THE KINETICS OF MILD ACID HYDROLYSIS OF GLUTEN AND THE FUNCTIONAL PROPERTIES OF THE MODIFIED PROTEINS AT VARIOUS LEVELS OF HYDROLYSIS

A Thesis presented in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Biotechnology at Massey University

JOHN JOSEPH HIGGINS

1988

ABSTRACT

Gluten is the mixture of proteins remaining in wheat flour after starch and water soluble components have been extracted by washing. Its unique dough forming properties are due to the structure of the proteins. A feature of the protein is the high glutamine content, about 30% of the total amino acids. A number of studies have shown that gluten's properties of insolubility and water-binding can be substantially modified by mild acid hydrolysis. The principal effect of the mild acid treatment is to hydrolyse the amide side chain of glutamine such that the amide group is replaced by a carboxyl group. In addition, it is known that hydrolysis of peptide bonds can have a large influence on the functional properties of proteins.

The aims of this study were to determine the kinetics of the acid catalysed deamidation and peptide bond hydrolysis reactions, and to comment on the resultant changes in functional properties.

A statistically designed experiment was used to determine the effect of temperature, hydrogen ion concentration and gluten concentration. An initial rate analysis of the results showed that reactions could be described by equations of the form:

Rate of amide bond hydrolysis $= k_1[\text{Amide}][H^+]$

and Rate of peptide bond hydrolysis $= k_2[\text{Peptide}][H^+]$

where $k = k_o e^{-\frac{E}{R} \cdot \frac{1}{T}}$

A stoichiometric analysis of the experimental data confirmed that hydrogen ions were consumed in both reactions.

A numerical solution was developed to predict the extent of reaction with time. A computer program incorporating the solution was used to simulate the reaction and test the

solution. The simulation results appeared to overestimate the progress of the reaction with time.

A series of ten gluten powders, hydrolysed to different extents was prepared at small pilot scale. The composition of the samples was determined and compared with the extent of hydrolysis predicted by the reaction simulation. Reasonable agreement was achieved.

A selection of the functional properties of the prepared samples was examined.

The quantity of alkali required to dissolve each preparation to the extent of its solubility at pH 7.6 increased markedly with the extent of hydrolysis due to the additional carboxyl groups requiring neutralization.

The flavour of each preparation was examined. A cereal flavour was found to decrease with the extent of hydrolysis. A lingering bitter flavour was found to increase with the extent of hydrolysis.

The solubility of all preparations at pH 7.6 in 0.1 M phosphate buffer increased with the extent of treatment so that the most hydrolysed samples were almost completely soluble. No (significant) difference was found between freeze dried and spray dried samples. Samples prepared without dialysis showed no solubility difference from those prepared with dialysis at a similar extent of hydrolysis.

The hydrophobicity of the preparations was measured using two different fluorescent probes and was found to increase with the extent of hydrolysis. The emulsion-forming properties of the preparations were found to depend on the oil used in the test, as would be expected if hydrophobicity was equivalent to the hydrophile lipophile balance, which is commonly used to classify emulsifying agents. The preparations did not, however, show the additivity properties of emulsifiers. It was also shown

that only the soluble portion of the preparations was responsible for emulsion formation.

The possibility of achieving deamidation of gluten using the enzymes peptidoglutaminase I and II was examined. No activity against gluten or partially hydrolysed gluten was found.

ACKNOWLEDGEMENTS

I am deeply grateful to the New Zealand Dairy Research Institute for giving me the opportunity to complete this work. I am indebted to many of my colleagues there for assistance with various aspects of this work and many helpful discussions. Particular mention must be made of Julie Anderson for her patient assistance with statistical design and interpretation.

My supervisors Professor Earle, Dr Maddox and Dr Sanderson have provided thoughtful guidance and shown commendable patience.

My family too have been tolerant throughout.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

* In fiche

SYMBOLS AND ABBREVIATIONS

a	activity (e.g. a_{H^+} is hydrogen ion activity)
A_{340}	Absorbance measured at a wavelength of 340 nm
ANS	8-anilino-1-naphthalenesulfonate
°C	degrees centigrade
cc	cubic centimetre
CI	confidence interval
CPA	cis parinaric acid
E	activation energy
E	the observed electrode potential in the Nernst equation
$E^{o'}$	the stable fixed potential including reference potential
F	the Faraday
GDH	L-glutamate dehydrogenase
g or gm	gram
HLB	hydrophile lipophile balance
k	reaction rate constant
°K	degrees Kelvin
L-glu	L-glutamic acid

ln	logarithm to base e
LOF	lack of fit
$\log_e$	logarithm to base e
$\log_{10}$	logarithm to base 10
M	molar
ml	millilitre
mm	millimetre
mM	millimolar
N_A	number of moles of component A
N_{Ao}	initial number of moles of component A
NAD	nicotinamide adenine denucleotide
NADH	nicotinamide adenine denucleotide reduced
nm	nanometre
PCA	pyrrolidone carboxylic acid
SDS	sodium dodecyl sulphate
t	statistic calculated in Students t test
T	temperature, °K
TN	total nitrogen content
TNBS	trinitrobenzene sulfonic acid

R	ideal gas law constant
W	watt
w/v	weight per unit volume
X_A	mole fraction of component A
v	the difference in voltage measured by a pH electrode over the range of pH
σ_{n-1}	standard deviation estimated using a population sample of size n
μ	ionic strength
μl	microlitre

1. INTRODUCTION AND LITERATURE REVIEW

1.1 Wheat gluten

1.1.1 Wheat gluten preparation

"Wheat gluten has been produced traditionally by two similar processes, the Martin and the batter processes. In the Martin process, flour is mixed into a stiff dough containing about 60% water. After hydration and development, the dough is kneaded with reciprocating rolls under water at high pressure. The kneading process washes out the starch and leaves the gluten as a coherent mass. The gluten is then dried, usually by a type of flash-drying, so as not to damage its native properties. This produces so-called vital gluten. Devitalized gluten can then be prepared by using higher drying temperatures to produce a desired degree of denaturation. In the batter process, the flour and water are fed continuously into a batter mixer. After a short rest period, the batter is pumped through a dispersal pump and washed with water on reciprocating or cylindrical screens. The gluten particles are collected from the washing screens and are processed into dry vital gluten as in the older Martin process. Other processes for isolating wheat gluten have been described but none has become important commercially". (Bushuk, 1981)

1.1.2 Chemistry of gluten

The chemistry of gluten has been extensively studied because of its great importance in dough formation and bread production. Gluten is not however a simple system but a complex of many proteins which interact with each other and other ingredients. Although there is a general understanding of its chemistry and mode of action much remains to be clarified. Because of the extensive study the subject has also been extensively reviewed. A brief outline only will be

given here, (Bushuk, 1981; Shukla, 1975; Kasarda et al, 1971; Schofield and Booth, 1983). Most cereal proteins have been described in terms of solubility using the method of classification developed by Osborne (1907). This divides cereal proteins into albumen, globulin, prolamine and glutelin based on solubility in water, dilute neutral salt solution, 70% ethyl alcohol and dilute acid or alkali respectively.

The major fractions in gluten (68% of wheat protein) are gliadin (prolamine) and glutenin (glutelin) (16% of wheat proteins). All four major solubility fractions have been found to be polydisperse and heterogeneous but represent chemically different groups of proteins which exhibit different physical properties.

Glutenin has an average molecular weight of about 2 million but consists of small sub units linked by interpolypeptide disulfide linkages. Gliadin proteins have intrapolypeptide disulfide linkages and an average molecular weight of about 28 000.

Gluten represents about 80% of the total protein of wheat flour and the dry solids contain 75 to 85% protein depending on the thoroughness of washing. Approximately 7% of the total protein of gluten is water soluble.

The chemistry and function of gluten are dominated by the very high content of glutamine, and, to a lesser extent, proline. The amino acid composition of gluten is shown in Table 1.1.

Glutamine has a side-chain amide group which is acid labile. Acid treatment converts the amide group to a carboxyl group, converting glutamine to glutamic acid, with the release of ammonia.

Table 1.1: Amino acid composition of gluten, gliadinm and glutenin[a] (moles amino acid per 10^5g)

Amino Acid	Gluten	Gliadin	Glutenin	Amino Acid	Gluten	Gliadin	Glutenin
Arginine	20	15	20	Valine	45	43	41
Histidine	15	15	13	Leucine	59	62	57
Lysine	9	5	13	Isoleucine	33	37	28
Threonine	21	18	26	Proline	137	148	114
Serine	40	38	50	Tyrosine	20	16	25
Aspartic acid	22	20	23	Phenylalanine	32	38	27
Glutamic acid	290	317	278	Tryptophan	6	5	8
Glycine	47	25	78	Cystine/2	14	10	10
Alanine	30	25	34	Methionine	12	12	12
				Ammonia	298	301	240

[a] Data of Wu and Dimler, (1963)

It is this reaction which forms the basis of the present study.

1.1.3 Functional properties of gluten

The functional properties of gluten with respect to its role in dough formation and bread loaf structure have been extensively studied because of the economic importance of bread and baking. The basic discovery for such studies was the demonstration of a strong positive correlation between the protein content of flour and loaf volume (Schofield and Booth, 1983). Other reviews are by Pyler (1983) and MacRitchie (1984). Starch-lipid-protein interactions have been reviewed by Pomeranz and Chung (1981).

The differences in functional properties between glutenin and gliadin have been summarized by Shukla (1975) as follows:

COMPARISON OF GLUTENIN AND GLIADIN PROPERTIES

Glutenin	Gliadin
1. Non-Newtonian fluid	Newtonian fluid
2. Considerable molecular attraction	Least molecular attraction
3. Molecules are random coils	Near globular molecules
4. Tensile strength 3.38 x 10^{-3} psi	Tensile strength 1.42 x 10^{-3} psi
5. Film elongation 63%	Film elongation 72%
6. Less extensible	Extensible
7. Elastic	Less elastic
8. Nonswelling	Swells in water

The structure of dough is a continuous matrix of protein with embedded starch granules. Mechanical work is important in establishing the protein matrix.

According to Kasarda et al (1971) "the hydrogen-bonding potential contributed by the many amide side chains of the gluten proteins is largely responsible for the cohesiveness of these proteins. The amide group is both an acceptor and donor of hydrogen bonds capable of interacting with other amide groups and with groups that are only acceptors or only donors. Water molecules perform an important function by satisfying some of the hydrogen-bonding sites and facilitating interchange of hydrogen bonds among proteins and other molecular species. Too much water, of course, will weaken a dough by occupying many sites normally involved in protein-protein interactions.

Hydrophobic and other apolar types of bonding probably contribute to the cohesiveness of the gluten proteins, since these proteins have many non polar side chains. There is, however, little direct evidence for such bonding. Areas of nonpolar character are probably involved in lipid binding as well as protein-protein interactions. The nonpolar part of phospholipid molecules may interact with nonpolar sites on the protein molecules, leaving the polar group free on the surface. Such polar groups can change the surface potential of the proteins by adding charges or by forming ionic bonds with oppositely charged side chains. Obviously, binding of either polar or nonpolar lipids can affect protein aggregation in a number of ways. Although the gluten proteins have relatively few ionizable side chains, ionic bonds between oppositely charged groups may be involved in protein-protein and other types of interactions".

Various models for the protein structure of dough have been proposed (Bushuk, 1981); the most recent being that of Graveland et al (1985).

1.1.4 Solubility of gluten

The solubility characteristics of gliadin were described by Holme and Briggs (1959).

"Gliadin, in contact with water, imbibes or combines physically with a considerable amount of water and yields the doughlike, hydrated mass characteristic of the protein at and near neutral pH values. This hydration tendency of the gliadin molecules is, however, not sufficient to overcome an inherent intermolecular attraction, probably through hydrogen bonds involving the amide groups on the protein and thus not sufficient to allow the gliadin to disperse molecularly in the aqueous solvent. Imposition of an electrical charge on the gliadin molecules, as by titration to pH values below 4, shifts the balance of hydration versus intermolecular binding tendencies in favour of the former, and the protein passes into what approaches molecular dispersion in water. A small increase in ionic strength of such a solution neutralizes the charge effect and the gliadin precipitates again as a hydrated mass. Independent of this peptizing action of a charge on the protein molecule, addition of substances such as urea, formamide or acetamide, which are stronger hydrogen bonders than water, serves, through competition with the intermolecular hydrogen bonds in the protein mass, to lower this intermolecular cross-linking tendency and to promote the passage of the protein into dispersion or solution. In a 2 to 3 molar solution of urea, for example, the intermolecular attraction of gliadin is so completely nullified that molecular or nearly molecular dispersions are attained in the urea-aqueous solvent independent of the pH and ionic strength of the solution".

"Partially deamidized crude gliadin, after 10-50% of the amide nitrogen has been removed under conditions accompanied by no detectable peptide bond hydrolysis, shows electrophoretically only a statistical heterogeneity

indicated by a spreading of a single peak greater than that due to diffusion. A marked change in solubility characteristics accompanies the deamidation, however; the deamidated gliadin becomes soluble at neutral pH range with little or no effect of changes in ionic strength".

The effect of amide groups of glutamine on gluten solubility was further studied by Beckwith et al (1963). Gluten was modified either by conversion of some of the side-chain amide groups to esters by reaction with methanol containing hydrogen chloride or by conversion of amide groups to carboxyl groups by digestion in 0.6 or 1.2 N HCl at 30°C for periods of up to 48 hours. There was very little peptide bond hydrolysis and no significant change in the secondary structure of the native proteins. The methyl ester derivatives were moderately soluble not only at pH 3, as is native gluten, but also at neutrality where native gluten is insoluble. Unlike the esterified proteins, a product insoluble at pH 3 but soluble at pH 8 was obtained when additional free carboxyl groups were produced in gluten as a result of amide hydrolysis. These results confirm the role of the side chain amide groups in intermolecular hydrogen bonding which contributes to the insolubility of the protein. A similar result was obtained from a study by Krull and Wall (1966) using synthetic polypeptides containing glutamine and glutamic acid. Measurement of solubility and optical rotary dispersion at various pH values established that the presence of amide groups increased the minimum pH for solubility and decreased the helical content of the polymers. It was concluded that hydrogen bonds and hydrophobic bonds act synergistically to maintain molecular structure and to reduce aggregation at the isoelectric point. Disturbing either type of bond may render the associations sufficiently unstable so that the solvating action of water may overcome the remaining interactions. A further study, using synthetic polypeptides containing side-chain amide groups showed that these groups may be associated with

indicated by a spreading of a single peak greater than that due to diffusion. A marked change in solubility characteristics accompanies the deamidation, however; the deamidated gliadin becomes soluble at neutral pH range with little or no effect of changes in ionic strength".

The effect of amide groups of glutamine on gluten solubility was further studied by Beckwith et al (1963). Gluten was modified either by conversion of some of the side-chain amide groups to esters by reaction with methanol containing hydrogen chloride or by conversion of amide groups to carboxyl groups by digestion in 0.6 or 1.2 N HCl at 30°C for periods of up to 48 hours. There was very little peptide bond hydrolysis and no significant change in the secondary structure of the native proteins. The methyl ester derivatives were moderately soluble not only at pH 3, as is native gluten, but also at neutrality where native gluten is insoluble. Unlike the esterified proteins, a product insoluble at pH 3 but soluble at pH 8 was obtained when additional free carboxyl groups were produced in gluten as a result of amide hydrolysis. These results confirm the role of the side chain amide groups in intermolecular hydrogen bonding which contributes to the insolubility of the protein. A similar result was obtained from a study by Krull and Wall (1966) using synthetic polypeptides containing glutamine and glutamic acid. Measurement of solubility and optical rotary dispersion at various pH values established that the presence of amide groups increased the minimum pH for solubility and decreased the helical content of the polymers. It was concluded that hydrogen bonds and hydrophobic bonds act synergistically to maintain molecular structure and to reduce aggregation at the isoelectric point. Disturbing either type of bond may render the associations sufficiently unstable so that the solvating action of water may overcome the remaining interactions. A further study, using synthetic polypeptides containing side-chain amide groups showed that these groups may be associated with

peptide amides or side-chain amides in the same or other molecules through hydrogen bonding to affect solubility and conformation of the protein (Krull et al, 1965). It was also found that polyglutamine of high molecular weight did not dissolve in water whereas low-molecular weight glutamine-containing polymers did. A similar effect is found for other known polymers containing hydrogen-bonding groups, such as amylose or polyacrylamide, the larger polymers being insoluble. This suggests a reason for the different solubility characteristics of the glutenin and gliadin fractions of gluten which are very similar apart from molecular weight.

An understanding of the factors affecting the solubility of proteins is fundamental to the study of proteins. The effects of pH, ionic strength, the phenomena of salting in and salting out, urea and the reduction of disulphide bonds are all well-known and have been applied to the study of the proteins of gluten.

Recent studies have shown that proteins can be rendered soluble depending on the energy used in dissolving them. This was shown for soy protein isolates by Shen (1976) and also by Furukawa and Ohta (1983) who examined the effect of ultrasonication and noted the importance of order of salt addition in avoiding salting out. The effect of high speed homogenization in rendering gluten proteins soluble was noted by Patey and Shearer (1980).

Proteins can be solubilized in the presence of surfactants and the use of sodium dodecyl sulfate (SDS) for this purpose has been extensively studied.

The work of Reynolds and Tanford (1970 a,b) established the conditions necessary for SDS to bind to proteins. These are that approximately 1.4 g SDS will bind to 1 g of protein. The binding depends on the concentration of SDS in the monomeric form and this must be >1.0 mM for maximum

binding. The ionic strength of the medium determines the critical micelle concentration (CMC) for SDS and thus the amount present in the monomeric form. The maximum binding of SDS takes place when ionic strength u _ 0.1.

Several studies have noted the effectiveness of SDS in solubilization of gluten proteins. Danno et al (1974) found that 76% of defatted wheat flour total nitrogen was solubilized by 1% SDS in 0.05M phosphate at pH 6.8, and Graveland et al (1974) solubilized 65-67% of total nitrogen with 1.5% SDS solution. When 0.069M SDS in 0.1M tris HCl, pH 8.0, was used, Bottomley et al (1982) achieved approximately 95% solubilization of flour and gluten proteins. Almost complete extraction of protein from wheat flour was achieved by Danno (1981) using two extractions with 0.5% SDS in 0.05M phosphate, pH 7.0, in combination with Waring Blender stirring.

It would appear that a combination of the factors SDS, pH and mechanical action are effective in solubilizing wheat flour proteins.

1.2 Acid Hydrolysis

1.2.1 Determination of kinetic data

The field of chemical reaction kinetics and design of reactors is described in a number of reviews and textbooks. Those used as a basis for the present study were by Laidler (1965) and Levenspiel (1982). Details of the techniques and difficulties of interpreting experimental data using a statistical approach are given by Kittrell (1970).

1.2.2 Acid hydrolysis of amides

Amides are susceptible to both acid and base catalysis and the phenomena have been extensively investigated. A general treatment of acid-base catalysis was given by Moore and Pearson (1981) and is summarised below.

There are several distinct types of acid-base catalysis, almost all of which involve a proton transfer in at least one step. These are treated, in general, in terms of substrate S or SH on which the acids or bases work, S^- the conjugate base of the substrate SH, B a general base, BH^+ its conjugate acid, HA a general acid, and A^- its conjugate base. B and A^- may or may not be the same as may BH^+ and HA. R is some other reactant not acting as a base or acid.

For acid hydrolysis of amides the appropriate general case is one in which prior equilibrium between substrate and hydrogen ion is followed by a rate-determining reaction with other reagent:

$$S + H^+ \rightleftharpoons SH^+ \qquad (1)$$

$$SH^+ + R \xrightarrow{\text{slow}} \text{products} \qquad (2)$$

$$\text{rate} = k[SH^+]\,[R] = kK_{eq}[S]\,[H^+]\,[R] \qquad (3)$$

where K_{eq} is the equilibrium constant for (1) and k is the rate constant for (2).

An important feature of this type of reaction is that the concentration of the complex SH^+ depends only on the hydrogen ion concentration in solution, regardless of the source of the hydrogen ion. Hence the rate also is dependent on the hydrogen ion only and not on the concentration of any other acids in the solution. This is an example of specific hydrogen ion catalysis. The observed kinetics depend a great deal on the value of the equilibrium constant of (1) and the relative concentrations of H^+ and S. If K_{eq} is large enough, S or H^+, whichever is present in least amount, is completely converted to SH^+. The observed kinetics are second order, first order in SH^+, and first order in R. The more usual case is for K_{eq} to be small so that S and H^+ are present in amounts initially added minus amounts that have reacted. The observed

kinetics are third order, first order in H^+, S, and R. Since H^+ is frequently constant, being regenerated in step (2), the kinetics may be psuedo-second order or pseudo-first order, depending on the change in R. The pseudo-constants, however, are dependent on the hydrogen ion concentration in solution.

In the case of amide hydrolysis H^+ is not regenerated (forms NH_4^+) and R is H_2O. In strong acids the rate of hydrolysis levels off and may decrease. This is due to the concentration of H_2O becoming a significant factor.

Also the acid hydrolysis of amides provides examples of K_{eq} large enough to cause the rate to level off at about 3M hydrogen ion concentrations.

Day and Ingold (1941) listed eight mechanisms for ester hydrolysis and suggested the following notation:

A acid hydrolysis	B base hydrolysis
1 unimolecular reaction	2 bimolecular reaction
' acyl-oxygen scission	" alkyl-oxygen scission

The mechanism for acid hydrolysis of amides is of the A'2 type

$$H_3O^+ + RCONH_2 \underset{}{\overset{\text{fast}}{\rightleftharpoons}} RCONH_3^+ + H_2O$$

$$H_2O + RCONH_3^+ \xrightarrow{\text{slow}} RCO\text{-----}O^+(H)(H) + NH_3$$

$$RCO\text{-----}O^+(H)(H) \xrightarrow{H_2O} RCOOH + H_3O^+$$

In acid solution NH_3 exists as NH_4^+

$$NH_3 + H_3O^+ \dashrightarrow NH_4^+ + H_2O \quad \text{(see Appendix 1.1)}$$

Several authors have discussed and reviewed the kinetics of amide hydrolysis. Laidler (1965) noted that the acid hydrolysis of amides was a second order reaction in dilute solution. At low acid concentrations a good correlation exists between pH and the logarithm of the rate constant.

"A complication exists, however, in that the rate ceases to be linear in (H^+) at higher acid concentrations, and actually reaches a maximum value at an acid concentration that is usually between 2 and 5M. This result was at one time explained in terms of the hypothesis that at high acid concentrations the protonated amide combined with the anion of the acid, forming an unhydrolysable complex. This, however, was shown to be incorrect by the fact that the position of the maximum rate was independent of the amide concentration. It has been suggested that the falling off of the rate at high acid concentrations is due to the decreased activity of the water. This hypothesis has been found to provide a satisfactory quantitative treatment of amide hydrolysis in acid solution. It is to be noted that the hypothesis is only consistent with an A'2 mechanism". (Laidler, 1965).

To account for these phenomena on a theoretical basis various acidity functions have been defined and were recently reviewed by Finston and Rychtman (1982). Laidler (1965) showed that the situation may be formulated in terms of the general A'2 mechanism.

$$H_3O^+ + S \overset{K1}{\rightleftharpoons} SH^+ + H_2O$$

$$SH^+ + H_2O \overset{K2}{\rightleftharpoons} X^* \overset{k}{\longrightarrow} \text{products}$$

Application of the steady-state treatment (Bailey and Ollis, 1977) leads to the result that the rate is

$$v = kK_2[SH^+]\,[H_2O]\,\frac{f_{SH^+} f_{H_2O}}{f_{X^*}} \qquad (1)$$

$$= kK_1K2\,[S][H^+]\,\frac{f_S\, f_{H^+}}{f_{X^*}} \qquad (2)$$

Where the f's are activity coefficients. Derivation of (1) and (2) is given in Appendix 1.2 (Laidler, 1965). The quantity $f_S \cdot f_{H^+} / f_{X^*}$ is believed to be fairly constant, so that the rate can be written as:

$$v = k'\,[S]\,[H^+] \qquad (3)$$

where k' is a second-order rate constant for the reaction. The experimentally determined first-order rate constant k^1, which expresses the variation in rate with acid concentration, is given in terms of the sum of the concentrations of S and SH^+,

$$v = k^1\,([S] + [SH^+]) \qquad (4)$$

Hammett's acidity function h_o is defined by

$$h_o = K_{SH+}\,\frac{[SH^+]}{[S]} \qquad (5)$$

alternatively, $H_o = -\log_{10} h_o = -\log_{10} a_{H+}\,\frac{f_B}{f_{B_H^+}}$

where a_{H+} equal to $[H^+]f_{H^+}$, is the activity of the hydrogen ion.

