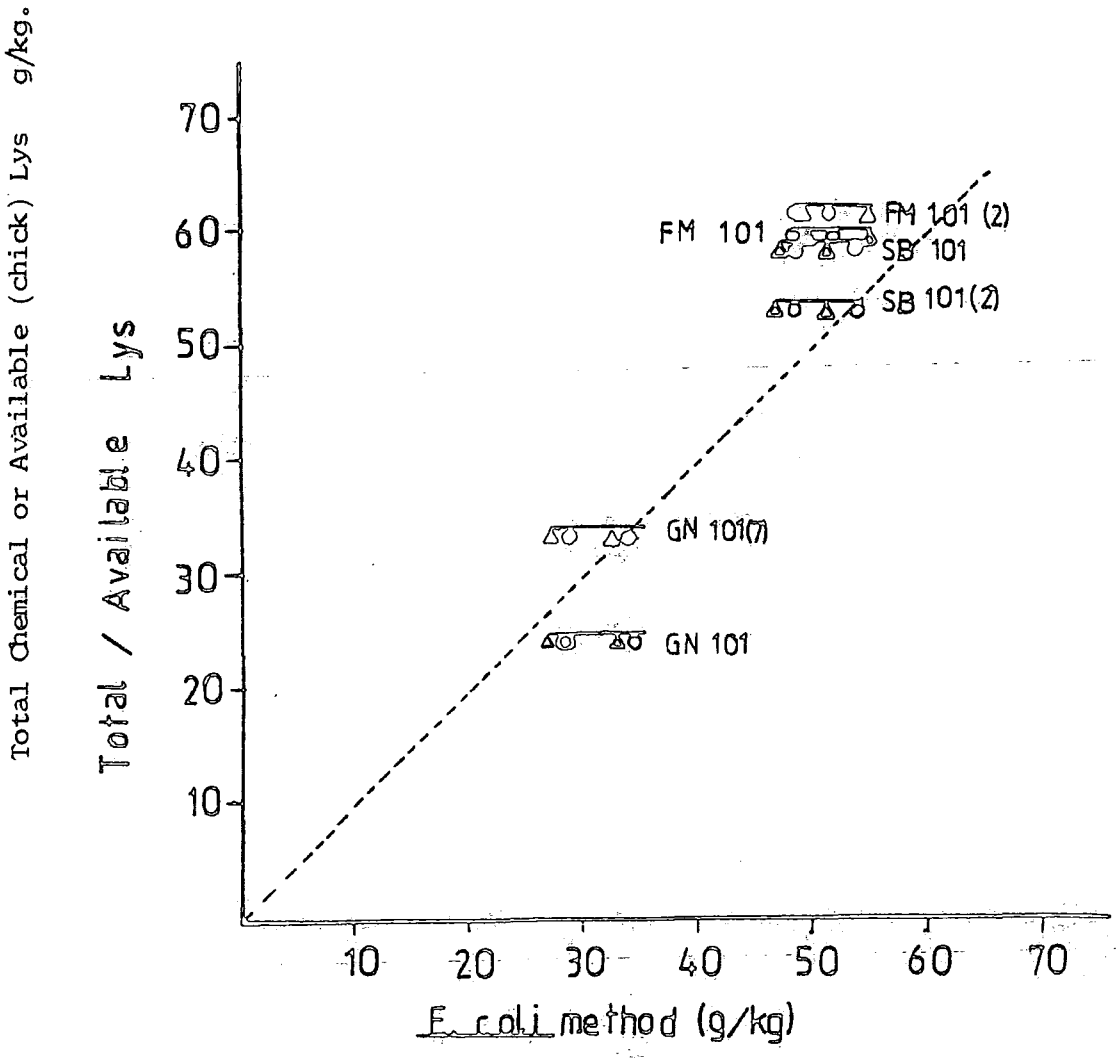


FIGURE 6.20 Lysine Assay Of Feed Meals.

Comparison of results from E coli assay calculated by (a) best eye fit ○ or (b) least squares best line △ with total Lys determined by chemical analysis (closed symbols). Data also compared, open symbols ◎ , ◬ , to published available Lys values from chick bioassay (Carpenter & Woodham, 1974). Figures in brackets represent no. of digests assayed.

b) Methionine

Results for Met showed a wider variation between digests for the same meal than did Lys results. Again the widest variation was in the results for GN101 but since this was the lowest of the three meals in terms of g Met Kg^{-1} meal, perhaps this was not surprising. Available values were calculated on the basis of total Met values determined microbiologically. In a collaborative trial in which twelve different meals were tested "total Met values were generally similar whether determined chemically or microbiologically although the latter were somewhat higher", (Carpenter & Woodham, 1974). Therefore, percentage availability figures quoted in terms of total chemical Met would be greater.

If the results taken individually, for each of the three digests assayed were used, the available Met contents of the three meals would be ranked in the same order as determined by chick or Strep. zymogenes assays.

Values obtained by the two methods of joining data points were very similar for FM and SB but there was more variation in the two values obtained with GN101. A graphical representation of the results is given in Fig. 6.21. See also tables 6.10, 6.11 and 6.12.

c) Tryptophan

Collaborative trials have shown that there is no single satisfactory method for the estimation of Trp - a view adequately supported by the multitude of analytical methods for its determination. The range of values obtained for Trp in the meals analysed here is quite high. However, reproducibility between assays with the same digest is good in all cases.

Tables 6.13, 6.14 and 6.15 show results from the assay of the meals for Trp. The results are presented in graphical form in Figure 6.22.

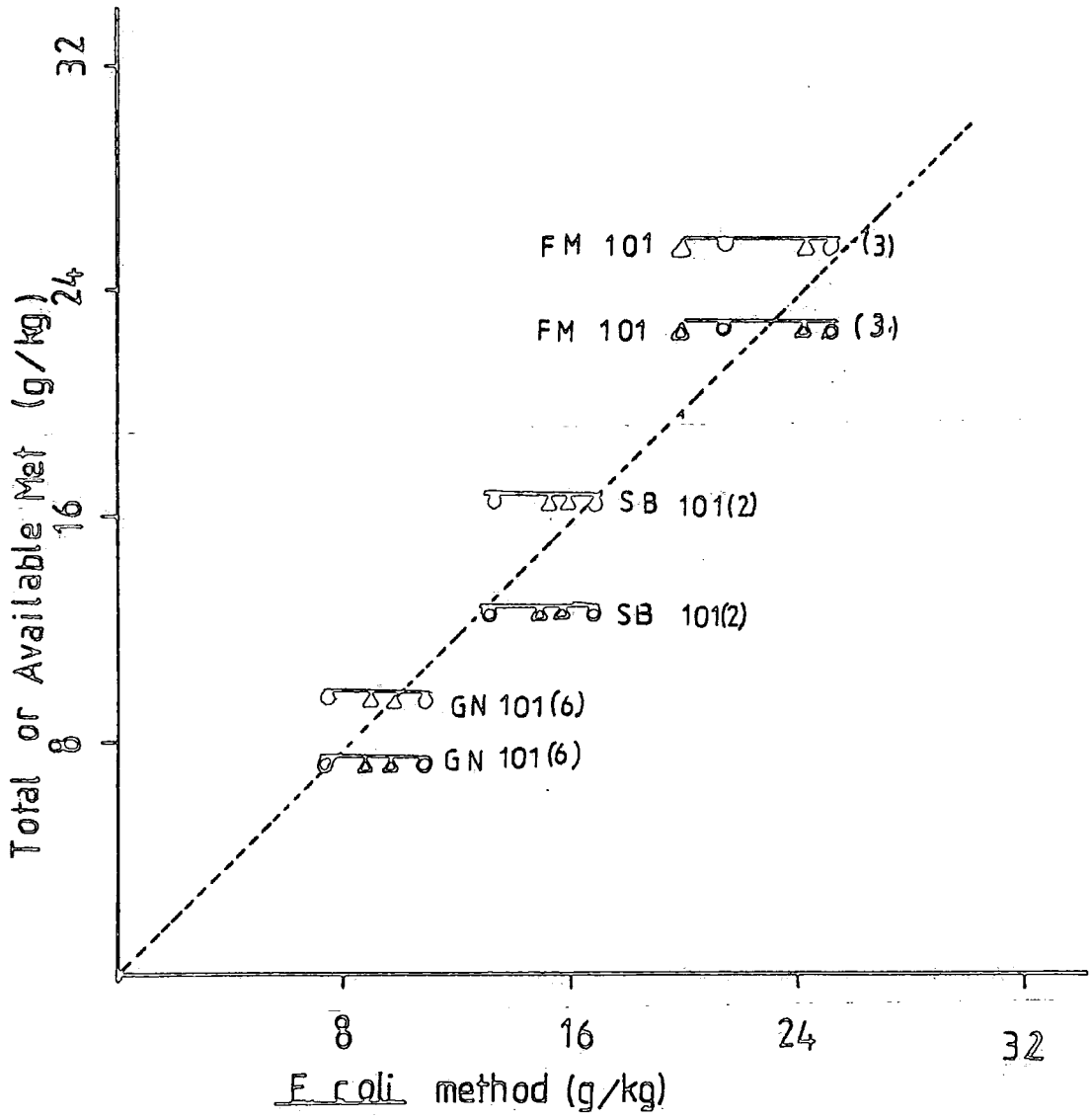


FIGURE 6.21 Methionine Assay Of Feed Meals

Comparison of results from E. coli assay of meals calculated by using method (a) ○—○ , or method (b), with total Met △—△ determined by microbiological method - closed symbols ○ , △ , or "available" Met -open symbols ◎ , ◬ , also determined by microbiological assay. Figures in brackets represent no. of assays on each sample.

Table 6.10

Results For Met Assays Of High Protein Feed Meals

Sample	Total Chem:	M'Biol	Available M'Biol:	Chick	Digest Results g Kg^{-1} 1	2	3
GN101	9.9	10	7.8	7.6	8.95	7.48	10.03
					8.89		
					8.47		
					σ = 0.91		
					m = 8.76		
					v = 0.85		
SB101	14	17	13	-	-	13.78	16.93
					σ = 2.22		
					m = 15.36		
					v = 4.97		
FM101	23	26	23	25	25.15	21.44	22.51
					σ = 1.90		
					m = 23.03		
					v = 3.64		

- Not determined

Total - acid hydrolysis

Available - Pre-digestion papain

σ = standard deviation

m = mean

v = variance

See Carpenter & Woodham (1974)

Published data is based on 900 g protein Kg^{-1}.

Digest results calculated on actual moisture contents as for Lys assays.

Table 6.11

Methionine Contents - Comparison Of Results Derived From Lines Joined By Eye[a] And Least Squares Analysis[b]

	1		2		3	
Sample	a	b	a	b	a	b
GN101	8.95	-	.48	9.66	10.00	10.26
	8.47	-	-	-	-	-
	8.89	-	-	-	-	-
SB101	-	-	13.78	14.47	16.93	15.92
FM101	25.15	24.72	21.44	20.14	22.51	22.28

Mean values g Kg^{-1}

	a	b
GN101	8.76 (89.6)	9.96 (99.6)
SB101	15.36 (90.4)	15.195 (89.4)
FM101	23.03 (88.6)	22.38 (86.1)

Values in Parenthesis Represent Percentage Availability Based on Published Data for Total Microbiological Assay (Acid Hydrolysis and Streptococcus zymogenes assay)

Assay procedure as section 3-4

Table 6.12

Percent Availability Of Met - Calculated From Met Determined By *Escherichia coli* Assay* Related To Total Microbiological Values

Sample	Total Score (M'Biol)	Digest 1 a	Digest 1 b	Digest 2 a	Digest 2 b	Digest 3 a	Digest 3 b
GN101	10	89.52	-	84.8	96.6	100.3	102.6
		84.7	-	-	-	-	-
		88.9	-	-	-	-	-
SB101	17	-	-	81.06	85.12	99.6	93.6
FM101	26	96.7	95.07	82.46	77.46	86.59	85.6

Mean Values (% availability)

	Method (a)	Method (b)
GN101	89.6	99.6
SB101	90.4	89.4
FM101	88.58	86.1

* Assay procedure as section3-4

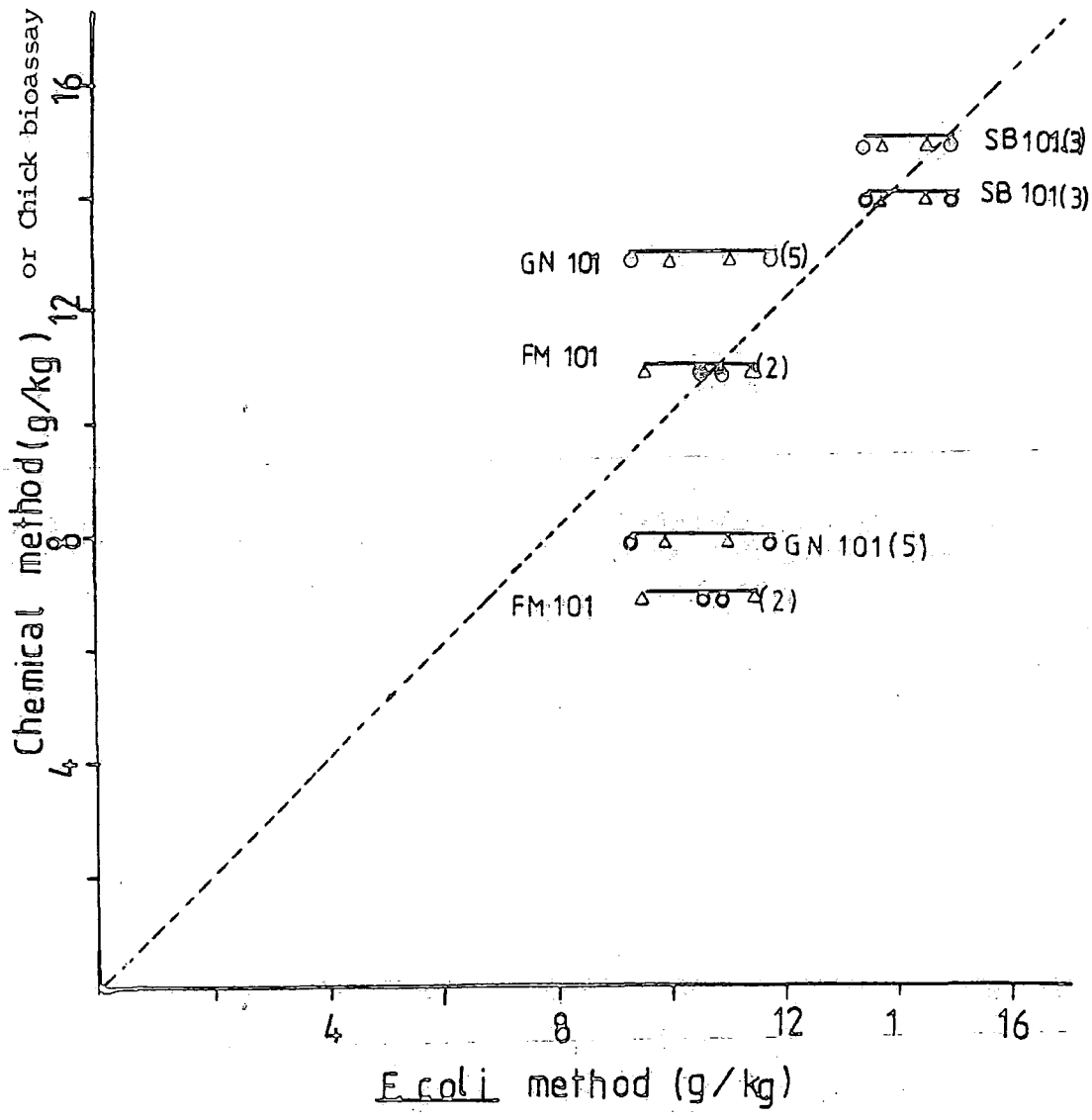


FIGURE 6.22 Tryptophan Assay Of Feed Meals

Comparison of measurements of "available" Trp determined with E. coli and published chemical results (solid symbols) and chick bioassay (open symbols). Figures in brackets represent number of assays on each sample.

Data calculated by method (a) ○ , ◎ ; or method (b) △ , ▲ .

Data presented in Table 6.14.

Availability was calculated in each case on the basis of published data for chemical assay. The values obtained for fish meal are of interest because from the limited data available the availability of Trp from this meal is very high. Inspite of published work in which the chemical assay gave values much greater than chick or microbiological assay results. If the results from the Escherichia coli assay are compared with those obtained by microbiological or chick assay then in almost every case they are higher with Escherichia coli. The published results show some discrepancy here, since strictly speaking, the ARC chick assay gives values for available Trp rather than total, but these are likely to be close in a good-quality feed. However, for GN101 and FM101 there is a large variation between total chemical and chick "available" results.

Since no set of results for Trp obtained by any method consistently agrees closely with any other set, caution must be used in drawing conclusions from the analytical results obtained with the limited data available.

When data for the same feed-stuff assayed for the three amino acids are compared, it can be seen (Table 6.16), that the availability of Lys and Met in a given sample is very close and the close correlation between 'available' and 'total' values was good with the former being 12% below the latter at the most but usually only about 10% less. This corroborates the findings published for these amino acids in these and other high protein feedstuffs by Carpenter & Woodham, 1974, where the mean value for available Met or Trp was quoted as being ca. 10% below the total in each instance.

Table 6.13

Results For Trp Assays Of High Protein Feed Meals

Sample	'Available' Trp *Chem	M'Biol	Chick assay	g.Kg^{-1} Digest 1	Digest 2	Digest 3
GN101	13	8.2	8.0	9.82	9.46	11.93
				9.25	9.57	
					σ = 1.095	
					m = 10.01	
					v = 1.199	
SB101	15	10.8	14	-		14.22
					14.66	13.98
					σ = 0.35	
					m = 14.29	
					v = .12	
FM101	11	5.2	6.8	-	10.79	11.14
					σ = 0.25	
					m = 10.97	
					v = 0.061	

Tryptophan values - calculated by method (a) points joined by eye.

\- not determined

* Data from Carpenter & Woodham, 1974

σ = standard deviation

m = mean

v = variance

Quoted data based on 900 g protein Kg^{-1}. Experimental Assay data based on actual moisture contents.

Table 6.14

Tryptophan Results - Comparison Of Results Obtained From Lines Joined By Eye[a] And By Least Squares Analysis[b]

	Digest 1		Digest 2		Digest 3	
Sample	a	b	a	b	a	b
GN101	9.82	-	9.46	10.78	11.93	10.44
	9.25	-	9.57	9.92	-	-
SB101	-	-	-	-	14.22	12.26
	-	-	14.66	15.19	13.98	12.97
FM101	-	-	10.79	9.81	11.14	11.86

Mean Values g Kg^{-1}

	Method (a)		Method (b)	
GN101	10.01	(77)	10.38	(79.8)
SN101	14.28	(95.24)	13.47	(89.82)
FM101	10.97	(99.7)	10.84	(98.5)

Figures in parentheses represent percentage availability based on published results from chemical analysis.

Table 6.15

Percentage Availability Of Trp Calculated From *Escherichia coli* *

Assay Related To Chemical Values For Trp

Sample	Chemical Score	Digest 1 a	Digest 1 b	Digest 2 a	Digest 2 b	Digest 3 a	Digest 3 b
GN101	13	75.53	-	72.7	82.89	91.6	80.31
		71.15	-	73.6	76.34	-	-
SB101	15	-	-	97.7	101.3	94.8	81.73
		-	-			93.2	83.01
FM101	11	-	-	98.09	89.8	101.2	107.8

Mean Values (% Availability)

	Method (a)	Method (b)
GN101	76.9	79.8
SB101	95.23	88.68
FM101	99.5	98.5

* Assay procedure as in section 3-4

Table 6.16

Summary Of Results For High Protein Meals

	LYS**			MET **			TRP **		
	Chem Assay *	E. coli assay mean g Kg^{-1}	% Avail mean	Total Met *	E. coli assay g Kg^{-1}	% Avail	Chem Assay *	E. coli assay g Kg^{-1}	% Avail
GN101	34	30.43	89.45	10	9.46	94.6	13	10.2	78.4
SB101	59	50.53	85.6	17	15.28	89.9	15	13.87	92.5
FM101	62	51.91	83.7	26	22.71	87.4	11	10.91	99.1

* Total scores from Carpenter & Woodham, 1974

All results are the mean values from all assays calculated by methods (a) and (b) Since there was good agreement between both methods of calculating the results, it was justifiable to use both sets of data in calculating the above means.

** Enzyme assayed by standard procedure (section 3-4)

Appendix 5 gives a worked example for the calculation of one result and the statistical data included. Appendix 6 gives a listing of the computer program used to calculate the best fit line. However, when this is done the best fit line does not necessarily pass through the origin, and a line has to be drawn parallel to it through this point, yet, sensibly, there must be an intercept at $x = 0$, $y = 0$ after blank values are subtracted. Therefore, as a check on this aspect, one set of data for each meal (all three amino acids plus their calibration curves), was recalculated using another computer program where the equation of the line was taken as $Y = mx + 0$ (to give a zero intercept and thus force the line through the origin of the graph). Data calculated in this way is referred to as constrained data.

Comparison of results for three samples calculated by best eye fit (a), by the conventional least squares method (b), and as discussed above (c), is given in Table 6.17. It can be seen that in most cases the constrained fit gives higher final values than the least squares fit. Variation in the methods was not obviously greater where the intercept obtained using method (b) was high; as would perhaps be expected. Table 6.18 shows the difference in values obtained by the two statistical methods (c - b), and the intercept values obtained from the best fit analysis of the pairs of points used to plot the best fit lines (b). The correlation between (c - b), and the intercepts (r) was 0.524. For 9 sets of points the minimum level for significance would be 0.670.

However, overall there was a very good correlation ($r = 0.997$), between the values obtained with the constrained data (c) and those from the simpler method of drawing the best line by eye.

Table 6.17

Comparison Of Graphical Points Joined By Eye (a), Joined By Least Squares Fit (b) Or By Using Constrained Data (c) For Nine Individual Assays

		Amino Acid Content (g Kg^{-1})		
Sample	Assay	a	* b	c
SB101	Lys	53.32	51.15	53.17
FM101	"	49.69	53.54	52.84
GN101	"	34.08	33.35	35.15
SB101	Met	16.93	15.92	16.75
FM101	"	25.15	24.02	25.70
GN101	"	10.00	10.26	8.55
SB101	Trp	14.22	11.26	16.51
FM101	"	10.79	9.81	12.08
GN101	"	9.46	10.78	10.35

All values quoted are calculated on g.Kg^{-1} dry weight meal and they are values taken from one individual assay with the results plotted and calculated in three ways.

* Results calculated by least squares method line drawn parallel to actual best fit line so that it passes through the origin.

Table 6.18

Relationship Of Difference Between Values Calculated By Method (c) And Method (b) And Intercept

Sample	Amino Acid	c - b*	Intercept (c)**
SB101	Lys	2.02	0.0113
FM101	"	-0.7	-0.0461
GN101	"	1.8	0.0137
SB101	Met	0.83	0.0527
FM101	"	1.68	-0.1278
GN101	"	-1.71	-0.0374
SB101	Trp	5.25	0.1097
FM101	"	2.27	0.0512
GN101	"	-0.43	0.0168

* From Table 6.17

** Intercept of best fit line

For these nine pairs of points correlation between (c - b) and i = 0.524

This is shown graphically in Fig. 6.23. Comparing (c) and (a) for individual amino acids for Lys r = 0.987, for Met r = 0.999 and for Trp r = 0.999.

It is very clear therefore that joining the lines by eye gives sensible, meaningful and reproducible results and the procedure is rapid and readily applicable to the routine analysis of large numbers of samples. Therefore, in subsequent calculations this was the sole method used.

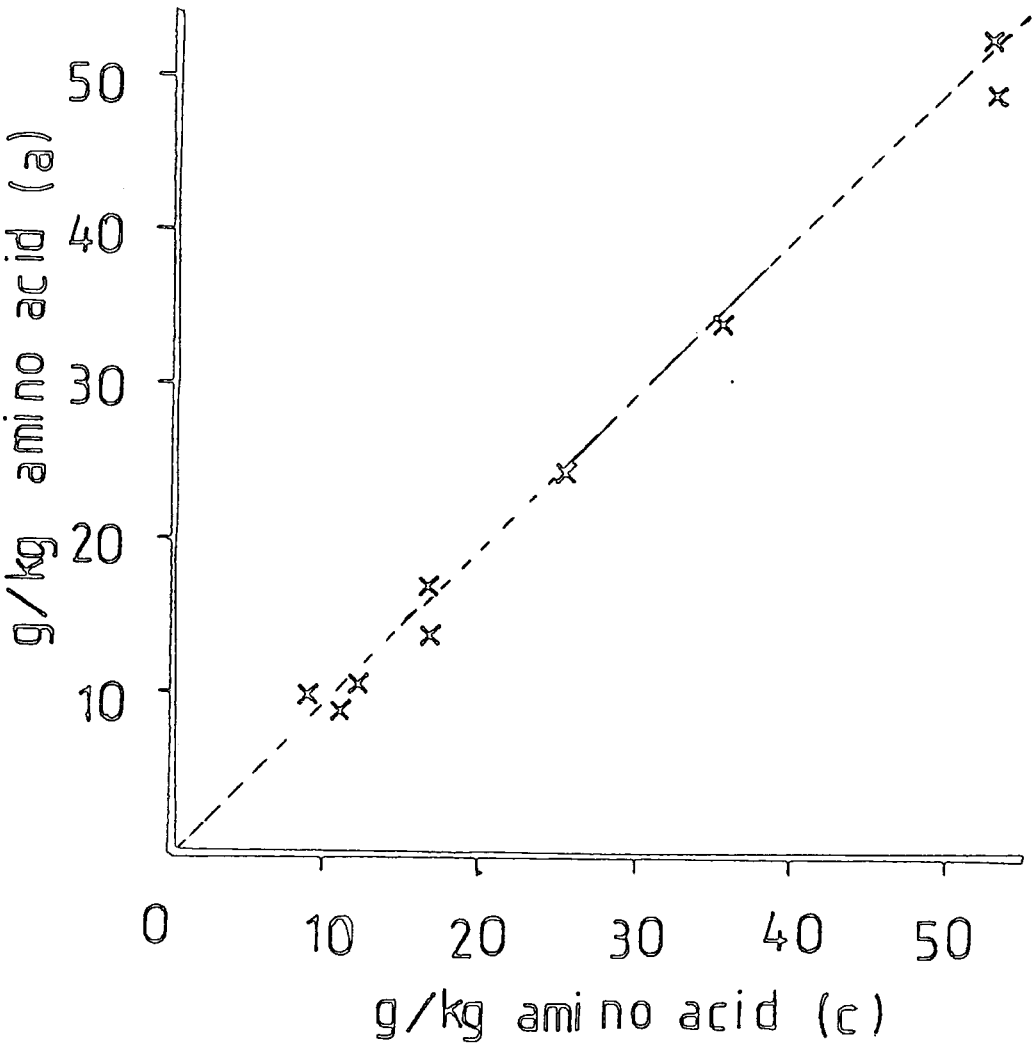


FIGURE 6.23 Variation In Results When Graphs Are Jioned By Eye (a) Or By Using Constrained Data (c).

Comparison of two methods of calculating results for each meal; data taken from Table 6.18. See text for method of calculation in each case.

6-7 Assay Of Rice (*Oryza sativa*, L) Samples

6-7-1 Introduction

Among the cereal proteins, rice has been found to be of relatively good quality (Bressani *et al.*, 1971), with a fairly good balance of essential amino acids. The main nutritional disadvantage of the rice grain is its low protein content (6 - 8%). The biological assessment of protein quality is difficult in samples that contain relatively little protein, assays such as NPU and PER require 9 - 10% dietary protein levels. The problems of assay of samples low in protein are particularly severe where that protein is of poor quality, and especially so if the protein is limiting in Lys (Hegsted & Juliano, 1974).

Analysis of Lys in rice is particularly important as it is the first limiting amino acid (Thr being second limiting). As the overall protein content of rice varieties is increased e.g., by breeding/selecting high protein cultivars, it has been a common observation that a decrease in the Lys content and hence the quality of the protein occurs.

To change rice nitrogen analyses to protein values the conversion used should be (N x 5.95), (Eggum & Juliano, 1973), but the factor 6.25 is more often used and results are therefore expressed as g.16 g N^{-1} to make it isonitrogenous with most proteins containing 16% Nitrogen. This is the usual procedure in most publications and therefore 16% N was usually used in the following calculations but care should be taken in interpreting data since occasionally composition is quoted as g 16.8 g N_2^{-1} (Juliano, 1966).

As with the meals (Section 6-6), rice samples were digested, assayed for Lys, Met and Trp by using the procedure described in Sections 3-4 and 2-8-3, and the results obtained were compared with published values (Hegsted & Juliano, 1974; Eggum & Juliano, 1973), and in personal communications by Dr. J. Ford (N.I.R.D.), and Dr. B. Juliano (I.R.R.I.), to Dr. J.W. Payne.

6-7-2 Methods

The digestion procedure and assay method have been described (Sections 2-8 and 3-4).

Two separate digests of each of four rice varieties were made, and each digest was assayed at least twice using not less than three different concentrations. Treatment of the digests prior to assay was as described for meals (Section 6-6-2). Digests were diluted as appropriate ($\frac{1}{4}$ usually), for assay, in 0.1M pH 7.2 buffer. One digest was assayed for Lys using M2626, the other using strain PA0110 Lys^{-} Trp^{-}. Both digests were also assayed for Met and Trp using the appropriate auxotrophs (PA0111 and PA0110, respectively). Controls showed that there were no inhibitors in the samples, and autolysis values at no time contributed more than 1% to the final assay values. Results were calculated and presented as described in Section 6-6-2 except that method (a) only was used i.e., the best lines between the various sets of duplicate points were drawn by eye. The justification for doing this was given in Section (6-6-3).

Final results were expressed as g/16 g N but comparison between these results and 16.8 g N values are given.

6-7-3 Results

a) Moisture Content

In the published literature, there are discrepancies in the moisture content quoted for milled rices. It is usually given as 12% but was quoted as 14% by Dr. B. Juliano (personal communication). These must be approximate values since it is unlikely that all varieties would have exactly identical moisture contents after milling. Percentage moisture content of the four varieties used in this study were determined experimentally and found to be: Intan, 10.7%; IR480-5-9, 10.9%; IR480-5-9 Brown, 12.2% and BP76-1 11.1%, and all results of Escherichia coli β-galactosidase assays are quoted on a dry weight basis to avoid confusion, since there was variation in moisture content amongst the samples.

Percentage protein values for the four rices are as follows: Intan 5.97; IR480-5-9 11.8; IR480-5-9 Brown, 10.9; and BP76-1, 15.2. These quoted values (N x 6.25), include 14% water (Dr. B. Juliano, personal communication).

b) Lysine Determinations

Comparison between total Lys (determined from amino acid analysis on acid hydrolysates), and available Lys determined by using either Escherichia coli growth assays or β-galactosidase assay are given in Table 6.19. It can be seen that in all cases the values obtained by the latter method were greater than those from Escherichia coli growth assays. However, values for the percentage availability of brown rice were lower than for the other samples.

Table 6.19 (cont.)

Sample	[a] % Protein N x 6.25	[a] % Lys	Total Lys g/16g N	[b] Available Lys g/16g N	[b] % Lys Available	[c] Available Lys g/16g N	[c] % Lys Available
BP76-1	15.2	.485	3.19	1.55	48.6	2.16*	66.7
						1.98	62.1
						1.94*	60.8
						2.44	76.5
						σ = 0.05	
						m = 2.13	
						v = 0.05	

σ = Standard deviation

m = Mean

v = Variance

a On total, not amino, N basis, assumes 14% water

b *Escherichia coli* growth assay J.W. Payne (1976)

c Determined by *Escherichia coli* β-galactosidase assay (dry weight basis)

All four results in column c are from separate assays.

Samples marked * are the same digest.

This could be caused by degradation of the sample during storage but this is unlikely since Dr. K.J. Carpenter verified 100% available Lys by the DNFB method after 6 months storage at room temperature (B.O. Juliano - personal communication). However, of the samples of rice BP76-1 and Intan were known to have been grown in 1969 (Bressani et al., 1971), and no information regarding the conditions under which they have been stored subsequently, for 14 years, is available. Another possible cause of low values for available Lys might be in the digestion. If the milling were insufficient, digestion could be incomplete since the particle size might limit accessibility of the enzymes to the proteins.

It must be stressed that great care is needed in interpreting literature values for rice since they are based on a single moisture value and different nitrogen values 6.25 or 5.95. Results for the enzyme assays were calculated on a dry weight basis and assuming 16% N, to make the sample isonitrogenous with other proteins. In Table 6.20, Lys availability values at N x 6.25 and N x 5.95 are compared and the latter gives values very close to those calculated in Table 6.19.

Finally, Table 6.21 compares total Lys values calculated at 14% moisture and on a dry weight basis and gives percentage availability figures for the enzyme assay calculated on dry weight values.

The data presented in Table 6.19 shows that the percentage Lys available in rice, especially in the case of brown rice, is lower than typically expected in samples higher in protein.

Table 6.20

Comparison Of Lys Results Calculated On Basis Of g 16 g N_2^{-1} And g 16.8 g N_2^{-1}

Sample	Total Lys g/16 g N_2	Total Lys g/16.8 g N_2	b Available Lys g/16 g.	b % Available	c Available Lys g/16.8 g N_2	c % Available
INTAN	4.07	4.27	3.12	73.1	3.36	78.7
					3.29	77.0
					3.33	78.0
					3.51	82.2
IR480-5-9	3.34	3.52	2.35	66.8	2.38	67.6
					2.59	73.6
					2.48	70.5
					2.51	71.3
IR480-5-9 (Brown)	3.59	3.77	2.11	56.0	2.02	53.6
					1.92	50.9
					1.68	44.6
					1.81	48.0
BP76-1	3.19	3.35	1.63	48.7	2.27	67.8
					2.08	62.1
					2.04	60.9
					2.56	76.4

b *Escherichia coli* growth assay (J.W. Payne, 1976)
c *Escherichia coli* β-galactosidase assay
These comparisons are illustrated in Fig. 6.24

Table 6.21

Lysine Results Calculated On Dry Weight Basis

Sample	Total Lys[a] g/16.8g N_2 14% H_2O	Total Lys[b] g/16.8g N_2 Dry weight	Available[c] Dry weight	% Available[d]
INTAN	4.27	4.85	3.36	69.3
			3.29	67.8
			3.33	68.7
			3.51	72.4
IR480-5-9	3.52	4.01	2.38	59.4
			2.59	64.6
			2.48	61.8
			2.51	62.6
IR480-5-9 (Brown)	3.77	4.29	2.02	47.1
			1.92	44.7
			1.68	39.2
			1.81	42.2
BP76-1	3.35	3.82	2.27	59.4
			2.08	54.5
			2.04	53.4
			2.56	67.0

a Published data - converted from N x 6.25 to N x 5.95

b Dry weight (b = a x 1.14)

c Determined by Escherichia coli enzyme assay

d % available determined by Escherichia coli assay on dry weight and g/16.8g N_2 basis

If these are underestimates, it is likely to be at least in part, a problem of digestion, since in two separate digests there is a difference of over 6% availability between the mean values of Lys.

It is possible that the digestion conditions, which were optimised with pure proteins and with high-protein meals, may need to be modified for milled rice samples, but this aspect was not examined further. Lys results are shown graphically in Fig. 6.24.

c) Methionine Determinations

Methionine values were calculated on a g/16g N_2 and dry weight basis. Table 6.22 compares values obtained for Met using Escherichia coli PA0111 with those determined for the same rice samples by Dr. J. Ford, using Streptococcus zymogenes assay, for total and available Met, and also with total Met values obtained by amino acid analysis after acid hydrolysis.

Table 6.23 gives a more direct comparison, based on dry weight values throughout. As for Lys, availability values are low in some cases; and once again there is a significant difference between the values obtained for the two separate digests of brown rice. The brown rice sample also gives values for availability considerably lower than the other three samples when assayed by Streptococcus zymogenes assay, again implying that digestibility may be a problem.

A graphical comparison of Met availability data obtained by Streptococcus zymogenes and Escherichia coli assays is given in Fig. 6.25.

d) Tryptophan Determinations

One of the problems in the evaluation of the results obtained for Trp contents of the tested rice samples, is the lack of published data on this amino acid.

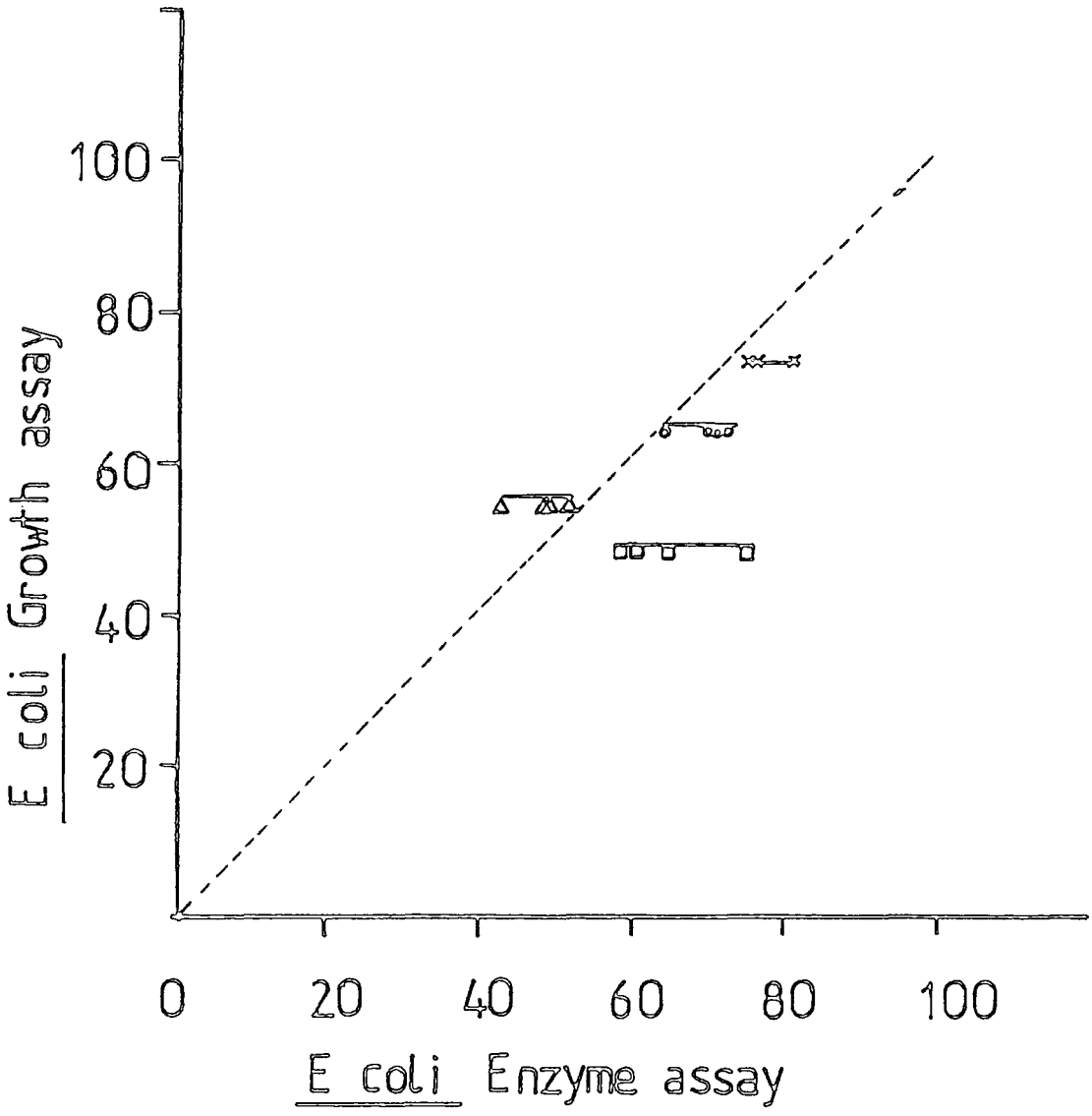


FIGURE 6.24 Assay Of Lys In Rice Meals

Comparison of availability determined by two E.coli assays for rice samples; Intan, ☆—☆ ; IR 480-5-9, ○—○; IR 480-5-9(brown), △—△ ; and BP 76-1, □—□ .

Data taken from Table 6.20 and expressed as % availability.

Table 6.22 (cont.)

Sample	% Protein	Met[a] g/16g N	Met g/16g N[b] Total	Met g/16g N[b] Available	%[c] Available	E. coli g/16g N_2	%[d] Available
BP76-1	15.2	1.71	1.55	1.33	86	1.17*	61.1
						1.05*	54.8
						1.37	71.5
						1.36	71.0
						σ = 0.16	
						m = 1.24	
						v = 0.02	

a Normal Acid Hydrolysis 12% moisture

b Data sent by Dr. J. Ford (1976), at 12% moisture

c Availability from St. zymogenes data

d Calculated on basis of total Met (column a), but converted to dry weight

* Samples from same digest - separate assays

σ = Standard deviation

m = Mean

v = Variance

Table 6.23

Comparison Of *Escherichia coli* And *Streptococcus zymogenes*

Values For Rice Samples Based On Dry Weights Throughout

Sample	St. zymog. g/16g N Dry wt.[a] Total	Avail.	% Avail.	E. coli Enz. Assay	% Available[b]
INTAN	2.95	2.38	80.6	2.23	75.6
				2.29	77.6
				2.17	73.6
				2.26	76.6
				σ = 0.05	
				m = 2.24	
				v = .003	
IR480-5-9	2.71	1.85	68.2	1.53	56.5
				1.58	58.3
				1.59	58.7
				1.58	58.3
				σ = 0.027	
				m = 1.57	
				v = 0.001	
IR480-5-9 (Brown)	2.68	1.57	58.6	1.04	38.8
				1.26	47.0
				1.37	51.1
				1.31	48.8
				σ = 0.14	
				m = 1.25	
				v = 0.02	
BP76-1	1.74	1.49	85.6	1.17	67.2
				1.05	60.3
				1.37	78.7
				1.36	78.2
				σ = 0.16	
				m = 1.24	
				v = 0.02	

a Data taken from Dr. J. Ford and multiplied by 12% to convert to dry weight

b Based on first column (Total Met - dry weight basis)

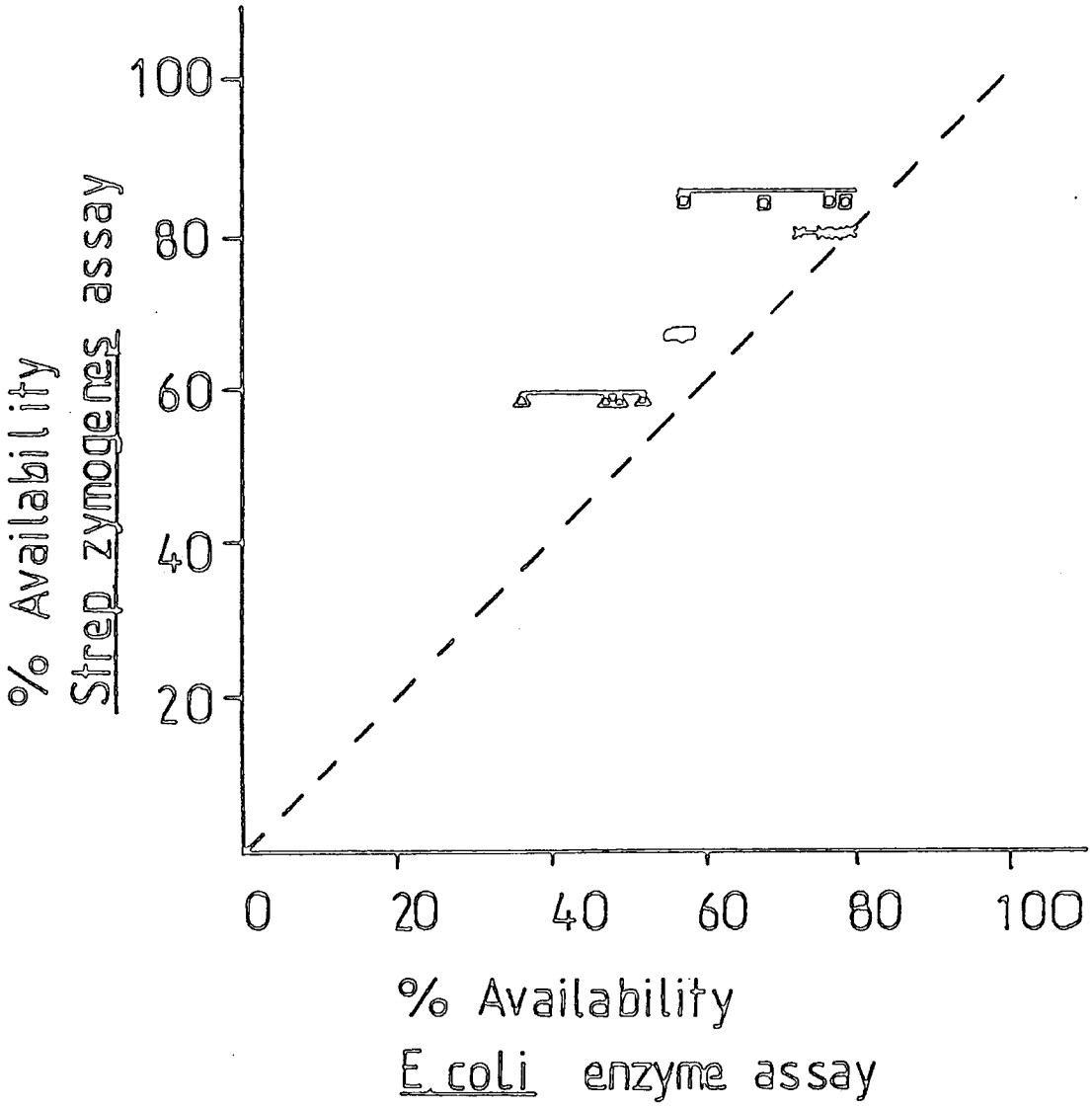


FIGURE 6.25 Assay Of Methionine In Rice Samples
Comparison Of Results Of Two Microbiological Assays

Comparison of values obtained from two different assays for available Met: Streptococcus zymogenes and E. coli from rice meals. Intan, ; IR 480-5-9, ; IR 480-5-9 (brown), ; and BP 76-1, .
All % availability values are calculated on a dry weight basis.

Amino acid profiles obtained from amino acid analysers after acid hydrolysis exclude Trp which is destroyed by the hydrolysis. However, published values for three of the samples are given in Table 6.24. No data was available for brown rice IR480-5-9, but Juliano (1966), gives Trp content for brown rice (unspecified varieties), determined by various authors to be in the range 1.21 - 1.58 g/16.8 g N. This is equivalent to 1.15 - 1.50 g/16.0 g N converted to the same base as the data in Table 6.24. In the absence of any information in the publication it is assumed here that the chemical data quoted by Juliano (1966), is on a dry weight basis. Values obtained for IR480-S-9 (Brown) were therefore compared to the maximum and minimum values for 'Brown' rice.

Comparison of percentage availability from Escherichia coli assay and published data is shown in Fig. 6.26.

In as much as the digestions and analytical procedures are the same here as for Lys and Met, there is every reason to believe that these Trp results are equally reliable, however, in the absence of comparative data it is difficult to verify this. Once again, the availability of the amino acid in the brown rice seems to be low; this could be due to an inadequate digestion procedure but it could also arise from lack of data on total Trp for this variety, for if the actual Trp contents for IR480-5-9BR are lower than other brown rice varieties the availability figures quoted would be correspondingly higher.

Results might have been higher if different enzymes or combinations of enzymes had been used, or if samples had been more finely ground.

Table 6.24

Tryptophan Content Of Rice Samples - Comparison Of Results

Sample	[a] Total g/16g N	[b] Total g/16g N	*Escherichia coli* enzyme assays g/16g N (dry wt.)
INTAN	1.35	1.29	1.16 1.19 1.19 σ = .02 m = 1.18 v = 0.0003
IR480-5-9	-	1.38	1.21 1.08 1.13 σ = 0.07 m = 1.14 v = .004
IR480-5-9[c]	-	-	0.691 0.950 0.745 σ = 0.14 m = 0.80 v = 0.019
BP76-1	0.97	0.92	0.948 0.895 0.764 σ = 0.1 m = 0.87 v = .009

a Bressani *et al.*, 1971 12% moisture

b Hegsted & Juliano, 1974 " " "

\- Not reported in literature

c No values for this variety.