Elimination of [S] and $[SH^+]$ from Eqs. (3), (4) and (5) then leads to

$$k^1 = \frac{k'K_{SH^+}[H^+]}{K_{SH^+} + h_o} \qquad (6)$$

The following special cases of this equation are of interest:

1. If the acid concentration is sufficiently small, $h_o << K_{SH^+}$ then the rate constant is given by

$$k^1 = k'K_{SH^+}[H^+] \qquad (7)$$

2. At sufficiently high acid concentrations, $h_o >> K_{SH^+}$ so that

$$k^1 = \frac{k'K_{SH^+}[H^+]}{h_o} \qquad (8)$$

At concentrations greater than about 2M, h_o increases much more rapidly than $[H^+]$, with the result that k^1 decreases with increasing acid concentration (Laidler, 1965).

The rate of amide hydrolysis by acids has also been reviewed by Challis and Challis (1970) who noted that for most amides

$$\text{Rate} = k\,[\text{Amide}][H_3O^+]$$

but with a rate maximum at some high acidity dependent on both the solvent acid and amide structure usually in the region of 2M to 5M for sulphuric acid. This is where most amides are extensively protonated on the oxygen atom and a logical deduction is that this species is the reactive intermediate.

Below the rate maximum increasing acidity raises the concentration of the reactive intermediate, whereas beyond

the rate maximum the chief effect of increasing acidity is to decrease the concentration or activity of water.

A relationship between the variables rate coefficient, acidity function and water activity was found to apply to a number of amides over a range of 0.6 to 10.1M HCl (Yates and Stevens, 1965; Yates and Riordan, 1965).

The acidity function was defined as

$$H_A = -\log a_{H^+} \cdot f_A / f_{AH^+} = pK_{AH^+} - \log [AH^+]/[A]$$

where A and AH^+ refer specifically to amides and their conjugate acids and $K_{AH^+} = a_{H^+} a_A / a_{AH^+}$ is the thermodynamic dissociation constant of the conjugate acid. It was shown that for hydrolysis of weakly basic amides i.e. present in the reaction medium as essentially an unprotonated amide, then

$$k_{obs} = k/K_{SH^+} h_A a_{H_2O}^r$$

Taking logarithms and rearranging gives

$$k_{obs} + H_A = r \log a_{H_2O} + \text{constant} \qquad (1)$$

and for more basic amides which are present as a mixture of the amide and its conjugate acid the corresponding relationship is;

$$\log k_{obs} - \log (h_A/K_{AH^+} + h_A) = r \log a_{H_2O} + \text{constant} \qquad (2)$$

In these equations k_{obs} is the observed pseudo first order rate constant, K_{AH^+} is the dissociation constant of protonated amide, and r is a hydration parameter which gives a direct measure of the number of water molecules needed to convert a protonated substrate molecule to the transition state.

If the assumptions involved in the derivation of Eqs (1) and (2) are justified, plots of either ($\log k_{obs} + H_A$) or $\log k_{obs} - \log (h_A/K_{SH^+} + h_A)$ against $\log a_{H_2O}$ should be linear.

Values of H_A in HCl were given by Yates and Riordan (1965) who also considered the effect of measurement of reaction rates at temperatures other than 25°C at which acidity functions and water activities are usually measured.

Mittal, et al (1982) studied amide hydrolysis with HCl. The concentration of the amides was varied over a range of 1-20M depending on their solubility. The concentration of HCl was kept constant at 0.1M. Rates increased to a maximum but peaked at higher concentration (ca 16M) than for acid concentration. It was shown that

$$k_2 = kK$$

where alternative descriptions are;

(a) $$\frac{-\, d[H^+]}{dt} = \frac{kK\ [H^+]\ [Amide]}{1 + K\ [Amide]}$$

(b) $$\text{rate} = k_2\ [Amide]\ [H^+]$$

(a) ---→ (b) when $1 >> K\ [Amide]$

The derivation of equation (a) above is given in Appendix 1.3.

Mittal et al (1982) also studied hydrolysis of amides with various carboxylic acids.

They showed that the catalysis was indeed due to proton transfer from a specific hydronium ion.

$$\text{Thus} \quad d[NH_4^+] = k_2 [H^+] [RCONH_2]$$

$$\text{or} \quad k_{obs} = k_2 [H^+]$$

Acid concentrations were 0.1 - 0.4M and include acetic acid, as used by Wu et al (1976) and by Aranyi and Hawrylewicz (1972) for gluten hydrolysis. $[H^+]$ was calculated from the dissociation constants.

1.2.3 Acid hydrolysis of proteins

Acid hydrolysis of proteins has not been intensively studied in recent years. As a consequence the reviews and most of the papers referred to are not recent. The reason for this is that most recent hydrolyses of proteins have used proteolytic enzymes because they give the specific partial hydrolysis required for amino acid sequence studies.

The hydrolysis of proteins by both acid and enzymatic methods has been reviewed by Hill (1965). Partial acid hydrolysis of proteins has been reviewed by Light (1967) and Kasper (1970).

In a comprehensive review, Leach (1953) examined measurement methods, reaction kinetics and amide and peptide bond hydrolysis mechanisms from a "rate of attack" point of view. Some points of particular interest are drawn from this review.

1.2.4 Acid hydrolysis of amide bonds in proteins

The amino acids glutamine and asparagine have amide side-chains which are relatively more acid labile than peptide bonds (Steinhardt and Fugitt, 1942; Leach and Lindley, 1953).

Other important points noted in the review by Leach (1953) are as follows:

(1) The rates of hydrolysis for glutamine and asparagine within peptide chains are not the same for all proteins but are similar.

(2) Comparative studies involving proteins and similar amides have shown similar reaction rates but free glutamine hydrolyses much more rapidly than its peptides. Glutamine is very much more labile than asparagine. Reviews of several studies have shown that the kinetics can be described by the logical assumption that rate of hydrolysis = k_1 [Amide][H^+]

There does not, however, appear to be a definitive demonstration of this point.

(3) The studies also suggest that the amino acid environment within the protein has little effect upon the rate of hydrolysis of the amide side chain.

(4) If the ratio of glutamine to asparagine were low (this is not the case with gluten, see Table 1.1) then the apparent kinetics may not be first order in [Amide] because of the difference in lability of glutamine and asparagine.

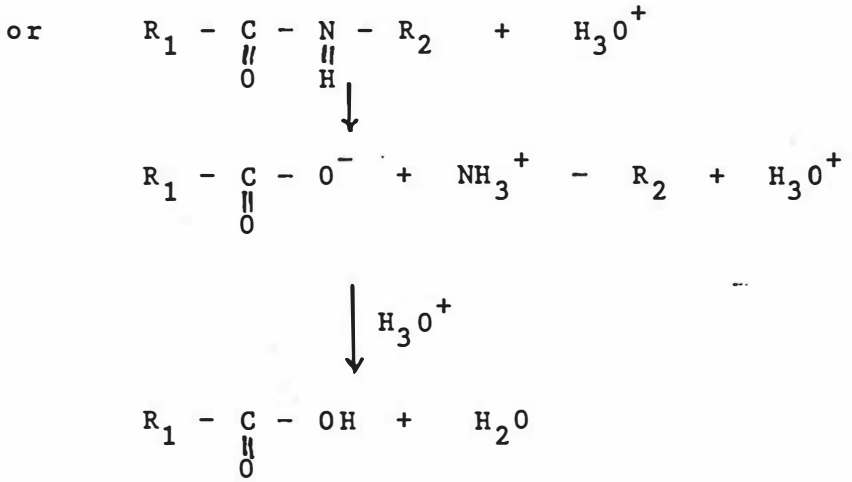
(5) The acid or other added anion can have a large effect on the reaction.

1.2.5 Acid hydrolysis of peptide bonds in proteins

The mechanism of acid hydrolysis of peptide bonds has been described by Lawrence and Moore (1971) and is illustrated here for a dipeptide.

$$^{+}H_3N\ CH(R_1)CONH\ CH(R_2)\ CO_2H \quad + \quad H_3O^{+}$$

$$\longrightarrow\ ^{+}H_3N\ CH(R_1)\ CO_2H \quad + \quad ^{+}H_3N\ CH(R_2)\ CO_2H$$



It should be noted that one hydrogen ion is consumed for each peptide bond broken. The stability of peptide bonds to hydrolysis varies considerably, the relative stability of the different bonds depending on the hydrogen ion concentration. Acid hydrolysis of peptide bonds can therefore be regarded as having a partial specificity (Leach, 1953). The preferential liberation of aspartic acid was studied by Blackburn and Lee (1954). Other apparently preferential liberations of amino acids by acid hydrolysis have been reviewed by Leach (1953) and more recently by Han et al (1983).

Studies of the kinetics of peptide bond hydrolysis of proteins have given mixed results and have been variously interpreted (Leach, 1953).

Careful studies of the acid hydrolysis of dipeptides showed that the reaction was first order in peptide concentration and that the reaction rate was directly proportional to the acid concentration (Harris et al, 1956) so that the

reaction rate could be represented by a second order equation (Lawrence and Moore, 1951)

$$\text{i.e. rate of hydrolysis} = k_2 [\text{peptide}][H^+]$$

Leach (1953) concluded that:

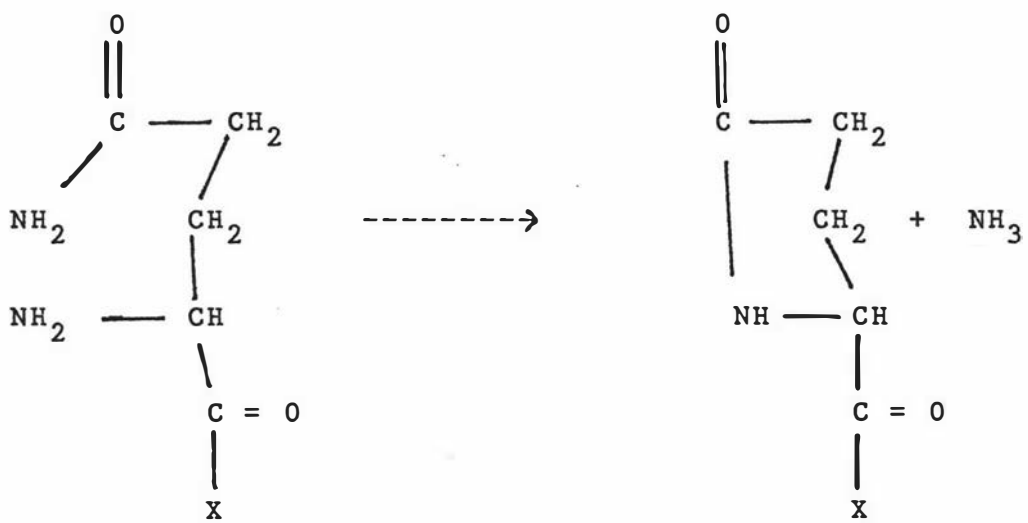
"In the light of more complete kinetic data on hydrolytic reactions in simple systems such as esters, amides and peptides, it seems most likely that protein hydrolysis at a constant pH is first-order throughout with respect to any particular peptide-bond type. However, it has already been pointed out that these bonds differ considerably in strength and the hydrolysis could not be expected to proceed at exactly the same rate throughout. The first rapid hydrolysis, involving mainly the most labile peptide bonds is comprised of a series of first-order reactions of gradually decreasing rate. After breakage of these bonds, the remaining ones may not differ so widely in reactivity and sometimes produce a linear first-order curve. Since the temperature and even the pH dependence of rate may vary with the nature of the bond to be broken, the change in slope of the first-order curve will depend not only upon the concentration of the labile bonds in any one protein, but upon the particular temperature and pH conditions chosen for the measurements. This may explain the apparent lack of agreement between the conclusions of different authors".

1.2.6 Acid hydrolysis and pyrrolidone carboxylic acid

A bitter taste is common in protein hydrolysates. The phenomenon appears to be related to peptide chain length and hydrophobicity (Adler-Nissen and Olsen, 1979). When reviewing the formation of bitter peptides in dairy products, Sullivan and Jago (1970) and Edwards and Kosikowski (1983) noted that high contents of glutamic acid and proline were consistent common factors. Glutamine and

proline are the most common amino acids in gluten (Table 1.1) and astringent off-flavours have been reported in acid hydrolysed gluten (Finley, 1975 and Wu et al, 1976). Under some conditions glutamine is susceptible to deamidation accompanied by a molecular rearrangement to the corresponding lactam, pyrrolid-2-one carboxylic acid (pyrrolidone carboxylic acid or PCA or pyroglutamic acid).

PCA is thought to be formed by cyclization of N-terminal glutamine (Sullivan and Jago, 1970).



Glutaminyl residue Pyrrolidonyl residue

Derivatives of glutamine in peptides were reviewed by Blombach (1967) who noted that the deamidation of glutamine at neutral and slightly acid or alkaline conditions is often accompanied by a molecular rearrangement to PCA.

The work of Melville (1938) showed that peptides with glutamine in the N-terminal position formed PCA.

Although there is little direct evidence, it seems likely that PCA could be formed during acid hydrolysis of gluten.

1.2.7 Review of previous studies of acid hydrolysis of gluten

One of the earliest and most comprehensive studies of the acid hydrolysis of proteins was made by Vickery (1922) who studied the rate of hydrolysis of wheat gliadin in HCl, H_2SO_4 and alkalis. The rate of liberation of ammonia from the hydrolysis of amide groups and formation of amino groups from the hydrolysis of peptide bonds were measured. When gliadin was boiled in 0.027M HCl it was found that ammonia was at first set free quite rapidly but that "neutralization of the acid by the ammonia set free is, in this case, the deciding factor in the rate of hydrolysis". Measurable peptide bond hydrolysis was detected during hydrolysis in 0.1M HCl.

Holme and Briggs (1959) studied the deamidation of gliadin in the course of studies of its physical characteristics. The rate of liberation of ammonia was recorded from a 1% gliadin suspension at 100°C with HCl concentrations of 0.008M, 0.02M and 0.04M i.e. conditions very similar to those used by Vickery (1922). It was noted that this hydrolysis appeared to follow second-order kinetics.

Gliadin was similarly hydrolyzed by McDonald and Pence (1961) who heated a 5.5% gliadin dispersion in 0.07M HCl for three hours at 96-98°C. This treatment removed about 28% of the total amide groups.

Aranyi and Hawrylewicz (1972) hydrolyzed gluten in a combination of 0.1M HCl plus 4M acetic acid. This combination was chosen because the gluten was solubilized initially and was compatible with subsequent analyses. Hydrolyses were carried out at 60°C for 24 hours. The effect of prior disruption of disulfide linkages in the protein by performic acid oxidation was also examined. After hydrolysis, it was found that starch could be eliminated as a sediment and lipid at the top of a centrifuge tube following centrifugation at 40 000 x g for

1 hour. It was concluded that the rates of ammonia production and peptide bond cleavage were similar in the untreated samples and that peptide bond cleavage was faster in the performic acid oxidized samples. That the rate of peptide hydrolysis and ammonia release is similar seems unlikely in that it is at variance with almost all other studies of amide hydrolysis.

A more extensive study of acid hydrolysis of gluten and the functional properties of the treated gluten was made by Wu et al (1976).

Gluten suspensions (5%) were heated in 0.02, 0.05, 0.1 and 0.5M HCl and 1.75, 3.5 and 8.75M acetic acid for up to one hour at 121°C and data for the residual amide level were obtained. The data were not, however, corrected for the effect of the approximately 15 minutes required for the autoclave to reach the reaction temperature or the cooling time of the autoclave (Wu, 1975).

Similar data in respect of ammonia release and effect of acid strength were obtained by Matsudomi et al (1981) using gluten suspensions (5%) heated in an autoclave at 120°C for 5-90 minutes in HCl, 0.05, 0.1 and 0.5M. The data, however, are insufficient for kinetic analysis.

The effect of acid strength, 0.33 and 0.67M HCl, and temperature 40, 50 and 60°C, were examined by Hamauzu et al (1982), while the effect of acid strength 0 - 1.0M HCl, temperature 50°C, 75°C and 95°C, at a fixed treatment time of 30 minutes were examined by Finley (1975).

Some of the data obtained by these authors are shown in Figure 1.1. A consistent pattern of increased reaction with both increasing acid concentration and increasing temperature can be seen. The data of Vickery (1922) for gliadin, are similar to those of Finley (1975) for gluten. Each author used a different source of gluten and these

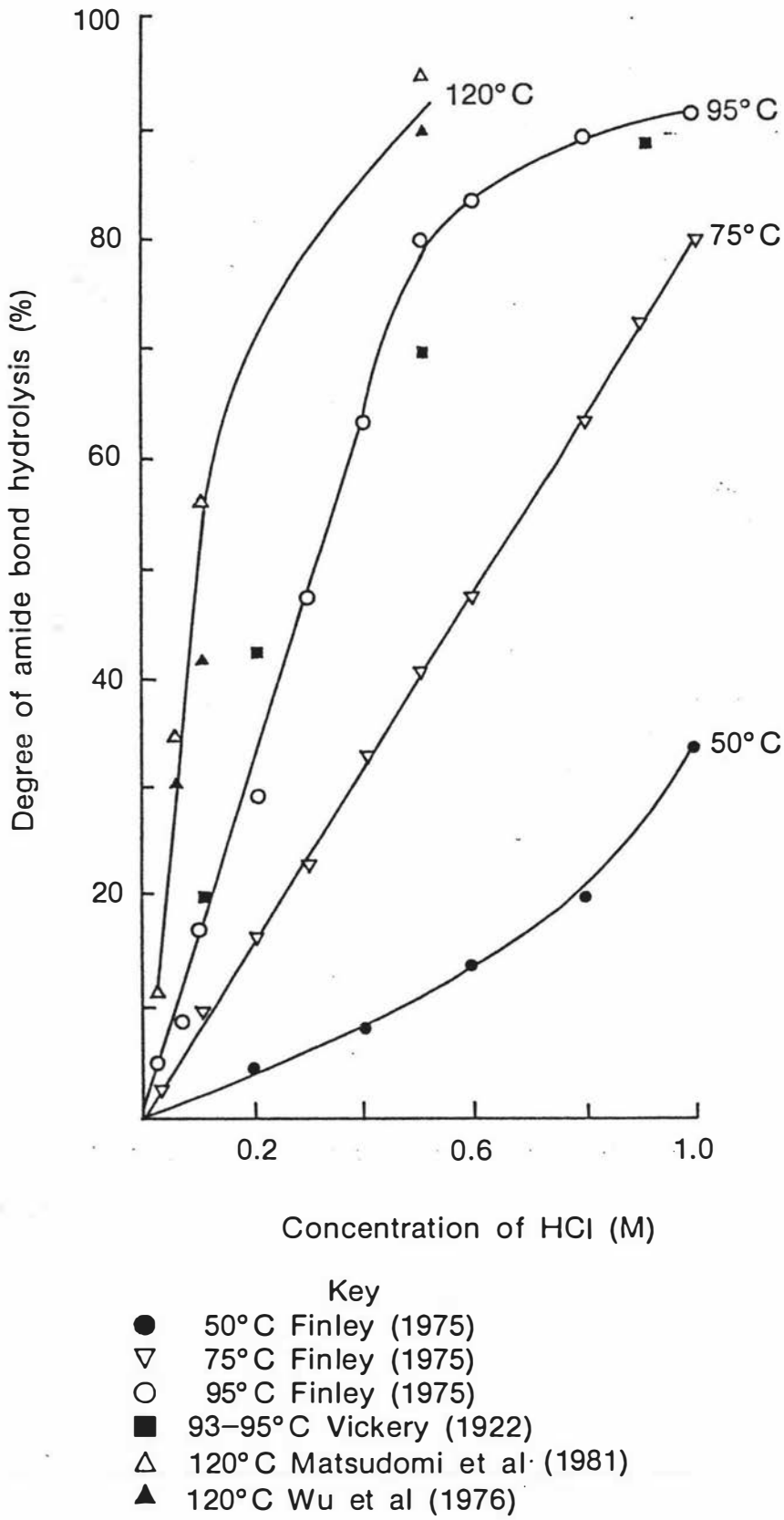


Figure 1.1 Extent of gluten deamidation after 30 minutes reaction (data from various authors).

data, because of their consistency, suggest that gluten from various sources behaves similarly in acid deamidation reactions.

The fit of these data to Arrhenius law was tested by defining a pseudo rate constant

K' = the extent of reaction after 30 minutes with 0.1M acid

A plot of k' against the reciprocal of absolute temperature on semi-logarithimic coordinates shows a straight line fit. (Figure 1.2) This confirms that the effect of temperature on reaction rate can be described by Arrhenius law (Levenspiel, 1972). There do not appear to be any studies which show the effect of varying the gluten concentration but, because of this result and the near linear dependence of the extent of hydrolysis on acid concentration shown in Figure 1.1, it seems likely that the acid hydrolysis of the glutamine amide group in proteins can be described by the kinetics of hydrolysis of amides as discussed in Section 1.2.2. This proposal has been tested in the present work.

1.3 Glutaminase enzymes

Glutamine has a central role in the nitrogen metabolism of biological organisms. Its function is that of a nitrogen or ammonia transport molecule. The amide NH_2 group of glutamine serves as the immediate source of nitrogen atoms in a wide range of biochemical substances, including purines and pyrimidines; the amino acids, tryptophan, histidine, asparagine, glutamic acid, and arginine; amino sugars; and the coenzymes folic acid and nicotinamide nucleotides (Hartman, 1973). The enzymes and pathways involved have consequently been the subject of numerous studies (e.g. Prusiner and Stadtman, 1973; Payne, 1980). Glutaminases, and especially L-glutamine amidohydrolase (E.C. 3.5.1.2) which catalyses the reaction:

$$\text{L-Glutamine} + H_2O \longrightarrow \text{L-glutamate} + NH_3$$

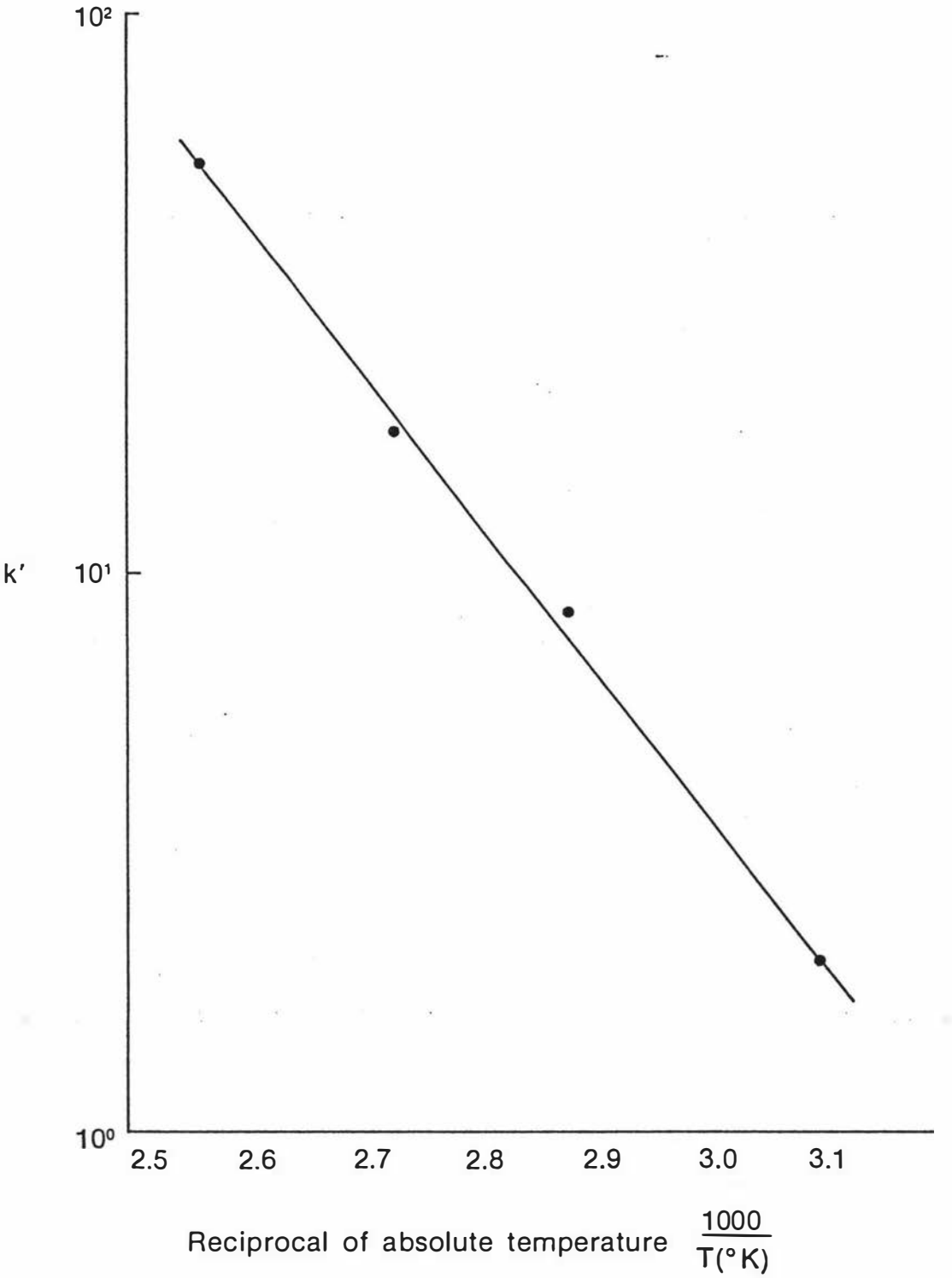
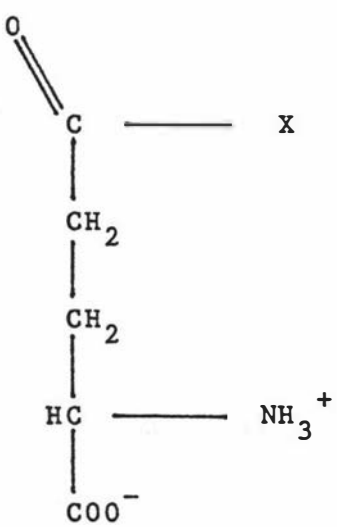


Figure 1.2 Effect of temperature on deamidation reaction (data from various authors).

have been studied (Prusiner, 1973), and this enzyme from E. coli is commercially available.

Hydrolysis of peptide bound amide of glutamine is the basis of the present study and if the reaction could be catalyzed enzymatically as well as by hydrogen ions an interesting comparison should arise.

There appear to have been few studies on the enzyme-catalyzed hydrolysis of peptide-bound glutamine, although the specificity of glutaminase from E. coli has been studied (Meister et al, 1955; Hartman, 1968). The enzyme catalyses reaction with L-glutamine and with α-methyl-DL-glutamine but not with O-glutamine, D or L asparagine or other amides. Other substrates have been examined and no reaction was found with various related compounds including N-acetyl-L-glutamine and L-glutaminyl-L asparagine), but some reaction with substrates substituting various groups, methyl, ethyl, etc, for the γ- amide (Hartman, 1968; Tower, 1967). It was thus found that all of these compounds, which act either as substrates or as competitive inhibitors have the common structural elements:

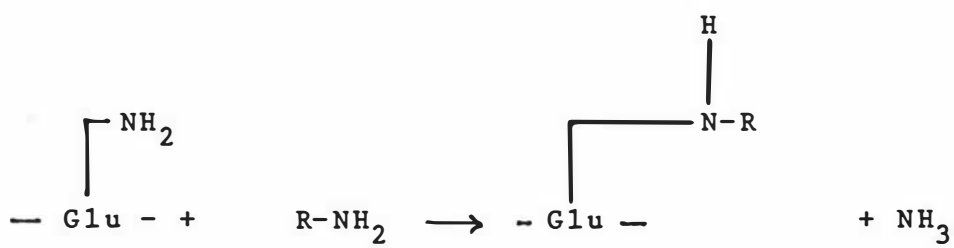


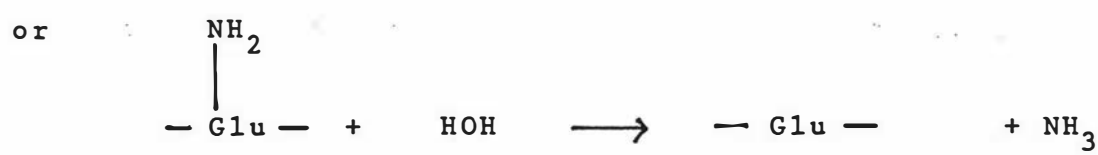
It appears unlikely, therefore, that this enzyme would react with peptide bound glutamine. Glutaminase from a number of sources has been used in the production of soy sauce to prevent liberated glutamine from being converted to pyroglutamic acid.

Increased levels of glutamic acid were shown in the final products. There was however, no indication of whether the deamidation took place on peptide bound or free glutamine (Yokotsuka et al, 1974, 1975).

The properties of enzymes and metabolic pathways for organic amides that have no special role in central metabolism but enable amides to be used by microorganisms for growth as carbon and/or nitrogen sources have been reviewed by Clarke (1980). A large number of amidases from a wide range of organisms have been described. The various substrates tested did not include polyamides.

Transglutaminases catalyze a Ca^{2+}-dependent acyl-transfer reaction in which the -carboxamide group of peptide-bound glutamine residues are the acyl donors. Primary amino groups in a variety of compounds may function as acyl acceptors with the subsequent formation of monosubstituted -amides of peptide-bound glutamic acid. In the presence of less than saturating levels of a primary amide or in the absence of an amine, water can act as the acyl acceptor with formation of peptide-bound glutamic acid.





Transglutaminase activity has been found to be widely distributed in animal tissues and organs. Many studies have dealt principally with transglutaminase from guinea pig liver because this organ offers a particularly rich source of enzyme.

There have been a number of studies of the enzyme and these have been reviewed by Folk and Chung (1973).

The use of this enzyme for gluten deamidation could be usefully investigated but was not pursued in this present study.

Kikuchi and Sakaguchi (1973) searched for a microorganism which would produce an enzyme to catalyze the deamidation of glutamine in protein or peptide. CBZ-L-glutamine (carbobenzoxy-L-gln) and t-AOC-L-glutaminyl-L-proline (tert-amyloxycarbonyl-Lgln-Lpro) were used as substrates for the screening of the enzymic activity for the reason that they are resistant to hydrolysis by proteases or peptidases and deamidation by already known L-glutaminases. Two new glutaminases were produced from a soil isolate microorganism identified as Bacillus circulans. They were named peptidoglutaminase because of their specificity toward peptide bound L-glutamine and their negligible action on free L-glutamine (Kikuchi et al, 1971). Peptidoglutaminase I was found to be specific for catalysing the hydrolysis of the γ- amide of L-glutamine with the carboxyl group free but the α- amino group substituted. Peptidoglutaminase II was specific for hydrolysis of the γ-amide group of L-glutamine that was substituted at the carboxyl position but the α-amino group could be either free or substituted. The reaction rates for both enzymes were affected by the substituent(s) forming the peptide bonds.

Conditions for enzyme production and isolation in homogeneous form have been studied (Kikuchi and Sakaguchi, 1973 a, b). The highest enzyme formation occurred during the stationary growth phase of the bacterium in a basal medium containing lactose (0.5%) and polypeptone (1%).

The pH dependence, maximum about pH 8, and temperature dependence, maximum about 50°C, of the enzyme activities were studied. Further substrate specificity studies showed that L-glutamine presented in polypeptide chains composed of more than four amino acids was a poor substrate. L-glutamine in native

insulin was scarcely deamidated by peptidoglutaminase II, but about 1.9 moles of ammonia per 1 mole of insulin were liberated by the enzyme when native insulin was oxidized with performic acid. When oxidized insulin A chain was incubated with peptidoglutaminase II, about 1.7 moles of ammonia per 1 mole of polypeptide chain were liberated. Under the same conditions almost no ammonia was liberated from the insulin B chain (Kikuchi and Sakaguchi, 1973 c).

The studies reviewed indicate that an examination of the potential of peptidoglutaminase for deamidation would be an interesting contrast to the study of acid-catalysed hydrolysis of amide bonds in gluten.

1.4 Functionality of proteins

1.4.1 Definition and review

Functionality is any property of a substance, besides its nutritional ones, that affects its utilization (Pour-El, 1976).

In recent years, in parallel with the development of a greater variety of processed foods and the development of a range of protein isolates as ingredients for these foods, the study of protein functionality has developed as a specialized subject separate from, but related to compositional and nutritional studies. An extensive discussion of the difficulties of definition and generalization is given in reviews by Kinsella (1976, 1982), and Pour-El (1981).

"Although the unique functional properties of several food proteins have been exploited and enjoyed for many years, it is only recently that research has begun to focus on the relationship between the physical properties of these proteins and their functional behaviour in food systems. Ironically, much of the impetus for this originated with attempts to simulate traditional products (meat, cheese) from less expensive oilseed proteins or to use these

proteins as functional ingredients to replace established sources, e.g. casein, egg white, gluten. There is an increasing realization of the critical nature of, and need for, comprehensive and organized information on functional properties. Long-term and systematic research is needed to elucidate the fundamental relationship between the structure and physical properties (Table 1.2). Such research requires the development and adoption of standardized methods for measuring specific functional properties. However, development of the best methodology depends upon an understanding of the physico-chemical basis of functionality".
(Kinsella, 1982).

Items three and four from Table 1.2 describe objectives, with respect to the study of functional properties, of the present study.

There are a large number of functional properties of proteins recognised and an even larger number of possible tests for each, almost none of which are recognised standard methods. These are summarised in Table 1.3 (Kinsella, 1982).

"The literature is replete with papers on the functional properties of proteins as measured by a variety of methods under differing conditions. The tendency has been for each investigator to devise methods and/or conditions to suit a particular situation with limited concern for an experimental design which would facilitate explanation of the physical property being studied. Much of the published data are of very limited value for practical or comparative purposes. This situation reflects the heterogeneous nature of the proteins involved, the very complex series of interactions, which are not amenable to easy measurement, involved in a particular functional property, the multiple interacting factors which impact on the resultant reactions, and the lack of appropriate measuring devices

Table 1.2 Research needed to develop an understanding of the physico-chemical basis of functionality

1. Definition of functional properties
2. Development of standardized methods based on physical properties
3. Relating functional properties to structural features and secondary interactions
4. Manipulation of protein structure by chemical or physical alterations
5. Assessment in food systems

Table 1.3 Functional properties of proteins in food applications

General property	Functional criteria
Organoleptic	Colour, Flavour, Odour
Kinesthetic	Texture, Mouthfeel, Smoothness, Grittiness, Turbidity
Hydration	Solubility, Wettability, Water Absorption, Swelling, Thickening, Gelling, Syneresis, Viscosity
Surface	Emulsification, Foaming (aeration, whipping), Film formation
Binding	lipid-binding, Flavour-binding
Structural	Elasticity, Cohesiveness, Chewiness, Adhesion, Network crossbinding, Aggregation, Dough formation, Texturisability, Fibre formation, Extrudability
Rheological	Viscosity, Gelation
Enzymatic	Coagulation (rennet) Tenderization (papain) Mellowing (proteinases)
'Blendability'	Complementarity (wheat-soy, gluten-casein)
Antioxidant	Off-flavour prevention (fluid emulsions).

accurately quantifying the results of these interactions in terms of the inherent properties of the protein and environmental factors".
(Kinsella, 1982).

Given that it is desirable to try to relate functional properties to protein structure, experiments must be restricted so as to take account of the environmental and process factors which also affect proteins. Some of these are listed in Table 1.4 (Kinsella, 1982).

All of these factors have been shown to be important for gluten and must be considered in preparation of samples for functional testing.

1.4.2 Review of previous studies of functionality of acid hydrolysed gluten

All the studies made of acid hydrolysis of gluten or gliadin have noted dramatic changes in functionality attributed to deamidation of glutamine. Matsudomi et al (1981) found that deamidation of up to 40% increased the emulsification and foaming ability of gluten. There was a linear relationship between deamidation and increased surface hydrophobicity (Matsudomi et al, 1982). Wu (1976) also found improved emulsification and foaming ability, while the foaming ability of gliadin was found to be improved by deamidation (McDonald and Pearce, 1961; de Meester, 1974).

The changes in viscosity resulting from deamidation of gluten were studied by Aranyi and Hawrylewicz (1972), Finley (1975) and Hamauzu et al (1982). It was found that viscosity under a variety of conditions decreased as the level of deamidation increased. The changes were explained in terms of the glutamine deamidation. The performance of deamidated gluten in complex food systems, in contrast to the model systems described above, has been investigated by various authors. Vidal (1972) treated wheat gluten by acid

Table 1.4 Some of the factors influencing the functional properties of proteins in foods

Intrinsic	Environmental factors	Process treatments
Composition of protein(s)	pH	Heating
Conformation of protein(s)	Redox status	pH
Mono- or Multi- component	Salts, Ions	Ionic strength
Homogeneity-heterogeneity	Water	Reducing agents
	Carbohydrates	Storage conditions
	Lipids	Drying
	Surfactants	Physical modification
		Chemical-enzymatic
	Flavours	modification

hydrolysis and examined its utility in bread and cakes. Gebhardt (1982) similarly concluded that deamidated gluten added to wheat flour was a potentially useful food ingredient.

Fung et al (1977) used acid hydrolysis to solubilize wheat flour and concluded that it had potential as a nutritious replacement for corn syrup.

Wheat gluten was deamidated and the acid hydrolysate mixed with milk to give a casein-wheat protein co-precipitate product. Functional properties equivalent to those of casein were claimed (Walker and Connolly, 1984).

1.5 Emulsions and emulsifiers

1.5.1 Definition and review

"An emulsion can be considered to be a dispersion of one liquid in another, that is, from the thermodynamic point of view, a two phase system, the dispersed phase consisting of microscopic droplets usually within the size range 0.1 to 100 μ in diameter. Such dispersions are never completely stable in the absolute sense, because the interface between phases is the seat of surface free energy, and if two droplets join together there is a net reduction in interfacial area. Hence, coalescence of drops is a thermodynamically spontaneous process whereas the reverse process requires expenditure of work and therefore does not occur spontaneously".
(Kitchener and Mussellwhite, 1968).

Many of the emulsions in which food proteins find application are of the oil in water type e.g. salad dressings and coffee whiteners. The present work and discussion of the work of other authors reviewed is confined to oil in water emulsions.

Operationally, a stable emulsion is one which is very slow to undergo the various processes which result in separation of oil and water phases. (Pearce and Kinsella, 1978).

There have been many studies of emulsions, and the subject has been reviewed by Becher (1965), Kitchener and Musselwhite, (1968) and Dickinson and Stainsby (1982).

To prepare a stable emulsion, material is added to prevent the close approach of the droplets. This material is an emulsifier and promotes stability by

(i) lowering the free energy of the interface and
(ii) forming an absorbed film around the liquid droplets.

An emulsifier is generally a surface-active material (surfactant) whose molecules are amphiphilic.

Griffin (1949) developed an empirical procedure for classifying surfactants according to their stability efficiency for a particular type of colloidal system. It is based upon the idea that, for a given oil and water system, there is an optimum balance between molecular hydrophilic and lipophilic character which leads to emulsification efficiency. This is the hydrophile-lipophile balance (HLB) method.

Use of the system for emulsifier selection has been explained by emulsifier manufacturers (ICI Americas Inc. 1980). Two key features are, first, that emulsifiers can be blended to give an HLB value which is an algebraic mean of the component HLB values. Secondly, oils can be classified in terms of their required HLB value i.e. the optimum emulsifier HLB value for that oil. The system has been reviewed by Dickinson and Stainsby (1982) and criticized by Riegelman and Pichon (1962). Further studies of the HLB postulate were made by Boyd et al (1972) and Ohba (1962a) and of the required HLB value of oils by Ohba

(1962b). A technique for rapid determination of HLB and required HLB values was prepared by Robbers and Bhatia (1961).

The HLB scale is from 0 to 20; and may be calculated as follows:

$$HLB = 20 \left(1 - \frac{S}{A}\right)$$

Where S = saponification number of ester
A = acid number of recovered acid

HLB may be approximated on the basis of dispersibility as follows:

	HLB
No dispersibility in water	1-4
Poor dispersion	3-6
Milky dispersion after shaking	6-8
Stable milky dispersion	8-10
Translucent to clear dispersion	10-13
Clear solution	13+

The general range of HLB values for various applications of surfactant is:

HLB Range	Application
4-6	W/O emulsifier
7-9	Wetting agent
8-18	O/W emulsifier
13-15	Detergents
10-18	Solubilizers

1.5.2 The emulsion stabilizing properties of proteins

Emulsion stabilization is an important functional property of proteins in food systems. The subject has been reviewed by Halling (1981), Graham and Phillips (1976), Dickinson and Stainsby (1982), Cherry et al (1979), Tornberg (1979), Morr (1981), and McWatters and Cherry (1981). Most of the studies reviewed relate to model systems and as with most functional property studies, a great deal of information is available but comparisons and conclusions are difficult. Halling (1981) and Fisher and Parker (1985) stated that there is no consensus on how proteins stabilize emulsions and no clear correlation between any interfacial properties of proteins and their effectiveness as emulsifiers. It has however, long been known that proteins can form a tough 'skin' around oil droplets and that the adsorption approaches equilibrium only slowly and is practically irreversible (Kitchener and Mussellwhite, 1968). The process of adsorption of proteins during emulsion formation was studied and described by Graham and Phillips (1976) as having three stages.

1. Diffusion of native proteins to the interface.

2. Penetration of the molecule into the interface and unfolding.

3. The absorbed, surface-denatured, protein molecules rearrange to the lowest possible free energy state.

In this and a further study (Graham and Phillips, 1979) the dependence of the surface orientation and packing on the flexibility of the proteins was noted. A marked selectivity of adsorption of individual whey proteins was found by Shimizu et al (1981), while Aoki et al (1984) found that adsorption of sodium caseinate to oil droplets effectively prevented soy protein isolate from being adsorbed.

Many factors, temperature, rate of oil addition and the mode and means of creating the dispersion as well as the pH, ionic strength, protein type and presence of other material influence the formation and stability of an emulsion. The effect of different types of emulsifying equipment was evaluated by Tornberg and Hermansson (1977).

A factor in the difficulty of studying emulsions is that, as with most functional testing, the methods are not standardized and this makes comparison of data between different workers difficult. Methodology for determination of emulsification properties was reviewed by Puski (1976). Peltonen (1982) discussed the phenomena to be considered when emulsions are centrifuged. A turbidemetric technique which indirectly measures the interfacial area of an emulsion was systematically evaluated by Pearce and Kinsella (1978), and this technique, with minor variations, is now in common use. The method suggests measurement of turbidity in a 0.1% SDS (sodium dodecyl sulphate) solution. Kamata et al (1984) pointed out the effect of SDS in dispersing flocculated oil droplets and used titration with SDS to define flocculation strength as an emulsion parameter.

In searching for means of relating protein structure to emulsification performance there have been various investigations of the surface activity and, more recently, of the hydrophobicity of proteins. These two approaches were brought together by Keshavarz and Nakai (1979) who showed a negative correlation between hydrophobicity and surface tension, thus indicating that more hydrophobic proteins may be more effective as emulsifiers since a lowering of surface tension contributes to emulsion stability. The work of Shimizu et al (1983) showed α_{s1}- casein is tightly adsorbed onto oil surfaces principally by its hydrophobic N-terminal region, and that it stabilizes the oil globules.

A number of methods have been proposed for the measurement of hydrophobicity of proteins. Bigelow (1967) showed how the average hydrophobicity of a protein could be calculated from the free energies of transfer of constituent amino acid side chains from an organic environment to an aqueous environment. It was, however, shown that hydrophobicity calculated by this method was not related to interfacial tension or effective hydrophobicity measured by hydrophobic chromatography or partition (Keshavarz and Nakai, 1979). Hydrophobic interaction chromatography was also used by Popineau and Godon (1982) to measure the surface hydrophobicity of gliadin components and the results showed a good correlation with hydrophobicity determined by the alkane binding method of Mohammadzadeh-K et al (1969). A technique for determination of surface hydrophobicity using a fluorescent probe was developed by Kato and Nakai (1980) and this technique was shown to give results proportional to those obtained by using SDS binding (Kato et al, 1984).

The significance of these techniques is discussed by the authors cited and the various methods have been reviewed by Nakai (1983).

A number of authors have attempted to relate the measured hydrophobicity properties of proteins to their emulsion forming properties. These studies can be divided into two groups. The first group used a single protein or protein mixture which was modified in some way in order to vary the surface hydrophobicity. Matsudomi et al (1982) showed that the surface hydrophobicity of gluten increased in proportion to deamidation and was linearly correlated with an improvement in emulsifying properties. Kato et al (1981) investigated the properties of partially denatured ovalbumin and lysozyme and showed that the emulsifying properties of the proteins increased with denaturation and correlated linearly with surface hydrophobicity. In a similar study, soybean 11S globulin was heated in different ionic strength solutions. The emulsion stability

correlated linearly with the surface hydrophobicity but a different correlation was obtained for each ionic strength (Matsudomi et al, 1985). Matorella and Richardson modified beta-lactoglobulin by amidation, ethyl and methyl-esterification. Hydrophobic properties were measured using cis-parinaric acid binding at different ionic strengths. Heptane binding and emulsifying properties were also measured. Correlations between these results varied considerably and it was concluded that measurements of effective hydrophobicity of proteins in low salt concentrations must be interpreted cautiously because of possible electrostatic interactions between the protein and cis-parinaric acid.

A second group of studies are those which consider a number of different proteins and attempt to correlate their functional and structural properties. The emulsifying properties of ovalbumin, 7S globulin, kappa-casein, beta-lactoglobulin and bovine serum albumin were followed during heat denaturation and correlated with the corresponding surface hydrophobicity (Kato et al, 1983). A reasonable correlation was established overall, but individual proteins showed considerable variation. A similar effect was found by Nakai et al (1980) when soy, sunflower and rapeseed proteins were treated with surfactants and Bacillus subtilis proteinase. Mangino et al (1985) took whey protein concentrates prepared from different wheys with varying heat treatments and measured emulsification properties and hydrophobicity using both cis-parinaric acid and pentane binding. Correlation coefficients varied depending on the protein preparation and hydrophobicity measurement method.

Much of this work, with an emphasis on the importance of protein hydrophobicity, was reviewed by Nakai (1983) who discussed the analogy between protein hydrophobicity and the hydrophile-lipophile balance (HLB) value of

surfactants. It was shown that these could be considered as ratios, viz

$$\text{hydrophobicity (protein)} = \frac{[\text{non polar}]}{[\text{non polar}] + [\text{polar}]}$$

$$\text{HLB (surfactant)} = \frac{[\text{polar}]}{[\text{non polar}] + [\text{polar}]}$$

$$\text{i.e. hydrophobicity (protein)} = 1 - \text{HLB (surfactant)}$$

Here, protein hydrophobicity and emulsifier HLB are defined as the proportion of non polar side chains and polar radicals in the molecules of protein and emulsifier, respectively. This definition does not exclude a further sub-classification of protein hydrophobicity to total or

surface (exposed) hydrophobicity which appears to be important for correlation with functonal properties.

Most of the authors quoted above appeared to recognize that hydrophobicity is only one of a number of factors which determine protein functionality. Other factors which control the conformation of proteins at interfaces are pH and ionic strength, the effect of which was demonstrated by Matsudomi et al (1985). The probable importance of protein flexibility was discussed by Shimizu et al (1981). Flexibility, determined by measuring susceptibility to protease digestion, was shown to be related to emulsification properties by Kato et al (1985).

No comprehensive study relating differing methods of determination of hydrophobicity and functional properties has been carried out. Small differences in technique and different ionic strengths and test oils for emulsions make comparison difficult. The measurement of protein hydrophobicity does, however, represent a major advance in the understanding of protein functionality.

1.5.3 The use of the HLB concept in food systems

Emulsifiers are commonly added to processed foods. The utility of the HLB system for selecting emulsifiers for these complex systems has been demonstrated. Govin and Leeder (1971) and Lin and Leeder (1974), showed that various measurements of the properties of ice cream depended on the HLB number of emulsifiers. Titus et al (1968) showed that the specific volume of cakes was dependent upon and had an optimum value of emulsifier HLB.

Bread volume and tenderness were shown to have an optimum value dependent on the HLB value of sucrose - fatty acid esters (Breyer and Walker, 1983).

1.5.4 Proteins and the HLB concept

In the past proteins have not been considered in terms of HLB values. Recent major reviews (Kinsella 1976, 1982; Pour-El, 1981) of the functional properties of proteins including emulsification make little or no mention of HLB.

Determinations of the HLB value of proteins have been made by van Eerd (1971), Aoki et al (1981) and Denoyer and Girard (1982).

The determinations were made by first determining the required HLB of oil or oil mixtures and then finding the mixture giving the best emulsification behaviour i.e. an HLB value was found by inference. The effect of using different oils with the same protein, rather than the common practice of testing various proteins against the same oil, has been investigated by Christian and Saffle (1967) who examined the effects of different oils and free fatty acids and found significant differences in the quantity of each emulsified by a fixed quantity of salt soluble meat protein.

1.5.5 Criticism of previous studies

In recent years there have been many studies in which actual or potential food proteins have been modified for functional improvement. Reviews by Feeney et al (1982) and Whitaker and Puigserver (1982) discuss the range of modifications and resulting protein property changes. In some of these studies changes in emulsification properties were measured. Examples are the work of Ma (1984) and Chen et al (1975). In each case the authors conclude that the protein modifications have improved the emulsifying properties of the protein. Such conclusions can be criticized for two reasons. The first is that no allowance is made for changes in solubility, i.e. changes in the soluble protein concentration, in the emulsification tests. The second is that there is no recognition of the consequences of using just one oil, i.e. it may be that the improvements are due to the protein becoming more suitable for the particular oil used but not necessarily for all oils. This criticism could be made of a large number of studies which compare the emulsification properties of proteins using a single oil.