Unspecified brown rices = 1.15 - 1.50 g Trp/16g N

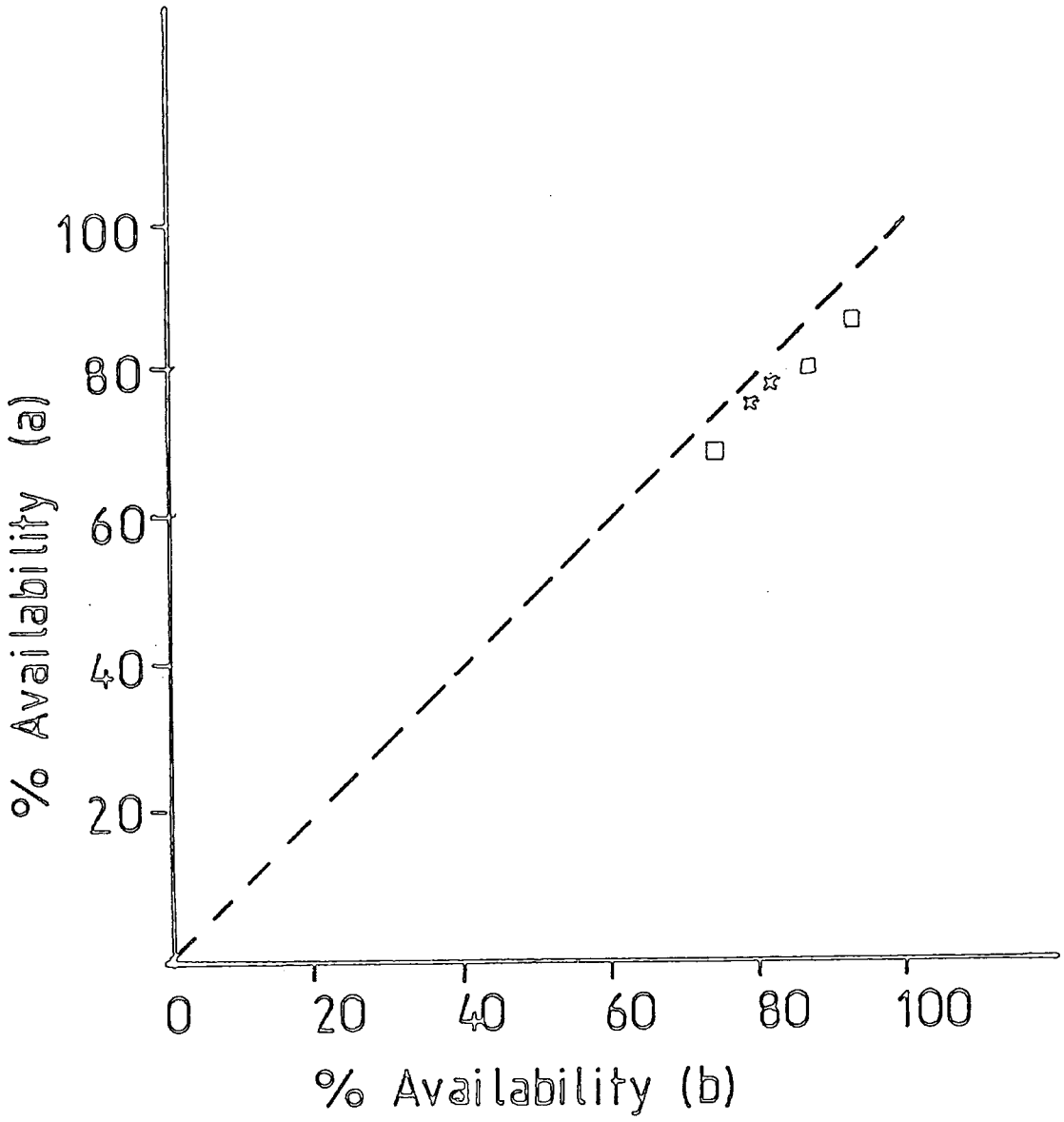


FIGURE 6.26 Assay Of Tryptophan In Rice Digests Comparison Of β -galactosidase Results With Published Data.

Comparison of available Trp in rice with published data. Values taken from Table 6.25: (a) Data from Bressani et.al. (1971)

(b) Data from Hegsted and Juliano (1974).

Intan, ✕—✕ ; BP 76-1, □—□ .

No published data is available for IR 480-5-9 or IR 480-5-9 (brown)

Table 6.25

Comparison Of *Escherichia coli* Enzyme Assay

And Published Data For Trp In Rice Samples (Dry Weight Basis)

Sample	a g/16g N	b g/16g N	*E. coli* Value	% Availability From a)	From b)
INTAN	1.51	1.44	1.16	76.8	80.6
			1.19	78.8	82.6
			1.19	78.8	82.6
IR480-5-9	-	1.54	1.21	-	78.6
			1.08	-	70.1
			1.13	-	73.4
IR480-5-9 (Brown)	-	-	.691	[c](60.1)	[d](46.1)
			.950	(82.6)	(63.3)
			.745	(64.8)	(49.7)
BP-76-1	1.09	1.03	.948	87.0	92.0
			.895	82.1	86.9
			.764	70.1	74.2

a Value from Table 6.24 converted to dry weight

b Value from Table 6.24 converted to dry weight

c Value calculated on basis of minimum for brown rice (1.15)

d Value calculated on basis of maximum for brown rice (1.50)

Ford (1964), showed that with whale-meat meals and fish meals, especially those of poorer quality, grinding finely increases the values for available amino acid (Lys, Met, Trp, Leu and Arg) content.

6-8 Assay of Vitamin B_{12}

6-8-1 Introduction

The mutants that were isolated as auxotrophic for Met also responded to B_{12}. It was therefore important to establish the comparative sensitivity to Met and to B_{12} to determine the limitations of the organism to assay particular materials that could contain both compounds (such as blood plasma and other biological fluids).

Earlier experiments (Section 4-3), established that the minimum concentration of vitamin B_{12} required to support good growth of strain PA0111 (Met^- Lys^-), was 10^{-10}M for plate growth and 10^{-12}M for tube growth.

6-8-2 β -Galactosidase Assay For Met and B_{12}

Strain PA0111 and strain 113-3, isolated by Davis & Mingioli (1950), (NCIB 8134, ATCC 10799), which has been extensively used by others, in Met assays; were tested simultaneously for β -galactosidase production in response to B_{12} or Met. Cells for Met assay were grown in Met. Both strains showed an identical response to Met (Fig. 6.27). However, using B_{12}-grown cells, a B_{12} assay was unsatisfactory because of high background enzyme levels (i.e., controls with no added B_{12}). It seemed possible that the normal sixty minute starvation period was inadequate for depletion of residual B_{12} in the cells, therefore an experiment was carried out to quantify the response to endogenous B_{12} after 50, 60 and 120 minutes starvation. After 60 minute starvation, 71.6% of the zero time response was obtained but this fell to background levels i.e., less than 1 percent after 100 minutes of starvation (results not shown).

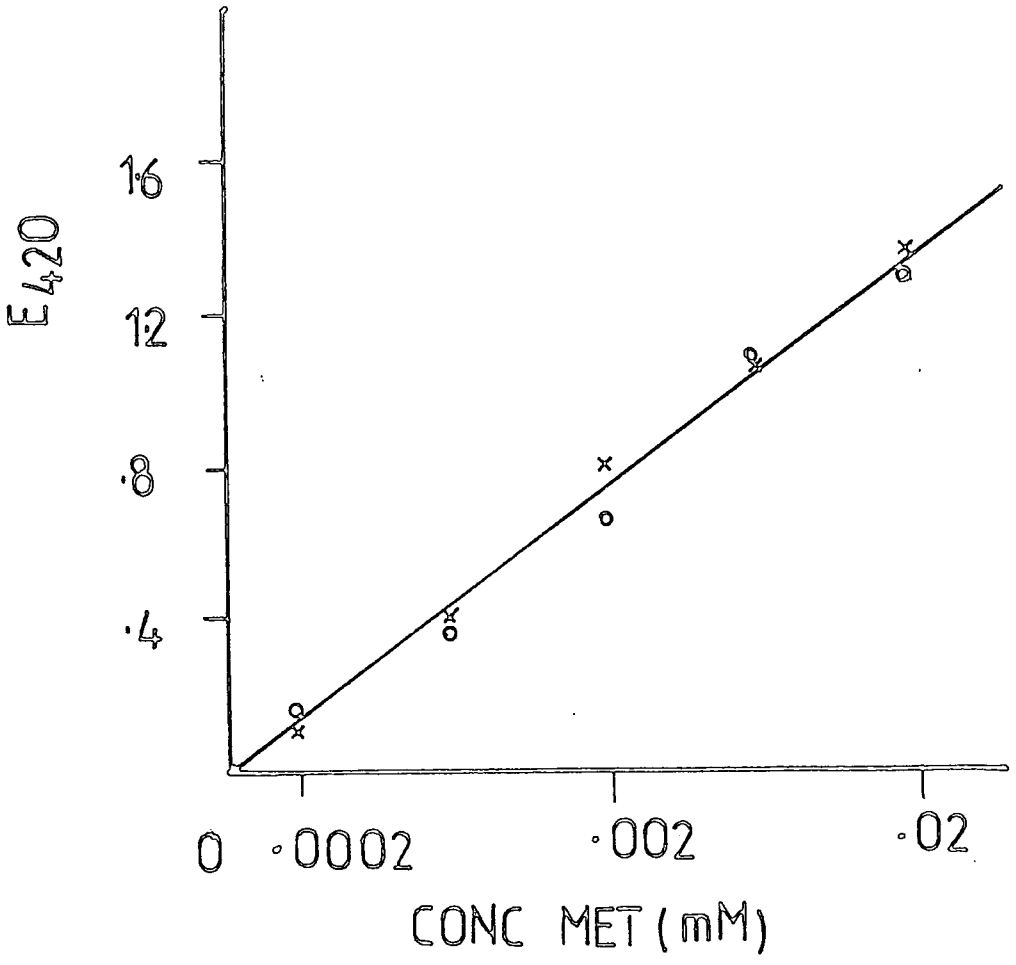


FIGURE 6.27 Response Of Two Methionine Auxotrophs To Met.

Response of strains 8134 (10799), Met^- o and PAO111, Lys^-Met^- x. Cells were grown in Met and starved for 60 min to remove response to endogenous Met and Met was assayed by the normal method (section3-4).

See also Fig. 6.10.

Thus, the normal starvation time is inadequate for B_{12} and high blanks will arise. Starvation periods of two hours or more, although adequate, would prolong the total assay time which is undesirable. Therefore, it was decided that even when assaying for B_{12}, cells would be grown on Met and starved for sixty minutes. However, the kinetics of enzyme synthesis in response to B_{12} was different with cells subject to prior cultivation in Met. After 90 minute protein synthesis there was only about 75% of the yield from a given B_{12} concentration as compared with cells grown in B_{12} and starved for 2 hours (Fig. 6.28). Therefore, it becomes unavoidable that the total assay period must be extended and so, arbitarily, to maintain sensitivity of the B_{12} assay, protein synthesis times were increased from 90 to 120 minutes.

6-8-3 Applications of B_{12} Assay

These studies showed that the mutants could be used to assay vitamin B_{12} by a modification of the standard method. In certain materials however, Met and B_{12} may both be present and whether either could be assayed individually in such samples would depend on their relative and absolute amounts taking into account the relative sensitivity of each assay. Thus, 0.1 ml of .002mM Met is sufficient to give a detectable enzyme yield whereas 5×10^{-14} M B_{12} will elicit a similar response (i.e., the assay is 2.5×10^{7} times more sensitive to B_{12} than it is to Met).

Preliminary studies arrived at applying the procedure to the assay of B_{12} in sera are described in the next Section (6-9).

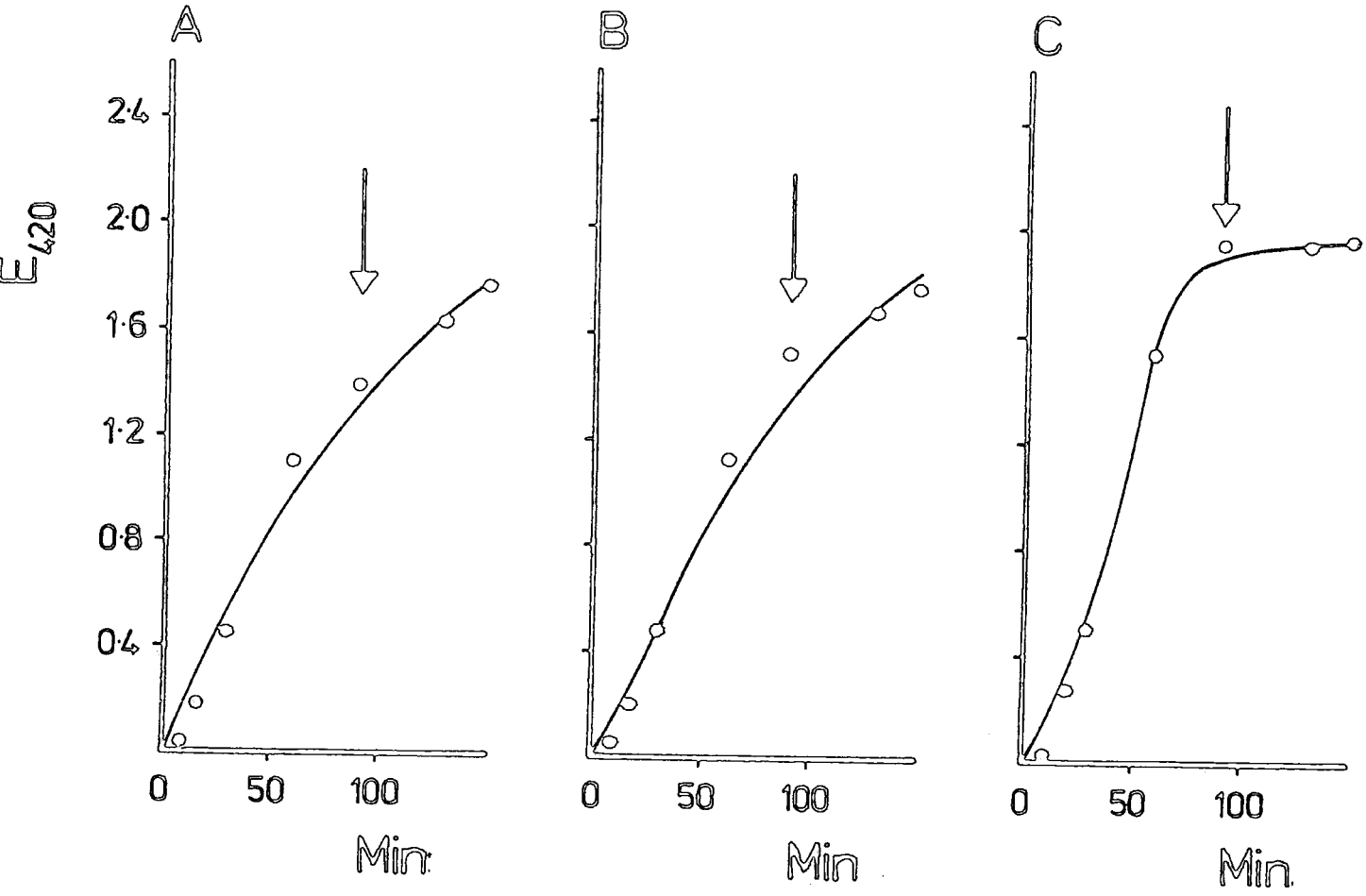


FIGURE 6.28 Kinetics Of Synthesis Of β-galactosidase In Response To B_{12} Using Cells Previously Grown In Met(A & B) Or In B_{12} (C).

Enzyme was assayed by standard procedure (section3-4). Arrow indicates 90min time . Graphs show that when cells were grown up in Met, to remove the requirement for long starvation periods, it is necessary to increase the time for protein synthesis from 90 to 120 min.

Strains used: (A) 8134, Met^-,

(B) PAO111 Lys^-Met^-

(C) PAO111Lys^-Met^-

6-9 Assay of Serum Vitamin B_{12}

6-9-1 Introduction

Vitamin B_{12} was first isolated in 1948 as cyanocobalamin, it is now thought (Matthews, 1979), that only very small amounts of this compound are found in the body, and that human serum contains several different cobalamins, the main ones being methyl- and hydroxy- forms.

Early attempts to assay blood sera for B_{12} levels involved the use of micro-organisms with a requirement for B_{12} for growth. Initial assay results were unreliable because of interference from other compounds in the sera. The various organisms used differ in their sensitivity and specificities to cobalamins, thus, to obtain specific and quantitative results, it is essential that the assay and sample treatment selected are capable of yielding the information required.

Vitamin B_{12} and many B_{12} analogues are wholly or partially bound to protein in sera. In the human stomach free vitamin B_{12} attaches itself to intrinsic factor-a mucoprotein. In many complexes the B_{12} is not available to micro-organisms and pretreatment by heating with cyanide under acid conditions is often used to release bound B_{12} and convert other compounds to cyanocobalamin (Girdwood, 1963). OH-cobalamin is readily inactivated by heat but in the CN- form it is heat stable.

Detailed accounts of microbiological assays for B_{12} are found in reviews by Hutner et al., (1958) and by Shaw & Bessel (1960).

The protozoan Ochromonas malhamensis is one of the most specific organisms known and has been used to measure B_{12} in crude extracts and in the presence of "B_{12}-like compounds", growth being estimated after 24 or 72 hours by turbidity (Ford, 1953). However, it is relatively insensitive.

Mutants of Escherichia coli, (113-3, (NCIB 8134), Davis & Mingioli, 1950), have been used to assay B_{12} by plate disc assay or by tube assay in which response to B_{12} was measured turbidometrically (Johansson, 1953). However, the organism is not specific for B_{12} in that it does not require the nucleotide portion in order to give a response (Shaw & Bessell, 1960), and problems caused by its dual response to Met as well as B_{12} have been discussed.

Lactobacillus leichmanii has been used to determine serum B_{12} levels, B_{12}-dependent growth is measured either turbidometrically after 24 hours, by acid production after 3 days incubation (Spray, 1955), or after 5 hours using a particle counter (Stuart & Sklaroff, 1966). However, the complex growth requirements, lack of sensitivity and specificity of these organisms make B_{12} determination difficult. Because this organism grows at low pH values, microbial contamination during the assay period is reduced and hence the sterilization step can be omitted allowing the study of unbound B_{12} - free from the bound form.

Euglena gracilis (strains t, bacillaris and especially z) have been widely used. They have the advantage of sensitivity and specificity but a 7 - 10 day incubation period is required (Chiao & Peterson, 1953). The z strain responds to less than 5 $\mu\mu$ g ml^{-1}. (5 pg ml^{-1}).

There are numerous references where modifications of these assay methods are discussed, results compared, etc. One recent reference suggested that the only way to increase the accuracy of Lactobacillus assays was by the use of an autodiluter to add samples to the plate cups, (Tennant & Withey, 1972). It seems that future improvements may result from the selection of suitable assay strains rather than development of mechanical devices to replace manual operations that should be accurate anyway!

A new approach to B_{12} assay was introduced by Barakat & Edkins in 1961. This involves the use of ^{57}Co-cyanocobalamin for a radioisotope dilution assay (RID). The principle of these assays is that serum B_{12} is extracted from its complex with protein by boiling at acid pH. A known amount of $^{57}Co\text{-}B_{12}$ is then allowed to compete with the extracted B_{12} for binding to a limited amount of vit B_{12}-binding protein (Intrinsic factor, chicken serum or toadfish serum). Finally, bound and free portions of vitamin B_{12} are separated by adsorption. Measurement of the amount of $^{57}Co\text{-}B_{12}$ that is bound allows determination of the degree of radioisotope dilution and hence the amount of serum B_{12} present, by reference to a curve constructed from a series of standards.

Many papers comparing RID and microbiological assays for B_{12} have appeared (Raven et al., 1972; Green et al., 1974). RID assays give a higher range of values for normal individuals than do microbiological assays and numerous modifications have been published.

The main problems seem to be: (a) the relative non-specificity of the binder used; (b) the possible presence in serum of biologically inactive analogues which may bind to protein; (c) the cost of the assay - which four years ago was in excess of £1.00 per test (Dr. Qureshni - personal communication); (d) the time required to carry out a batch of RID assays is fairly long, (one complete working day).

It is apparent, therefore, that these assays have disadvantages and there is a general reluctance within the medical profession to use them (Dr. Qureshni - personal communication).

An anonymous contributor to Nutritional Reviews (1979), stated "Such (RID) assays have been commonly employed for several years and yet only now are their pitfalls, and the biochemical basis thereof, becoming truly understood".

Apparently therefore, there is still the need to improve B_{12} assay and because the mutant strain PAO111 responded satisfactorily to both B_{12} and to Met, a preliminary investigation was carried out to determine if the β-galactosidase procedure could be used to assay serum B_{12} levels. Selective acid or alkaline degredation of B_{12} in sera was not feasible (see Section 6.10), nor were initial attempts at genetic manipulation to obtain a strain responding only to one of these alternate growth requirements.

6-9-2 Materials

Crystalline B_{12} (Cyanocobalamin), was supplied by Sigma Ltd. Therapeutic doses of B_{12} (250 μg ampoules), Cytamen, produced by Glaxo were kindly supplied by Dr. Qureshni, Haematologist, Freeman Hospital, Newcastle Upon Tyne; as were a variety of human sera that had been assayed using the RID method.

6-9-3 Methods and Results

Dilutions of the Cytamen standard were made and their activity compared with the normal standard prepared from crystalline B_{12}. At the appropriate, equivalent dilutions they both had the same activity in the β -galactosidase assay; indicating that there were no interfering compounds in the B_{12} standard.

By taking the average range of B_{12} (500 pg ml^{-1}, 0.36889 x 10^{-9}M) and Met (3.4 μg ml^{-1}, 0.228 x 10^{-7}M) levels in normal sera, it can be calculated that when such a sera is assayed at $^{1}/_{100}$ dilution using strain PA0111 about 80% of the enzyme response will arise from Met and 20% from the B_{12}. However, because both can vary independently in sera it is theoretically impossible to measure each alone.

In the preliminary study, six human sera were supplied; these had been assayed for B_{12} using a RID method but their Met levels were unknown. When they were each diluted $^{1}/_{1000}$ and known amounts of B_{12} were added to each there was a good quantitative response to these incremental values of B_{12} (90 - 95%), in all but one case. This sample was subsequently assayed at $^{1}/_{10}$ and $^{1}/_{100}$ dilution but no enzyme response was obtained; the sample clearly contained an inhibitor, possibly the patient had been on antibiotic or drug therapy that inhibited enzyme production.

One sample, later found to have a very high B_{12} content, (RID result), was assayed at a dilution of $^{1}/_{1000}$ and a linear response was obtained over three concentrations. On the assumption that the response arose from B_{12} alone, the value derived from the β -galactosidase assay was very close to the hospital value.

It seems likely that because the sample was high in B_{12}, its free Met content had been diluted out below the sensitivity of the assay for Met and therefore, the response was essentially that of B_{12} - but without actual values for Met in the sample it was impossible to confirm this speculation.

Because most of the B_{12} in sera is bound, the vitamin was first extracted: 1 ml of sera was boiled with 1 ml HCl in the presence of 20 mg L^{-1} KCN to release bound B_{12} and convert all cobalamins to cyanocobalamin. Solutions were then neutralised with known volumes of pH 10.0 buffer, prior to assay.

6-9-4 Conclusion

Further work needs to be carried out to determine whether the β -galactosidase assay of serum vitamin B_{12} is feasible. The method does have certain inherent advantages over other microbiological assays in that the sensitivity is greatly increased over growth methods allowing higher dilutions to be employed. Another important advantage that Escherichia coli has over certain other proteolytic B_{12}-assay strains is that it will not liberate bound vitamin. This capacity, which varies in different organisms, may mean that part, but not all, of the bound vitamin may be liberated. This will not occur when Escherichia coli is the assay organism. The extraction procedure described can be carefully controlled.

The sensitivity of the β -galactosidase assay for B_{12} warrants further study. Problems in assaying either Met or B_{12} alone when they are both present has been discussed (Section 6-8-2).

In the following Section (6-10), work is described in which selective degradation of either Met or B_{12} is examined. Following this, another approach, also described, is the genetic manipulation of strain PA0111 to obtain an assay organism that would respond solely to B_{12}.

6-10 Attempt To Devise A Selective Assay For Either Met Or B_{12}

6-10-1 Introduction

Strain PA0111, and each of the other three Met auxotrophs isolated originally, responded to both B_{12} and to Met. Therefore, although the biological distribution of B_{12} is limited, in samples such as mammalian body fluids, where both compounds may be present in various relative concentrations, results of assays for Met would be impossible to interpret (Section 6-9), consequently, a variety of methods were investigated with the aim of selectively destroying the biological activity of either Met or B_{12} in various test samples.

6-10-2 Selective Degradation of Met or B_{12} In Blood Sera

Performic acid is a strong oxidising agent which will convert Met to methionine sulphone (Met (O_2)). This compound will not replace Met for growth or enzyme synthesis in strain PA0111. For descriptions of preparation methods for performic acid oxidation of Met see Section 5-2. Met and B_{12} were subjected to performic acid oxidation to determine their relative stability.

In a series of experiments, it was found that 10^{-4}M was the minimum concentration of performic acid able to destroy the activity of a 10^{-11}M solution of B_{12} (0.4 ml of 10^{-4}M performic acid added to 4.6 ml 10^{-11}M B_{12} reacted at 0°C for 90 minutes) (Fig. 6.29). However, this treatment caused partial oxidation of a 0.004mM solution of Met. The maximum concentration of performic acid that did not lower Met activity under these conditions was 10^{-8}M (Fig.6.30).

Furthermore, when 10^{-4}M performic acid-treated samples were subsequently diluted and used for β-galactosidase assays, carry over of the oxidising agent affected the enzyme assay.

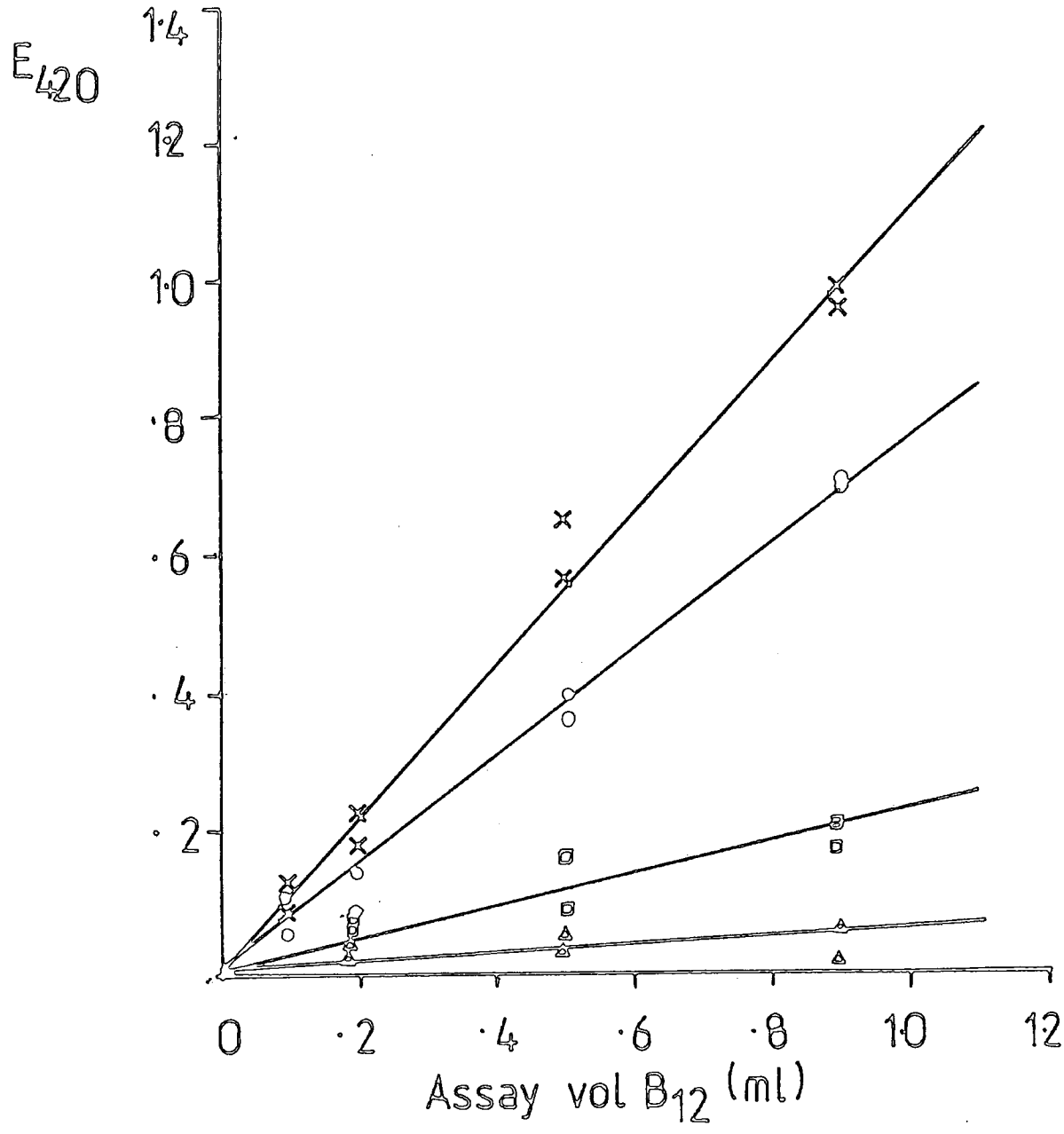


FIGURE 6.29 Effect Of Performic Acid On Loss Of Activity Of Vitamin B_{12} in β-galactosidase Assay.

Volumes of B_{12} were assayed; untreated ✕—✕, 0.4ml performic acid at various concentration was added to 4.6 ml 10^{-11}M B_{12} solution, reacted for 90 min at room temp (22°C), neutralised and assayed by standard method. Performic acid concentrations used were 10^{-4}M △—△, 10^{-6}M ○—○ ,and 10^{-8}M □—□ .

All solutions were diluted 1/1000 after treatment and prior to assay.

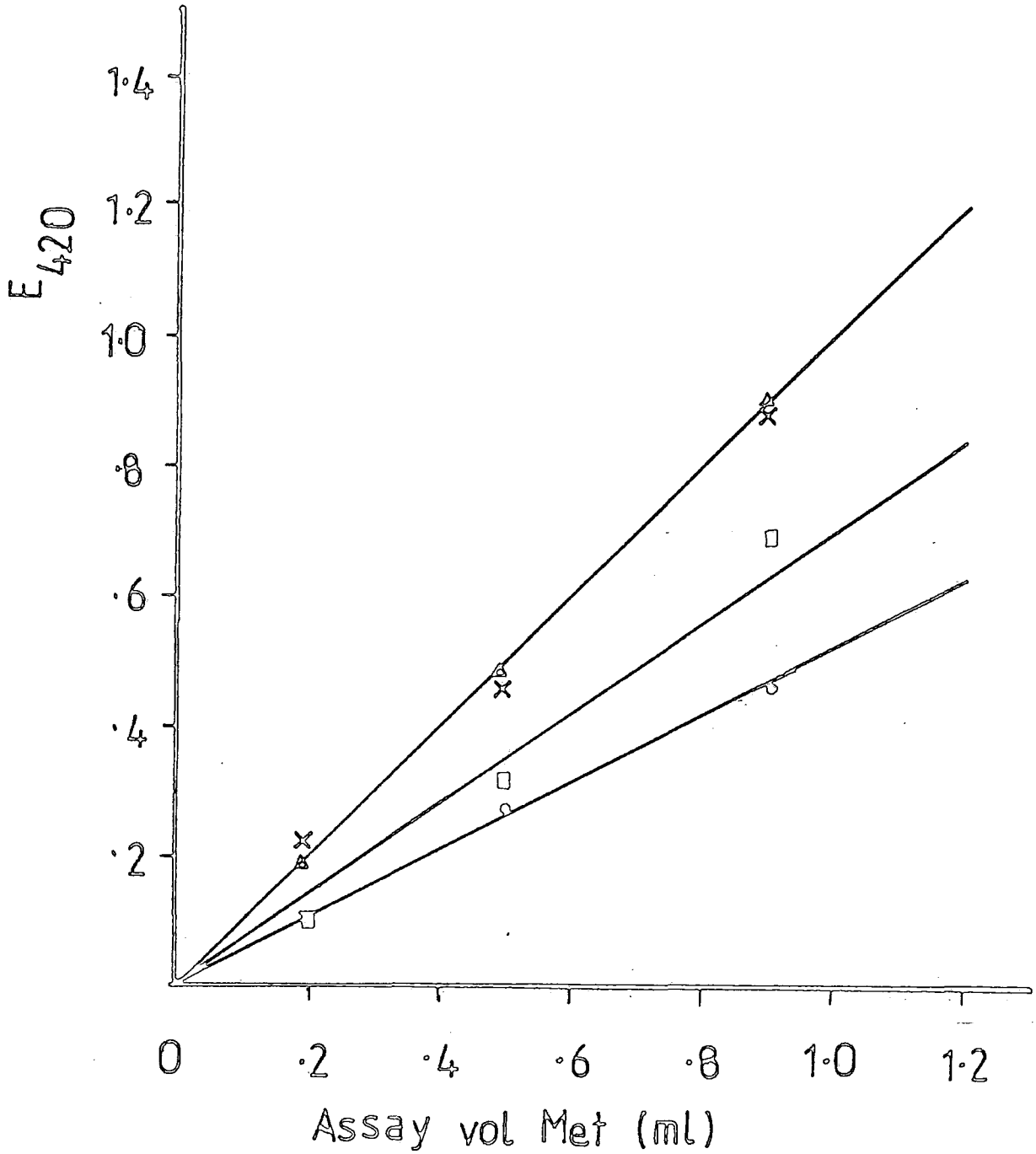


FIGURE 6.30 Effect Of Performic Acid On Loss Of Activity Of Met In β-galactosidase Assay.

Volumes of Met (4.6ml, 4nM) untreated ✕—✕ , and reacted with 0.4ml performic acid at stated concentrations at room temperature (20-22°C) were neutralised and assayed by standard procedure (section 3-4). Performic acid concentrations used were: 10^{-4}M ○—○, 10^{-5}M □—□, and 10^{-8}M △—△. All assays were carried out in duplicate but for clarity mean values only are shown.

This approach was, therefore, not pursued, but alkaline degradation of B_{12} was investigated.

B_{12} is known to be labile in the presence of alkalis (Sebbrell & Harris, 1954). 0.015M NaOH is quoted as causing a 90% decrease in vitamin B_{12} at room temperature in 23 hours. Therefore, to try and selectively destroy B_{12}, 1 ml of 1M NaOH was reacted in duplicate with B_{12} solution (4.0 ml), at 10^{-6}, 10^{-7} and 10^{-8}M. One duplicate was incubated at 37°C for 90 minutes, the other at room temperature (20°C), for 90 minutes. Solutions were then diluted for β -galactosidase assay; when diluted, the three had pH values of 7.0, 8.0 and 9.0 respectively. Results showed that by using the highest alkali concentration it was possible to degrade selectively and completely vitamin B_{12} at room temperature (i.e., zero response in β -galactosidase assay). At a reaction pH of 13.0 the time of contact with alkali could be decreased to 30 minutes at 37°C. Under the conditions described, Met was stable (Fig. 6.31). Potentially, therefore, this procedure could be applied to test samples whose composition approximated to that of the controls. However, when blood sera containing Met and B_{12} were treated in this way, there was precipitation of blood proteins which interfered with the results. Furthermore, the levels of alkali that were necessary to destroy known amounts if B_{12} added to sera of patients with pernicious anaemia (known low B_{12} levels), were such that on dilution for enzyme assay there was unacceptable enhancement of the colour of the liberated σ -nitrophenol.

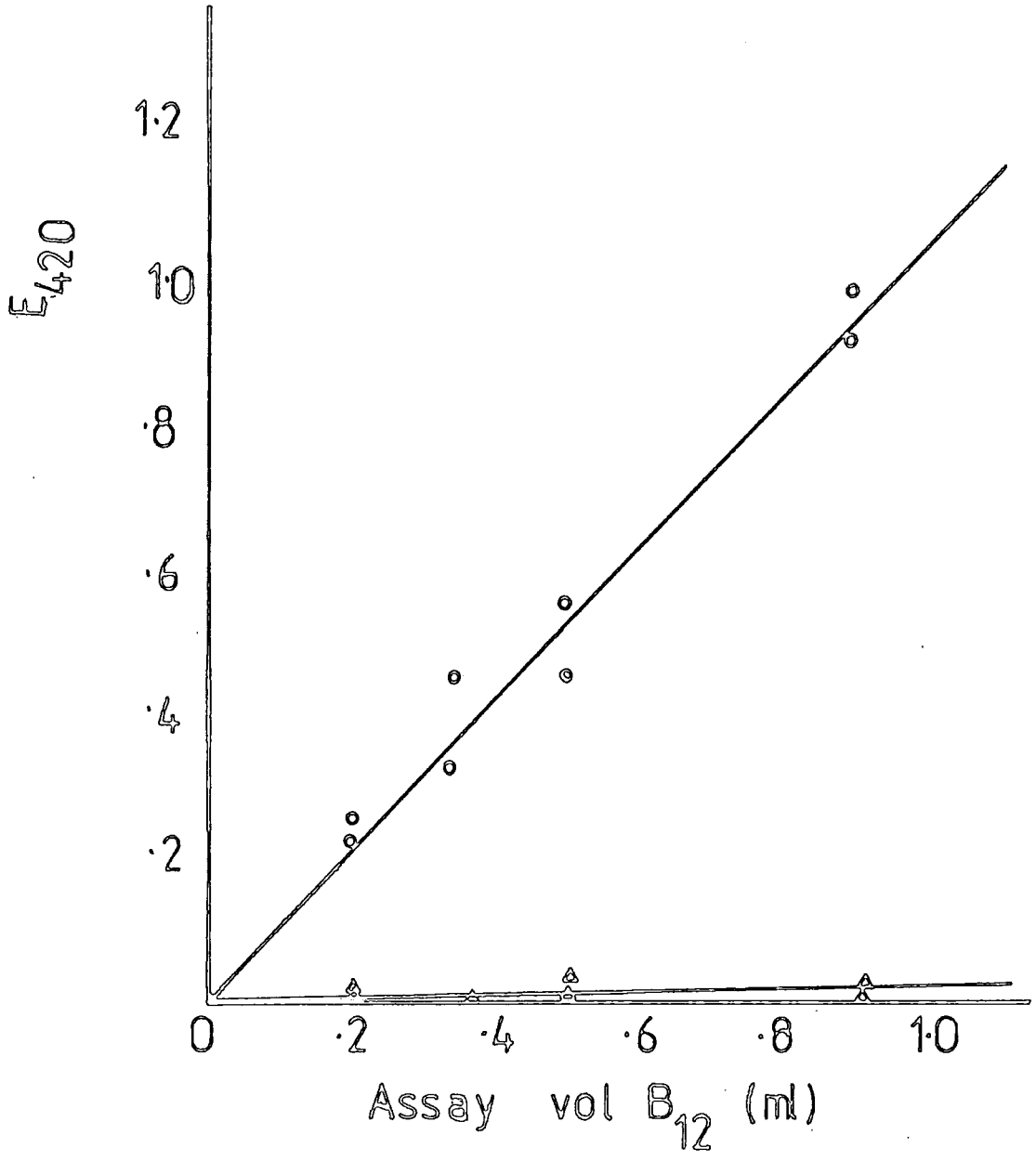


FIGURE 6.31 Assay Of B_{12} After Treatment With Sodium Hydroxide.

1 ml 1M NaOH was reacted with 4.0 ml 10^{-8}M B_{12} at room temperature (20-22°C), for 90 min. The solution was then diluted to give a 10^{-12}M solution (if there had been no destruction of the B_{12} activity this would be ca.10^{3} times too concentrated to assay)- and the enzyme response was assayed in the normal way (section 3-4). Treated B_{12} △—△ , Untreated control - assayed at 10^{-15}M ◎—◎. 4 mM Met treated in the same way and assayed after dilution lost none of its activity (data not shown - see text for details)

It was concluded, therefore, that the selective degradation of B_{12} worked out for control solutions was not applicable to blood samples, although it might be possible to assay for Met after destruction of B_{12} in other material of lower protein content, provided the alkali was neutralised prior to the enzyme assay. Thus, an alternative approach was investigated.

6-10-3 Genetic Manipulation To Obtain Assay Strain

An alternative to trying to selectively degrade either Met or B_{12} is to try and obtain a strain that has lost the ability to respond to one or other of these nutrients. One way in which this might occur is in a transport mutant that fails to take up the nutrient. It is known that the gene (ton B), which is associated with vitamin B_{12} uptake and utilisation (Kadner & Bassford, 1978; Kadner & McElhaney, 1978), maps near the opp (oligopeptide permease), at 27 minutes on the Escherichia coli chromosome (Bachmann & Brooks-Low, 1980). Therefore, an attempt was made to isolate an opp$^-$ strain from PAO111 and to test whether its response to the vitamin was lost.

10^8 cells of PAO111 grown to exponential phase were spread on plates containing Lys & Met (0.5mM), and allowed to dry for 15 minutes at 37°C. Triornithine (Orn_3), at an inhibitory concentration 10 μ L, 10mM (Payne, 1968), was spread onto the centre of each plate and after incubation overnight at 37°C, each plate showed confluent growth with a central region of inhibition around the Orn_3, that contained many small colonies. The surface only of each of 34 of these colonies was removed (to avoid background growth), and they were each resuspended in 0.25 ml sterile distilled water.

0.01 ml samples from each were then plated out, in triplicate, onto Lys plus Met or Lys plus B_{12} plates (0.5mM amino acids and 10^{-10}M B_{12}), i.e., minimum concentrations that will support good growth on plates. All of the triornithine resistant colonies grew on this concentration of B_{12}, indicating that the mutation had presumably not affected the ton B locus and the cells, therefore, were able to utilise B_{12}.

Subsequently, an attempt was made to isolate a strain that responded to Met but not to B_{12} by two Ampicillin enrichments (200 μ g ml^{-1}), in the presence of B_{12} (minimum concentration that would support growth in tubes 10^{-12}M), and imtermediate growth in Met each time (Section 4-2-2). Treated cells were plated onto Lys plus Met (0.2mM), plates and replicated onto B_{12} (10^{-10}M). The experiment was repeated several times but no colonies responding to Met but not B_{12} were isolated.

The uptake of vitamin B_{12} is complex and various components are involved, also the β -galactosidase assay is very sensitive (0.1 ml of 10^{-14}M B_{12} can be detected). In retrospect, it seems likely that even if the main uptake sites were impaired, sufficient molecules of the vitamin might still enter the cell to elicit a response. Therefore, as an alternative it was decided to try and obtain a mutant that had selectively lost the ability to respond to Met.

6-10-4 Met Uptake In *Escherichia coli*

The uptake of Met is fairly well documented. There are thought to be two uptake systems; the Met D locus codes for the high affinity L-Met transport system (K_m = 0.13 μ M), this lies between the ton A and pro A loci and is also responsible for the transport of D-Met and N-acetyl Met (Kadner & Watson, 1974).

The low affinity transport site (Met P), has a K_m of 20 μM and, as yet, is not mapped (Kadner, 1974, 1977).

Cells with a mutation in Met D no longer grow on D-Met or N-acetyl methionine as a Met source and the high affinity uptake of L-Met is lost. However, although L-Met is a potent inhibitor of D-Met uptake, D-Met has little or no effect on the uptake of the L isomer. Competition between L and D Met indicates that there are two separate binding sites. The D-Met site also appears to be involved in the binding of methionine sulphoxide, methionine sulphoximine and N-acetyl methionine. Cells defective in the Met D site frequently show analogue-resistant properties - being resistant to 50 μg Met sulphoximine ml^{-1} and 500 μg α-methyl Met ml^{-1}.

The aim, therefore, was to select a mutant defective in both the Met D and Met P sites, which would not respond to Met but the response to B_{12} would, hopefully, be intact.

6-10-5 Methods and Results

During selection for transport-deficient mutants, the minimum concentration of L-Met, D-Met and B_{12}, which support good growth, were used. These were for L and D-Met in tubes 10^{-5}M and 2×10^{-5}M in plates and for B_{12} 10^{-12}M and 10^{-10}M for tubes and plates respectively. It was demonstrated prior to replicating the mutants that when replicating from a plate containing 10^{-10}M B_{12} to a minimal plate any carry-over of B_{12} was insufficient to support visible growth of the auxotrophs.

The selection procedure for strains able to respond to B_{12} but not to D or L-Met was carried out in two parts.

First, cells were mutagenised with nitrous acid as described previously (Section 4-2-2), with two Ampicillin enrichments and the cells were selected for their inability to grow on D-Met by replica plating. Cells were grown before, and between enrichments in L-Met (so that cells unable to utilise the D isomer grew), and they were selected by incubation in 0.01mM D-Met for three hours, before enriching with Ampicillin (200 $\mu g\ ml^{-1}$).

After two cycles of enrichment and selection, cells were plated onto agar containing 10^{-10}M B_{12} and replicated onto plates containing 2×10^{-5}M D-Met. Several presumptive colonies, unable to grow on D-Met were obtained. However, some of these grew when transferred from the master plate onto fresh D-Met plates. Other colonies grew very slightly on this concentration of D-Met. However, when approximately 10^8 cells, regrown from each of these latter colonies were added to separate tubes containing A medium, Lys (1mM), and B_{12} (10^{-10}M), together with inhibitors (Met sulphoximine, 50 $\mu g\ ml^{-1}$, α -Methyl Met, 500 $\mu g\ ml^{-1}$), growth occured in each case, suggesting that the cells were inhibitor resistant.

Further plates were prepared containing A medium, Lys and B_{12}, as above and after thorough drying, these were spread with approximately 10^8 cells from the colonies under test. After 45 minutes, to allow thorough drying of the inoculated plates, 5 μL spots of inhibitors (500 and 100 μg Met sulphoximine ml^{-1} and 5000 and 1000 μg α-methyl Met ml^{-1}), were added to the plates. After 24 hours incubation at 37°C, zones of inhibition were evident around the methionine sulphoximine (highest concentration only), spot. The experiment was repeated but using this inhibitor only at a concentration of 500 μL of 500 $\mu g\ ml^{-1}$ spread onto the surface of the plate.

After incubation, inhibitor-resistant colonies were seen. These were picked from the plate, purified (since the inhibitor is bacteriostatic and background cells were picked up), and its growth on D-and L-Met rechecked. Tests were also carried out to ensure that the sulphoximine-resistant cells were not deficient in the glutamine uptake site.

After repeating the tests several colonies were isolated, resistant to α-methyl Met and Met sulphoximine at the concentrations described. However, none showed complete lack of growth on D or L Met. When they were used for β -galactosidase assays they showed a response (enzyme yield), to Met equivalent to that of the parent strain. This indicated that although in these mutants their Met D uptake sites may have become defective, sufficient Met could still be accumulated to allow the enzyme to be synthesised. Further attempts to isolate the desired mutants were made on two separate batches of mutagenised cells without success.

6-10-6 Conclusions

It proved to be impossible using simple selection procedures to isolate a mutant strain that utilised B_{12} for its auxotrophic requirement whilst being unable to utilise Met.

The strain required would have to carry multiple mutations since Met uptake is controlled by genes in at least two distinct loci. There is also tentative evidence that other uptake routes may be used, for example, Met can enter via Leu permease. Since a specific selection procedure was unavailable, this work was discontinued.

No further attempt was made to try and isolate the opposite mutation (response to Met but not B_{12} Section 6-10-3), since the sensitivity of the enzyme assay to B_{12} is such that the uptake of B_{12} is unlikely to be adequately blocked (Di Masi et al., 1973; Kadner, 1978).

However, this double response does lead to certain limitations in the use of the strain PA0111 to assay Met in particular circumstances. These problems were discussed in Sections 6-8 and 6-9.

6-11 General Conclusions

A strain of Escherichia coli, (M2626), auxotrophic for Lys has been mutagenised and from this two mutants, each with a double auxotrophic requirement have been isolated. One strain (PA0110), is a double mutant auxotrophic for Lys and Trp. The other (strain PA0111), is auxotrophic for Lys and Met. Both strains can be used to assay for their respective amino acids by the β -galactosidase procedure. The final enzyme yield has been shown to be unaffected by concentrations of nutrients in excess of those likely to be encountered in a digest (see Section 6-1). The strain PA0111 has the potential disadvantage that it responds both to Met and to B_{12} and thus, if both substances occur in a sample, interpretation of the results is not at present possible.

CHAPTER SEVEN

CONCLUDING DISCUSSION

Concluding Discussion

7-1 Introduction

There is a frequent need to assess the nutritional quality of a protein. This value being dependent on the quantity, availability and proportions of the essential amino acids comprising it, and for optimum utilisation, the presence of sufficient non-essential amino acids. Total protein is assayed chemically, usually by the Kjeldahl procedure, but quality can be assayed by using a variety of chemical or bioassay methods.

The advantages and disadvantages of many of the commonly used methods were discussed in Chapter 1. Biological methods are inherently slow, imprecise, labour intensive, subject to many uncontrollable variables and expensive; thus there are potential advantages in alternative microbiological methods.

A problem that is always likely to be present with any protein assay is its value as a predictor of nutritional value for a particular species. Thus some might argue that a microbiological assay would not predict the value of a food source for say the rat, chick or for humans.

However, similarities between the amino acid and peptide transport systems of the intestinal bacterium Escherichia coli and those of the mammalian gut (Section 3-1-1), recommend this organism as a tool for assaying nutritional value. Results described for the assay of heat-treated Lys samples (Section 6-3), show a response is obtained only to "available" residues as would be found for animals.

7-2 The Use Of Escherichia coli As An Assay Organism

Various 'natural' amino acid auxotrophs have been used for amino acid assay and these are discussed in Chapter 1; (Sections 1-5-2-1 and 1-5-2-2); on balance Escherichia coli has a number of advantages for amino acid assays.

There has, however, been a general reluctance for scientists engaged in biologically related amino acid analysis, e.g., in quality control, to use microbiological assays. In part, this arises because of problems associated with using unfamiliar organisms, such as the protozoan, Tetrahymena. Other difficulties arise from the complexities of the growth media required, e.g., with the Lactobacilliaceae; and the variations in response to a given amino acid arising from changes in media conposition.

Many of these difficulties are removed if Escherichia coli is used as the test organism. It is easy to grow on simple media, non-pathogenic, much of its metabolism is understood and perhaps most importantly, it is readily subjected to genetic manipulation. Hence, characteristics can be introduced to tailor it as a specific assay organism.

In this thesis assays for Lys, Met and Trp were described but given the ease of genetic manipulation and the detailed knowledge of its linkage-map and metabolism it is possible to select other amino acid auxotrophs of interest e.g., Thr and Ile. Ideally, the strain should have a block in the last enzyme of the particular amino acid biosynthetic pathway so no metabolic precursors could substitute.