The basis of this criticism is most clearly demonstrated in the work of Wu (1975). Gluten deamidated to varying extents by acid hydrolysis was used to prepare emulsions. Emulsifying capacity and stability were measured and the data are plotted as shown in Figure 1.3. The data show an optimum level of deamidation and Wu (1975) claimed that this was the best emulsifier. An alternative view, however, is that the optimum is for the oil used, i.e. the gluten is a protein probe of the oil properties. If, instead of gluten, emulsifier mixtures of various HLB values were used, the same sort of data could very well be produced but would be interpreted as showing the required HLB of the oil and not that a particular emulsifier was superior to others. If the hydrophobicity of the gluten increases with deamidation, as shown by Matsudomi et al (1982), then the data of Figure 1.3 should show a poor

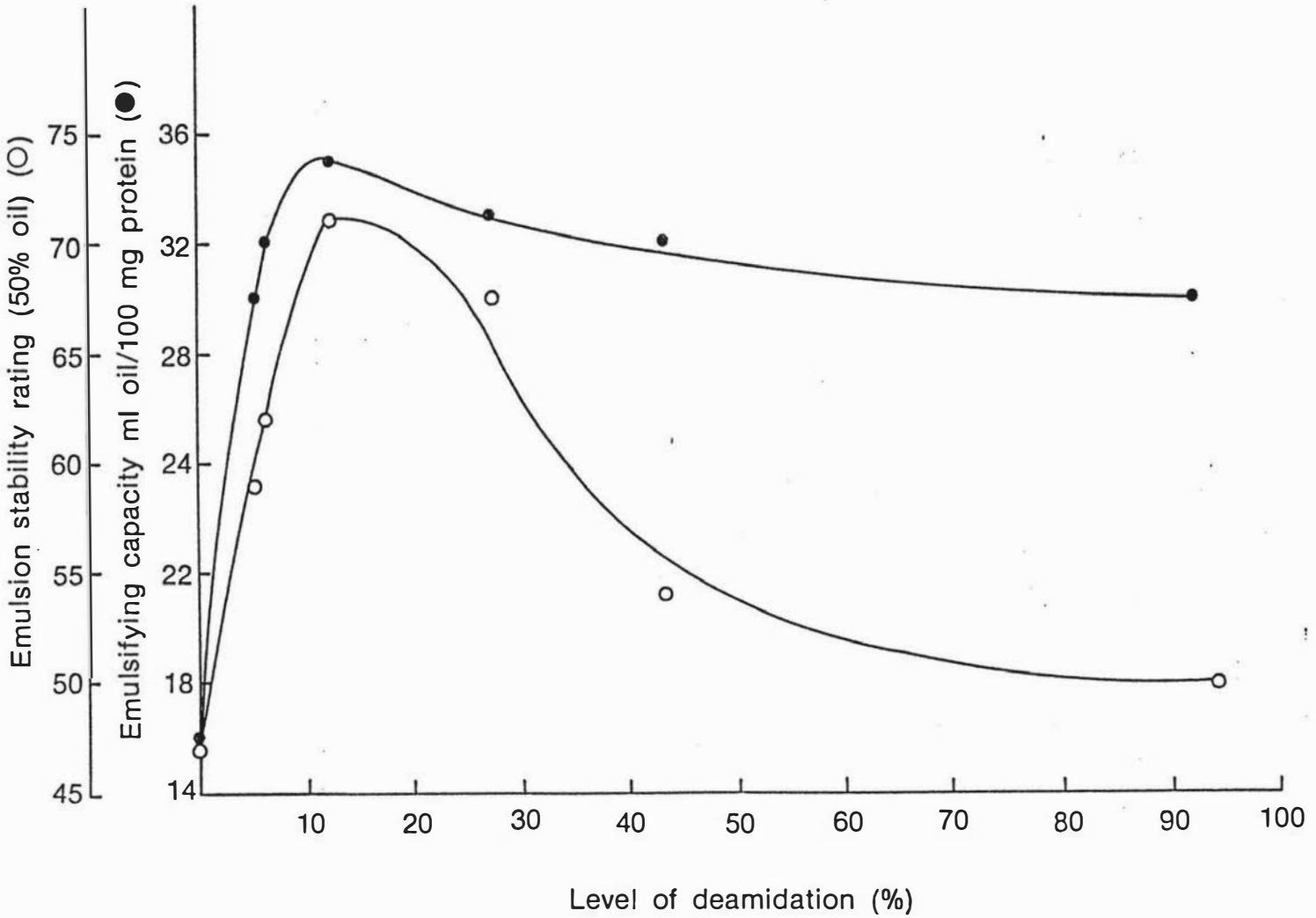


Figure 1.3 Emulsification properties of deamidated gluten (data from Tables 2 and 4 of Wu (1975)).

positive correlation of emulsification properties and hydrophobicity. Clearly, however, factors other than hydrophobicity are involved. If, however, hydrophobicity is analogous to HLB, as suggested by Nakai (1983), then the apparent optimum shown in Figure 1.3 would be expected.

1.5.6 Hypothesis for test in this work

It is proposed that the data of some earlier studies, cited above, where changes in emulsion characteristics were interpreted as implying changes in the emulsifying power of the proteins, might equally be interpreted as a measure of the characteristics of the oil/water system. If the analogy between protein hydrophobicity and HLB suggested by Nakai (1983) is correct then linear correlations between hydrophobicity and emulsifying properties of proteins are theoretically incorrect.

That the analogy between protein hydrophobicity and emulsifier HLB values is valid has been taken as an hypothesis for test in the present work.

1.6 Statement of research objectives

The objectives of this work were, first, to determine the kinetics of the acid catalyzed deamidation of gluten. Measurements of peptide bond hydrolysis were also made. A second objective was to prepare gluten deamidated to various extents and to characterize the functional properties with emphasis on the emulsifying properties in relation to hydrophobicity and its analogy to the HLB values of emulsifiers.

2. EXPERIMENTAL MATERIALS AND METHODS

2.1 Materials

Vital wheat gluten was supplied by Manildra Starches Pty Ltd (The Crescent, Auburn NSW 2144, Australia).

Hydrochloric acid, for laboratory experiments, was prepared from concentrated volumetric solution ampoules designed for the preparation of 1.0N solution (BDH Chemicals Ltd, Poole, England).

For pilot scale experiments the hydrochloric acid was commercial grade (packed by Scientific Service Laboratories Ltd., Napier, New Zealand).

ANS (8-anilino-1-naphthalenesulfonate) was supplied by Sigma Chemical Company, (St Louis Mo, 63178, USA). CPA (9, 11, 13, 15-cis trans trans cis octadecatetraenoic acid (cis parinaric acid) was supplied by Molecular Probes Inc., Junction City, Or., 97448, USA). Butylated hydroxytoluene (2, 6-di-tert-butyl-p-cresol) (BHT) was supplied by BDH Chemicals Ltd, Poole, BH124NN, England. All other chemicals used were reagent grade. Soyabean oil was supplied by Andrew Industries Ltd, Takapuna, New Zealand. Castor oil was supplied by the National Dairy Association of New Zealand. For laboratory experiments and preparation of reagent solutions, water purified by reverse osmosis using a Millipore RO system was further purified with a Milli-Q apparatus (MQ) (Millipore Corporation, Bedford Massachusetts USA).

2.2 Measurement of pH

Measurements of pH were made using a glass combination pH electrode and a digital pH meter (Metrohm AG CH-9100 Heresau, Switzerland). The same electrode was used throughout the experiments. When not in use the electrode was stored in 3M KCl solution and the internal reservoir was kept filled with

3M KCl. The electrode was further maintained by occasional soaking in a pepsin/HCl mixture to remove protein residues. The electrode was calibrated using two buffers, either pH 2 and pH 3 or, pH 4 and pH 7, depending on the sample range. The buffers were guaranteed to be pH 2.00 ± 0.02, pH 3.00 ± 0.02, pH 4.00 ± 0.02 and pH 7.00 ± 0.02 (BDH Chemicals Ltd, Poole England). The pH meter was equipped with a built in temperature compensation system with a resistance thermometer probe.

Buffers and samples were held in a waterbath maintained at 25±1°C and the resistance thermometer was also immersed in the bath.

2.3 Preparation of buffer solutions

Phosphate buffer was prepared at the required pH by mixing 0.2125M Na_2HPO_4 and 0.2125M NaH_2PO_4. This concentration of buffer was required for the determination of N-terminal amino groups using TNBS (see Section 2.6.2) and it was found to be convenient to use, suitably diluted, for other determinations.

2.4 Preparation of SDS solutions

Solutions of sodium dodecyl sulphate (SDS) of approximately 1% w/v were prepared by weighing 10 g SDS and adding water to a total volume of approximately 1000 ml. Gentle stirring and warming were continued until the SDS was completely dissolved.

2.5 Determination of ammonia

The determination procedure was based on the reaction.

$$NH_4^+ + \text{alpha-ketoglutarate} + NADH \xrightarrow{GDH} \text{L-glutamate} + NAD^+ + H_2O$$

where GDH is L-glutamate dehydrogenase
NAD is nicotinamide adenine dinucleotide
NADH is nicotinamide adenine dinucleotide, reduced

The concentration of ammonia can be found by determining the decrease in absorbance at 340 nm due to the decrease in NADH concentration.

First, a reagent mixture was prepared. The quantities required for 100 determinations were:

5.0 ml	(1.0M) alpha-keto glutarate adjusted to pH 7.6
50 ml	0.2125M buffer phosphate (pH 7.6)
24 ml	Water
1.0 ml	GDH (Boehringer, Mannheim GmbH, West Germany)
10 ml	NADH 2.4 x 10^{-3} M dissolved in 0.05M $NaHCO_3$ adjusted to pH 10 with NaOH (Lowry et al 1961)

A 0.1M ammonium chloride solution was prepared and then diluted to give a 4.0 mM standard solution. Varying quantities of this solution, 0, 5, 20, 30, 40, 50 and 60 µl, were used and water added to make a total of 100 µl. Samples were either 100 µl or some smaller quantity with water added to make 100 µl. The samples and standards were contained in small test tubes and 0.9 ml of the reagent mixture was added. The tube contents were mixed and then held in a waterbath controlled at 25 $\pm$ 1°C for 45 minutes. The absorbance of the samples was read at 340 nm using a Perkin Elmer spectrophotometer (Coleman Instruments Division, Oak Brook, Illinois 60521, USA).

During initial experiments for determination of ammonia and N-terminal amino groups using TNBS a different spectrophotometer was used, (Gilford 250, Model 1084, equipped with UV or visible lamp, recorder/printer, cuvette selector and sampler with temperature control of the cuvette. Gilford Instrument Lab Inc., Oberkin Ohio USA).

The calibration of this machine was found to drift in an unsatisfactory manner, see Appendices 3.5, 3.6, 3.7, and a change to the Perkin Elmer spectrophotometer was made.

A similar procedure was used by Ikura et al (1980), while the same reaction can be used for determination of submicro quantities of ammonia (Levitzki, 1970). The enzyme from Boehringer was preferred because of its low ammonia content compared to that from Sigma which was used for some preliminary experiments.

2.6 Determination of N-terminal amino groups

2.6.1 Determination using fluorescamine

Udenfriend et al (1972) described the use of fluorescamine (4-phenylspiro [furan-2 (3H), 1'phthalan] - 3, 3'-dione) for this purpose. The reagent reacts directly with primary amines to form fluorophors.

Several factors make fluorescamine suitable for determination of primary amines, including amino acids, peptides and proteins. At pH 9, reaction with primary amines proceeds at room temperature, with a half time of a fraction of a second. Excess reagent is concomitantly hydrolysed with a half-time of several seconds.

Fluorescamine, as well as its hydrolysis products, is non-fluorescent. Studies with small peptides have shown that the reaction goes to near completion (about 80 to 95% of theoretical yield) even when fluorescamine is not present in large excess.

For determination, primary amines are first buffered to an appropriate pH (> 7) and then fluorescamine, dissolved in acetone, a water miscible nonhydroxylic solvent, is added. In a fraction of a second at room temperature, the reaction is complete and in less than a minute excess reagent is destroyed. The resulting fluorescence is proportional to

the amine concentration and the fluorophors are stable over several hours.

Standards for the determination of N-terminal amino groups using fluorescamine were prepared using 2.5 mM L-glutamic acid (L-glu) dissolved in 1% SDS. Varying quantities of this solution, 0, 10, 20, 30, 40, 50 and 60 µl, were added to test tubes and the volume was made up to 200 µl with 1% SDS solution. Samples taken from the diluted hydrolysate were either 200 µl or some smaller quantity with 1% SDS solution added to make 200 µl. To each tube 1.5 ml of 0.2125M pH 8.2 buffer was added. A solution of fluorescamine, 30 mg in 100 ml of acetone, was prepared fresh for each set of assays. Fluorescence was determined using a Perkin Elmer MPF-2A fluorescence spectrometer fitted with a 150 W Xenon lamp and a temperature controlled cuvette holder. The samples were held in a water bath controlled at 25 $\pm$ 1°C. Immediately before fluorescence measurement the tube contents were mixed on a vortex mixer (Vortex Genie, Scientific Industries Inc, Queens Village 29, New York, USA), and 0.5 ml of fluorescamine solution was injected into the vortex. The fluorescence of each sample was read with an excitation wavelength of 390 nm, slitwidth 5 nm, and an emission wavelength of 475 nm and slit width 20 nm. The results were recorded as peak heights on a recorder chart set so that correction for ammonia levels could be made (described in Section 3.6). Bohlen et al (1973) stated that different proteins will give a different response to fluorescamine since not all proteins have the same proportion of amino groups nor is it certain that all amino groups in proteins react completely or that all fluorescamine substituted amino groups exhibit the same quantum yield of fluorescence. It was found that raising or lowering the concentration of phosphate buffer had little effect on sensitivity. Changing the buffer pH between 7 and 9 had a marked effect which varied with different proteins.

The reaction of fluorescamine was similarly studied for analysis of peptides by Nakai et al (1974). Peptides were produced by hydrolysis with proteases and separated by Dowex-50 chromatography. Peptides which did not react with fluorescamine were found to correspond to a large peptide with glutamine at the NH_2 terminus. Formation of pyroglutamic acid was thought to be responsible for the poor reactivity. Low fluorescence was found for peptides with aspartic acid, asparagine and glutamic acid at the NH_2 terminus.

The fluorescence due to individual amino acids was examined by Stein et al (1983). Glutamic acid but not glutamine was included in the experiments. Glycine amide however gave a fluorescence similar to that of glycine suggesting that the amide group did not show much reaction with fluorescamine.

It should be noted that fluorescamine does not react with proline or hydroxyproline. This disadvantage may be overcome by introducing an appropriate intermediate step to convert these amino acids to primary amines which can be determined using fluorescamine (Weigele et al, 1973).

2.6.2 Determination using 2, 4, 6-trinitrobenzenesulfonic acid (TNBS)

Adler-Nissen (1979) further developed existing techniques to measure the degree of hydrolysis of food proteins. To use the method a series of tubes was prepared, each containing 2.0 ml phosphate buffer 0.2125M (pH 8.2) and 0.25 ml of sample or standard. Standards were prepared using 2.5 mM L-glu dissolved in 1% SDS. Varying quantities of this solution, 0, 20, 50, 100, 150 and 250 µl, were used and 1% SDS solution was added to make a total of 250 µl. Samples taken from the diluted hydrolysate were either 250 µl or some smaller quantity with 1% SDS solution added to make the volume up to 250 µl. TNBS solution, 0.1% w/v, was freshly prepared for each set of determinations and protected from light. Two ml of TNBS solution were added

to each tube, the contents mixed and then placed in a waterbath controlled at 50 ± 1°C for 60 minutes. Light was excluded during the incubation by covering the bath with aluminium foil. After the tubes were removed from the water bath, 3.5 ml of 0.1M HCl was added to each tube and the contents mixed. Absorbance of the tube contents was read at 340 nm against a water blank.

The use of TNBS for measuring amino groups in proteins and peptides was first developed by Fields (1971). Absorbance was measured at 420 nm. Goldfarb (1966) showed that the absorbance at 420 nm was very dependent on the level of added sulphite but that the dependence was less at a second peak at about 340 nm. The method developed by Adler-Nissen (1979) does not require added suplhite and reaction is taken to completion to avoid kinetic analysis. Because food proteins are not always fully soluble SDS is used. A quantitative equivalence to amino groups was demonstrated with a different standard curve for each protein. The reaction of TNBS with ammonia has been studied by Whitaker et al (1980). An extinction coefficient similar to those for alpha and epsilon-amino groups was found. Clegg et al (1982) found that with equal molar concentrations of leucine nitrogen and ammoniacal nitrogen, ammonia gave 20% of the absorbance of that of leucine. The effect was additive when combined.

2.7 Determination of total and non-protein nitrogen

Total nitrogen (TN) and non-protein nitrogen (NPN) (nitrogen soluble in 12% trichloroacetic acid) were determined using an automated Kjeldahl procedure. ("Kjel-Foss Automatic") 16210 A/S N. Foss Electric, Hillerod, Denmark).

3. EXPERIMENTS TO VERIFY ANALYTICAL METHODS

The unique properties of gluten, described in Section 1, give rise to some peculiar difficulties in analysis. An understanding of the chemistry of gluten has been dependent on the discovery of suitable solvent systems for the measurements required. For this present work the determination of N-terminal amino groups is dependent on solubilization of the gluten using SDS. The experiments described below were designed to check that SDS was effective in solubilization. In addition, it was necessary to use buffer solutions of sufficient strength to neutralize the acidity of the samples but the effect of the SDS was reported to be dependent on ionic strength (see Section 1.1.4). The effect of ionic strength and experimental procedure was also considered.

A further complication was the possible interference of ammonia in the N-terminal amino group determinations and this too was investigated.

3.1 The effect of SDS in solubilization of gluten

Solubilization of gluten protein by SDS is required in the determination of N-terminal amino groups. An experiment to check the solubilization was prepared.

3.1.1 Experimental method

Two hydrolysed gluten preparations A and C were prepared. For A, 90 ml of 0.1M HCl, and for C, 90 ml of approximately 0.03M HCl, were added to 10.0 g gluten in a screw-top pyrex jar. Preparation A was held in a waterbath at 70°C for 6.2 hours and then held, with preparation C, under refrigeration for two weeks until required. For these experiments untreated gluten was labelled D. Preparation A was diluted 10^{-1} with

(1) 1% w/v SDS solution in water or,

(2) 1% w/v SDS solution in 0.02M phosphate buffer, pH 8.0 or,

(3) 1% SDS solution in 0.02M phosphate buffer (pH 8.0) containing 0.005M mercaptoethanol.

Preparations C and D were diluted as in (2) above only. The diluted suspensions were held overnight at 4°C and then a portion of each was placed in an MSE centrifuge and spun at 3000 rpm for 25 minutes and the supernatant recovered (MSE Scientific Instruments, Manor Royal, Crawley, Sussex, England).

The supernatants produced were slightly cloudy. Samples of whole suspensions and supernatants were subjected to TN and NPN determination.

Protein solubility was calculated as:

$$\frac{\text{TN - NPN (supernatant)}}{\text{TN - NPN (total suspension)}} \times 100$$

The pH of each sample and diluent type was determined.

3.1.2 Results

Each analysis, i.e. of total and supernatant fractions, was performed in duplicate. The detailed results are shown in Appendix 3.1. The summarized results are shown in Table 3.1. The results show that protein solubility is substantially complete except for the untreated gluten.

3.1.3 Discussion

The addition of mercaptoethanol, as suggested by Danno et al (1974), appears to be unnecessary. Most gluten protein, more than 95%, is in solution after sampling from acid suspension. The technique, then, appears to be suitable for sampling for determination of N-terminal amino groups.

Table 3.1 Protein solubility for each gluten source and diluent

Gluten source (diluent)	A(1)	A(2)	A(3)	C(2)	D(2)
Protein solubility (%)	97.5	100	98	99	88

Table 3.2 The effect of buffer pH on the determination of N-terminal groups using fluorescence determined with fluorescamine

ADDITION OF[1] NaH_2PO_4 (ml)	pH OF BUFFER SOLUTION	PEAK HEIGHT (mm)
nil	9.08	193,173
2	8.10	103,111
4	7.77	74,77
6	7.57	56,56

Note (1)

Na_2HPO_4 was added to give a total volume of 50 ml. The concentration of both Na_2HPO_4 and NaH_2PO_4 solutions was 0.2125 M

The SDS is not completely effective in solubilizing dry gluten. This may be because of the total quantity of SDS is barely sufficient to bind to the protein (Reynolds and Tanford, 1970 a, b). For determination of N-terminal amino groups using TNBS or fluorescamine much greater dilutions of the protein, and hence an excess of SDS, would be available.

3.2 The effect of buffer pH on the determination of N-terminal amino groups using fluorescamine

3.2.1 Experimental method

Buffer solutions, 0.2125M of varying pH, were prepared by mixing various amounts of 0.2125M NaH_2PO_4 with 0.2125M Na_2HPO_4 to a total volume of 50 ml as shown in Table 3.2. Each buffer was used with fluorescamine, as described in Section 2.6.1, using 200 µl of 0.75 mM L-glu as sample.

3.2.2 Results

The results are shown in Table 3.2. The arbitrary units arise because the results are peak heights on the fluorimeter output chart, with arbitrary scaling to give a measurable output.

3.2.3 Discussion

A strong pH effect is shown. This was similarly described by Bohlen et al (1973) for various proteins. The effect may vary for different proteins and amino acids. In this study, where proteins are modified, the relationship between fluorescence intensity and pH value may change. The results cannot therefore be claimed to represent absolute values of N-terminal amino group content.

3.3 The effect of buffer concentration and the use of SDS on the determination of N-terminal amino groups using fluorescamine

The use of SDS is an effective means of solubilizing gluten as shown in Section 3.1. Reynolds and Tanford (1970 a, b) showed that the maximum binding of SDS takes place when ionic strength μ = 0.1. Calculation of the molarity of Na_2HPO_4 to give μ = 0.1 shows that the buffer concentration should be circa = 0.03M (Glasstone and Lewis, 1960).

The effect of buffer concentration and use of SDS was investigated in a series of experiments. The object was to ensure that gluten was fully solubilized by the SDS when sampled for determination of N-terminal amino groups using fluorescamine and TNBS.

3.3.1 The effect of buffer concentration on the fluorescence response from a standard sample (L-glu)

3.3.1.1 Experimental method

Buffer solutions Na_2HPO_4 and NaH_2PO_4, 0.2M, were prepared. Portions of these were diluted to 0.1M and 0.02M, and then mixed to give pH 8.0. One half of each of the three buffers had SDS added to give 1% w/v. The determination was performed using 200 ul of 0.5 mM L-glu as sample. Triplicate determinations were made, as described in Section 2.6.1 but using the buffers described above.

3.3.1.2 Results

Peak height data, shown in Table 3.3 indicate a large difference due to buffer concentration and a slight depression of the result due to the SDS. There was good agreement between triplicate samples.

Table 3.3 The effect of buffer concentration on the fluorescence response from a standard sample

BUFFER	PEAK HEIGHT (mm)			MEAN (mm)
0.02M no SDS	61	63	63	62
0.1M no SDS	85	82	83	83
0.2M no SDS	90	92	91	91
0.02M 1% SDS	60	59	54	58
0.1M 1% SDS	75	77	76	76
0.2M 1% SDS	92	90	87	90

3.3.2 The effect of buffer concentration on the fluorescence response of gluten with fluorescence

3.3.2.1 Experimental method

A hydrolysed gluten preparation, B, was prepared by adding 90 ml of 0.03M HCl to 10.0 g of gluten in a screw-top pyrex jar and holding in a waterbath at 70°C for 6.2 hours, and then held under refrigeration until required as described in Section 3.1.1 for preparations A and C. Gluten samples A, B and C were diluted 10^{-3}. The diluent was either water or 1% w/v SDS solution. Samples, 200 ul, from the final dilution were used to determine fluorescence response as described in Section 2.6.1 except that various buffer strengths, as described in Section 3.3.1.1, were used in the determination. Samples diluted in water were determined using buffer without SDS and samples diluted in 1% w/v SDS were determined using buffer containing 1% w/v SDS. All determinations were made in duplicate.

3.3.2.2 Results

The peak height data obtained are given in Table 3.4. The mean effect of each factor in the experiment is also shown in Table 3.4.

3.3.2.3 Discussion

For samples of gluten the variation between duplicates was much greater than for L-glutamic acid samples as described in Section 3.3.1. Because of this variation no clear effect of the buffer strength can be observed. The effect of SDS is, however, quite clear; the fluorescence response is greater in its presence, presumably because of the solubilization effect.