For a given amount of the required amino acid, the extent to which it is catabolised will influence the level of protein synthesis. Hence, in an auxotroph specific degradative enzymes for the amino acid may be repressed and thus catabolism maintained at a minimum. For example, it may be advantageous to construct an auxotroph with an additional mutation in the first step of, say, the Lys catabolic pathway (Saccharopine dehydrogenase); provided that such catabolism is not required to produce an essential metabolite.

Other mutations could be introduced into the test strain; for example, antibiotic resistance: if the strain were resistant to, say, tetracyclines or chloramphenicol then e.g. the initial digestion of test sample, amino acid starvation, and the amino acid-dependent protein synthesis could usefully be carried out in the presence of the relevant antibiotic. Hence, incubation times could be increased without danger of contamination.

Ideally, the assay strain must be stable and deletion mutations are therefore ideal. However, point mutations, for example to amino acid auxotrophy, have typical reversion rates of 1 in 5×10^7; at this frequency there are no problems for use as a test organism provided the cultures are maintained in the presence of excess amino acid.

7-3 Choice Of Assay Enzyme

The advantages of measuring amino acid-dependent enzyme synthesis rather than growth have been discussed (Section 3-3). The inducible enzyme β-galactosidase was selected for this study and it was found (Fig. 4.2), that the relative sensitivity of the assay to Lys, Met and Trp was inversely proportional to the distribution of these amino acids in the β-galactosidase molecule.

The ratios of Lys : Met : Trp residues in β-galactosidase are 29 : 24 : 35 (1 : 0.83 : 1.21), (Table 4.1). Hence, for a given number of residues of, say Trp, in a test protein there will be fewer β-galactosidase molecules synthesised than from an equal number of Lys or Met residues, since the enzyme contains more Trp residues. This assumes that identical proportions of each amino acid supplied are used to synthesise the enzyme.

In Figure 4.2 the ratio of the slopes (A) for Met : Lys are 2.1 : 1.7 = 1.24 : 1 and the ratio of Lys : Met residues in the enzyme are 29 : 24 = 1.25 : 1. Similarly, ratios of the slopes (B) for Lys : Trp are 1.75 : 1.37 = 1.3 : 1 and the ratio of these residues in the enzyme molecule are 35 : 29 = 1.21 : 1.

Seemingly, therefore a similar proportion of each amino acid is used to synthesise the enzyme.

How closely this relationship between the sensitivity of the assay and the concentration of that amino acid in the β-galactosidase molecule would hold for other amino acids is not known, but it might reasonably be expected to be a fairly general observation.

Furthermore, the response to a given amount of Lys, Met or Trp in free form or as small peptides, supplied alone or with a complex mutrient mixture, is constant.

Thus, eliminating the need for complete protein digestion and allowing assay of typical biological samples e.g. rices, meals and similar samples high in carbohydrate or fats, etc.

Other enzymes could be monitored which may lead to increased sensitivity. It has already been demonstrated that amino acid dependent synthesis of derepressible alkaline phosphatase can be carried out, the enzyme being assayed with σ-nitrophenol phosphate as substrate (J.W. Payne - personal communication). The sensitivity of the assay could be increased by the use of fluorogenic, rather than chromogenic substrates.

Radio-active incorporation methods (Section 1-5-3-2), have a sensitivity comparable with those based on enzymic assays. Extensive investigation followed by suitable collaborative trials would be necessary to assess the value of these alternatives.

7-4 Automation

If an assay method is to gain acceptance as a routine screening procedure it must not only be reliable, specific and sensitive but the technique should be as rapid and simple as possible. The procedure described in this thesis has the advantage that it can in principle be automated, thus allowing a steady throughput of samples. Given a supply of amino acid starved cells a digest can be assayed in under 2½ hours, hence at least two batches of sample could be analysed during one working day.

7-5 Other Applications

The emphasis herein has been upon amino acid assay and nutritional availability of feed proteins. However, amino acid levels in mammalian body fluids may be of interest in diagnosis or research. To give one example, Trp levels in human serum may be of interest to a clinician - Trp is unusual amongst amino acids in that it is transported in blood largely bound to serum albumin ((9 - 16 mg L^{-1} blood, 90% bound). Salicylates and fatty acids compete with Trp for albumin binding sites and drug-induced diabetes leads to Trp accumulation on fasting.

The range of medical applications of the β -galactosidase amino acid assay need to be considered more fully although the presence of antibiotics or other drugs in the serum might render the method inapplicable for certain patients.

7-6 Assay Of Serum B_{12}

The results of initial work indicate that the assay described could be a useful method to measure serum B_{12}. However, the problem of interference of B_{12}-dependent protein synthesis by Met-dependent protein remains. In principle, if a mutant were isolated that responded to B_{12} but not to Met the problem would be overcome providing a very sensitive assay for B_{12} Ca. 100 pg will elicit a B_{12}-dependent enzyme response (Section 6-8-3).

7-7 Summary

With the mutants described, the assay herein can be used to detect available Lys, Met or Trp in free form, as small peptides or in compound feedstuffs. The isolation of other auxotrophs would allow the method to be used for assay of other amino acids. The assay is rapid and has the potential for automation. The sensitivity is of the same order for each of the three amino acids assayed with ca. 1 -3 x 10^{-} g eliciting a detectable response. This compares very favourable with a typical tube dilution assay (Section 1-5-2-3), using Escherichia coli which would generally detect $1-2\times10^{-5}$ g .02 of amino acid.

In a feed meal, such as those described in Section 6-6, in which there is ca. 50% protein 0.5mg meal is adequate to assay for all three amino acids, in duplicate at several concentrations. Similarly in a rice sample that is say, 5 - 10% protein, less than 5.0 mg is theoretically required for similar analysis. It is of interest to note that the sensitivity of the assay is such that there would be sufficient protein in a single barley grain (weighing approximately 0.04 g), to assay for available Lys, Met and Trp by the β -galactosidase method.

STATISTICAL TREATMENT See Section 6-3-4 page 206

Having read E_{420} and E_{550} and calculated the correct values as shown in preceeding table a least squares regression analysis was used to find true values of m (gradient) and c (intercept) for each line. Hence, the % decrease in Lys could be calculated for each sample.

$$\text{Gradient (m)} = \frac{\Sigma xy - \frac{\Sigma x \, \Sigma y}{N}}{\Sigma x^2 - \frac{(\Sigma x)^2}{N}}$$

$$\text{Intercept (c)} = \frac{\Sigma x \, \Sigma xy - \Sigma y \, \Sigma x^2}{(\Sigma)^2 - N\Sigma x^2}$$

x	Unauto-claved y	10 psi x1,10m y	10 psi x2,10m y	15 psi x1,30m y	15 psi x1,40m y
0.1	0.187	0.088	0.216	-	0.070
0.1	0.200	0.217	0.203	0.123	0.195
0.3	0.715	0.553	0.394	0.528	0.369
0.3	0.641	0.480	0.500	0.457	0.491
0.5	1.208	0.918	0.934	0.573	0.636
0.5	1.176	0.922	1.022	0.793	0.709
0.7	1.398	1.393	1.424	1.097	1.006
0.7	1.412	1.363	1.279	1.090	1.094
0.9	1.722	1.693	1.722	1.429	1.372
0.9	1.962	1.832	1.736	1.310	1.439
Σx	= 5.0				
Σx^2	= 3.3				
r	0.987	0.996	0.992	0.985	0.991
R^2(%)	97.400	99.240	98.440	99.010	98.200
m	2.0125	2.04075	1.97075	1.6085	1.5830
c	0.05595	-0.0745	-0.0428	-0.0643	-0.0534
$y = mx + c$					
If x = 0					
y =	0.5595	-0.0745	-0.0428	-0.0643	-0.0534
If x = 0.9					
y =	1.8672	1.7610	1.7309	1.3830	1.3713
% Lys remaining		94.31	92.70	74.07	73.44
% Lys loss		5.69	7.37	25.93	26.56

r=Correlation coefficient. % variance explained (R^2).

APPENDIX 2 Actual results from an assay; see Section 6-3-4

TUBE	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Unautoclaved Tle assay medium 1/200	0.1	0.3	0.5	0.7	0.9																									
10 psi 10 min x 1						0.1	0.3	0.5	0.7	0.9																				
10 psi 10 min x 2											0.1	0.3	0.5	0.7	0.9															
15 psi 30 min x 1																0.1	0.3	0.5	0.7	0.9										
15 psi 45 min x 1																					0.1	0.3	0.5	0.7	0.9					
0.02 m Mys																										0.1	0.5	0.9	X	X
Corrected OD's	0.187	0.715	1.208	1.398	1.722	0.008	0.553	0.918	1.398	1.693	0.216	0.394	0.934	1.420	1.722	-	0.528	0.573	1.097	1.423	0.070	0.369	0.636	1.006	1.372	0.239	0.825	1.242	Subtracted from corrected values	
	0.200	0.641	1.176	1.412	1.963	0.217	0.480	0.992	1.363	1.822	0.203	0.500	1.072	1.279	1.736	0.123	0.457	0.713	1.020	1.310	0.195	0.491	0.709	1.094	1.439	0.389	0.978	1.320		

All volumes made up to 2 mls. All treated at 1/100 dilution then diluted as required to assay

APPENDIX 3 Calculation Of Lysine In Myoglobin Digest

Molecular weight of Myoglobin = 17,816
$= 1.7816 \times 10^4$

Conc. Protein in digest = 1 mg ml^{-1}

But there are 19 moles Lys.mole $myoglobin^{-1}$,

Hence no. of nmol Lys $= \frac{10^6}{1.7816 \times 10^4} \times 19$

$= 1.066 \times 10^3$

From Fig. 6.18

O D 0.2 = 0.55 ml digest

O D 0.2 = 5.6 nmol Lys ml^{-1} (final Volume)

Hence 1 ml digest contains $\frac{5.6}{.55}$ nmol Lys = 1.02 x 10 nmol Lys

at a dilution of $\frac{1}{100}$

1 ml neat digest therefore contains 1.02×10^3 nmol

Percentage Lysine detected $= \frac{1.02 \times 10^3}{1.077 \times 10^3}$

$= 94.7\%$

APPENDIX 4 Calculation Of Methionine In Myoglobin Digest

Molecular wt Myoglobin = 17,816
$= 1.7816 \times 10^4$

Conc. Myo.in original digest = 1 mg ml^{-1}

There are 2 moles of Met per mole Myoglobin,

hence no. of nmol Met $= \frac{2 \times 10^6}{1.7816 \times 10^4}$

$= \underline{\underline{1.122 \times 10^2}}$

From Figure 6.19

O D of 0.2 = .45 ml of 0.004 mM Met
= 1.8 nmol ml^{-1}

O D of 0.2 = .205 of Myoglobin digest $(\frac{1}{10})$ dilution

Hence, .205 ml of digest (diluted for assay) contained 1.8 nmol Met

1.0 ml of neat digest therefore contained $\frac{1.8 \times 10}{.205}$ nmol Met

$= 8.78 \times 10$ nmol

Percentage Met determined $= \frac{8.78 \times 10}{1.122 \times 10^2} \times 100$

$= \underline{\underline{78.3\ \%}}$

APPENDIX 5 Worked example for calculation of result from a meal digest (GN101)

Sample	GN101
Digest	3, Assay 4
Assay Organism	PA0110, $Lys^{-}Trp^{-}$
Amino Acid assayed	Trp
Chemical Score	34 g Kg^{-1} crude protein (N x 6.25)

From assay result (plotted graphically)		Fron statistical analysis	
x	y (i.e. E_{420})	mean y	predicted y
0.4	0.087, 0.103	0.095	.0969
0.6	0.182 0.199	0.1905	.1867
0.8	0.251 0.295	0.2745	.2764

Plotting lines and also plotting calibration curve for Lys:

E_{420} = 0.2 = 0.003 µmol Lys ml^{-1} (final concentration)

E_{420} = 0.2 = 0.525 ml ($^{1}/_{10}$) GN101 Digest in 2 ml final volume.

0.525 ml GN101 (1/ 10) = 0.006 µmol Lys

= 0.876 µg Lys

0.525 ml GN101 (Neat) = 8.76 µg Lys

1 ml GN101 = 16.69 µg Lys

Since solution was 1 mg ml^{-1} then 1 mg GN101 contained 16.69 µg Lys

BUT crude protein content = 503 g 900 g^{-1} = 558.89 g Kg^{-1} (dry)

1 mg of GN101 protein contained $\frac{16.69}{.559}$ µg Lys

= 29.86 µg Lys

Therefore 1 Kg protein = 29.86 g Lys

APPENDIX 5 ctd....

But sample contained 5.99% moisture

Hence, actual protein present $= \frac{558.89}{1.0599}$

$= 527.31 \text{ g Kg}^{-1}$

1 Kg Sample contained 527.31 g protein

Lysine determined in GN101 $= \frac{16.69}{.527}$

$= \underline{31.67 \text{ g Kg}^{-1} \text{ Crude protein}}$

Using Data from least squares analysis :

E_{420} 0.2 = 0.0033 µmol ml^{-1} Lys

E_{420} 0.2 = 0.56 ml GN101

Calculation as above:

Lysine determined in GN101 = $\underline{31.96 \text{ g Kg}^{-1}}$ Crude protein

APPENDIX 6(a) Listing Of Linear Regression Program

(NOT constrained data)

```
900 DIM D(20,6)
910 M$(2)=">=80" :M$(3)=">=90": M$(4)=">=95" : M$(5)=">=98":M$(6)=">=99"
920        FOR I=4 TO 20
930          FOR J=1 TO 6
940            READ D(I,J)
950          NEXT J
960        NEXT I
1000 PRINT CHR$(147)
1100 PRINT "[CLR][DOWN][DOWN][DOWN][DOWN][DOWN]";"LINEAR REGRESSION PROGRAM"
1200 FOR I=1 TO 800:NEXT I
1300 S1=0 :S2=0: S3=0:S4=0
1350 PRINT CHR$(147)
1400 INPUT "ENTER TITLE OF DATA";T$
1500 INPUT "HOW MANY PAIRS OF POINTS";N
1600      FOR I=1 TO N
1700        PRINT "ENTER X ";I;
1800        INPUT X(I)
1900 PRINT "ENTER 1ST Y VALUE";
2000 INPUT D1
2005 PRINT "ENTER 2ND Y VALUE";
2006 INPUT D2
2008 Y(I)=(D1+D2)/2
2100 S1=S1+X(I) : S2=S2+Y(I)
2200       S3=S3+X(I)*X(I)
2300       S4=S4+X(I)*Y(I)
2400         S5=S5+Y(I)*Y(I)
2500     NEXT I
2600 REM NOW TO FIND THE SLOPE
2700 M=(N*S4-S1*S2)/(N*S3-S1*S1)
2800 REM NOW FOR THE INTERCEPT
2900 C=(S3*S2-S1*S4)/(N*S3-S1*S1)
3000 XB=S1/N : YB=S2/N
3100 DX=SQR((S3-(S1*S1)/N)/(N-1))
3200 DY=SQR((S5-(S2*S2)/N)/(N-1))
3300 R=M*DX/DY
3400 PRINT CHR$(147)
3500 PRINT T$
3600 PRINT "  X";TAB(8);"Y";TAB(14);"PRED Y";TAB(27);"DEVN"
3700 PRINT :PRINT
3800 FOR I=1 TO N
3900 PRINT X(I);TAB(6);Y(I);TAB(12); M*X(I)+C;TAB(25);
4000 Z=Y(I)-(M*X(I)+C)
4100 Z9=Z9+Z*Z
4200 REM CALCULATE RMS ERROR OF FIT
4300 PRINT Z
4400 NEXT I
4500 Z8=SQR(Z9/N)
4600 PRINT:PRINT
4700 PRINT "MEAN X:";XB;TAB(20);"MEAN Y:";YB
4800 PRINT :PRINT "X DEVN: ";DX;TAB(20);"Y DEVN: ";DY
```

ctd....

APPENDIX 6(a) ctd.....

```
4900 PRINT:PRINT "SLOPE: ";M;TAB(20);"INTERCEPT: ";C
5000 PRINT:PRINT "CORRLN COEFF: ";INT(R*1000+0.5)/1000;  ;TAB(20);
5100 PRINT "R SQUARED: "; INT(R*R*100+0.5);"%"
5200 PRINT :PRINT "RMS ERROR: ";Z8
5300 PRINT:PRINT"THE EQUATION IS:"
5400 PRINT:PRINT:PRINT TAB(10);
5500 IF M=1 THEN PRINT "Y=X";: GOTO 5700
5600 PRINT "Y=";M;" *X ";
5700 IF C>=0 THEN PRINT "+";C
5800 IF C<0 THEN PRINT " ";C
5900 PRINT:PRINT
6000 INPUT "HAVE YOU A PRINTER";P$
6100 IF LEFT$(P$,1)="Y" THEN GOSUB 6600
6150 PRINT CHR$(147)
6200 PRINT "PRESS ANY KEY TO END"
6300 GET A$:IF A$="" THEN 6300
6400 CLOSE2
6500 END
6600 REM PRINTING SUBROUTINE
6700 OPEN 2,4
6720 FOR T=1 TO 75
6740 PRINT#2,CHR$(163);
6760 NEXT T
6770 PRINT#2
6800 PRINT#2,T$:PRINT#2:PRINT#2
6900 PRINT#2, "  X";TAB(8);"Y";TAB(14);"PRED Y";TAB(27);"DEVN"
7000 PRINT#2,:PRINT#2
7100 FOR I=1 TO N
7200 PRINT#2, X(I);TAB(6);Y(I);TAB(12); M*X(I)+C;TAB(25);
7300 Z=Y(I)-(M*X(I)+C)
7400 Z9=Z9+Z*Z
7500 REM CALCULATE RMS ERROR OF FIT
7600 PRINT#2, Z
7700 NEXT I
7800 Z8=SQR(Z9/N)
7900 PRINT#2,:PRINT#2
8000 PRINT#2, "MEAN X:";XB;TAB(20);"MEAN Y:";YB
8100 PRINT#2, :PRINT#2, "X DEVN: ";DX;TAB(19);"Y DEVN: ";DY
8200 PRINT#2,:PRINT#2, "SLOPE: ";M;TAB(21);"INTERCEPT: ";C
8300 PRINT#2,:PRINT#2, "CORRLN COEFF: ";INT(R*1000+0.5)/1000;  ;TAB(20
8400 PRINT#2, "R SQUARED: "; INT(R*R*100+0.5);"%"
8500 PRINT#2, :PRINT#2, "RMS ERROR: ";Z8
8600 PRINT#2,:PRINT#2,"THE EQUATION IS:"
8700 PRINT#2, TAB(16);
8800 IF M=1 THEN PRINT#2, "Y=X";: GOTO 9000
8900 PRINT#2, "Y=";M;" *X ";
9000 IF C>=0 THEN PRINT#2, "+";C
9100 IF C<0 THEN PRINT#2, " ";C
9200 PRINT#2 :PRINT#2
9300 PRINT#2,"SIGNIFICANCE LEVEL"
```

APPENDIX 6(a) ctd...

```
9350 PRINT#2:PRINT#2
9360 GOSUB 11000
9400 PRINT#2:PRINT#2
9600 CLOSE 2
9700 RETURN
10000 REM SIGNIFICANCE ROUTINE
10500 INPUT N,R
10600 OPEN 2,4
11000 IF N<4 THEN PRINT#2, "TOO FEW POINTS FOR SIGNIFICANCE":RETURN
12000 IF N>20 THEN PRINT#2, "TOO MANY POINTS FOR ME!":RETURN
14000 IF R<D(N,1) THEN PRINT#2,"< 80% SIGNIFICANT":RETURN
15000 IF R>D(N,6) THEN PRINT#2, ">99.8% SIGNIFICANT" : RETURN
16000 FOR J=2 TO 6
17000 IF R>=D(N,J-1) AND R<=D(N,J) THEN PRINT#2,M$(J);" %SIGNIFICANT":RETURN
18000 NEXT J
19000 RETURN
20000 DATA .8,.9,.95,.98,.99,.998
21000 DATA .687,.805,.878,.934,.959,.986
22000 DATA .608,.729,.811,.882,.917,.963
23000 DATA .551,.669,.754,.833,.875,.935
24000 DATA .507,.621,.707,.789,.834,.905
25000 DATA .472,.582,.666,.750,.798,.875
26000 DATA .443,.549,.632,.715,.765,.847
27000 DATA .419,.521,.602,.685,.735,.820
28000 DATA .398,.497,.576,.658,.708,.772
29000 DATA .380,.476,.553,.634,.684,.772
30000 DATA .365,.458,.532,.612,.661,.750
31000 DATA .351,.441,.514,.592,.641,.730
32000 DATA .338,.426,.497,.574,.623,.711
33000 DATA .327,.410,.482,.558,.606,.694
34000 DATA .317,.400,.468,.543,.590,.678
35000 DATA .308,.389,.456,.529,.575,.662
36000 DATA .299,.378,.444,.516,.561,.648
```

APPENDIX 6(b) Results obtained in assay and analysis of results by linear regression. See section 6-6-2 (page 234)

X = volume of Lys assayed, Y = E_{420} reading . Program listing given in Appendix 6(a).

ASSAY 4 LYS CAL CURVE USING STRAIN PAO 110

X	Y	PRED Y	DEVN
0.2	0.215	0.25695	-0.0419500003
0.4	0.5785	0.4929	0.0855999998
0.6	0.6835	0.72885	-0.04535
0.8	0.9665	0.9648	1.6999997E -03

MEAN X:	0.5	MEAN Y:	0.610875
X DEVN:	0.25819889	Y DEVN:	0.31065
SLOPE:	1.17975	INTERCEPT:	0.021.
CORRLN COEFF:	0.981	R SQUARED:	96%
RMS ERROR:	0.0747		

THE EQUATION IS: Y = 1.17975 *X + 0.021

SIGNIFICANCE LEVEL

= 98 %SIGNIFICANT

APPENDIX 6(c) Results obtained in assay of GN 101 and statistical treatment by linear regression.

See Appendices 6(a) for programme used and 6(b) for calibration curve; further details in Section 6-6-2.

ASSAY 4 LYS GN 101 USING STRAIN PAO 110

X	Y	PRED Y	DEVN
0.4	0.0950	0.0969166663	-1.91666622E -03
0.6	0.1905	0.186666666	3.83333379E -03
0.8	0.2745	0.276416666	-1.91666622E -03

MEAN X:	0.5	MEAN Y:	0.18667
X DEVN:	0.2	Y DEVN:	0.08981
SLOPE:	0.44875	INTERCEPT:	-0.08258
CORRLN COEFF:	0.999	R SQUARED:	100%
RMS ERROR:	3.833E -03		

THE EQUATION IS: Y = 0.44875 *X -0.08258

SIGNIFICANCE LEVEL

TOO FEW POINTS FOR SIGNIFICANCE

REFERENCES

References

ADAMS, M.W. (1974)
The need for rapid assays of protein quality.
1974, J. Agri. Food Chem., 22, 556-559.

ADAMSON, D.C.M. and SIMPSON, J.S. (1959)
Microbiological assay of vitamins and amino acids.
1958, Proceedings of International Symposium on Microchemistry, Birmingham University.
1959, Pergamon Press, London.

ADRIAN, J. (1974)
Nutritional and physiological consequences of the Mailliard reaction.
1974, World Rev. Nutr. Dietet., 19, 71-122.

AHRENS, R.A., WILSON, J.E. Jnr. and WOMACK, M. (1966)
Utilization of calories and nitrogen by rats fed diets containing purified casein versus a mixture of amino acids simulating casein.
1966, J. Nutr., 88, 219-227.

AKESON, W.R. and STAHMANN, M.A.
A pepsin pancreation digest index of protein quality evaluation.
1964, J. Nutr., 83, 257-261.

ALMOG, R. and BERNS, D.S. (1981)
A sensitive assay for proteins and biliproteins.
1981, Anal. Biochem., 114, 336-341.

ANDERSON, G.H., ASHLEY, D.V.M. and JONES, J.D. (1976)
Utilization of L-methionine sulphoxide, L-methionine sulphone and cysteic acid by the weanling rat.
1976, J. Nutr., 106, 1108-1114.

ANDERSON, M.E. and WILLIAMS, H.H. (1951)
Microbiological evaluation of protein quality 1.
A colorimetric method for the determination of the growth of Tetrahymena geleii W in protein suspensions.
1951, J. Nutr., 44, 335-343.

ANON. (1979)

Pitfalls in the diagnosis of vitamin B_{12} deficiency by radiodilution assay.

1979, Nutr. Rev., 37, 313-315.

ASSOCIATION OF OFFICIAL ANALYTICAL CHEMISTS (A.O.A.C.) (1975)

Methods of Analysis, 12th edition, no. 2.046, 15-16.

AYMARD, C., SEYER, L. and CHEFTEL, J.C. (1979)

Enzymatic reduction of methionine sulphoxide. In vitro experiments with rat and liver kidney.

1979, Agric. Biol. Chem., 43, 1869-1872.

BACHMANN, B.J. and BROOKS-LOW, K. (1980)

Linkage map of Escherichia coli K-12, Edition 6.

1980, Microbiol. Rev., 44, 1-56.

BAILEY, J.E. and OLLIS, D.F.

Biochemical Engineering Fundamentals.

McGraw Hill Kogakusha Ltd., Tokyo, p.550.

BARAKAT, R.M. and EKINS, R.P. (1961)

Assay of vitamin B_{12} in blood; a simple method.

1961, Lancet, ii, 25-31.

BARTON-WRIGHT, E.C. (1972)

The microbiological assay of the essential amino acids in compound feeding stuffs.

1972, Analyst (London), 97, 138-141.

BARTON-WRIGHT, E.C. (1963)

Practical methods for the microbiological assay of vitamin B-complex and amino acids.

1963, United Trade Press, London.

BARTON-WRIGHT, E.C. (1952)

The microbiological assay of the vitamin B complex and amino acids.

1952, Pitman, London.

BASSLER, K.H., FEKL, W. and LANG, K. (1976)

Proceedings of 3rd International Symposium on Balanced Nutrition in Therapy.

1976, Springer-Verlag, Berlin, Heidelberg & New York.

BATTERHAM, E.S., MURISON, R.D. and LEWIS, C.E. (1979)
Availability of Lysine in protein concentrates as determined by slope-ratio assay techniques with growing pigs and rats, and by chemical techniques.
1979, Proc. Nutr. Soc., 41, 383-391.

BELIKOV, V.M. and BABAYAN, T.L. (1971)
Mixtures of amino acids in human nutrition.
1971, Russian Chem. Rev., 40, 441-456.

BELL, G., HIGGINS, C.F., PAYNE, G.M. and PAYNE, J.W. (1977)
Use of Escherichia coli to determine available lysine in plant proteins.
Nutritional Evaluation of Cereal Mutants.
1977, I.A.E.A., Vienna, 107-123.

BENDER, A.E. (1978)
Proteins In Food Processing and Nutrition.
1978, Academic Press, London, 59-79.

BENDER, A.E. (1973)
Chemical scores and availability of amino acids.
Proteins in Human Nutrition, J.W.G. Porter and B.A. Rolls (eds).
1973, Academic Press, London & New York, 167-178.

BENDER, A.E. (1971)
Processing damage to protein foods.
Protein Advisory Group Bulletin, Vol.13, 10-19.
1971, United Nations, New York.

BENDER, A.E. (1961)
Determinations of the nutritive value of proteins by chemical analysis.
1961, Nat. Res. Council Publication, 834, 407-424.

BENDER, A.E. (1958)
Biological methods for evaluating protein quality.
1958, Proc. Nutr. Soc., 17, 85-91.

BERGNER, H. (1978)
Protein evaluation and protein metabolism.
Biochemical Aspects of New Protein Foods. F.E.B.S. Symposium No. 44, J.A. Nissen, B.O. Eggum, L. Munck and H.S. Olsen (eds).
1978, Pergamon Press, Oxford, 149-160.

BESSELL, C.J., HARRISON, E. and LEES, K.A. (1950)
Assay of vitamin B_{12} with a mutant of Escherichia coli.
1950, Chemistry & Industry, 28, 561.

BEST, R., HOWELL, E., DECILLIS, A. and SCHRAY, S.H.
A novel method for protein quantitation using a competitive binding assay.
1982, Anal. Biochem., 119, 299-303.

BHAT, K.S. (1982)
Changes in lens proteins in under nourished and well nourished patients with cataract.
1982, Br. J. Nutr., 47, 483-488.

BIGWOOD, E.J. (1972)
Protein and Amino Acid Functions.
1972, Pergamon Press, Oxford.

BIO-RAD LABORATORIES (1979)
Bio-Rad Laboratories, California.
1979, Technical Bulletin, 78-0791.

BJARNSON, J. and CARPENTER, K.J. (1970)
Mechanism of Heat Damage in Proteins.
2. Chemical changes in pure proteins.
1970, Br. J. Nutr., 24, 313-329.

BJARNSON, J. and CARPENTER, K.J. (1969)
Mechanisms of Heat Damage to Proteins.
1. Models with acylated lysine units.
1969, Br. J. Nutr., 23, 859-868.

BLACKBURN, S. (1968)
Amino Acid Determinations.
1968, Marcel Dekker, New York.

BLOCK, R.J. and MITCHELL, H.H. (1947)
The correlation of the amino acid composition of proteins with their nutritive value.
1947, J. Sci. Food Agri., 5, 305-318.

BLOCK, R.J. and WEISS, K.W. (1956)
Amino Acid Handbook.
Methods and results of protein analysis. C.C. Thomas (ed)
1956, Springfield III.

BODWELL, C.E. (1977)
Use of enzymic assays for the determination of nutritional quality.
Nutritional Evaluation Of Cereal Mutants.
1977, I.A.E.A., Vienna, p.87-105.

BODWELL, C.E. (1976)
Evaluation Of Proteins For Humans.
1976, Avi Publishing Co., Westport, Connecticut, p.327.

BODWELL, C.E., ADKINS, J.S. and HOPKINS, D.T. (1981)
Protein Quality In Humans.
Assessment and in vitro estimation.
1981, Avi Publishing Co., Westport, Connecticut.

BOLINDER, A.E. (1972)
Large plate assays for amino acids.
Analytical Microbiology Vol.2, F. Kavanagh (ed.).
1972, Academic Press, New York & London, 479-591.

BOLINDER, A.E. (1970a)
Plate assay methods for amino acids. 9. Threonine.
1970, Acta. Pharm. Suec., 7, 47-54.

BOLINDER, A.E. (1970b)
Plate assay methods for amino acids. 10. Argenine and Histidine.
1970, Acta. Pharm. Suec., 7, 113-124.

BOLINDER, A.E. (1970c)
Plate assay methods for amino acids. 11. Further studies on Lysine.
1970, Acta. Pharm. Suec., 7, 125-132.

BOLINDER, A.E. (1970d)
Plate assay methods for amino acids. 12. Cystine.
1970, Acta. Pharm. Suec., 7, 201-208.

BOLINDER, A.E. (1970e)
Plate assay methods for amino acids.
1970, Acta Pharm. Suec., 7, 209-238.

BOLINDER, A.E. (1970f)
Purity of amino acids.
1970, Anal. Biochem., 35, 212-226.

BOLINDER, A.E. (1969)
Plate assay methods for amino acids. 8. Tryptophan.
1969, Acta. Pharm. Suec., 6, 257-270.

BOLINDER, A.E. (1968a)
Plate assay methods for amino acids. 4. Usefulness of five strains of lactic acid bacteria for the assay of six amino acids.
1968, Acta. Pharm. Suec., 5, 117-134.

BOLINDER, A.E. (1968b)
Plate assay methods for amino acids. 5. Usefulness of nine strains of lactic acid bacteria for the assay of methionine.
1968, Acta. Pharm. Suec., 5, 401-416.

BOLINDER, A.E. (1968c)
Plate assay methods for amino acids. 6. Comparative assays for methionine carried out by the plate method, the tube method and the ion-exchange column chromatographic method.
1968, Acta. Pharm. Suec., 5, 417-428.

BOOTH, V.H. (1971)
Problems in the determination of FDNB-available lysine.
1971, J. Sci. Food Agric., 22, 658-666.

BOYNE, A.W., FORD, J.E., HEWITT, D. and SHRIMPTON, D.H. (1975)
Protein quality of feeding stuffs 7. Collaborative studies on the microbiological assay of available amino acids.
1975, Br. J. Nutr., 34, 153-162.

BOYNE, A.W., PRICE, S.A., ROSEN, G.D. and STOTT, J.A. (1967)
Protein quality of feeding stuffs 4. Progress report on collaborative studies on the microbiological assay of available amino acids.
1967, Br. J. Nutr., 21, 108-206.

BOYNE, A.W., CARPENTER, K.J. and WOODHAM, A.A. (1961)
Progress report on an assessment of laboratory proceedures suggested as indicators of protein quality in feeding stuffs.
1961, J. Sci. Food Agric., 12, 832-848.

BRADFORD, M.M. (1976)
A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding.
1976, Anal. Biochem., 72, 248-254.

BRAND, E., SAIDEL, L.J., GOLDWATER, W.H., KESSEL, B, and RYAN, F.J. (1945)
The empirical formula of β-lactoglobulin.
1945, J. Am. Chem. Soc., 67, 1524-1532.

BRESSANI, R., ELIAS, L.G. and JULIANO, B.O. (1971)
Evaluation of protein quality of milled rices of differing protein content.
1971, Agric. Food Chem., 19, 1028-1034.

BRIDSON, E.Y. and BRECKER, A. (1970)
Design and formulation of microbial culture media.
Methods In Microbiology, Vol.3A. J.R. Norris & D.W. Ribbons (eds)
1970, Academic Press, London & New York, p.229-295.

BROT, N. and WEISSBACH, H. (1982)
The biochemistry of methionine sulphoxide residues in proteins.
1982, T.I.B.S., 7, 137-139.

BROT, N., WERTH, J., KOSTER, D. and WEISSBACH, H. (1982)
Reduction of N-acetyl methionine sulphoxide: A simple assay for peptide methionine sulphoxide reductase.
1982, Anal. Biochem., 122, 291-294.

BROT, N., WERTH, J. and WEISSBACH, H. (1981)
Enzymatic reduction of protein-bound methionine sulphoxide.
1981, P.N.A.S., U.S.A., 78, 2155-2158.

BRUNS, P.J., BRUSSARD, T.B. and KAVKA, A.B. (1976)
Isolation of homozygous mutants, after induced self fertilization in Tetrahymena.
1976, P.N.A.S., U.S.A., 73, 3243-3247.

BUNYAN, J. and PRICE, S.A. (1960)
Studies on protein concentrates for animal feeding.
1960, J. Sci. Food Agri., 11, 25-37.

BUNYAN, J. and WOODHAM, A.A. (1964)
Protein quality of feeding stuffs. 2. The comparative assessment of protein quality in three fish meals by microbiological and other laboratory tests, and by biological evaluation with chicks and rats.
1964, Br. J. Nutr., 18, 537-544.

CANNON, P.R., STEFFEE, C.H., FRAZIER, L.J., ROWLEY, D.A. and STEPTO, R.C. (1947)
The influence of time of ingestion of essential amino acids upon the utilization in tissue synthesis.
1947, Fed. Proc. Fed. Am. Soc. Exp. Biol., 6, 390.

CARP, H., MILLER, F., HOIDAL, J. and JANOFF, A. (1982)
Potential mechanism of emphysema: α_1,-Proteinase inhibitor recovered from lungs of cigarette smokers contains oxidised methionine and has decreased elastase inhibitory capacity.
1982, P.N.A.S., U.S.A., 79, 2041-2045.

CARPENTER, K.J. (1960)
The estimation of available lysine in animal-protein foods.
1960, Biochem. J., 77, 604-610.

CARPENTER, K.J. and BOOTH, V.H. (1973)
Damage to lysine in food processing: its measurement and significance.
1973, Nutr. Rev., 43, 424-457.

CARPENTER, K.J. and WOODHAM, A.A. (1974)
Protein quality of feeding stuffs 6. Comparison of the results of collaborative biological assays for amino acids with those of other methods.
1974, Br. J. Nutr., 32, 647-660.

CARPENTER, K.J., HARTLEY, A.W. and WARD, L.D. (1964)
Hydroxylysine as a stimulant in microbiological assays for lysine.
1964, Proc. Nutr. Soc., 23, XL-XLI.

CASCIERI, T. and MALLETTE, M.F. (1976)
Peptide utilisation by Pseudomonas putida and Pseudomonas mallophila.
1976, J. Gen. Microbiol., 92, 282-295.

CHEN, R.F., SMITH, P.D. and MALY, M. (1978)
The fluorescence of fluorescamine amino acids.
1978, Arch. Biochem. Biophys., 189, 241-250.

CHIAO, J.S. and PETERSON, W.H. (1953)
Microbiological assay of vitamin B_{12} with a mutant strain of Escherichia coli.
1953, Appl. Microbiol., 1, 42-46.

CHONG MIN LEE, TUNG-CHING, L. and CHICHESTER, C.O. (1977)
The effect of Mailliard reaction products on the absorption of Tryptophan.
1977, Comp. Biochem. Physiol., 56A, 473-476.

CHRISTENSEN, B.G. (1981)
β -Lactam Antibiotics. Salton, G. and Shockman, G.P. (eds)
1981, Academic Press, New York, p.103.

CHRISTENSEN, H.N. (1968)
Relevance of transport across the plasma membrane to the interpretation of the plasma amino acid pattern.
Protein Nutrition And Free Amino Acid Patterns. J.H. Leathen (ed)
1968, Rutgers University Press, U.S.A., 41-52.

C.I.B.A. SYMPOSIUM No.50 (1977)
Peptide transport and hydrolysis.
1977, Elsevier, Amsterdam.

C.I.B.A. SYMPOSIUM No.4 (1972)
Peptide transport in bacteria and mammalian gut.
1972, Elsevier, Amsterdam.

CLEGG, K.M. (1978)
Dietary enzymic hydrolysates of protein.
Biochemical Aspects Of New Protein Foods.
FEBS Symposium 44. J.A. Nissen, B.O. Eggum, L. Munck and H.S. Olsen (eds)
1978, Pergamon Press, Oxford, 109-118.

COHEN, J.S., YARIV, J., KALB, A.J., JACOBSON, L. and SHECHTER, Y. (1979)
^{13}C NMR analysis of methionine sulphoxide in protein.
1979, Biochem. Biophys. Methods, 1, 145-151.

COLE, D.A.J., BOORMAN, K.N., BUTTERY, P.J., LEWIS, D., NEALE, R.J. SWAN, H. (eds).
Protein Metabolism And Nutrition.
1976, Butterworth, London.

CONCON, J.M. (1975)
Chemical determination of critical amino acids in cereal grains and other foodstuffs.
Protein Nutritional Quality Of Foods And Feeds.
Part 1. Assay Methods. M. Friedman (ed)
1975, Marcel Dekker, New York, 311-380.

COOPER, K.E. (1972)
Analytical Microbiology Vol.2. F.W. Kavanagh (ed)
1972, Academic Press, New York.

COOPER, K.E. (1963)
Analytical Microbiology Vol.1. F.W. Kavanagh (ed)
1963, Academic Press, New York.

CRAVEN, G., STEERS, E. and ANFINSEN, C.B. (1965)
Purification, composition and molecular weight of the β -galactosidase of Escherichia coli K12.
1965, J. Biol. Chem., 240, 2468-2477.

CROFT, L.R. (1980)
Structure of α -chymotrypsinogen (bovine).
Handbook Of Protein Sequence Analysis.
1980, J. Wiley & Sons, Chichester, U.K., p.193.

CUQ, J.L., BESANCON, P., CHARTIER, L. and CHEFTEL, C. (1978)
Availability of methionine sulphoxide residues.
1978, Food Chem., 3, 85-102.

CUQ, J.L., PROVANSAL, M., GUILLEUX, F. and CHEFTEL, C. (1973)
Oxidation of methionine residues of casein by hydrogen peroxide.
1973, J. Food Sci., 38, 11-13.

DAS, M. and RADHAKRISHNAN, A.N. (1976)
Role of peptidases and peptide transport in the intestinal absorption of proteins.
1976, World Rev. Nutr. Diet., 24, 58-87.

DAVIS, B.D. and MINGIOLI, E.S. (1950)
Mutants of Escherichia coli requiring methionine or vitamin B_{12}.
1950, J. Bact., 60, 17-28.

DELTORT-LAVAL, J. (1976)
Biological criteria of protein evaluation.
Protein Metabolism And Nutrition. D.J.A. Cole, K.N. Borman, P.J. Buttery, D. Lewis, R.J. Neale and H. Swan (eds)
1976, Butterworth, London, 232-47.

DERSE, P.H. (1969)
Amino acid analysis employing microbiological assay and automated analysers.
1969, Feedstuffs, 41, 44.

DESGRES, J., BOISSON, D., PADIEU, P. (1979)
Gas liquid chromatography of isobutyl ester, N(O)-hepta fluorobutyrate derivatives of amino acids on a glass capillary column for quantitative separation in clinical biochemistry.
1979, J. Chromatogr., 162, 133-152.

DICKINSON, L. (1959)
Experiences with the plate assay of phenylalanine with a mutant of Escherichia coli.
1959, Analyst, (London), 83, 647-648.

DIFCO LABORATORIES (1953)

Difco Manual Of Dehydrated Culture Media Reagents For Microbiological And Clinical Laboratory Procedures. 9th Edition.

1953, Difco Laboratories, Detroit, Michigan.

DIMASI, D.R., WHITE, J.C., SCHNAITMAN, C.A. and BRADBEER, C. (1973)

Transport of vitamin B_{12} in Escherichia coli: Common receptor sites for vitamin B_{12} and the Escherichia colicins on the outer membrane of the cell envelope.

1973, J. Bact., 115, 506-513.

DREYER, J.J. (1973)

Methods of protein evaluation with rats 2.

Proteins In Human Nutrition. J.W.G. Porter & B.A. Rolls (eds)

1973, Academic Press, London & New York, 245-261.

DÜMMER, H.W. (1971)

Availability of amino acids in heat damaged protein.

1971, Nutr. Abst. Rev., 41, No.5250.

EGGUM, B.O. (1978)

Biological aspect of protein adequacy.

Biochemical Aspects Of New Protein Foods.

FEBS Symposium No.44. J.A. Nissen, B.O. Eggum, L. Munck and H.S. Olsen (eds)

1978, Pergamon Press, Oxford, 161-170.

EGGUM, B.O. (1975)

Indirect measures of protein adequacy.

Protein Metabolism And Nutrition. D.J.A. Cole, K.N. Borman, P.J. Bultery, D. Lewis, P.J. Neale and H. Swan (eds)

1975, Butterworth, London, 249-258.

EGGUM, B.O. (1973a)

A study of certain factors influencing protein utilization in rats and pigs.

Contribution No.406.

1973, Institute of Animal Science, Copenhagen.

EGGUM, B.O. (1973b)
The levels of blood amino acids and blood urea as indicators of protein quality.
Proteins In Human Nutrition. J.W.G. Porter and B.A. Rolls (eds)
1973, Academic Press, London & New York, 317-327.

EGGUM, B.O. and JULIANO, B.O. (1973)
Nitrogen balance in rats fed rices differing in protein content.
1973, J. Sci. Food Agric., 24, 921-927.

EJIRI, S.I., WEISSBACH, H. and BROT, N. (1979)
Reduction of methionine sulphoxide to methionine by Escherichia coli.
1979, J. Bacteriol., 139, 161-164.

EJIRI, S.I., WEISSBACH, H. and BROT, N. (1980)
The purification of methionine sulphoxide reductase from Escherichia coli.
1980, Anal. Biochem., 102, 393-398.

ELLINGER, G.M. and DUNCAN, A. (1976)
The determination of methionine in proteins by gas-liquid chromatography.
1976, Biochem. J., 155, 615-621.

ELLINGER, G.M. and PALMER, R. (1969)
The biological availability of methionine sulphoxide.
1969, Proc. Nutr. Soc., 28, 42A.

ERBERSDOBLER, H.F. (1977)
Biological significance of carbohydrate-Lysine cross linking during heat treatment.
1977, Adv. Exptal. Biol. Med., 86, 367-378.

ERBERSDOBLER, H. (1976)
Amino acid availability.
Protein Metabolism And Nutrition. D.J.A. Cole, K.N. Boorman, P.J. Buttery, D. Lewis, R.J. Neale and H. Swan (eds)
1976, Butterworth, London, 139-159.

ERBERSDOBLER, H. (1973)

The normal course of digestion in food proteins.

Proteins In Human Nutrition. J.W.G. Porter and B.A. Rolls (eds)

1973, Academic Press, New York, 453-469.

ERICSON, L.E. and CARLSON, B. (1953)

Studies on the occurrence of amino acids, niacin and pantothenic acid in marine algae.

1953, Ark. Kemi., 6, 511-522.

EVANCHO, G.M., HURT, H.D., DEVLIN, P.A., LANDERS, R.E. and ASHTON, D.H. (1977)

Comparison of Tetrahymena pyriformis W and rat bioassays for the determination of protein quality.

1977, J. Food Sci., 42, 444-448.

EVANS, I.M., FORD, J.E., HANNAH, L.C. and BOULTER, D. (1976)

Comparison of chemical and microbiological methods in the estimation of methionine in cowpea (Vigna unguiculata) seeds.

1976, Br. J. Nutr., 36, 289-293.

EVANS, E. and WITTY, R. (1978)

An assessment of methods used to determine protein quality.

1978, World Rev. Nutr. Diet., 32, 1-26.

F.A.O. (1973)

F.A.O. Nutrition Reports Series No.52.

1973, Food & Agriculture Organisation, Rome.

FASMAN, G.D. (1976)

Structure of bovine chymotrypsinogen.

CRC Handbook. The Proteins Vol.III

1976, CRC Press Inc., U.S.A., 437-440.

FEENEY, R., BLANKENHORN, G. and DIXON, H.B.F. (1975)

Carbonyl-amine reactions in protein chemistry.

Advances In Protein Chemistry Vol.29. Anfinsen, C.B., Edsall, J.T. and Richards, F.M. (eds)

1975, Academic Press, New York, 135-203.

FERNELL, W.R. and ROSEN, G.D. (1956)
Microbiological evaluation of protein quality with Tetrahymena pyriformis W.
Characteristics of growth of the organism and determination of relative nutritive values of intact proteins.
1956, Br. J. Nutr., 10, 143-156.

FINLEY, J.W. and FRIEDMAN, M. (1973)
Chemical methods for available lysine.
1973, Cereal Chem., 50, 101-107.

FINOT, P.A. (1973)
Non-enzymic browning.
Proteins In Human Nutrition. Porter, J.W.G. and Rolls, B.A. (eds)
1973, Academic Press, London, 501-514.

FINOT, P.A., MAGNENAT, G., GUIGNARD, G. and HURRELL, R.F. (1982)
The behaviour of tryptophan during "early" and "advanced" Mailliard reactions.
1982, Int. J. Vitam. Nutr. Res., 53, 226.

FINOT, P.A., MAGENAT, E., MOTTU, F. and BIYARD, E. (1978)
Biological availability and metabolic transit of amino acids modified by industrial processes.
1978, Ann. Nutr. Aliment., 32, 325-338.

FORD, J.E. (1981)
Microbial methods for protein quality assessment.
Chapter 16, Protein Quality In Humans: Assessment And In Vitro Estimation. Bodwell, C.E., Adkins, J.S. and Hopkins, D.T. (eds)
1981, Avi Publishing Co. Inc., Westport, Connecticut, 278-305.

FORD, J.E. (1977)
Microbiological procedures for protein quality assessment.
Nutritional Value Of Cereal Mutants.
1977, I.A.E.A., Vienna, 73-85.

FORD, J.E. (1975)
Some effects of overheating of protein on its digestion and absorption.
Peptide Transport In Protein Nutrition. D.M. Matthews and J.W. Payne (eds)
1975, North Holland, Amsterdam, 183-203.

FORD, J.E. (1965)
A microbiological method for assessing the nutritional value of proteins. 4. Analysis of enzymically digested food proteins by Sephadex-gel filtration.
1965, Br. J. Nutr., 19, 277-293.

FORD, J.E. (1964)
A microbiological method for assessing the nutritional value of proteins. 3. Further studies on the measurement of available amino acids.
1964, Br. J. Nutr., 18, 449-460.

FORD, J.E. (1962)
A microbiological method for assessing the nutritional value of protein. 2. The measurement of 'available' methionine, leucine, isoleucine, arginine, histidine, tryptophan and valine.
1962, Br. J. Nutr., 16, 409-425.

FORD, J.E. (1960)
A microbiological method for assessing the nutritional value of proteins.
1960, Br. J. Nutr., 14, 485-497.

FORD, J.E. (1953)
The microbiological assay of vitamin B_{12}. The specificity of the requirement of Ochromonas malhamensis for cyanocobalamin.
1953, Br. J. Nutr., 7, 299-306.

FORD, J.E. and SALTER, D.N. (1966)
Analysis of enzymically digested food proteins by Sephadex-gel filtration.
1966, Br. J. Nutr., 20, 843-859.

FORD, J.E. and SHORROCK, C. (1978)
Metabolism of heat-damaged proteins in the rat. Inhibition of amino acid uptake by 'unavailable peptides' isolated from enzymic digests of heat-damaged cod fillet.
1978, Br. J. Nutr., 40, 185-191.

FORD, J.E. and SHORROCK, C. (1971)
Metabolism of heat-damaged proteins in the rat.
1971, Br. J. Nutr., 26, 311-322.

FRANK, O., BAKER, H., HUTNER, S.H., RUSOFF, I.I., and MORCK, R.A. (1975)
Evaluation of protein quality with the phagotrophic protozoan Tetrahymena.
Protein Nutritional Quality Of Foods And Feeds. Part 1. Assay Methods. M. Friedman (ed)
1975, Marcel Dekker, New York, 203-209.

FRIEDMAN, M. (1975)
Protein Nutritional Quality Of Foods And Feeds. Parts 1 & 2.
1975, Marcel Dekker, New York.