Table 3.4 The effect of buffer concentration on the fluorescence response from gluten

A Peak height data

SAMPLE	PEAK HEIGHTS (mm)		MEAN
Gluten A 0.02M no SDS	5.9	9.6	7.8
" " 0.1M " "	8.5	11.0	9.8
" " 0.2M " "	8.4	11.7	10.1
" " 0.02M + SDS	11.0	16.9	14.0
" " 0.1M " "	14.2	24.1*	19.2
" " 0.2M " "	12.4	26.9	19.7
Gluten B 0.02M no SDS	8.5	12.8	10.7
" " 0.1M " "	9.3	9.6	9.5
" " 0.2M " "	9.9	9.7	9.8
" " 0.02M + SDS	11.0	4.3	7.7
" " 0.1M " "	14.7	12.0	13.4
" " 0.2M " "	11.2	14.0	12.6
Gluten C 0.02M no SDS	6.7	7.6	7.2
" " 0.1M " "	5.6	4.2	4.9
" " 0.2M " "	4.6	4.2	4.4
" " 0.02M + SDS	13.6	21.6	17.6
" " 0.1M " "	15.0	25.7*	20.5
" " 0.2M " "	24.0	23.8*	23.9

* data converted to equivalent reading at sensitivity 3, when actually off scale, from readings at sensitivity 2, using the following data.

Setting on fluorimeter recorder:

Sensitivity 2	Sensitivity 3	(Ratio $\frac{3}{2}$)
6.3	21.6	3.43
3.8	12.8	3.37
	MEAN	3.4

Table 3.4 (continued)

B Mean peak height data for various buffers (mm)

	(no SDS)			(plus SDS)			Gluten
	0.02M	0.1M	0.2M	0.02M	0.1M	0.2M	Mean
Gluten A	7.8	9.8	10.1	14.0	19.2	19.7	13.4
Gluten B	10.7	9.5	9.8	7.7	13.4	12.6	10.6
Gluten C	7.2	4.9	4.4	17.6	20.5	23.9	13.1
Buffer mean	8.6	8.1	8.1	13.1	17.7	18.7	
SDS mean		8.3			16.5		

3.3.3 Further experiment with buffer concentration on the fluorescence response from gluten

3.3.3.1 Experimental method

The experimental procedure was the same as that described in Sections 3.3.1.1 and 3.3.2.1 except that triplicate dilutions of gluten sample A were made using the same set of buffers, 0.02M, 0.1M and 0.2M phosphate buffer (pH 8.0) with and without SDS. Also water and 1% w/v SDS in water was included in the experiment. A 200 ul sample was taken from each dilution for fluorescence determination which was made in duplicate using 0.2M buffer without SDS, the technique otherwise being as described in Section 2.6.1.

3.3.3.2 Results

The peak height data obtained are given in part A of Table 3.5 and the mean peak height for each determination is given in part B of Table 3.5

3.3.3.3 Discussion

Agreement between duplicate determinations was good whereas agreement between the triplicate dilutions was poor. This variation probably arises as a consequence of the difficulties of sampling a flocculent dispersion. It can be concluded that SDS in the dilution medium has a positive effect in solubilizing gluten. Because of the variation, no clear effect of buffer strength can be observed.

3.3.4 The effect of buffer concentration, mercapto-ethanol and centrifugation on the fluorescence response from gluten

In this set of experiments the composition of the buffer used for the determination of N-terminal amino groups with fluorescamine was examined. The hydrolysed gluten samples used were diluted in buffer containing SDS so that the gluten was already solubilized.

Table 3.5 The effect of buffer concentration on the fluorescence response for gluten samples (no SDS in determination buffer)

A Peak height data

DILUTION BUFFER	PEAK HEIGHTS[1] (mm)						MEAN PEAK HEIGHT $\pm \sigma_{n-1}$
Water	24	30	22	21	34	19	25 ± 5.8
1% SDS	23	29	30	59	54	54	41.5 ± 15.8
0.02M + SDS	33	33	32	31	34	35	33 ± 1.4
0.1M + SDS	74[2]	22	19	20	25	22	21.6 ± 2.3 (n=5)
							30.3 ± 21.5 (n=6)
0.2M + SDS	24	24	33	33	34	23	28.5 ± 5.3
0.02M	27	25	37	32	33	36	32 ± 53
0.1M	19	24	12	12	33	30	22 ± 8.9
2M	12	13	19	18			15.5 ± 3.5 (n=4)

B Mean peak height data

Sample diluent	PEAK HEIGHTS (mm) WATER	0.02M	0.1M	0.2M	AVERAGE
With SDS	42	33	22	29	32
Without SDS	25	32	22	16	24

Note (1)
Reading from left to right the peak heights are triplicate dilutions presented as duplicate determinations.

Note (2)
This appears to be a spurious result and was ignored in determination of mean values.

3.3.4.1 Experimental method

Gluten samples were prepared by taking 10^{-3} dilutions in 1% SDS of gluten samples A, B, C, as described in Section 3.3.2.1, and mixing them together. Fluorescence of samples, 200 µl, was determined using buffers with and without added SDS as described in Section 3.3.1, for final dilution. A further portion of the gluten suspension was centrifuged (10 minutes, 3000 rpm MSE centrifuge). The supernatant was sampled into the same set of buffers as above. A further portion of the gluten suspension, 9.5 ml, had 10 µl of diluted mercapto-ethanol solution added such that the final concentration was 0.005M (Danno et al (1974). After three minutes this was sampled and fluorescence determined in 0.2M pH 8.0 buffer (without SDS). The technique was otherwise as described in Section 2.6.1. All determinations were made in duplicate.

3.3.4.2 Results

The peak height data obtained are given in Table 3.6. If it is assumed that centrifugation makes little or no difference, the effect of SDS and buffer strength can be seen as shown in Table 3.7.

3.3.4.3 Discussion

The data in Table 3.6 show that centrifugation has little or no effect, i.e. that the SDS in the dilution medium is effective in solubilizing the gluten. The mercapto-ethanol did not improve on the action of SDS in the dilution medium. There were quite large differences between duplicate determinations. No systematic effect of fluorescence determination buffer concentration could be seen, as shown in Table 3.7, in contrast to a marked buffer concentration effect with L-glu (Table 3.3). Dilution of the sample in 1% SDS appears to be effective in solubilization of the gluten and adding SDS to the determination buffer is not required as indicated by the data in Table 3.7.

Table 3.6 The effect of buffer concentration, mercaptoethanol and centrifugation on the fluorescence response from gluten

ANALYSIS BUFFER	PEAK HEIGHT (mm) NOT CENTRIFUGED	MEAN	CENTRIFUGED	MEAN
0.02M no SDS	38, 37	38	34, 38	36
0.1M no SDS	38, 53	46	36, 181(1)	36
0.2M no SDS	42, 36	39	47, 38	43
0.02M 1% SDS	34, 33	34	38, 31	35
0.1M 1% SDS	49, 52	51	39, 34	37
0.2M no SDS (0.005M M.E.)	41, 45	43	38, 41	40
Mean	41		38	
σ_{n-1}	7 (n=12)		5 (n=11)	

Note (1)

This appears to be a spurious result and was ignored in determination of mean values.

Table 3.7 The effect of the concentration of the determination buffer on the fluorescence response of gluten.

	PEAK HEIGHT (mm)			
Buffer Concentration	0.02M	0.1M	0.2M	MEAN
plus SDS	35	44	40	40
no SDS	37	41	41	40
Mean	36	43	41	

3.4 Conclusions from experiments described in Sections 3.1 to 3.3

(a) The sampling of gluten suspensions is difficult and appears to give rise to substantial analytical variation.

(b) The use of SDS is effective in solubilizing gluten protein

(c) Centrifugation has little effect implying that gluten protein is almost completely solubilized

(d) There is no need for SDS in the measurement buffer - implying that once SDS is bound to protein it does not easily come off again

(e) The determination of N-terminal amino groups using fluorescamine is affected by the pH and strength of the buffer used but the effect on the standard, L-glu, is different to that for protein samples

3.5 Measurement of gluten concentration using fluorescamine

This experiment was carried out to demonstrate that the fluorescence obtained was proportional to the N-terminal amino groups present.

3.5.1 Experimental Method

The gluten used was from Sample A, described in Section 3.1.1, diluted 10^{-2} in 0.02M phosphate buffer (pH 8.0) containing 1% SDS. Various quantities, 25, 50, 75, 100 and 150 µl were added in duplicate to test tubes and the volume was made up to 200 µl with 1% SDS solution; 0.2125M phosphate buffer (pH 8.2) was added and the determination was carried out as previously described in Section 2.6.1.

A standard curve was prepared using as samples, in duplicate, 200 µl 1% SDS and 50, 100, 150 and 200 µl of 0.5 mM L-glutamic acid solution (in 1% SDS). A further set of

samples were prepared in which 50 µl of gluten as described above was added to a series of sample tubes with further addition of 20, 50, 100 and 150 µl of 0.5 mM L-glu. The samples were made up to 200 µl with 1% SDS and the determination carried out as described above.

3.5.2 Results

The peak height data obtained are given in Appendix 3.2. In Figure 3.1 the data for gluten are depicted in terms of a relative concentration where a 200 µl sample is equal to 1.0. A satisfactory straight line was obtained. In Figure 3.2 the standard curve also shows a satisfactory straight line and when gluten is added a parallel line was obtained.

The data also show that content of N-terminal amino groups of gluten preparation A, diluted 10^{-1}, is equivalent to 5.6 mM L-glu.

3.6 The effect of ammonia on the determination of N-terminal amino groups using fluorescamine

The possible reaction of fluorescamine with ammonia, in contrast to N-terminal amino groups, was investigated as follows.

3.6.1 Experimental method

Two separate experiments were conducted. In the first experiment, samples were prepared with 0, 10, 20, 40, 60 and 100 µl of 4 mM, NH_4Cl alone, added to 50 µl of gluten sample A diluted to 10^{-2} (described in Sections 3.1.1 and 3.5.1) and, added to 100 µl of 0.5 mM L-glu. Sample volumes were made up to 200 µl with 1% w/v SDS solution. The samples were prepared in duplicate and the fluorescence was determined using fluorescamine as described in Section 2.6.1. In the second experiment ammonia was included in the buffer, (1.5 ml) added in addition to the sample, see Section 2.6.1. The desired concentrations were achieved by preparing buffer which was 0.1M in NH_4Cl and mixing it in various proportions with buffer without NH_4Cl. Because of

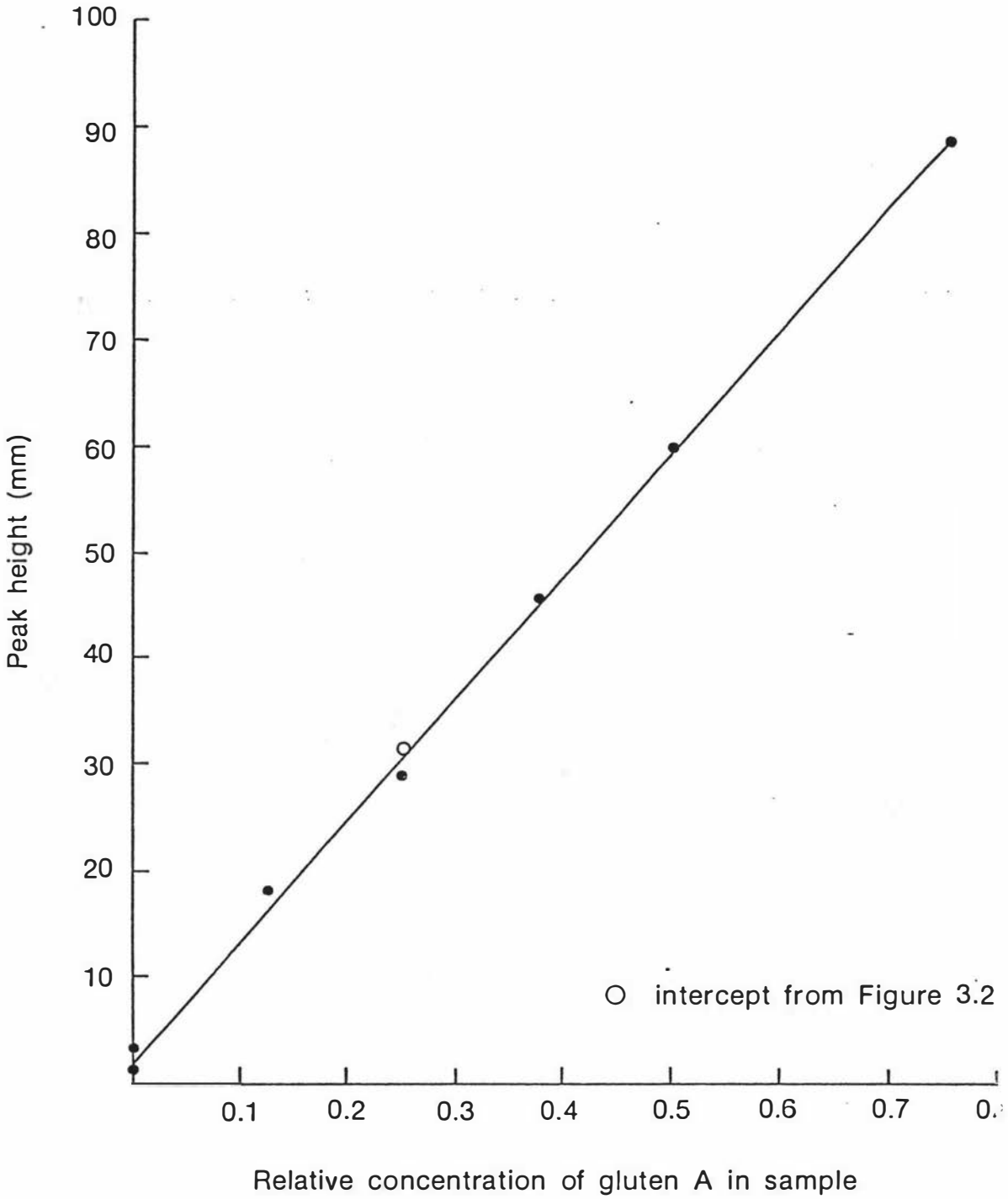


Figure 3.1 The fluorescence response obtained with gluten an fluorescamine.

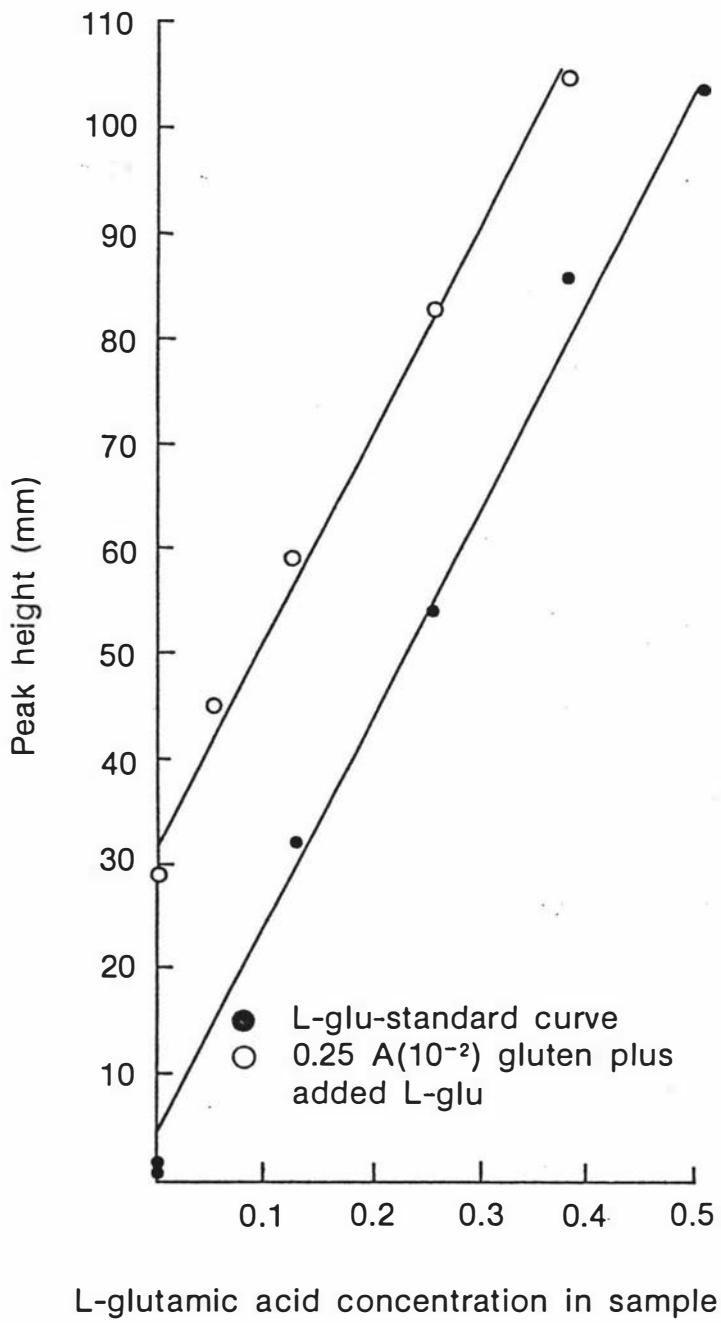


Figure 3.2 The fluorescence response obtained with L-glu and L-glu plus gluten and fluorescamine.

the volume ratios, 1.5 ml, compared to 0.2 ml of sample, ammonia concentrations of 0.01, 0.05, 0.1 and 0.5M in the sample were simulated by using a buffer solution 0.1M in NH_4Cl. The mixtures used to achieve the desired concentrations were:

CONCENTRATION OF NH_4Cl IN SAMPLE (M)	BUFFER A (0.1M NH_4Cl) (ml)	BUFFER B (ml)
0.5	1.0	0.5
0.1	0.2	1.3
0.05	0.1	1.4
0.01	0.02	1.5
nil	nil	1.5

The NH_4Cl affected the pH of the buffer
i.e. Buffer A pH 8.017
Buffer B pH 8.086

A sample of 200 µl of 0.75 mM L-glu was added to each tube and the fluorescence determined as otherwise described in Section 2.6.1.

3.6.2 Results

The peak height data from the first experiment are recorded in Appendix 3.3. These data are presented in Figure 3.3. The ammonia concentrations, calculated to be the likely maximum in hydrolysis experiments can be seen to have a slight, almost negligible effect. The data of the second experiment, covering much higher ammonia concentrations, are shown in Appendix 3.4. Both sets of data are combined in Figure 3.4. The graph can be used to correct fluorescence readings for the effect of ammonia providing that the fluorimeter is set to read 93 mm peak height for a 0.75 mM L-glu sample (see Appendix 3.4).

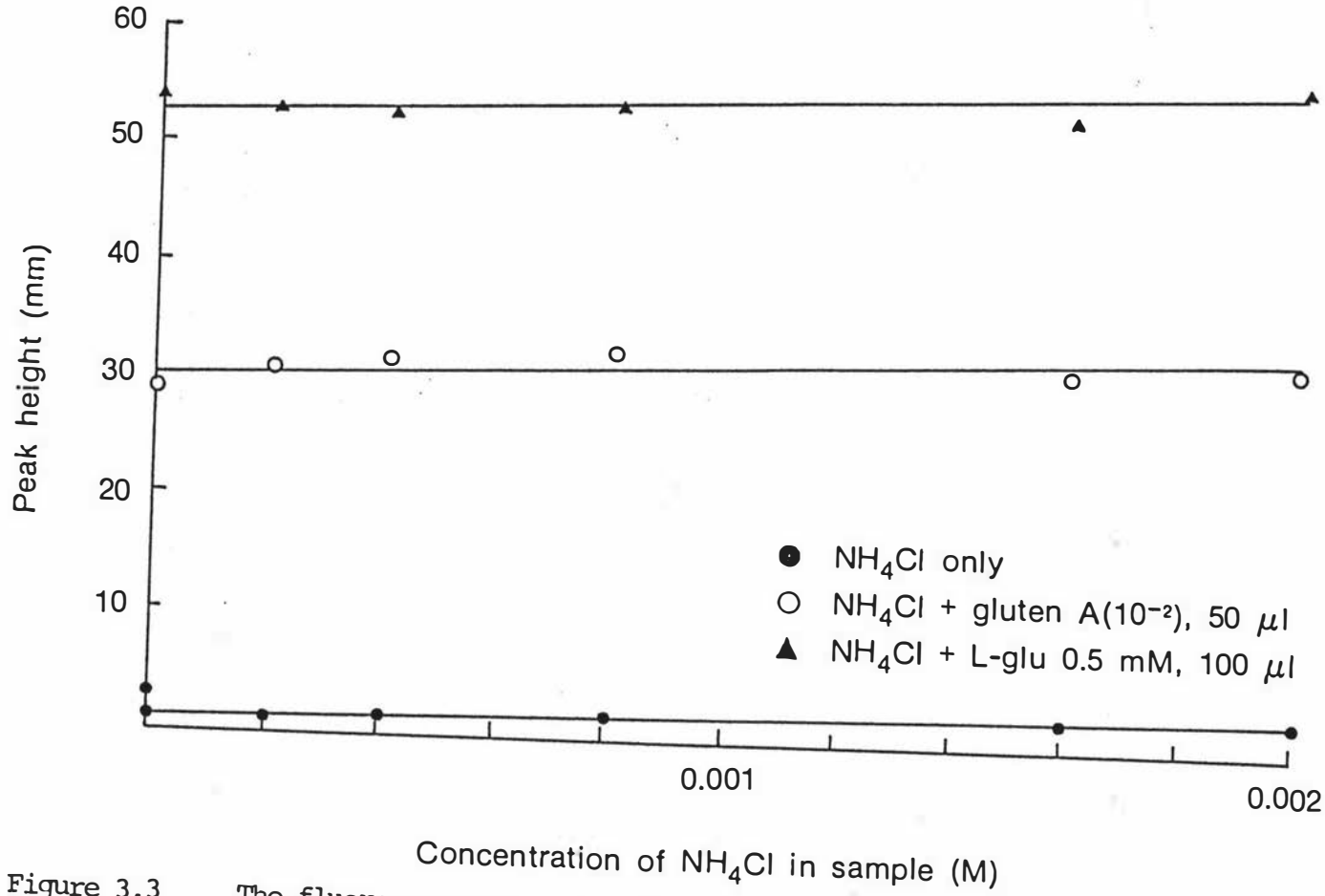


Figure 3.3 The fluorescence response for ammonia with fluorescamine.

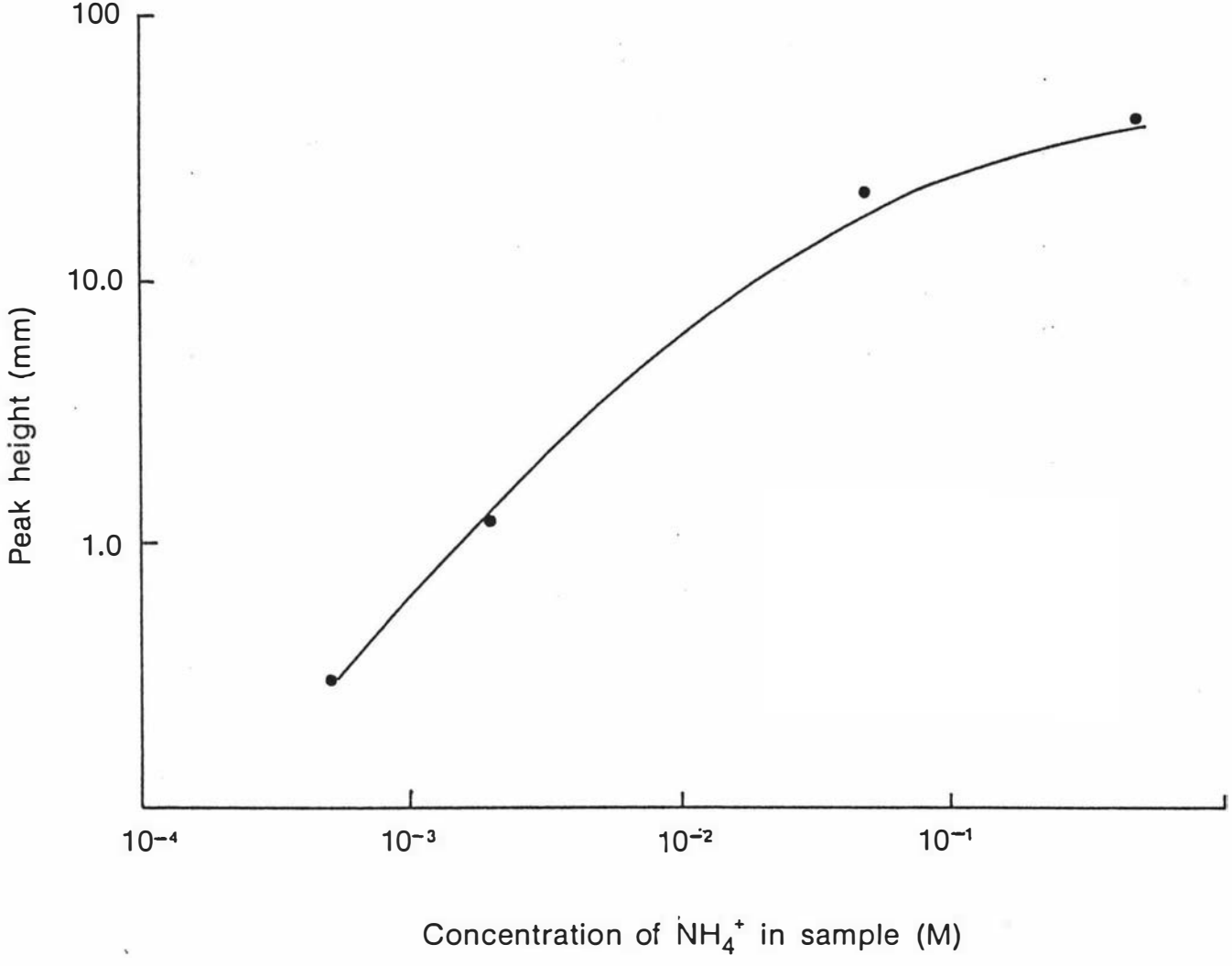


Figure 3.4 The fluorescence response for ammonia with fluorescamine.