FRIEDMAN, M. and FINLEY, J.W. (1975)
Evaluation of methods for tryptophan analysis in proteins.
Protein Nutritional Quality Of Foods And Feeds. Part 1: Assay Methods. M. Friedman (ed)
1975, Marcel Dekker, New York, 423-452.

FRIEDMAN, M. and FINLEY, J.W. (1971)
Methods of tryptophan analysis.
1971, J. Agric. Food Chem., 19, 626-633.

FUJII, N., SASAKI, T., FUNAKOSHI, S., IRIE, H. and YAJIMA, H. (1978)
Studies on peptides. LXXIV.
Convenient procedure for the preparation of methionine sulphoxide derivatives.
1978, Chem. Pharm. Bull., 26, 250-253.

GARNER, M.H. and SPECTOR, A. (1980)
Selective oxidation of cysteine and methionine in normal and senile cataractous lenses.
1980, P.N.A.S., U.S.A., 77, 1274-1277.

GAVIN, J.J. (1957a)
Microbiological Process Report, 2. The diffusion methods.
1957, Appl. Microbiol., 5, 25-33.

GAVIN, J.J. (1957b)
Microbiological Process Report, 3. Turbidometric methods.
1957, Appl. Microbiol., 5, 235-243.

GAVIN, J.J. (1956)
Microbiological Process Report, 1. The test organism.
1956, Appl. Microbiol., 5, 25-33.

GILVARG, C. and KATCHALSKI, E. (1965)
Peptide utilisation in Escherichia coli.
1965, J. Biol. Chem., 240, 3093-3098.

GIRDWOOD, R.H. (1963)
The microbiological assay of vitamins and amino acids.
1963, J. Medical Lab. Technol., 20, 26-33.

GITLER, C. (1964)
Protein digestion and absorption in non ruminants.
Mammalian Protein Metabolism, Vol.1. H.N. Munro and J.B. Allison (eds)
1964, Academic Press, New York & London, 35-69.

GOLDBERG, A.L. and BOCHES, F.S. (1982)
Oxidised proteins in erythrocytes are rapidly degraded by the adenosine triphosphate-dependent proteolytic system.
1982, Science, 215, 1107-1109.

GOL'DFARB, D.M. and NESTEROVA, G.F. et al. (1968)
Auxotrophic Escherichia coli strains as indicators of amino acids in human urine and blood plasma.
1968, Genetika, 4, 88. (Chem. Abstr., 69, 84019).

GOODNO, C.C., SWAISGOOD, H.E. and CATIGNANI, G.L. (1981)
A fluorimetric assay for available lysine in proteins.
1981, Anal. Biochem., 115, 203-211.

GREEN, R., NEWMARK, P.A., MUSSO, A.M. and MOLLIN, D.L. (1974)
The use of chicken serum for measurement of serum vitamin B_{12} concentration by radio-isotope dilution: Description of method and comparison with microbiological assay results.
1974, Br. J. Haematol., 27, 507-526.

GREENAWAY, W.T. and DAVIS, G. (1975)
An improved five minute biuret protein method for wheat and other grains.
Protein Nutritional Quality Of Foods And Feeds. Part 1: Assay Methods. M. Friedman (ed)
1975, Marcel Dekker, New York, 403-410.

GREENSTEIN, J.P. and WINITZ, M. (1961)
Chemistry Of The Amino Acids, Vol.1.
1961, John Wiley, New York & London, 245-432.

GREY, S.J., ADIBI, S.A. and MENDEN, E. (1968)
Intestinal absorption of amino acids in man.
Protein Nutrition And Free Amino Acid Patterns. J.H. Leathem (ed)
1968, Rutgers University Press, 53-79.

GUIRARD, B.M. and SNELL, E.E. (1962)
Nutritional requirements of microorganisms.
The Bacteria Vol.4. I.C. Gunsalus and R.Y. Stanier (eds)
1962, Academic Press, London & New York.

GUTHRIE, R. (1969a)
Multiple Laboratory Screening. E.S. Benson and P.E. Strandjord (eds)
1969, Academic Press, New York, 131-146.

GUTHRIE, R. (1969b)
Screening for phenylketonuria.
1969, Triangle, 9, 104-109.

GUTHRIE, R. and SUSI, A. (1963)
A simple phenylalanine method for detecting phenylketonuria in large populations of newborn infants.
1963, Paediatrics, 32, 338-343.

HACKLER, L.R. (1977)
In vivo Indices: Relationships to estimating protein values for the human.
Evaluation Of Proteins For Humans. Bodwell, C.E. (ed)
1977, Avi Publishing Co., Westport, Connecticut, 55-66.

HAENEL, H. and KHARAYTAN, S.G. (1973)
Some observations on the use of microbiological techniques for the determination of protein quality.
Proteins In Human Nutrition. J.W.G. Porter and B.A. Rolls (eds)
1973, Academic Press, New York & London, 195-206.

HARTLEY, A.W., WARD, L.D. and CARPENTER, K.H. (1965)
Hydroxylysine as a growth stimulant in microbiological assays for lysine.
1965, Analyst (London), 90, 600-605.

HAYES, W. (1968)
DNA as the genetic material. The nature of mutation.
The Genetics Of Bacteria And Their Viruses. 2nd Edition.
1968, Blackwell Scientific, Oxford, 297-388.

HEGSTED, D.M. (1971)
Nutritional Research On The Value Of Amino Acid Fortification.
N.S. Scrimshaw and A.M. Altschul (eds)
1971, M.I.T. Press, Cambridge, Massachusets, 157-178.

HEGSTED, D.M. and JULIANO, B.O.
Difficulties in assessing the nutritional quality of rice protein.
1974, J. Nutr., 104, 772-781.

HEIMANN, W. (1980)
Fundamentals Of Food Chemistry.
1980, Ellis Horwood, Chichester, England, 43-45.

HELMS, P. and ROLLE, G. (1970)
Nutritive evaluation of protein quality with Tetrahymena pyriformis W.
Evaluation Of Novel Food Protein Products.
Wenner-Gren Centre, International Symposium Series Vol.14.
A.E. Bender, R. Kihlberg, B. Lofgvist and L. Munch (eds)
1970, Pergamon Press, Oxford, 259-264.

HENRY, K.M. and FORD, J.E. (1965)
The nutritive value of leaf protein concentrates determined in biological tests with rats and by microbiological methods.
1965, J. Sci. Food Agric., 16, 425-432.

HEPFER, C. and KANEY, A.R. (1976)
Isolation of auxotrophic mutants of Tetrahymena pyriformis.
1976, J. Protozool., 23, 22A.

HERMSDØRF, C.L. and SIMMONDS, S. (1980)
Role of peptidases in utilisation and transport of peptides by bacteria.
Microorganisms And Nitrogen Sources. J.W. Payne (ed)
1980, Wiley, Chichester, 301-335.

HEWITT, W. (1977)
Microbiological Assay: An Introduction To Quantitative Principles And Evaluation.
1977, Academic Press, New York & London.

HODSON, A.Z. and KRUEGER, G.M. (1947)
Changes in the essential amino acid content of proteins of dry skim milk on prolonged storage.
1947, Arch. Biochem., 12, 51-55.

HOLSINGER, V.H. and POSATI, L.P. (1975)
Chemical estimation of available lysine in dehydrated dairy products.
Protein Nutritional Quality Of Foods And Feeds Part 1.
Assay Methods. M. Friedman (ed)
1975, Marcel Dekker, New York, 479-503.

HORN, J.M., LINCHTENSTEIN, H. and WOMACK, M. (1968)
A methionine-fructose compound and its availability to microorganisms and rats.
1968, Agr. Food Chem., 16, 741-745.

HOUGHTON, R. and HAO LI, C. (1979)
Reduction of sulphoxides in peptides and proteins.
1979, Anal. Biochem., 98, 36-46.

HURRELL, R.F. and CARPENTER, K.J. (1978)
Digestibility and Lysine values of proteins heated with formaldehyde or glucose.
1978, J. Agric. Food Chem., 26, 796-802.

HURT, H.D., FORSYTHE, R.H. and KRIEGER, C.H. (1975)
Factors which influence the biological evaluation of protein quality by the protein efficiency ratio method.
Protein Nutritional Quality Of Foods And Feeds Part 1.
Assay Methods. M. Friedman (ed)
1975, Marcel Dekker, New York, 87-112.

HUTNER, H.S., CURY, A. and BAKER, H. (1958)
Microbiological assays.
1958, Rev. Fundamental Dev. Anal., 36, 849-867.

I.A.E.A. (1977)
Conclusions and recommendations.
Nutritional Evaluation Of Cereal Mutants.
1977, I.A.E.A., Vienna, 151-187.

I.A.E.A. (1975)
Recommendations of an ad hoc panel on analytical screening methods for seed protein content and quality.
Breeding For Seed Protein Improvement Using Nuclear Techniques.
1975, I.A.E.A., Vienna, Annexe III, 193-209.

JACKSON, R.W. and DEMOSS, J.A. (1965)
Effects of toluene on Escherichia coli.
1965, J. Bact., 90, 1420-1425.

JACQUOT, R. and PERET, J. (1972)
Protein efficiency ratio and related methods.
Protein And Amino Acid Functions. E.J. Bigwood (ed)
1972, Pergamon, Oxford, 317-347.

JOHANNSSON, K.R. (1953)
Response to and assay of vitamin B_{12} by a mutant of Escherichia coli.
1953, Proc. Soc. Exp. Biol. Med., 83, 448-453.

JOHNSON, D.A. (1980)
Ozone inactivation of human α_1,-protinase inhibitor.
1980, American Review of Respiratory Disease, 121, 1031-1038.

JONES, A. and BURNS, S.M. (1958)
Experiences with the plate assay of L-phenylalanine with a mutant of Escherichia coli.
1958, Analyst (London), 83, 647-648.

JULIANO, B.O. (1966)
Physicochemical data on the rice (Oryza sitiva L) grain.
1966, International Rice Research Institute, Technical Bulletin 6.

KADNER, R.J. (1978)
Repression of synthesis of vitamin B_{12} receptor in Escherichia coli.
1978, J. Bact., 136, 1050-1057.

KADNER, R.J. (1977)
Transport and utilisation of D-methionine and other methionine sources in Escherichia coli.
1977, J. Bact., 129, 207-216.

KADNER, R.J. (1974)
Transport systems for L-methionine in Escherichia coli.
1974, J. Bact., 117, 232-241.

KADNER, R.J. and BASSFORD, P.J. (1978)
Bacterial Transport. B.P. Rosen (ed)
1978, Dekker Ltd., U.K., p.465.

KADNER, R.J. and McELHANEY, E. (1978)
Outer membrane-dependemt transport systems in Escherichia coli: turnover of ton B function.
1978, J. Bact., 134, 1020-1029.

KADNER, R.J. and WATSON, W.J. (1974)
Methionine transport in Escherichia coli. Physiological and genetic evidence for two uptake systems.
1974, J. Bact., 119, 401-409.

KAESTNER, H.W., KAUL, A.K. and NIEMANN, E.G. (1976)
Use of Tetrahymena pyriformis to evaluate the relative nutritive value (RNV) of some crop cultivars and processed cereals.
1976, Qual. Plant-Plant Foods Hum. Nutr., 25, 261-373.

KAKADE, M.L. and LIENER, I.E. (1969)
Determination of available lysine in foods.
1969, Anal. Biochem., 27, 273-279.

KATA, Y., WATANABE, K. and SATO, Y. (1981)
Effect of some metals on the Mailliard reaction of ovalbumin.
1981, J. Agric. Food Chem., 29, 540-543.

KAVANAGH, F.W. (1972)
Analytical Microbiology Vol.2.
1972, Academic Press, New York.

KAVANAGH, F.W. (1963)
Analytical Microbiology Vol.1.
1963, Academic Press, New York.

KAVANAGH, F.W. (1960)
A commentry on microbiological assaying.
1960, Adv. Appl. Microbiol., 2, 65-93.

KIDDER, G.W. and DEWEY, V.C. (1951)
The biochemistry of ciliates in pure culture.
Biochemistry And Physiology Of Protozoa Vol.1. A. Lwoff (ed)
1951, Academic Press, New York, 323-401.

KIHARA, H. and SNELL, E.E. (1960a)
Peptides and bacterial growth. VIII. The nature of streptogenin.
1960, J. Biol. Chem., 235, 1409-1414.

KIHARA, H. and SNELL, E.E. (1960b)
Peptides and bacterial growth. IX. Release of double inhibitions by single peptides.
1960, J. Biol. Chem., 235, 1415-1418.

KIKUCHI, Y. and TAMIYA, N. (1981)
Methionine sulphoxide in the resilium protein of surf clams.
1981, J. Biochem., 89, 1975-1976.

KNIPFEL, J.E., AITKEN, J.R., HILL, D.C., McDONALD, B.E. and OWEN, B.D. (1975)
Amino acid composition of food proteins: inter and intra laboratory variation.
1975, J. Assoc. Off. Anal. Chem., 54, 777.

KOJIMA, H. (1965)
1965, Chem. Abstr., 62, 9448a.

KONOVA, I.U. and VERBINA, N.M. (1966)
Quantitative assay of tryptophan by the diffusion method using Escherichia coli and Bacillus subtilis.
1966, Microbiology, 35, 906-911.

KUSAKABE, H., KODAMA, K, KUNINAKA, A, YOSHINO, H. and SODA, K. (1979)
Sensitive and simple methods for determination of L-lysine with L-lysine α-oxidase.
1979, Agri. Biol. Chem., 43, 1749-1752.

LANDERS, R.E. (1975)
Relationship between protein efficiency ratio of foods and relative nutritive value measured by Tetrahymena pyriformis W bioassay techniques.
Protein Nutritional Quality Of Foods And Feeds; Part 1. Assay Methods. M. Friedman (ed)
1975, Marcel Dekker, New York, 185-202.

LAW, B.A. (1978)
Peptide utilisation by Group N Streptococci.
1978, J. Gen. Microbiol., 105, 113-118.

LAWRIE, N.R. (1937)
Studies on the metabolism of protozoa. III. Some properties of a proteolytic extract obtained from Glaucoma pyriformis.
1937, Biochem. J., 31, 789-791.

LEA, C.H. and HANNAN, R.S. (1949)
Studies on the reaction between protein and reducing sugars in the 'dry' state.
1. The effect of activity of water, of pH and of temperature on the primary reaction between casein and glucose.
1949, Biochem. Biophys. Acta., 3, 313.

LEDERBERG, J. (1950)
The β-D galactosidase of Escherichia coli, strain K-12.
1950, J. Bact., 60, 381-392.

LEDERBERG, J. (1956)
Bacterial protoplasts induced by penecillin.
1956, Proc. Nat. Acad. Sci. Wash., 42, 574-577.

LEDERBERG, J. and LEDERBERG, E.M. (1952)
Replica plating and indirect selection of bacterial mutants.
1952, J. Bact., 63, 399-406.

LEES, B.A. and TOOTHILL, J.P.R. (1955a)
Microbiological assay on large plates. 1. Routine laboratory assays.
1955, Analyst (London), 80, 95-109.

LEES, K.A. and TOOTHILL, J.P.R. (1955b)
Microbiological assay on large plates. 2. Methods for precision assays.
1955, Analyst (London), 80, 110-118.

LEES, K.A. and TOOTHILL, J.P.R. (1955c)
Microbiological assays on large plates. 3. High throughput, low precision assays.
1955, Analyst (London), 80, 531-535.

LEGGETT-BAILEY, J. (1967)
Techniques In Protein Chemistry.
1967, Elsevier, Amsterdam.

LEVINE, R.L., OLIVER, C.N., FULKS, R.M. and STADTMAN, E.R. (1980)
Turnover of bacterial glutamine synthetase: oxidative inactivation precedes proteolysis.
1980, P.N.A.S., U.S.A., 78, 2120-2124.

LINDSTEDT, S. (1953)
On the inability of Hydroxylysine to promote growth in rats fed a lysine-deficient diet.
1953, Acta. Phys. Scandinav., 27, 377-379.

LIPTON, S.H. and BODWELL, C.E. (1975)
Chemical approaches for estimating nutritionally available methionine.
Protein Nutritional Quality Of Foods And Feeds Part 1. Assay Methods. M. Friedman (ed)
1975, Marcel-Dekker, New York, 569-597.

LONGENECKER, J.B. (1973)
Ion exchange chromatography for amino acid assay: application for determining amino acid requirements in the rat.
Proteins In Human Nutrition. J.W.G. Porter and B.A. Rolls (eds)
1973, Academic Press, London & New York, 155-167.

LOUW, A.I. and CARLSSON, F.H.H. (1978)
The oxidation of methionine and its effects on the properties of cardiotoxin V^{11} from Naja melanoleuca venom.
1978, Biochem. Biophys. Acta., 534, 322-327.

LOW, K. BROOKS- (1972)
Escherichia coli K12. F prime factors, old and new.
1972, Bact. Rev., 36, 587-607.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. and RANDALL, R.J. (1951)
Protein measurement with the Folin Phenol Reagent.
1951, J. Biol. Chem., 193, 265-275.

MACIEJEWICZ-RYS,J and ANTONIEWICZ, A.M. (1978)
2-Aminoethylphosphonic acid as an indicator of Tetrahymena pyriformis W growth in protein-quality evaluation assay.
1978, Br. J. Nutr., 40, 83-90.

MARABLE, N.L. and SANZONE, G. (1981)
In vitro assays of protein quality using enzymatic hydrolyses.
Protein Quality In Humans Assessment And In Vitro Estimations.
Bodwell, C.E., Adkins, J.S. and Hopkins, D.T. (eds)
1981, Avi Pub. Co., Westport, Connecticut, 261-277.

MATTHEWS, D.M. (1979)
Distribution of cobalamins in the animal body.
Vitamin B_{12}. Proceedings Of Third International Symposium.
Zagalak, B. and Friedrich, W.
1979, deGruyter, Berlin and New York.

MATTHEWS, D.M. (1977)
Memorial Lecture. Protein absorption - then and now.
1977, Gastroenterology, 73, 1267-1279.

MATTHEWS, D.M. (1975)
Intestinal absorption of peptides.
1975, Physiol. Rev., 55, 537-608.

MATTHEWS, D.M. and ADIBI, S.A. (1976)
Peptide absorption.
1976, Gastroenterology, 71, 151-161.

MATTHEWS, D.M. and PAYNE, J.W. (1980)
Transmembrane transport of small peptides.
1980, Curr. Top. Membr. Trans., 14, 331-425.

MATTHEWS, D.M. and PAYNE, J.W. (1975a)
Peptide Transport In Protein Nutrition.
1975, North Holland, American Elsevier Amsterdam, New York.

MATTHEWS, D.M. and PAYNE, J.W. (1975b)
Peptides in the nutrition of microorganisms and peptides in relation to animal nutrition.
Peptide Transport In Protein Nutrition.
1975, North Holland, American Elsevier Amsterdam, New York. 1-60.

MAURON, J. (1973)
The analyses of food proteins, amino acid composition and nutritive value.
Proteins In Human Nutrition. J.W.G. Porter & B.A. Rolls (eds)
1973, Academic Press, New York & London, 139-154.

MAURON, J. (1972)
Influence of industrial and household handling on food protein quality.
Protein And Amino Acid Functions. E.J. Bigwood (ed)
1972, Pergamon Press, Oxford, 417-473.

MAURON, J. (1970a)
Nutritional evaluation of proteins by enzymic methods.
Evaluation Of Novel Protein Products. A.E. Bender, R. Kihlberg, B. Lofquist and L. Munck (eds)
1970, Pergamon Press, London, p.211.

MAURON, J. (1970b)
Nutritional evaluation of proteins by enzymic methods.
Improving Plant Proteins By Nuclear Techniques.
1970, I.A.E.A., Vienna, p.203.

McHUGH, W. SANDMANN, R.A., HANEY, W.G., SOOD, S.P. and WITTMER, M (1976)
Characterisation of selected fluorescamine-amino acid reaction products by high-performance liquid chromatography.
1976, J. Chromatogr., 124, 376-380.

McLAUGHLAN, J.M. and KEITH, M.O. (1975)
Bioassay of protein quality.
Protein Nutritional Quality Of Foods And Feeds. Part 1. Assay Methods. M. Friedman (ed)
1975, Marcel Dekker, New York, 79-85.

McLAUGHLAN, J.M. and CAMPBELL, J.A. (1969)
Methodology of protein evaluation.
Mammalian Protein Metabolism Vol.3. H.N. Munro (ed)
1969, Academic Press, New York & London, 391-422.

MEISTER, A. (1965)
Biochemistry Of The Amino Acids Vol.1.
1965, Academic Press, New York & London, 201-230.

MILLER, E.L., CARPENTER, K.J. and MILNER, C.K. (1965)
Availability of sulphur amino acids in protein foods.
3. Chemical and nutritional changes in heated cod muscle.
1965, Br. J. Nutr., 19, 547-564.

MILLER, J.H. (1972a)
Experiments In Molecular Genetics.
1972, Cold Spring Harbour Lab., Cold Spring Harbour, New York, p.117.

MILLER, J.H. (1972b)
Experiments In Molecular Genetics.
1972, Cold Spring Harbour Lab., Cold Spring Harbour, New York, p.57.

MILLER, M.W. (1961)
Pfizer Handbook Of Microbial Metabolites.
1961, McGraw Hill, New York, p.460.

MILLER, D.S. and SAMUEL, P.D. (1970)
Effects of the addition of sulphur compounds to the diet on utilisation of protein in young growing rats.
1970, J. Sci. Food Agric., 21, 616-618.

MOLHOLT, B. (1967)
Isolation of amino acid auxotroph of Escherichia coli using ampicillin enrichment.
1967, Microb. Genet. Bull., 27, 8-9.

MOODY, C.S. and HASSAN, H.M. (1982)
Mutagenicity of oxygen-free radicals.
1982, P.N.A.S., U.S.A., 79, 2855-2859

MORRISON, A.B. and McLAUGHLAN, J.M. (1972)
Availability of amino acids in foods.
Protein And Amino Acid Functions. E.J. Bigwood (ed)
1972, Pergamon, Oxford, 389-416.

MOYHUDDIN, M., SHARMA, T.R., DHAR, P., KAUL, A.K. and NIEMANN, E.G. (1977)
Rapid assays of protein quality in cultivars of cereals using Aspergillus flavus.
Nutritional Evaluation Of Cereal Mutants.
1977, I.A.E.A., Vienna, 125-145.

MOYHUDDIN, M., KAUL, A.K., SHARMA, T.R. and NIEMANN, E.G. (1976)
The use of two fungal strains as test organisms for the evaluation of relative nutritive value in cereals and legumes.
1976, Qual. Plant-Plant Foods Hum. Nutr., 25, 317-329.

MOZZARI, G.F., NEIDERBERGER, P. and HÜTTER, R. (1978)
Permeabilisation of microorganisms by Triton X-100.
1978, Anal. Biochem., 90, 220-233.

NAKAMURA, H. and PISANO, J.J. (1976a)
Derivitization of compounds at the origin of thin-layer plates with fluorescamine.
1976, J. Chromatogr., 121, 33-40.

NAKAMURA, H. and PISANO, J.J. (1976b)
Detection of compounds with primary amino groups on thin-layer plates by dipping in a fluorescamine solution.
1976, J. Chromatogr., 121, 79-81.

NATIONAL ACADEMY OF SCIENCES (1980)
Recommended Dietary Allowances. 9th Revised Edition.
1980, N.A.S., Washington D.C.

NEUMANN, N.P. (1972)
Oxidation with Hydrogen Peroxide. Hirs, C.H.W. & Timasheff, S.N. (eds)
1972, Methods In Enzymol., 25, Part B, 393-399.

NEUMANN, N.P., MOORE, S. and STEIN, W.H. (1961)
Modification of the methionine residues in ribonuclease.
1961, Biochem., 1, 68-75.

NGONGE, C. and MURRAY-BALLANCE, G. (1982)
A sensitive colorimetric procedure for nitrogen determination in Micro-Kjeldahl digests.
1982, J. Agri. Food Chem., 30, 416-420.

NIKAIDO, H., BOVOIL, P. and HIROTA, Y. (1977)
Outer membranes of gram-negative bacteria XV. Transmembrane diffusion rates in lipo protein-deficient mutants of Escherichia coli.
1977, J. Bact., 132, 1045-1047.

NISBET, T.M. (1980)
Aspects of peptide transport in Saccharomyces cerevsiae and Streptococcus Spp.
1980, PhD. Thesis (Durham).

NISBET, T.M. and PAYNE, J.W. (1979)
Peptide transport in Saccharomyces cerevsiae; characteristics of transport system shared by di and tri-peptides.
1979, J. Gen. Microbiol., 115, 127-133.

NJAA, L.R. (1980)
A method for determination of unoxidised and total methionine in protein concentrates with special reference to fish meals.
1980, Br. J. Nutr., 43, 339-348.

NJAA, L.R. (1977)
Biological methods for evaluating protein quality.
Nutritional Evaluation Of Cereal Mutants.
1977, I.A.E.A., Vienna, 59-72.

NJAA, L.R. (1962)
Utilisation of methionine sulphoxide and methionine sulphone by the young rat.
1962, Br. J. Nutr., 16, 571-577.

NURSTEN, H.E. (1980)
Recent developments in studies of the Mailliard Reaction.
1980, Food Chemistry, 6, 263-277.

ORIAS, E. and BRUNS, P.J. (1976)
Induction and isolation of mutants in Tetrahymena.
Methods In Cell Biology Vol.13. D.M. Prescott (ed)
1976, Academic Press, New York & London, 247-282.

ORIAS, E. and RASMUSSEN, L. (1979)
Dual capacity for nutrient uptake in Tetrahymena.
V. Utilisation of amino acids and proteins.
1979, J. Cell. Sci., 36, 343-351.

OSNER, R.C. and JOHNSON, R.M. (1974)
Nutritional and chemical changes in heated casein.
I. Preliminary study of solubility, gel filtration pattern and amino acid pattern.
1974, J. Fd. Technol., 9, 301-308.

OSNER, R.C. and JOHNSON, R.M. (1975)
Nutritional changes in heated casein.
II. Net protein utilisation pepsin digestibility and available amino acid content.
1975, J. Fd. Technol., 10, 133-136.

PROTEIN ADVISORY GROUP (P.A.G.) (1975)
Guidelines on protein methods for cereal breeders, as related to human nutritional requirements.
P.A.G. Guideline No.16.
1975, P.A.G. Bulletin, 5, 22-48.

PAULIS, J.W. and WALL, J.S. (1975)
Protein quality in cereals evaluated by rapid estimation of specific protein fractions.
Protein Nutritional Quality Of Foods And Feeds. M. Freidman (ed)
1975, Marcel Dekker, New York, 381-402.

PAYNE, J.W. (1980a)
Microorganisms And Nitrogen Sources.
1980, J. Wiley, Chichester, Sections 3.12 & 3.15.

PAYNE, J.W. (1980b)
Microorganisms And Nitrogen Sources.
Part 2.1 Transport and utilisation of peptides by bacteria.
1980, Wiley, Chichester, 211-257.

PAYNE, J.W. (1977)
Transport and hydrolysis of peptides by microorganisms.
Peptide Transport And Hydrolysis. Ciba Foundation Symposium 50 (new series)
1977, Elsevier/Excerpta Medica North-Holland, 305-334.

PAYNE, J.W. (1976)
Peptides and microorganisms.
1976, Adv. Microb. Physiol., 13, 55-113.

PAYNE, J.W. (1975)
Transport of peptides in microorganisms.
Peptide Transport In Protein Nutrition. Matthews, D.M. and Payne, J.W. (eds)
1975, North Holland & American Elsevier, Amsterdam & New York, 283-364.

PAYNE, J.W. (1968)
Oligopeptide transport in Escherichia coli: specificity with respect to side chain and distinction from dipeptide transport.
1968, J. Biol. Chem., 243, 3395-3404.

PAYNE, J.W. and BELL, G. (1979)
Direct determination of the properties of peptide transport systems in Escherichia coli using a fluorescent-labelling procedure.
1979, J. Bact., 137, 447-455.

PAYNE, J.W., BELL, G. and HIGGINS, C.F. (1977)
The use of an Escherichia coli Lys$^-$ auxotroph to assay nutritionally available lysine in biological materials.
1977, J. Appl. Bacteriol., 42, 165-177.

PAYNE, J.W. and GILVARG, C. (1968)
Size restriction on peptide utilisation in Escherichia coli.
1968, J. Biol. Chem., 243, 6291-6299.

PAYNE, J.W. and NISBET, T.M. (1981)
Continuous monitoring of substrate uptake by microorganisms using fluorescamine. Application to peptide transport by Saccharomyes cerevisiae and Streptococcus faecalis.
1981, J. Appl. Biochem., 3, 447-458.

PAYNE, J.W. and TUFFNELL, J.M. (1980)
Assays for amino acids, peptides and proteins.
Microorganisms And Nitrogen Sources.
1980, J. Wiley, Chichester, 727-765.

PAYNE, J.W. and TUFFNELL, J.M. (1981)
Cleavage of peptide-bound methionine sulphoxide and methionine sulphone: possible role in enhanced proteolysis in Escherichia coli.
1981, FEMS Microbiol. Letters, 12, 279-282.

PEELING, J., CLARK, D.T., EVANS, I.M. and BOULTER, D. (1976)
Evaluation of the ESCA technique as a screening method for the estimation of protein content and quality in seed meals.
1976, J. Sci. Fd. Agri., 27, 331-340.

PELLETT, P.L. (1978)
Protein quality evaluation revisited.
1978, Food Technol. (Chicago), 32, 60-119.

PELLETT, P.L. (1973)
Methods of protein evaluation with rats 1.
Proteins In Human Nutrition. J.W.G. Porter & B.A. Rolls (eds)
1973, Academic Press, London & New York, 225-244.

PETERSON, G. (1979)
Review of the Folin Protein Quantitation Method of Lowry, Rosebrough, Farr and Randall.
1979, Anal. Biochem., 100, 201-220.

PINE, M.J. (1980)
Turnover of microbial cell proteins and their peptide residues.
Microorganisms And Nitrogen Sources. Payne, J.W. (ed)
1980, Wiley, Chichester, 459-490.

PION, R. (1973)
The relationship between the levels of free amino acids in blood and muscle and the nutritive value of proteins.
Proteins And Human Nutrition. J.W.G. Porter & B.A. Rolls (eds)
1973, Academic Press, London & New York, 329-342.

PRESCOTT, J.M., PETERS, V.J. and SNELL, E.E. (1953)
Peptides and bacterial growth . V. Serine peptides and growth of Lactobacillus delbruecki 9649.
1953, J. Biol. Chem., 203, 533-540.

PURI, S.P. and MULLEN, K. (1980)
Applied Statistics For Food And Agricultural Scientists.
1980, G.K. Hall Medical Publishers, Boston, Mass.

PUTNAM, S.L. and KOCH, A.L. (1975)
Complications in the simplest cellular enzyme assay: Lysis of Escherichia coli for the assay of β-galactosidase.
1975, Anal. Biochem., 63, 350-360.

RAMASARMA, G.B., LIENDERSON, L.M. and ELVEHJEM, C.A. (1949)
Purified amino acids as a source of nitrogen for the growing rat.
1949, J. Nutr., 38, 177-188.

RANHOTRA, G.S. and JOHNSON, B.C. (1965)
Effect of feeding different amino acid diets on growth of rats and nitrogen retention of weanling rats.
1965, Proc. Soc. Exp. Biol. Med., 118, 1197-1203.

RASMUSSEN, L. (1976)
Nutrient uptake in Tetrahymena.
1976, Carlsberg Res. Commun., 41, 143-167.

RAVEN, J.L., ROBSON, M.B., MORGAN, J.O. and HOFFBRAND, A.V. (1972)
Comparison of three methods for measuring vitamin B_{12} in serum: Radioisotopic, Euglena gracilis and Lactobacillus leichmannii.
1972, Br. J. Haematol., 22, 21-31.

RECHCIGL, M. (1978)
Culture media for microorganisms and plants.
Handbook Of Nutrition And Food Vol. III.
1978, CRC Press, Florida, Section G.

REGNERY, D.C. (1944)
A leucineless mutant of Neurospora crassa.
1944, J. Biol. Chem., 154, 151-160.

REISS, U. and GERSHON, D. (1978)
Methionine sulphoxide reductase: a novel protective enzyme in liver and its potentially significant role in ageing. Liver And Ageing. K. Kitani (ed)
1978, Elsevier/North Holland Biomedical Press, Amsterdam, 55-64.

REYNOLDS, H. (1970)
Effect of type of carbohydrate on amino acid accumulation and utilization by Tetrahymena.
1970, J. Bacteriol., 104, 719-725.

RICE, G.H., STEWART, F.H.C., HILLIER, M.H. and JAGO, G.R. (1978)
The uptake of amino acids and peptides by Streptococcus lactis.
1978, J. Dairy Res., 45, 93-107.

RICKENBERG, H.V. (1959)
The effect of metal ions and proteins on the stability of the β-galactosidase of Escherichia coli.
1959, Biochem. Biophys. Acta, 35, 122-129.

ROBBINS, K. and BAKER, D.H. (1980)
Evaluation of resistance of Lysine sulfite to Mailliard destruction.
1980, J. Agric. Food Chem., 28, 25-28.

ROCKLAND, L.B. and DUNN, M.S. (1959)
Determination of the biological value of proteins with Tetrahymena geleii H.
1959, Food Technol., 3, 289-292.

ROCKLAND, L.B. and DUNN, M.S. (1946)
The microbiological determination of tryptophan in unhydrolysed casein with Tetrahymena geleii H.
1946, Arch. Biochem., 11, 541-543.

ROLLE, G. (1976)
Research concerning the usefulness of Tetrahymena pyriformis W as a test organism for the evaluation of protein quality.
1976, Acta. Agric. Scand., 26, 282-286.

ROLLE, G. and EGGUM, B.O. (1971)
A comparative protein evaluation with Tetrahymena pyriformis W and rats.
1971, Acta. Agric. Scand., 21, 69-72.

ROSE, W.C. (1937)
The nutritive significance of the amino acids.
1937, Physiol. Rev., 18, 109.

ROSE, W.C., COON, M.J. and LAMBERT, G.F. (1954)
The amino acid requirements of man. VI. The role of calorie intake.
1954, J. Biol. Chem., 210, 331-337.

ROSEN, G.D. and FERNELL, W.R. (1956)
Microbiological evaluation of protein quality with Tetrahymena pyriformis W. 2. Relative nutritive value of proteins in foodstuffs.
1956, Br. J. Nutr., 10, 156-169.

ROSSI, J.J. and BERG, C.M. (1971)
Differential recovery of auxotrophs after penecillin enrichment in Escherichia coli.
1971, J. Bact., 106, 297-300.

ROWLEY, D.A. and HALLIWELL, B. (1982)
Superoxide-dependent formation of hydroxyl radicals from NADH and NADPH in the presence of iron salts.
1982, FEBS Letters, 142, 39-41.

RUDEMO, M., MASON, V.C. and BECH-ANDERSON, S. (1979)
Hydrolysate preparation for amino acid determinations in feed constituents. 3. Statistical description of amino acid determinations.
1979, Z. Tierphysiol. Tierernahr., 41, 254-264.

RYAN, F.J. (1946)
The application of Neurospora to bioassay.
1946, Fed. Proc. Fed. Am. Soc. Exp. Biol., 5, 366-369.

SANO, K., KANAMORI, K., SHIBA, A. and NAKAO, M. (1981)
Automatic assay of urinary protein using Coomassie Brilliant Blue G-250.
1981, Anal. Biochem., 113, 197-201.

SATTERLEE, L.D., MARSHALL, H.F. and TENNYSON, J.M. (1979)
Measuring protein quality.
1979, J. Am. Oil Chemists Soc., 56, 103-109.

SAUBERLICH, H.E. (1961)
Growth of rats fed protein free diets supplemented with purified amino acid mixtures.
1961, J. Nutr., 74, 298-305.

SAVIGE, W.E. and FONTANA, A. (1977)
Interconversion of methionine and methionine sulphoxide.
1977, Methods In Enzymology, 47, E, 453-459.

SCHWARTZ, D.O. and BECKWITH, J.R. (1969)
Mutagens which cause deletions in Escherichia coli.
1969, Genetics, 61, 371-376.

SCRIMSHAW, N.S. and YOUNG, V.R. (1977)
Evaluation Of Proteins For Humans. Bodwell, C.E. (ed)
1977, Avi Publishing Co., Connecticut, p.9.

SEBEK, I.K. (1965)
Microbiological method for the determination of tryptophan.
1965, J. Bacteriol., 90, 1026-1031.

SEBBRELL, W.H. and HARRIS, R.S. (1954)
The Vitamins Vol.1.
1954, Academic Press, New York, p.401.

SEILER, N. (1970)
Use of the dansyl reaction in biochemical analysis.
1970, Methods of Biochem. Anal., 18, 259-337.

SHAW, W.H.C. and BESSELL, C.J. (1960)
The determination of vitamin B_{12}. A critical review.
1960, J. Lab. Clin. Med., 85, 389-409.

SHEKLETON, M.C. and HAYNES, W.C. (1959)
Microbiological assays with several strains of Leuconostoc mesenteroides.
1959, J. Bacteriol., 77, 114-115.

SHEFFNER, A.L. (1967)
In vitro protein evaluations.
Newer Methods Of Nutritional Biochemistry Vol.3.
A.A. Albanese (ed)
1967, Academic Press, New York, p.125.

SHEPHERD, N.D., TAYLOR, T.G. and WILTON, D.C. (1975)
An improved method for the determination of the biological availability of lysine in proteins using the microorganism Tetrahymena pyriformis.
1975, Proc. Nutr. Soc., 34, 100A-101A.

SHOCKMAN, G.D. (1963)
Amino acids.
Analytical Microbiology. F. Kavanagh (ed)
1963, Academic Press, New York & London, 568-674.

SHORROCK, C. (1976)
An improved procedure for the assay of available lysine and methionine in feedstuffs using Tetrahymena pyriformis W.
1976, Br. J. Nutr., 35, 333-341.

SHORROCK, C. (1972)
Studies on the biological availability of amino acids in feedstuffs.
1972, PhD Thesis, Reading University.

SHORROCK, C. and FORD, J.E. (1978)
Metabolism of heat-damaged proteins in the rat.
1978, Br. J. Nutr., 40, 185-191.

SHORROCK, C. and FORD, J.E. (1973)
An improved procedure for the determination of available lysine and methionine with Tetrahymena.
Proteins In Human Nutrition. J.W.G. Porter and B.A. Rolls (eds)
1973, Academic Press, London & New York, 207-223.

SILANO, V. (1977)
Factors affecting digestability and availability of proteins in cereals.
Nutritional Evaluation Of Cereal Mutants.
1977, I.A.E.A., Vienna, 13-47.

SILK, D.B.A., CLARK, M.L., MARRS, T.C., ADDISON, J.M., BURSTON, D., MATTHEWS, D.M. and CLEGG, K.M. (1975)
Jejunal absorption of an amino acid mixture simulating casein and an enzymic hydrolysate of casein prepared for oral administration to normal adults.
1975, Br. J. Nutr., 33, 95-100.

SIMPSON, I.A. and SONNE, O. (1982)
A simple, rapid and sensitive method for measuring protein concentration in subcellular membrane fractions prepared by sucrose density centrifugation.
1982, Anal. Biochem., 119, 424-427.

SKOGBERG, D. and RICHARDSON, T. (1979)
Preparation and use of an enzyme electrode for specific analysis of L-lysine in cereal grains.
1979, Cereal Chem., 56, 147-152.

SKÖLD, O. and WALLENBORG, C. (1969)
A microbiological method for the determination of asparagine in blood serum.
1969, Acta. Pharm. Suec., 6, 407-412.

SLUMP, P. and SCHREUDER, H.A.W. (1973)
Oxidation of methionine and cystine in foods treated with hydrogen peroxide.
1973, J. Sci. Fd. Agric., 24, 657-661.

SLUMP, P. and VAN BEEK, L. (1975)
Amino acids in faeces related to digestibility of food proteins.
Protein Nutritional Quality Of Foods And Feeds. Part 1.
Assay Methods. M. Friedman (ed)
1975, Marcel Dekker, New York, 67-78.

SNOW, J.T., FINLEY, J.W. and KOHLER, G.O. (1976)
A kinetic study of the bisulphite reduction of methionine sulphoxide to methionine.
1976, J. Sci. Fd. Agric., 27, 649-654.

SOLBERG, M., BERKOWITZ, K.A., BLASCHEK, H.P. and CURRAN, J.M. (1979)
Rapid evaluation of protein quality of foods using Clostridium perfringens.
1979, J. Food Sci., 44, 1335-1337.

SOTELO, A. and SONSA, V. (1976)
Disc microbiological method for the determination of Lysine or methionine in protein hydrolysates.
1976, Nurt. Rep. Intl., 14, 337-344.

SPRAY, G.H. (1955)
An improved method for the rapid estimation of vitamin B_{12} in serum.
1955, Clinical Science, 14, 661-667.

SRINIVAS, H., VAKIL, U.K. and SREENIVANSAN, A. (1975)
Evaluation of protein quality of inradiated food using Tetrahymena pyriformis W and rat assay.
1975, J. Food Sci., 40, 65-69.

STAHMANN, M.A. and WOLDEGIORGIS, G. (1975)
Enzymatic methods for protein quality determination.
Protein Nutritional Quality Of Foods And Feeds. Part 1.
Assay Methods. M. Friedman (ed)
1975, Marcel Dekker, New York, 211-234.

STEERS, E. and CUATRECASAS, P. (1974)
Methods In Enzymology XXXIV. Jakoby, W. and Wichek, M (eds)
1974, Academic Press, New York, 350-358.

STEIN, S., BÖHLEN, P., STONE, J., DAIRMAN, W. and UNDENFRIEND, S. (1973)
Amino acid analysis with fluorescamine at picomole level.
1973, Arch. Biochem. Biophys., 155, 203-212.

STEINHART, H. (1978)
A direct fluorimetric method for the determination of tryptophan from hydrolysates for foodstuffs and feeds.
1978, Z. Tierphysiol. Tienernahr., 41, 48-56.

STOTT, J.A., SMITH, H. and ROSEN, G.D. (1963)
Microbiological evaluation of protein quality with Tetrahymena pyriformis W.
3. A simplified assay procedure.
1963, Br. J. Nutr., 17, 227-233.

STUART, J. and SKLAROFF, S.A. (1966)
Rapid microbiological assay of serum vitamin B_{12} by electronic counter.
1966, J. Clin. Path., 19, 46-50.

SYKES, D.A. and EVANS, C.J. (1975)
Some methods for microbiological assay.
Soc. Appl. Bact. Technical Bulletin No.8. Board, R.G. and Lovelock, D.W. (eds)
1975, Academic Press, London. 56-62.

TAJIMA, M., TANDOKORO-YASUI, S., SUZUKI, T., SHINODA-KENMOCHI, K., KITANO, T., TSUCHIYA, K. and FUKUSCHIMA, H. (1978)
Comparative study between gas chromatography and ion-exchange chromatography on amino acid analysis of food.
1978, Agric. Biol. Chem. (Tokyo), 42, 1949-1952.

TANAKA, H., IMAHARA, H., ESAKI, N. and SODA, K. (1980)
Selective determination of L-methionine and L-cysteine with bacterial L-Met-γ-Lysase and antitumour activity of the enzyme.
1980, J. Appl. Biochem., 2, 439-444.

TANAKA, M., KIMIAGAR, M, TUNG-CHING, L. and CHICHESTER, C.O. (1977)
Effect of Mailliard browning on nutritional quality of protein.
1977, Adv. Exp. Biol. Med., 86, 321-341.

TENNANT, G.B. (1979)
Whole serum assay of L-methionine using a chloramphenicol-resistant strain of Pediococcis acidilactici.
1979, J. Appl. Bact., 47, 395-399.

TENNANT, G.B. and WITHEY, J.L. (1972)
An assessment of work simplified procedures for the microbiological assay of serum vitamin B_{12} and serum folate.
1972, Med. Lab. Technol., 29, 171-181.

TEUNISSON, D.J. (1971a)
Elutriation and coulter counts of Tetrahymena pyriformis grown in peanut and cottonseed meal media.
1971, Appl. Microbiol., 21, 878-887.

TEUNISSON, D.J. (1971b)
Microbiological assay of intact proteins using Tetrahymena pyriformis W. 1. Survey of protein concentrates.
1971, Anal. Biochem., 2, 405-420.

TOENNIES, G. and KOLB, J.J. (1941)
Methionine studies VI. dl-methionine sulphone.
1941, J. Biol. Chem., 140, 131-134.

TONZETICH, J. (1976)
Chromatographic separation of methionine, methionine sulphoxide, methionine sulphone and their products of oral metabolism.
1976, Anal. Biochem., 73, 290-300.

TSAN, M.F. and CHEN, J.W. (1980)
Oxidation of methionine by human polymorphonuclear leucocyctes.
1980, J. Clin. Invest., 65, 1041-1050.

TUFFNELL, J.M. and PAYNE, J.W. (1981)
Methionine sulphone production by Escherichia coli: influence of physiological status on oxidation and reduction of methionine sulphoxide.
1981, FEMS Microbiol. Letters., 12, 273-277.

UDENFRIEND, S. (1978)
Fluorescent methods for isolation, characterisation and assay of peptides and proteins.
Versatility Of Proteins. Choh Hao Li (1975).
Proc. Intl. Symp. On Proteins, Taipei, 1978.
1978, Academic Press, New York.

VARMA, S.D., ETS, T.K. and RICHARDS, R.D. (1977)
Protection against superoxide radicals in rat lens.
1977, Ophthal. Res., 9, 421-431.

VISWANTHA, T. and LIENER, I.E. (1956)
Isolation and properties of a proteinase from Tetrahymena pyriformis W.
1956, Arch. Biochem. Biophys., 61, 410-421.

WALL, L.L. and GEHRKE, C.W. (1974)
Automated enzymatic determination of L-lysine in grain.
1974, J. Assoc. Off. Anal. Chem., 57, 1098-1103.

WALSH, K.A. and NEURATH H. (1964)
Composition of α -chymotrypsinogen.
The Proteins Vol.1. Composition, Structure And Function.
Neurath, H. (ed)
1964, Academic Press, New York.

WILLIAMS, A.P., HEWITT, D. and COCKBURN, J.E. (1979)
A collaborative study on the determination of cyst(e)ine in feedingstuffs.
1979, J. Sci. Food Agric., 30, 469-474.

WINITZ, M. and ADAMS, R.F. (1971)
Chemically defined diets.
Balanced Nutrition And Therapy. K. Lang, W. Fekl and G. Berg (eds)
1971, Georg. Thieme. Verlag., Stuttgart, 46-66.

WINITZ, M., SEEDMAN, D.A. and GRAFF, J. (1970)
Studies in metabolic nutrition employing chemically defined diets. I. Extended feeding of human adult males.
1970, Am. J. Clin. Nutr., 23, 525-537.

UDENFRIEND, S. (1978)
Fluorescent methods for isolation, characterisation and assay of peptides and proteins.
Versatility Of Proteins. Choh Hao Li (1975).
Proc. Intl. Symp. On Proteins, Taipei, 1978.
1978, Academic Press, New York.

VARMA, S.D., ETS, T.K. and RICHARDS, R.D. (1977)
Protection against superoxide radicals in rat lens.
1977, Ophthal. Res., 9, 421-431.

VISWANTHA, T. and LIENER, I.E. (1956)
Isolation and properties of a proteinase from Tetrahymena pyriformis W.
1956, Arch. Biochem. Biophys., 61, 410-421.

WALL, L.L. and GEHRKE, C.W. (1974)
Automated enzymatic determination of L-lysine in grain.
1974, J. Assoc. Off. Anal. Chem., 57, 1098-1103.

WALSH, K.A. and NEURATH H. (1964)
Composition of α -chymotrypsinogen.
The Proteins Vol.1. Composition, Structure And Function.
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1964, Academic Press, New York.

WILLIAMS, A.P., HEWITT, D. and COCKBURN, J.E. (1979)
A collaborative study on the determination of cyst(e)ine in feedingstuffs.
1979, J. Sci. Food Agric., 30, 469-474.

WINITZ, M. and ADAMS, R.F. (1971)
Chemically defined diets.
Balanced Nutrition And Therapy. K. Lang, W. Fekl and G. Berg (eds)
1971, Georg. Thieme. Verlag., Stuttgart, 46-66.

WINITZ, M., SEEDMAN, D.A. and GRAFF, J. (1970)
Studies in metabolic nutrition employing chemically defined diets. I. Extended feeding of human adult males.
1970, Am. J. Clin. Nutr., 23, 525-537.

WOODHAM, A.A. (1978)
The nutritive value of mixed proteins.
Nutritional Improvement Of Food And Feed Proteins.
M. Friedman (ed)
1978, Plenum Press, New York, 365-378.

WOLDRINGH, C.L. (1973)
Effects of toluene and phenethyl alcohol on the ultra structure of Escherichia coli.
1973, J. Bact., 114, 1359-1361.

WOLZAK, A., ELIAS, L.G. and BRESSANI, R. (1981)
Protein quality of vegetable proteins as determined by traditional biological methods and rapid chemical assays.
1981, J. Agri. Food Chem., 29, 1063-1068.

ZAGALAK, B. and FRIEDRICH, W. (1979)
Vitamin B_{12}.
Proceedings of Third European Symposium.
1979, Walter de Gruyter & Co., New York.

ZDANOWSKI, M.K and RASMUSSEN, L. (1979b)
Tyrosine requirement in Tetrahymena revealed by nurtitional amino acid imbalances.
1979, Comp. Biochem. Biophys., 62, 331-334.

ZDANOWSKI, M.K. and RASMUSSEN, L. (1979b)
Peptidase activity in Tetrahymena.
1979, J. Cell Physiol., 100, 407-412.