3.6.3 Discussion

At low concentrations of ammonia the likely contribution to the total fluoresence is practically negligible. At the very high concentrations shown in Figure 3.4 the ammonia concentration is in excess of the fluorescamine and this probably contributes to the non-linear response.

3.7 The determination of N-terminal amino groups of gluten using TNBS

3.7.1 Experimental method

Gluten samples were taken from preparations A and B as described in Sections 3.3.1 and 3.3.2.1. To dilute these samples 0.5 ml 0.2125M phosphate buffer (pH 8.2) and 4.0 ml 1% SDS solution were mixed. Then 0.5 ml of the sample was added.

Dilutions 10^{-1} and 10^{-2} were prepared.

In a first experiment samples of gluten B diluted 10^{-1} were used. When 4.0 ml 0.1M HCl was used to quench the reaction (as described by Adler-Nissen (1979), a cloudy solution was obtained. Thus the amount of acid was varied as shown in Table 3.8.

In a further experiment, samples of gluten A, 0.125 ml, diluted 10^{-1} and 10^{-2} were added to 0.125 ml of varying strengths of L-glu solution. The determination was made, as described in Section 2.6.2.

3.7.2 Results

The effect of the volume of 0.1M HCl added to quench the reaction with TNBS is shown in Table 3.8. The addition of 4.0 ml 0.1N HCl gave cloudy solutions but a lower quantity left a satisfactory clear solution. Adler-Nissen (1979) notes that cloudy solutions can be a problem when the pH is too low. The pH of each final solution was determined as shown in Table 3.8. Adler-Nissen (1979) also states that

Table 3.8 The effect of the volume of HCl added to quench the reaction with TNBS

VOLUME OF 0.1 N HCl ADDED (ml)	pH	SOLUTION APPEARANCE
Nil	8.053	clear
2	6.642	clear
3	6.103	clear
3.5	5.610	clear
4	3.335	cloudy

any pH below neutral is acceptable. The addition was therefore altered to 3.5 ml, as described in Section 2.6.2. When subsequently some cloudiness appeared it was found that it could be cleared by mixing the tube contents.

The results of the experiment using gluten sample A and L-glu are shown in Figure 3.5. The raw data are given in Appendix 3.5. A straight line standard curve can be seen. The effects of gluten on L-glu appear to be additive. The reaction with TNBS appears to give a satisfactory measurement of gluten and L-glu is a suitable standard. The data also show that the content of N-terminal amino groups of gluten preparation A, diluted 10^{-1}, is equivalent to 1.8 mM L-glu.

3.7.3 Discussion

The data show that the N-terminal amino groups in gluten can be satisfactorily determined using TNBS. The determination used is that proposed by Adler-Nissen (1979) with the minor modification of a reduced quantity of 0.1M MCl to quench the reaction and the use of L-glu as a standard. The content of N-terminal amino groups of gluten preparation A is apparently less when measured with TNBS than with fluorescamine as reported in Section 3.5.2. This is not an unexpected result as was concluded in Section 3.4. No similar effects of buffer pH and concentration have been reported for the reaction with TNBS but have not been excluded in this present work.

3.8 The effect of ammonia on the determination of N-terminal amino groups using TNBS

Ammonia reacts with TNBS as discussed in Section 2.6.2. The experiments which follow were designed to quantify the effect so that, knowing the level of ammonia, the reaction of TNBS with N-terminal groups could be separately determined.

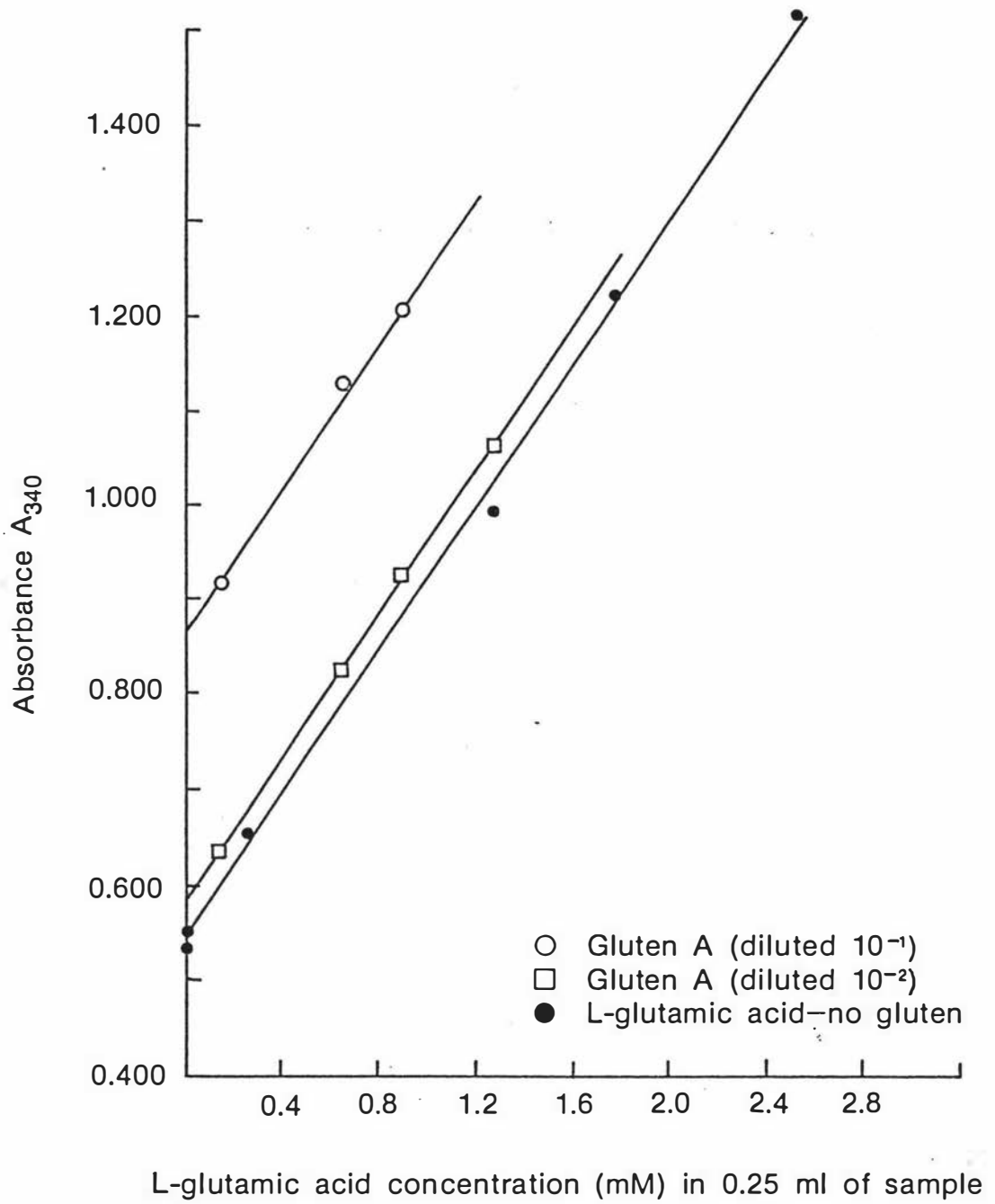


Figure 3.5 The reaction of L-glu and L-glu plus gluten with 2, 4, 6 - trinitrobenzene sulfonic acid (TNBS).

3.8.1 Experimental method

In a first experiment varying quantities, 5-100 µl, of 0.05 M NH_4Cl were mixed with 150 µl of 2.5 mM L-glu and the volume of each sample made to 250 µl using 1% SDS solution. In a further experiment a similar procedure was followed except that the ammonia solution was also added to 150 µl of gluten sample A diluted 10^{-1}.

In both experiments the reaction and determination were made as absorbed in Section 2.6.2.

3.8.2 Results

Absorbance data from the first and second experiments are recorded in Appendices 3.6 and 3.7, respectively, and are shown in Figure 3.6. The difference in absorbance between the three curves appears to be constant throughout the range indicating that the absorbance due to sample and ammonia are measured independently.

The same data are replotted in Figure 3.7 in the form of a correction to the absorbance for the contribution of ammonia.

A straight line fit on logarithmic coordinates is obtained.

3.8.3 Discussion

The substantial additive effect of ammonia on the absorbance due to N-terminal amino groups as shown by Whitaker et al (1980) and Clegg et al (1982) has been confirmed. The effect is not however linear, as shown in Figure 3.7, whereas the response to N-terminal amino groups is linear as shown in Figure 3.5. A correction can be made by using the data of Figure 3.7 which can be extrapolated to lower concentrations. The correction plainly cannot be made by assuming that the absorbance due to ammonia is simply 20% of that due to N-terminal amino groups as stated by Clegg et al (1982).

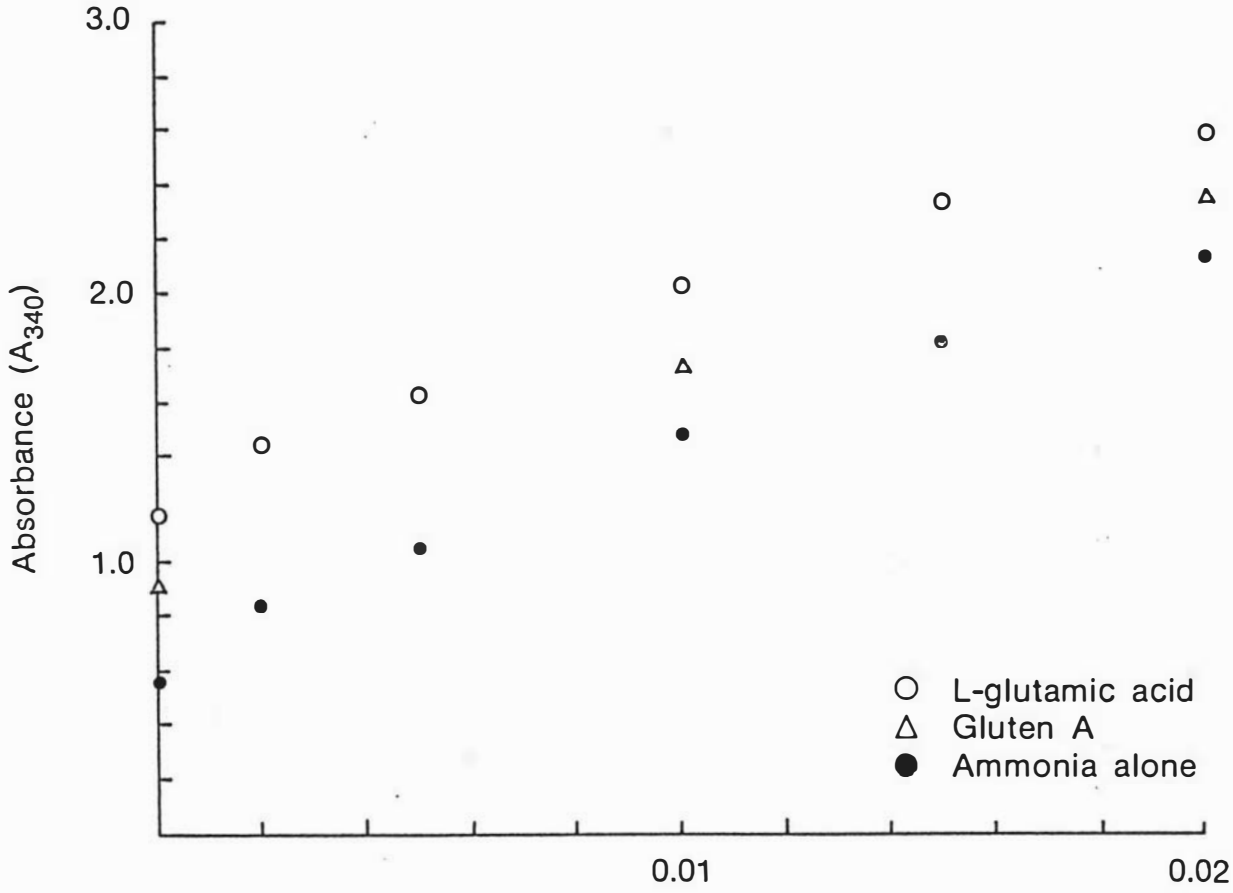


Figure 3.6 The effect of ammonia on the determination of N-terminal amino groups using TNBS.

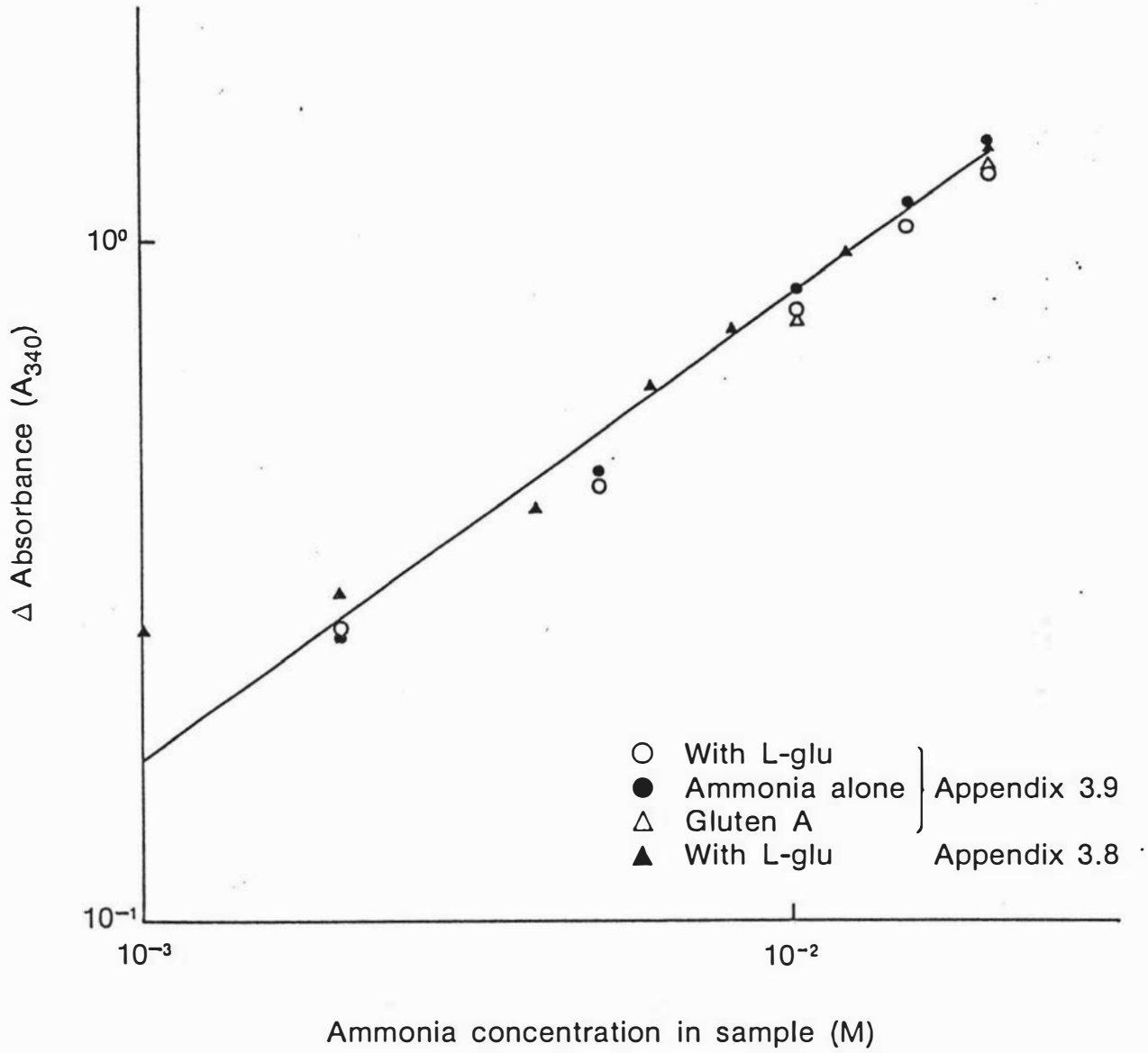


Figure 3.7 A correction for the effect of ammonia on the determination of N-terminal amino groups using TNBS.

The correction to absorbance is an absolute figure measured in absorbance units. This is in contrast to the correction for the effect of ammonia when using fluorescamine for the determination of N-terminal amino groups which must be related to an arbitrarily defined scale as described in Section 3.6.

3.9 Conclusions from experiments described in Sections 3.5 to 3.8

Both fluorescamine and TNBS act satisfactorily in giving measurements of the N-terminal amino group content of gluten. The absolute values obtained are not, however, the same.

Both reagents react to some extent with ammonia and the effect has been characterized. The effect for TNBS is an absolute measurement and the correction developed could be used for any system where an independent determination of ammonia was available. For fluorescamine, however, the correction depends on an arbitrarily determined scaling. Because, however, the system is very sensitive to protein and not to ammonia, its effect can for practical purposes, in this present work be neglected.

4. EXPERIMENTAL DETERMINATION OF THE KINETICS OF ACID HYDROLYSIS OF GLUTEN

4.1 General Approach

Decisions on the type and scale of experiment were based on the following considerations.

A likely option for a commercial process would be acid treatment of gluten as an integral part of the gluten production process, i.e. the gluten may be in a dough form and the acid added in a concentrated form, since low acid and solids concentrations could give rise to higher costs for the subsequent separation and drying operations. It was therefore important to include concentrations of acids and gluten as high as was practical within the experimental conditions.

That the kinetics of deamidation would follow those for acid hydrolysis of amides was taken as a working hypothesis (Leach, 1953; Mittal et al, 1982). To test this hypothesis it was necessary to vary the concentration of acid and amide (gluten) over as wide a range as possible while also taking into consideration the effect of temperature. It was decided to try to minimise the number of experiments but to try to gain a large amount of information from each experiment. Another criterion was that the kinetic experiments should initially be on a bench scale with simple apparatus.

As a result of these considerations a scheme was adopted whereby the reactions were conducted in a series of Bijou bottles which could be immersed in baths at the appropriate reaction temperatures and removed after various reaction times. Further details of the system are given in the following sections.

Several preliminary experiments were necessary in order to define experimental conditions for the hydrolysis proper.

4.2 Preliminary Experiments

4.2.1 Determination of maximum gluten concentration

The maximum gluten concentration was determined as that which could be reliably dispersed in the reaction bottles. Trials were made of concentrations up to 16% w/v in 5 ml. This was achieved by adding 0.8 g of gluten and making the volume up to 3 ml with deionised water, i.e. 2 ml were allowed for acid addition. Dispersion was achieved by placing each bottle on a vortex mixer. It was found that the maximum concentration for reliable dispersion was 14% w/v gluten.

4.2.2 Determination of the specific volume of gluten

The specific volume of gluten was determined by measuring the displacement of water in a pycnometer.

A 50 ml pycnometer containing a small magnetic stirring bar was dried and weighed. The pycnometer was filled with degassed deionised water and allowed to equilibrate at 25°C in a water bath. The pycnometer was carefully dried and reweighed. These measurements served to establish the exact volume of the pycnometer.

Most of the water was then withdrawn with a pipette and the pycnometer reweighed. About 4 gms of gluten were added and the weight was again recorded. A little more water was added and the gluten mixed with the water with the aid of the magnetic stirring bar. The pycnometer was then filled to the top with degassed water and equilibrated and reweighed as before. Two determinations were made and the results were:

0.7525 cc/gm and 0.7523 cc/gm

The values are similar to those found for other proteins by Munro (1980) and Bell et al (1982). These values were used to determine the quantitites of gluten and acid to be added

for a constant reaction volume of 5 ml. The protein density might be expected to vary both with the initial acidity and as the reaction proceeded due to changes in protein conformation (Ifft, 1976; Kuntz and Kauzmann, 1974). No account was taken of these changes in calculation of ammonia and N-terminal amino groups. The reaction volume was assumed to remain constant.

4.2.3 Determination of the heating rate in reaction bottles

One reaction bottle with a copper-constantan thermocouple sealed through the cap was filled with 5.0 ml of water. The thermocouple was arranged so that it was immersed in the water. The bottle was placed in a bath controlled at the appropriate temperature. The temperature in the bottle was measured using an electronic thermometer for copper-constantan with an internal reference (Comark Electronics, Sussex, England). The calibration was checked by testing against a potentiometer source.

The change in temperature with time is shown in Figure 4.1. This shows that three to four minutes were required for the bottle contents to reach the temperature of the bath. Most of the change, 90%, was complete in less than two minutes for the lower temperatures and within three minutes for the highest temperature of 130°C.

Only one of the visually variable bottles was used for these tests. The results are, however, consistent with samples taken after two minutes reaction time appearing to be slightly below the curve in some ammonia vs time plots. The initial rate data, Section 4.8.1 were taken from the total curve, that is a visual allowance was made for the delay in initial reaction due to the bottle contents not instantaneously reaching the reaction temperature. No further correction was warranted.

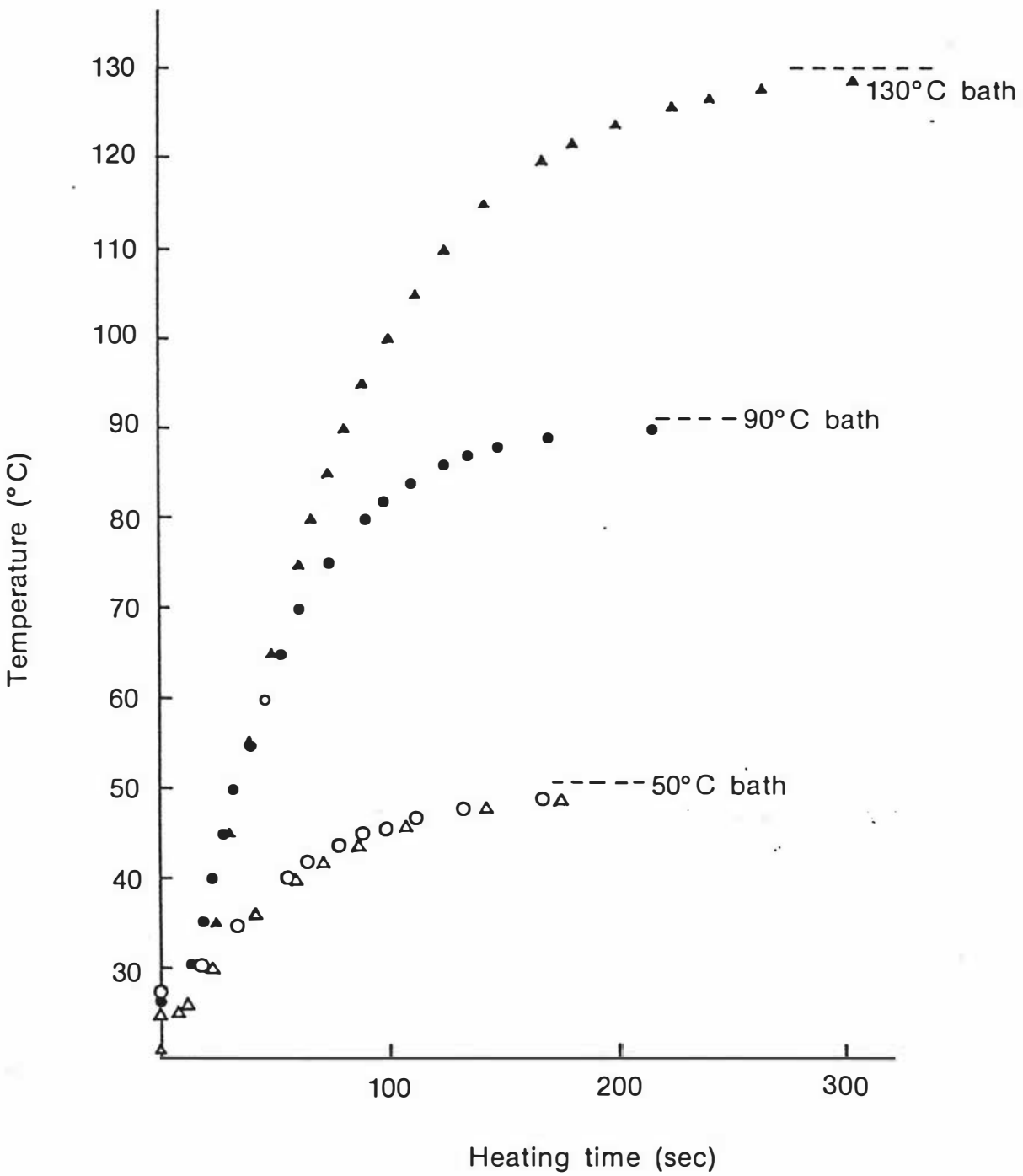


Figure 4.1 The rate at which bottle contents are heated to bath temperature.

4.2.4 Monitoring of gluten quality for the duration of the experiments

The bag of gluten was stored at room temperature in the laboratory and opened and resealed each time gluten was withdrawn.

Chemical analysis of duplicate samples of the bag contents was made at the beginning and end of the period of hydrolysis experiments. Moisture content was determined by drying in an oven at 103±1°C for 5 hours. Kjeldahl nitrogen analysis was used to determine the proportion of protein nitrogen. The results are shown in Table 4.1.

Water absorption was chosen as a functional property of the gluten which:

(i) might be expected to change if properties affecting the kinetic measurements were to change

(ii) could be reproducibly measured.

Water absorption measurements were made using the method of Knightbridge and Goldman (1975). The results are shown in Table 4.2. The results show that while there was a small increase in the moisture content of the stored gluten there was little change in an important functional property. The variation between water absorption values in Table 4.2 is within the standard error of the method (Cooper, 1985).

4.2.5 Characterization of pH electrode

The various possible kinetic models describe reaction rates in terms of concentration of reactants. From previous studies of the acid hydrolysis of amides it was expected that the concentration of hydrogen ion would be important (Ali and Capindale, 1975; Crocker, 1907; Mittal et al, 1981, 1982). The hydrolysis experiments which follow were designed to test the effect of hydrogen ion concentration.

Table 4.1: Chemical analysis of the gluten used for determination of hydrolysis kinetics

SAMPLE DATE (Result date)	SAMPLE NO	MOISTURE (%)		TOTAL NITROGEN (%)		NON-PROTEIN NITROGEN (%)
13 Jan 84	A	7.73		12.69		0.14
		7.70	7.72	12.67	12.68	
24 Jan 84	B	7.63		12.63		0.12
		7.62	7.63			
7 Sept 84	A	8.20		12.55		0.08
		8.20	8.20	12.54	12.55	
21 Sept 84	B	8.17		12.54		0.07
		8.17	8.17	12.60	12.57	

Table 4.2: Water absorption measurements on the stored gluten used in experiments for determination of hydrolysis kinetics

DATE MEASURED	WATER ABSORPTION (%)
8 March 1984	128.0
1 June 1984	129.8
4 September 1984	125.2
6 December 1984	118.7
21 March 1985	131.1

A common technique in acid hydrolysis experiments is to use a low concentration of the compound to be hydrolysed in a known concentration of acid so that the acid concentration remains essentially constant. That technique was not appropriate in this case for two reasons. First, gluten, in common with other proteins, combines with a certain amount of hydrogen ion which has the effect of lowering the hydrogen ion concentration of the acid solution. The change on binding is dependent on hydrogen ion concentration (Edsall, 1943). Second, it was desired in this work to test the effect of varying the gluten concentration. It was also known that hydrogen ions were consumed in the reaction. It was therefore necessary to measure the hydrogen ion concentration for each experiment. The pH meter (see Section 2.2), was specified to operate between pH values of 0 to 14. When tested, however, it appeared to operate quite satisfactorily at the pH values a little below zero required for these experiments.

It is generally assumed that the glass electrode functions as a hydrogen electrode only within a restricted range of acidity. In aqueous solutions of strong acids the pH response of the glass electrode shows a departure from a hydrogen electrode function. This departure depends on the acid and electrode used. Various theories have been proposed but the origin of the acid error is not clear (Beck and Wynne-Jones, 1952). To characterize the electrode used in the present experiments the following measurements were made.

Two molar HCl was prepared from a concentrated volumetric solution ampoule (BDH Chemicals Ltd., Poole, England). The accuracy is guaranteed to be within the limits 0.999 and 1.001 when 1.0M solutions are prepared. The concentration of the two molar HCl was measured using sodium carbonate as described by Vogel (1961). Three titrations were made and the mean value of the normality was found to be 1.9697, with sigma (n-1) = 0.0033

i.e. 1.970 ± 0.006

This value was used in subsequent calculations.

The two molar HCl was then used to prepare 0.2, 0.02 and 0.002M HCl by dilution. It was also used to prepare pH 1.0, pH 1.5 and pH 2.0 buffer solutions by mixing HCl with 0.2M KCl (Bates and Paabo, 1968). The pH meter was then calibrated using pH 2.0 and pH 3.0 buffers at 25±1°C (BDH Chemicals Ltd, Poole, England). These buffers are stated to be correct to ±0.02 pH units at 20°C.

The pH of each of the prepared solutions was then determined at 25°C. Between readings, the electrode was rinsed in water purified by reverse osmosis and dried with a tissue. The electrode was immersed in each solution for five minutes before a pH measurement was recorded.

The pH values are shown in Table 4.3 and are presented in chronological order. It can be seen that there was some drift in the calibration of the electrode.

The "correct" pH values included in Table 4.3 were calculated from data for HCl with a hydrogen electrode (Harned and Ehlers, 1935; Randall and Young, 1928). The data are summarised in Figure 4.2 where data from other sources are also included (Hubbard et al, 1939; MacInnes and Belcher, 1931; Dole, 1932; Sinclair and Martell, 1950). Details of the calculation of pH values from other authors are given in Appendix 4.1.

The data show that the acid error of glass electrodes was experienced with the electrode used in these experiments. The level of error was similar to that found by other authors, as shown in Figure 4.2. The phenomenon of drift in strong acid solutions noted by Sinclair and Martell (1950) and Beck et al, (1963) also occurred. The pH values determined from the experiments described below were used

Table 4.3: pH measurements obtained in pH electrode characterization experiments

CALIBRATION CONSTANTS	PREPARATION SOURCE	NOMINAL CONCTN (g-mole l^{-1})	'TRUE' CONCTN (g-mole l^{-1})	MEASURED pH	'TRUE' ACTIVITY COEFFICIENT	'TRUE' pH	MEASURED ACTIVITY (g-mole l^{-1})	'TRUE' ACTIVITY (g-mole l^{-1})
S_{rel} = 1.038	BDH	pH 2.0	-	1.998		2.0		
	BDH	pH 3.0	-	2.995		3.0		
pH 6.734	Diluted							
	2M HCl	0.002	0.00197	2.732	0.95(1)	2.728	0.00185	0.00187
	"	0.02	0.0197	1.792	0.88	1.761	0.0161	0.017
	"	0.2	0.197	0.870	0.767	0.820	0.135	0.151
	"	0.2(1)	0.97	0.861	0.767	0.820	0.138	0.151
	"	0.2(2)	0.197	0.861	0.767	0.820	0.138	0.151
	"	2.0	1.970	-0.125	1.000	-0.294	1.33	1.97
	"	2.0(3)	1.970	-0.120	1.000	-0.294	1.32	1.97
	BDH	pH 2.0	-	2.000		2.0		
	BDH	pH 2.0(4)	-	2.002		2.0		
	KCl/2M	pH 2.0	0.0128	1.979	0.896	1.940	0.010	0.011
		pH 1.5	0.0408	1.518	0.842	1.462	0.030	0.035
		pH 1.0	0.132	1.033	0.784	0.985	0.093	0.104
	BDH	pH 4.0(5)		3.939		4.0		
	BDH	pH 7.0		6.787		7.0		
	BDH	pH 7.0(6)		6.789	7.0			
	BDH	pH 3.0		3.018		3.0		
	BDH	pH 3.0(7)		3.016		3.0		

	BDH	pH 2.0		2.011		2.0		
	BDH	pH 2.0[8]		2.011		2.0		
NOTES: (1)	reading taken after 10 minutes						1.82[10]	3.16
(2)	reading taken after 15 minutes						2.81[10]	5.62
(3)	reading taken after 20 minutes						3.16[10]	6.76
(4)	reading taken after 10 minutes						4.22[10]	10.0
(5)	reading taken after 15 minutes							
(6)	reading taken after 10 minutes							
(7)	reading taken after 10 minutes							
(8)	reading taken after 10 minutes							
(9)	guessed value							
(10)	data from Figure 4.2 by interpolation							
S_{rel} = 1.034	BDN	pH 2.0	-	1.997		2.0		
	BDH	pH 2.0[1]	-	2.004		2.0		
pH 6.744	HCl/2M	pH 1.0	0.132	1.033	0.784	0.985	0.093	0.104
	Diluted							
	24 HCl	0.2	0.197	0.871	0.767	0.820	0.134	0.151
	"	2.0	1.970	-0.116	1.000	-0.294	1.31	1.97
	"	2.0[2]	1.970	-0.117	1.000	-0.294	1.31	1.97
	5.ON HCl	5.0[3]		-0.718	3.162	-1.199	5.22	15.81
	5.ON HCl	5.0[4]		-0.722	3.162	-1.199	5.27	15.81
	1.ON HCl	1.0[5]		0.211	0.813	0.090	0.615	0.813
	BDH	pH 2.0		2.018		2.0		
	BDH	pH 3.0		3.039		3.0		

S_{rel} = 1.049	BDH	pH 2.0		1.998		2.0		
pH 6.655	BDH	pH 3.0		2.996		3.0		
	0.5N HCl	0.5[6]		0.523	0.758	0.421	0.300	0.379
	1.0N HCl	1.0[7]		0.226	0.813	0.090	0.594	0.813
	5.0N HCl	5.0[8]	-0.700	3.162		-1.199	5.01	15.81
	5.0N HCl	5.0[9]		-0.701	3.162	-1.199	5.02	15.81
	BDH	pH 2.0		1.977		2.0		
	Diluted							
	24 HCl	1.0	0.985	0.224	0.810	0.098	0.567	0.798
	HCl/2M	pH 2.0		1.966		2.0		

NOTES (1) reading taken after 60 minutes

(2) reading taken after 10 minutes

(3) sample prepared previously ex BDH CVS

(4) sample prepared previously ex BDH CVS (Different from (3) above)

(5) " " " " "

(6) " " " " "

(7) " " " " " (repeat of (5) above)

(8) " " " " " (repeat of (4) above)

(9) reading taken after 15 minutes

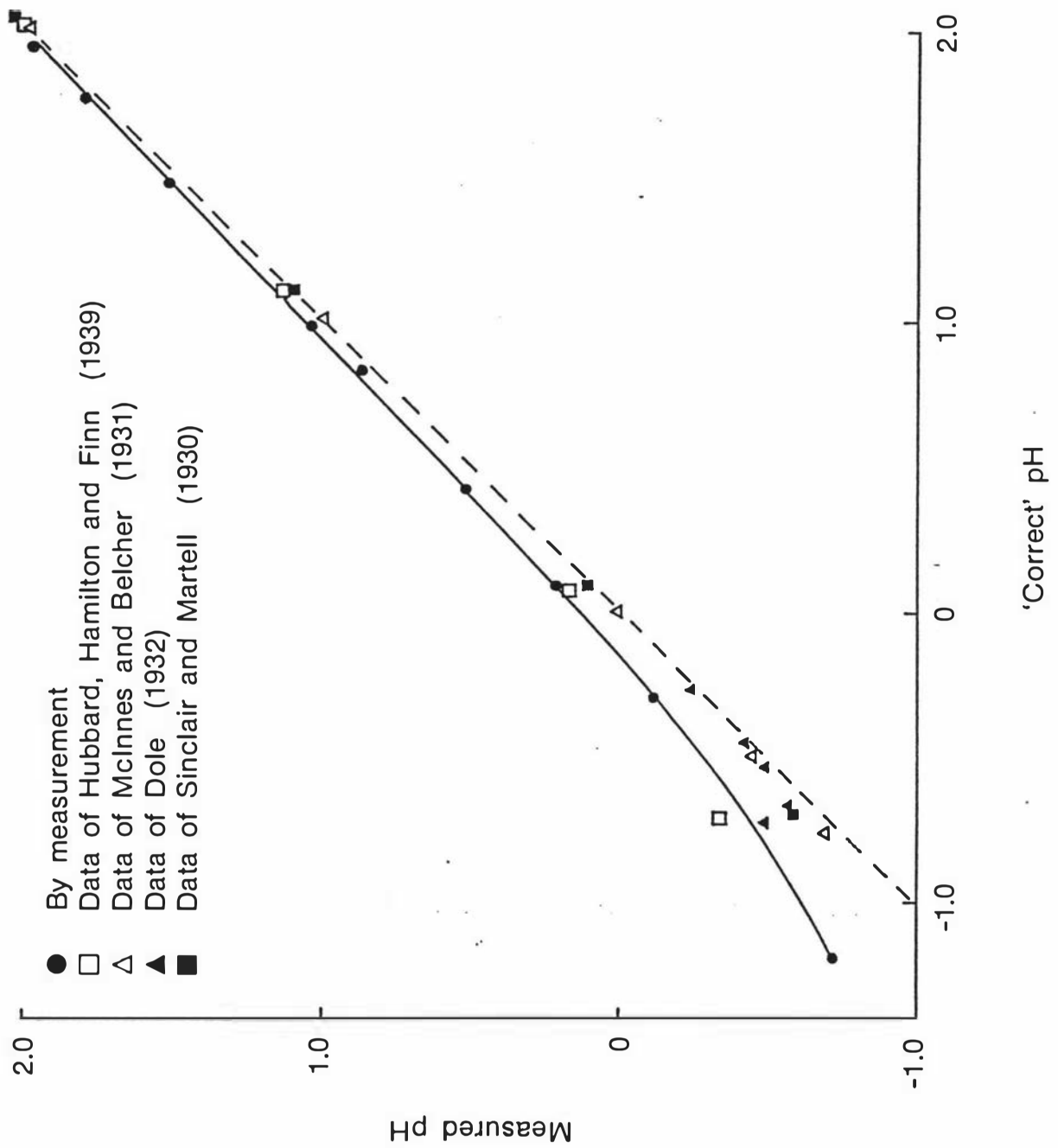


Figure 4.2 Illustration of acid error data for pH electrode.

in subsequent kinetic analysis without correction for these effects. The consequences of using the data in this form are discussed in Section 4.11.

4.3 Experimental design for hydrolysis experiments

It was desired to cover a range of conditions with variables:

Acid strength	0.02 - 2M
Temperature	50-130°C
Gluten concentration	1-14% w/v

The acid strength was chosen so as to cover a wide range of concentrations between that which would give a measurable deamidation and that which would give a rapid deamidation but without charring of the protein. The temperature upper limit is a convenient level for oil bath operation. The lower temperature limit was known to give a reasonable reaction rate at the lowest acid strength. The chosen levels are shown in Table 4.4.

These were used in an experimental design which required 15 runs including 3 repetitions (John, 1981). This design represented a substantial reduction from the possible 27 combinations of the variables if all possible combinations were examined without repetition. The design is shown in Table 4.5. The performance order was randomised. Because, on some occasions, the oil bath used to achieve 130°C was not available, the next experiment in the sequence was performed. The intended and actual performance orders are shown in Table 4.5.

4.4 Experimental methods

4.4.1 Acid hydrolysis of gluten

Hydrochloric acid solutions at 5.0M and 1.0M were made by adjusting the final volume of solutions containing the contents of concentrated volumetric solution ampoules designed for the preparation of 1.0M solution.

Table 4.4: Selected levels for each independent variable

Level		-1	0	1
Acid Strength	(M)	0.02	0.2	2.0
Temperature	(°C)	50	90	130
Gluten Concentration	(% w/v)	1	7.5	14.0

Table 4.5: Experimental design for determination of the kinetics of acid hydrolysis of gluten

DESIGN ORDER	RUN NO.	CODE	ACID CONCN. (M)	TEMP (°C)	GLUTEN CONCN. (% w/v)	PERFORMANCE ORDER
15	1	-1,-1,0	0.02	50	7.5	13
4	2	+1,-1,0	2.0	50	7.5	7
11	3	-1,+1,0	0.02	130	7.5	12
3	4	+1,+1,0	2.0	130	7.5	14
12	5	-1,0,-1	0.02	90	1.0	5
5	6	+1,0,-1	2.0	90	1.0	1
7	7	-1,0,+1	0.02	90	14.0	3
8	8	+1,0,+1	3.0	90	14.0	4
14	9	0,-1,-1	0.2	50	1.0	9
9	10	0,+1,-1	0.2	130	1.0	10
6	11	0,-1,+1	0.2	50	14.0	2
2	12	0,+1,+1	0.2	130	14.0	11
13	13	0,0,0	0.2	90	7.5	8
1	14	0,0,0	0.2	90	7.5	15
10	15	0,0,0	0.2	90	7.5	6

Gluten was weighed into dried Bijou bottles. The weights required were 0.050, 0.375 or 0.700 gm and weighing accuracy was ± 0.0005 gm. Actual weights were recorded. Twelve bottles were prepared in this way for each experiment.

Water was added to each bottle and the contents mixed on a vortex mixer to disperse the gluten. It was important to carry out the mixing soon after water addition to avoid having spots of dry gluten sealed to the bottle walls by a firm dough. A short time before heating began hydrochloric acid was added to give the required concentration and the bottle contents were mixed again. Both water and acid were added using an adjustable 5.0 ml pipette (±0.002 ml) (Gilson, France).

The gluten was assumed to have a specific volume of 0.752 cc/gm (Section 4.2.2). This information was used to calculate the volume of acid and water required as shown in Table 4.6.

Heating of the reaction bottles was achieved by immersion in a water or oil bath controlled at the required temperature of 50, 90 or 130±1°C. Reaction time was reckoned as beginning from immersion.

Reaction times varied, (see Tables 4.7 to 4.21) and were selected so that the reaction progress could be easily followed with the samples taken. To assist in determination of the initial rate of reaction the total reaction time was divided using a logarithmic scale so that more samples were taken during the initial, more rapid reaction stage.

Sample bottles were removed from the reaction bath and allowed to cool at room temperature. It was found that more rapid cooling by plunging the bottles into an ice bath usually led to the bottles cracking and the contents being

Table 4.6: Quantities of gluten, water and hydrochloric acid required for a total volume of 5.0 ml

Weight of gluten (g)			0.7	0.375	0.05
NOMINAL ACID CONCENTRATION IN REACTION (M)	CONCENTRATION OF ACID ADDED (M)	VOLUME OF ACID ADDED (ml)	VOLUME OF WATER ADDED (ml)		
2.0	5.0	2.0	2.474	2.718	2.962
0.2	1.0	1.0	3.474	3.718	3.962
0.02	1.0	0.1	4.374	4.618	4.862

lost. Depending on the total reaction time, bottles remained on the bench at room temperature for periods of up to 8 hours before sampling took place.

4.4.2 Analysis of hydrolysate

For most of the experimental runs a blank reaction bottle containing water and acid but no gluten was prepared. The pH of this was determined first. For very low pH values a considerable period, about 10 minutes, was required for a stable reading to be obtained. In some cases, particularly for very low pH values or high gluten concentrations, a steady pH was never obtained. For all runs, once an initial pH determination had been made, the pH was measured by immersing the electrode in the sample bottle, mixing gently several times and then recording the reading after 2 minutes. After pH measurements had been completed the reaction bottles could be sampled for further analysis.

The physical form of the gluten suspensions varied depending on the pH and degree of hydrolysis. Some were smooth suspensions, others contained a few flocs, and some a ball of dough surrounded by liquid. A small magnetic stirrer bar was placed in each bottle and the bottle was placed on a magnetic stirrer. Stirring assisted in maintaining an even suspension during sampling.

Samples, 0.5 ml, were required from the reaction bottles for determination of ammonia, and N-terminal amino groups. To assist in obtaining representative samples, disposable pipette tips with the ends cut off to make a large opening were used. In some cases, where there were single balls of dough, only a liquid sample was taken to be used for ammonia analysis.

Two 0.5 ml samples were removed from each reaction bottle for ammonia determination. The samples were transferred to centrifuge tubes containing 0.5 ml, 0.2125M phosphate

buffer (pH 8.2) and 4.0 ml water. The tube contents were mixed.

Two 0.5 ml samples were removed from each reaction bottle for determination of N-terminal amino groups using fluorescamine and TNBS. The samples were transferred to centrifuge tubes containing 0.5 ml 0.2125M phosphate buffer (pH 8.2) and 4.0 ml 1% SDS. The tube contents were mixed. Further dilutions were made, as necessary, to obtain a concentration suitable for assay.

The centrifuge tubes containing the required dilution were placed in an MSE centrifuge and spun at 3000 rpm for 25 minutes. The supernatant liquid was then used as a sample in the determination.

4.5 Results of hydrolysis experiments

4.5.1 Presentation of data

To determine the kinetics of acid hydrolysis of amide and peptide bonds in gluten, 15 experiments, using the conditions shown in Table 4.5, were performed. For each reaction time in each experiment two reaction bottles were prepared as described in Section 4.4.1. After the reaction the pH of each reaction bottle was determined and then the contents were sampled for determination of ammonia and N-terminal amino groups as described in Section 4.4.2. Because of the duplication of reaction bottles, samples from each bottle and determinations made on each sample, eight determinations were made for each variable at each reaction point. A detailed record of these measurements is included in the microfiche attached as Appendix 4.2. The mean results for these multiple determinations, representing the consumption of hydrogen ions, appearance of ammonia and increase in N-terminal amino groups are shown for each experiment in Tables 4.7 through 4.21.

Table 4.7: Summary of results of hydrolysis experiment, Run 1

REACTION TIME (mins)	AMMONIA (mM)	pH	L-glu (TNBS) (mM)	L-glu (FLUORESCAMINE) (mM)
0	0.16	3.132	5	13
10	0.07	3.205	4	11.7
25	0.08	3.193	4	11.4
62	0.08	3.221	4.5	12.2
163	0.11	3.275	5.3	14.4
360	0.22	3.233	6.25	17.6

Table 4.8: Summary of results of hydrolysis experiment, Run 2

REACTION TIME (mins)	AMMONIA (mM)	pH	L-glu[1] (TNBS) (mM)	L-glu (FLUORESCAMINE) (mM)
0	14.0	-0.179		-
10	27.2	-0.177		7
25.0	35.2	-0.183		49.5
60	60 (52)[2]	-0.177		45
360	110	-0.156		-

(1) all values were negative and decreasing

(2) from repeat analysis

Table 4.9: Summary of results of hydrolysis experiment, Run 3

REACTION TIME (mins)	AMMONIA (mM)	pH	L-glu (TNBS) (mM)	L-glu (FLUORESCAMINE) (mM)
0	0.3	3.208	5.8	27
11	1.2	3.318	8.9	32
27	2.3	3.502	8.9	35
60	3.3	3.630	8.9	37
150	8.9	3.811	10.9	38
360	16.1	4.112	12.0	43

Table 4.10: Summary of results of hydrolysis experiment, Run 4

REACTION TIME (mins)	AMMONIA (mM)	pH	L-glu (TNBS) (mM)	L-glu (FLUORESCAMINE) (mM)
0	10	-0.230	-	-
2	76	-0.214	1.5	36
3.95	90	-0.200 (-0.158)	17	78
7.7	110	-0.191 (-0.145)	77.5	147
15.1	115	-0.179 (-0.140)	159	289
30	130	-0.166 (-0.118)	226	406

Table 4.11: Summary of results of hydrolysis experiment, Run 5

REACTION TIME (mins)	AMMONIA (mM)	pH	L-glu (TNBS) (mM)	L-glu (FLUORESCAMINE) (mM)
0	0	1.842	0.8	3.2
10	0	1.852	1.0	3.3
24.5	0.36	1.865	1.0	3.6
62	0.92	1.880	1.0	3.6
252	2.08	1.925	1.3	3.9
360	3.56	2.013	2.7	4.4

Table 4.12: Summary of results of hydrolysis experiment, Run 6

REACTION TIME (mins)	AMMONIA (mM)	pH	L-glu (TNBS) (mM)	L-glu (FLUORESCAMINE) (mM)
0	5.6	-0.231	-	-
2	10.8	-0.228	(1)	3.4
5.33	14.6	-0.230	(1)	4.5
13	17.0	-0.230	(1)	9.8
26	18.8	-0.217	(1)	17.5
60	21.8	-0.224	3	33.4
120	21.9	-0.228	4.25	53.5
240	22.2	-0.220	10.25	69.2
360	21.7	-0.222	14.5	75.8

(1) Negative values obtained

Table 4.13: Summary of results of hydrolysis experiment, Run 7

REACTION TIME (mins)	AMMONIA (mM)	pH	L-glu (TNBS) (mM)	L-glu (FLUORESCAMINE) (mM)
0	0.54	3.937	17	48
10	0.36	3.898	14	37
26	0.51	3.927	14	39
60	0.72	3.988	15	40
149	1.23	3.971	19	44
360	1.92	4.030	17	41

Table 4.14: Summary of results of hydrolysis experiment, Run 8

REACTION TIME (mins)	AMMONIA (mM)	pH	L-glu (TNBS) (mM)	L-glu (FLUORESCAMINE) (mM)
0	35	-0.29	0	20
2	82	-0.304	3.8	24
4.7	141	-0.289	7.5	60
13	230	-0.278	3.8	104
26	326	-0.260	18.8	260
60	348	-0.254	62.5	496

Table 4.15: Summary of results of hydrolysis experiment, Run 9

REACTION TIME (mins)	AMMONIA (mM)	pH	L-glu (TNBS) (mM)	L-glu (FLUORESCAMINE) (mM)
0	0	0.868	0.05	0.7
10.5	0.12	0.868	0.10	1.08
25.0	0.30	0.870	0.113	1.36
60	0.56	0.869	0.03	1.78
149	1.28	0.871	0.02	1.68
360	2.59	0.873	0.09	1.88

Table 4.16: Summary of results of hydrolysis experiment, Run 10

REACTION TIME (mins)	AMMONIA (mM)	pH	L-glu (TNBS) (mM)	L-glu (FLUORESCAMINE) (mM)
0	0	0.872	0.063 (low sample)	0.7
2	1.0	0.875	0.75	2.6
4.7	6.1	0.886	1.0	5.0
13	13.9	6.907	2.44	11.0
27	17.1	0.928	4.25	19.3
60	19.0	0.935	8.94	37.0
	-	0.865	-	-

Table 4.17: Summary of results of hydrolysis experiment, Run 11

REACTION TIME (mins)	AMMONIA (mM)	pH	L-glu (TNBS) (mM)	L-glu (FLUORESCAMINE) (mM)
0	7.2	0.905	2.0	57
10	7.2	0.913	4.0	47
25	7.3	0.915	3.25	30
60	8.5	0.919	5.75	24
149	12.4	0.929	5.25	20
379	23	0.957	5.5	16

Table 4.18: Summary of results of hydrolysis experiment, Run 12

REACTION TIME (mins)	AMMONIA (mM)	pH	L-glu (TNBS) (mM)	L-glu (FLUORESCAMINE) (mM)
0	0	0.888	4.4	23.5
2	14.4 16.3	0.982	6.0	28.3
4.7	61	1.202	9.5	4.6
13	119 126	1.769	16	75 64
26	117 122	2.281	24	80
61	119 145	2.749	27	90

Table 4.19: Summary of results of hydrolysis experiment, Run 13

REACTION TIME (mins)	AMMONIA (mM)	pH	L-glu (TNBS) (mM)	L-glu (FLUORESCAMINE) (mM)
0	0	0.910	4.25	16.5
5	8	0.912	4.88	21.0
12.5	18.6	0.943	5.25	20.3
30	4.23	0.994	7.13	40.4
74	56.5	1.073	10	33.5
180	78.5	1.196	12.75	43.4

Table 4.20: Summary of results of hydrolysis experiment, Run 14

REACTION TIME (mins)	AMMONIA (mM)	pH	L-glu (TNBS) (mM)	L-glu (FLUORESCAMINE) (mM)
0	0.3	0.870	3.25	14.3
5	8.1	0.887	3.25	15.5
12.25	18.8	0.910	3.75	19.3
30	32.5	0.955	4.25	20.5
74	49.5	1.033	7.13	23.7
183	80	1.172	9.5	38.2

Table 4.21: Summary of results of hydrolysis experiment, Run 15

REACTION TIME (mins)	AMMONIA (mM)	pH	L-glu (TNBS) (mM)	L-glu (FLUORESCAMINE) (mM)
0	1.0	0.897	3.4	19.3
5	5.0	0.907	4.6	24.5
12.25	9.4	0.935	5.6	31.2
30	18.2	1.005	7.2	30.8
77	50	1.075	8	37.0
180	68	1.235	12.5	52.0

The data for the determination of N-terminal amino groups using fluorescamine and TNBS were corrected for the effect of ammonia as described in Sections 3.6 and 3.8. The details of the correction are included in Appendix 4.2.

For each experiment it was found that the required correction for the determination of N-terminal amino groups using fluorescamine was negligible but for determination using TNBS the correction was substantial.

At low levels of gluten there was some over-correction as occurred in Table 4.12. The values of L-glu from using TNBS were approximately half those obtained from using fluorescamine. Although the determination using TNBS appeared to follow the hydrolysis of peptide bonds in a satisfactory manner, it was decided not to include the data in further analysis. This was due to:

(i) little benefit from duplication of analysis
(ii) the unsatisfactory ammonia correction
(iii) the apparently correct number of peptide bonds given by the determination using fluorescamine (see Section 4.7.3)

Various features of the experiments are illustrated in Figures 4.3 and 4.4 which show data from Tables 4.12 and 4.18.

Figure 4.3 shows that when deamidation is complete the peptide bond hydrolysis reaction, as measured by the fluorescamine reaction and the consumption of hydrogen ions, continues. Figure 4.4 shows that with a high level of gluten and a moderate level of acid the reaction continues until nearly all of the acid is consumed.

4.5.2 Discussion of some unexpected features of the data

Although most of the data followed the expected pattern of change with time, temperature and acid concentration, there

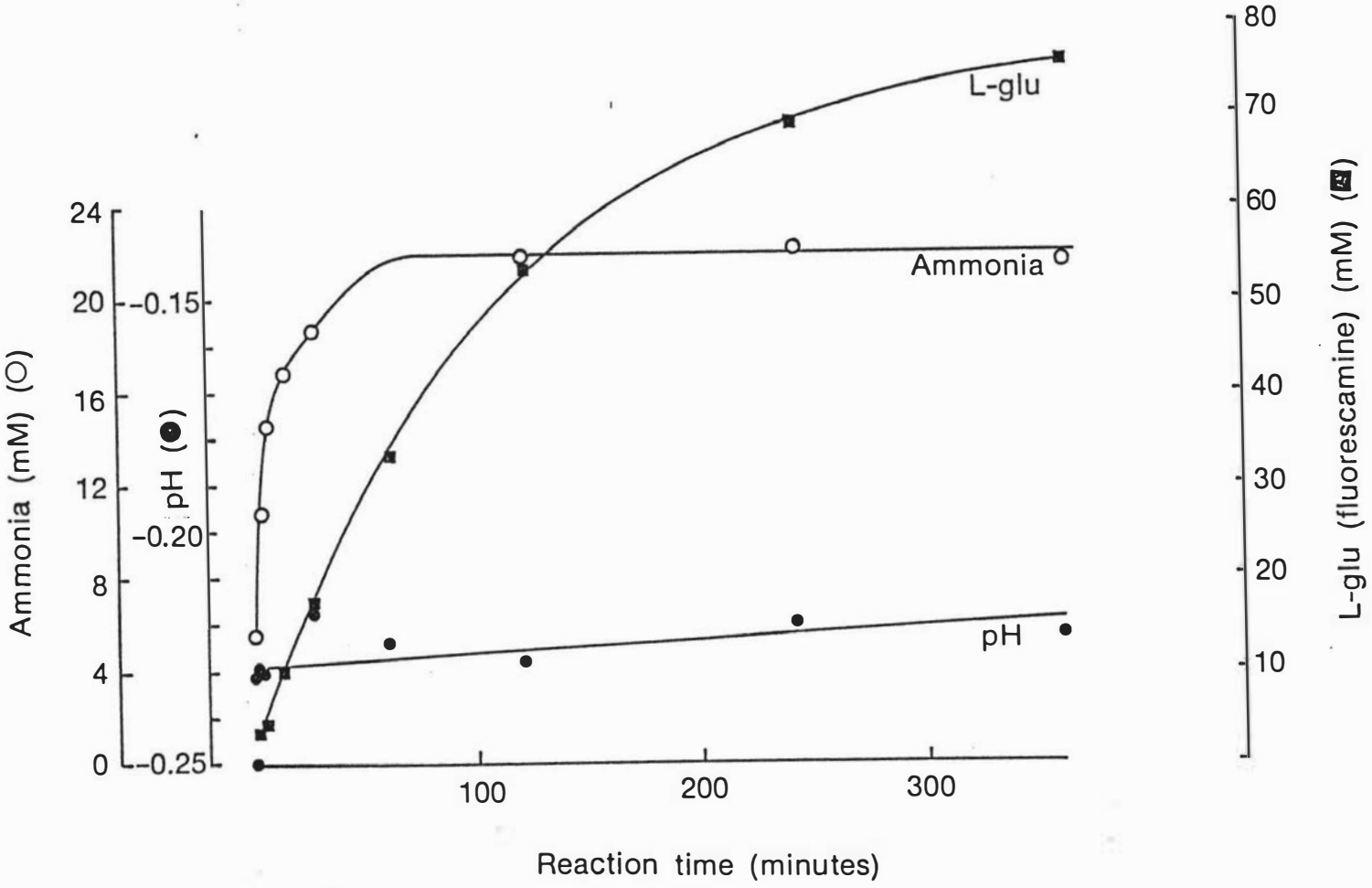


Figure 4.3 Illustration of experimental data from Run 6.

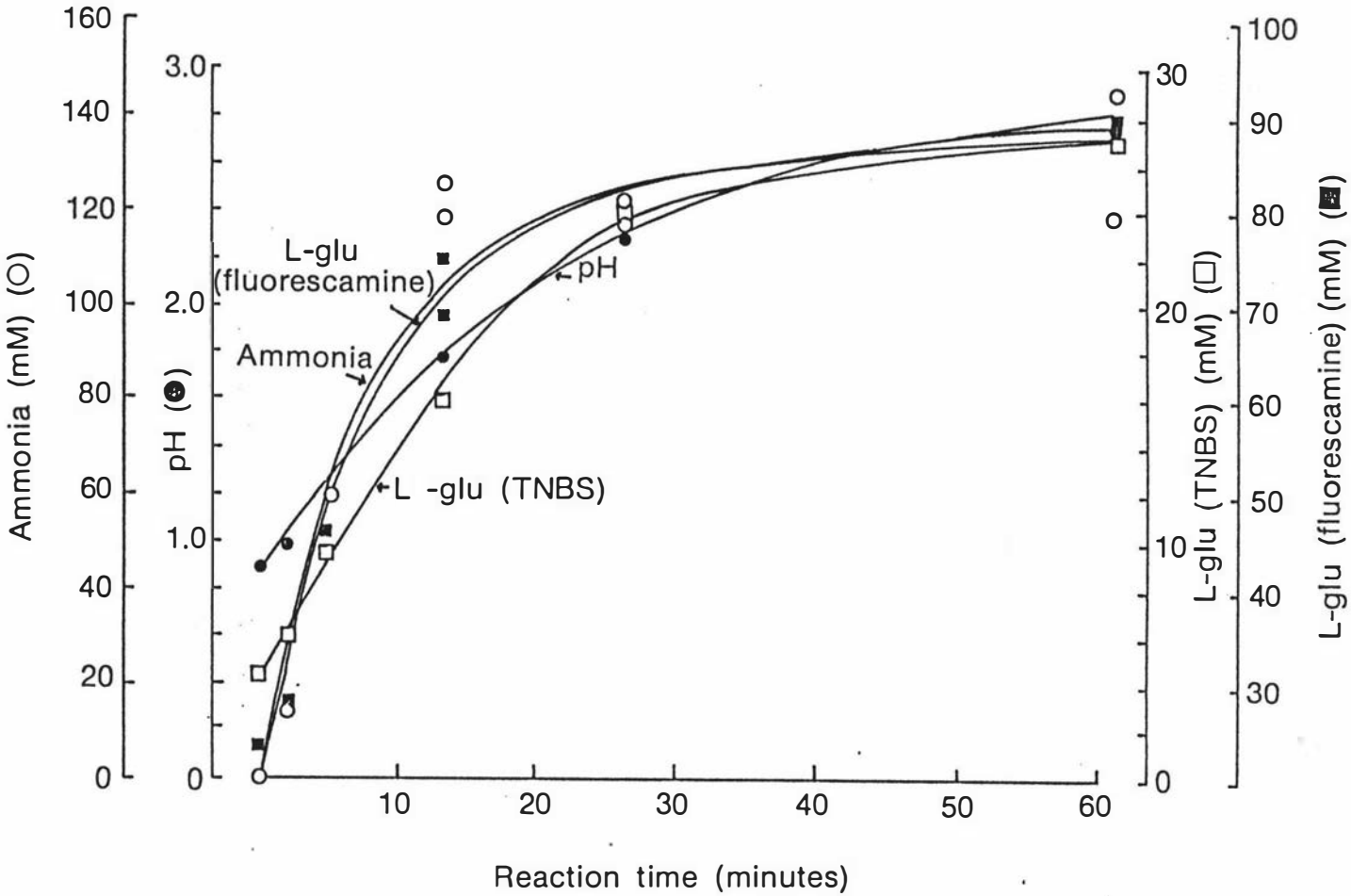


Figure 4.4 Illustration of experimental data from Run 12.

were some exceptions as noted here. The experimental conditions to which reference is made are shown in Table 4.5, and the experimental data for runs 1-15 are given in Tables 4.7 through 4.21. Further reference can be made to the detailed data in Appendix 4.2 in the attached fiche.

For runs 2, 4, 6, and 8, the initial level of ammonia is greater than the expected zero level. This is attributed to the deamidation reaction beginning and continuing during those times at which the samples were held at room temperature. This would of course occur for all samples but is clearly observed in the samples from these runs which are those with 2M acid. Some further discussion of the extent of reaction expected at room temperature is given in Appendix 4.3. One other run shows an appreciable initial level of ammonia, that is run 11 where the acid concentration was 0.2M. For this run the reaction bottles were held overnight in refrigerated storage and this extra holding time may have contributed to the amount of 'bench reaction' experienced. For all other runs sampling took place immediately after the required pH determinations had been made. The effect of this reaction on the reaction rate determinations is discussed in Section 4.8.1.1.

For most of the experiments the level of free amino groups, determined with the use of fluorescamine increases throughout the experiment as expected. For runs 2 and 11, however, the levels decreased throughout the experiment. In run 2 the free amino groups determined with TNBS also decreased but in run 11 show a slight increase. Both of these runs were at 50°C which meant that there was little reaction compared to those runs at higher temperature. The other run at 50°C, run 1 also has some odd features. Here, the change in ammonia concentration is about 0.15 mM, which would correspond to an increase from pH 3.2 to pH 3.3, assuming that 1 mole H^+ is consumed for 1 mole of NH_3 produced, which is what occurs although the pH measurements in this run were particularly unstable and did not reach

steady values. The change in free amino groups, however, of approximately 5 mM as determined by fluorescamine is apparently very much larger than the change in ammonia concentration. It follows that all of the data for determination of free amino groups using fluorescamine for runs at 50°C must be treated with some caution. At higher temperatures the results appear to be more sensible. The effect of these problems on the reaction rate determinations are discussed in Section 4.8.1.2.

The determination of N-terminal groups using TNBS gave, in every case, a useful measurement which followed the progress of the reaction. It could be recommended as a means of monitoring this type of hydrolysis. The data obtained are, however, a result of reaction with both N-terminal amino groups and ammonia. The correction for ammonia, discussed in Section 3.8, introduces some error into the measurements. For runs 2 and 6 the correction for ammonia gave negative values for concentration of free amino groups. For run 9 the data were very scattered after correction. Apart from these difficulties the data follow the same trends as for the determination with fluorescamine.

The difficulty in determining pH in run 1 was mentioned above and similar difficulty with similar circumstances i.e. high pH and high gluten concentration, was experienced with run 7 where the changes in ammonia and free N-terminal groups are not matched by a corresponding change in pH. The effect of these problems on reaction rate determination and stoichiometry measurements are discussed in Sections 4.8.1.3 and 4.8.2.3.

4.6 Analysis of experimental error in hydrolysis experiments

The data presented in Appendix 4.2 show the standard deviation of each set of readings. A further analysis of the data was

undertaken to try to show the source of the variation. Three possible sources of variation can be identified:

(i) between duplicate determinations on one sample from one bottle
(ii) between duplicate samples from one bottle
(iii) between bottles

Substantial variability was noted in some initial experiments described in Section 3.3.2.3 and it was because of this that the large number of determinations at each point was made.

The causes of this variation are many and include errors in weighing, pipetting, timing, etc. Observations during the experiments suggested that there might be greater variation due to sampling for determination of N-terminal amino groups. With samples where the gluten was present as a number of flocs, or in some cases a single ball of dough, the number of flocs collected in a 0.5 ml sample varied considerably. For the ammonia determination there was little effect since the ammonia was entirely in the liquid portion of the sample whereas the protein measured in determination of N-terminal amino groups is partly contained in the solid.

To quantify the variation caused by these effects the technique of hierarchical analysis of variance was used (Davies, 1967). This is a method of analysing the variance to which a response is subject into its various components corresponding to the sources of variation which can be identified. To apply this analysis the total data sets from the ammonia and N-terminal amino group determination using fluorescamine were considered.

The calculations were made with the aid of the STATS ANOVA statistical package available on the New Zealand Dairy Research Institute's computer system.

Table 4.22 shows how the hierarchy of variance is calculated. Tables 4.23 and 4.24 show the calculated values for the ammonia

Table 4.22: Quantities calculated in hierachical analysis of variance

15 runs
6 times
2 bottles
2 samples
2 determinations

Analysis of Variance (if all runs and times were random)

Source	df	Quantity estimated by MS
Runs	14	$\sigma_d^2 + 2\sigma_s^2 + 4\sigma_b^2 + 8\sigma_t^2 + 48\sigma_r^2$
Times	5	$\sigma_d^2 + 2\sigma_s^2 + 4\sigma_b^2 + 8\sigma_t^2$
Runs x Times	70	
Bottles within times	90	$\sigma_d^2 + 2\sigma_s^2 + 4\sigma_b^2$
Samples within bottles	180	$\sigma_d^2 + 2\sigma_s^2$
Determinations within samples	360	σ_d^2

and N-terminal amino group determination data. From these data, estimates of the standard deviation identified by component, i.e. for determinations, sampling or bottles, were calculated. These standard deviations cannot be tested for significant differences from each other using the F test, which is usually used with this technique, because they are not really independent. For this reason, the following approach was adopted. First, confidence intervals were calculated for each standard deviation using the technique given by Davies (1967). The calculated values are shown in Table 4.25 and details of the calculation in Appendix 4.4. These data make possible a comparison of standard deviations within each type of determination. Then, to compare variation between types of determination a coefficient of variation was calculated, i.e. the standard deviation expressed as a percentage of the arithmetc mean. In this instance the mean used is that for all data as shown in Tables 4.23 and 4.24. When the confidence intervals are also calculated, Table 4.25, it can be seen that:

(i) the variation for samples and bottles for N-terminal amino group determination using fluorescamine indicates that the difficulties experienced in sampling flocculated material appear to have had a substantial effect on the uncertainty of the estimated values

(ii) there is significantly greater difference between bottles than for sampling or determinations for the ammonia determination. This may reflect minor differences in timing in very fast reactions or possibly small amounts of gluten caught up on the walls of the bottle and therefore effectively removed from the reaction.

4.7 Determination of initial concentrations of peptide and amide bonds

Reaction kinetics are usually described in terms of the initial concentration of the reactants. In this instance it was necessary to determine the initial or total concentrations of

Table 4.23: Values calculated by analysis of variance of ammonia determination data

SOURCE	DF (M.V.)	S.S.(1)	M.S.S.(1)	F RATIO	MS	DF
RUN	14	309112.88	22079.49	2.88**		
TIME	5	103976.06	20795.21	2.71*		
Error = run x time	70	537137.50	7673.39			
BOTTLE	1	906.30	906.30	5.75*		
Error = RunxB+TxB+RxTxB	88(1)	13866.67	157.58		165.99 (bottles)	89
SAMPLE	1	29.12	29.12	1.02 ns		
Error	177(2)	5067.25	28.63		28.63 (samples)	178
Subsamples	344	4235.28	12.31		12.31 (assays)	344

Missing cell estimates:

Cel no.	Estimated value
138	192.42
265	-0.23
266	0.23

Estimated overall mean 181.20

Note (1) Raw data was multiplied by 100 and SS and MS by 10,000

Table 4.24: Values calculated by analysis of variance for determination of N-terminal amino groups using fluorescamine

SOURCE	DF (MV)	SS	MSS	F RATIO	MS	df	
RUN	14	378028.50	27002.04	12.60***			
TIME	5	68150.98	13632.20	6.36***			
Error	67(3)	143557.47	2142.65				
BOTTLE	1	438.19	438.19	1.83ns	241.765	85	bottles
Error	84(5)	20111.83	239.43				
SAMPLE	1	23.90	23.90	0.23ns			
Error	169(10)	17802.13	105.34		104.859	170	samples
Subsamples	342	1077.00	3.13		3.15	342	assays

Missing cell estimates:

Cell no.	Estimated value
49	0.19
50	-0.19
51	0.19
52	-0.19
73	0.19
74	-0.19
75	0.19
76	-0.19
93	0.19
94	-0.19
95	0.19
96	-0.19
113	0.19
114	-0.19

194	11.64
261	0.19
262	-0.19
267	51.37

Estimated overall mean 42.70

Table 4.25: Coefficient of variation and confidence intervals for sources of variation in determination of ammonia and N-terminal amino groups using fluorescamine

Determination	Source	CI	CV(%)	CI
Ammonia	Determinations	3.51 (3.26 3.82)	1.94	(1.80,2.11)
Ammonia	Samples	2.354 (2227, 3435)	1.58	(1.26,1.90)
Ammonia	Bottles	5.862 (4925,6977)	3.24	(2.72,3.85)
N-terminal amino groups	Determinations	1.775 (1.65,1.93)	4.16	(3.86,4.52)
"	Samples	7.13 (6.32,8.09)	16.7	(14.80,18.76)
"	Bottles	5.85 (4.42,7.42)	13.70	(10.35,17.38)

Mean for ammonia = 181.2 (raw data multiplied by 100)

Mean for N-terminal amino groups = 42.7

Note: The coefficient of variation is the standard deviation expressed as a percentage of the arithmetic mean

amide and peptide bonds available for hydrolysis. This was achieved by taking each reaction to completion and measuring the ammonia or N-terminal amino groups in each case.

4.7.1 Determination of total available peptide bonds

Approximately 10 mg of gluten were placed in a vacuum hydrolysis tube (Pierce Chemical, Rockford Il., USA). The gluten was weighed exactly using a 5 figure analytical balance. Two ml of 6M HCl were added and the tube was sealed and held for 24 hours at 108°C. When cooled, the acid and black residue were drawn off and the tube was rinsed several times. The acid and rinsings were adjusted to approximately pH 2 with 8M NaOH and the volume made up to 25.0 ml. From the flask containing the 25 ml, 0.5 ml were withdrawn and diluted with 4.5 ml of buffer solution which was prepared from 0.5 ml of 0.2125M phosphate buffer at pH 8.2 and 4.0 ml 1.0% SDS solution. Samples of varying size were taken from this dilution and the number of N-terminal amino groups determined using fluorescamine as described in Section 2.6.1. The results of the analysis are given in Table 4.26. Data for the preparation of the standard curve are included in the attached fiche as Appendix 4.5. The peak height and sample size data were plotted as shown in Figure 4.5 to check for linearity. The variation in the calculated L-glutamic acid content shown in Table 4.26 reflects the uncertainties of the standard curve. The last 3 figures, i.e. for the highest sample sizes, correspond to the mid-range of the standard curve. Their mean was taken for calculation of the available peptide bonds as shown in the lower part of Table 4.26.

4.7.2 Determination of the level of free amino groups in gluten

The determination of the total number of free amino groups after total hydrolysis, described in Section 4.7.1, is not the number of free amino groups available for hydrolysis

Table 4.26: Peak heights and equivalent concentration of L-glutamic acid resulting from total hydrolysis of duplicate samples of gluten

SAMPLE SIZE (ul)	PEAK HEIGHT SAMPLE A (mm)	L-GLU IN HYDROLYSIS (mM)	PEAK HEIGHT SAMPLE B (mm)	L-GLU IN HYDROLYSIS (mM)
10	7	70	6	60
	7		6	
50	28	62.5	19	41.5
	28		19	
100	53	62.5	36	41
	54		37	
150	78	64.5	55	42.3
	78		53	
200	102	66.9	70	42.5
	101		70	
Mean value of last three measurements		64.6		41.9
(standard deviation)		(2.2)		(0.8)
Moles of L-glu in total weight of sample		1.29×10^{-4}		8.38×10^{-5}
Total moles divided by sample weight		1.29×10^{-4}		8.38×10^{-5}
		1.535×10^{-2}		1.070×10^{-2}
= moles per gram		8.40×10^{-3}		7.83×10^{-3}

The mean value was taken as 8.12×10^{-3} moles per gram

After correction for the initial level of free amino groups in gluten (1.92×10^{-4} moles per gram), the value used for further calculations was 7.93×10^{-3} moles per gram.

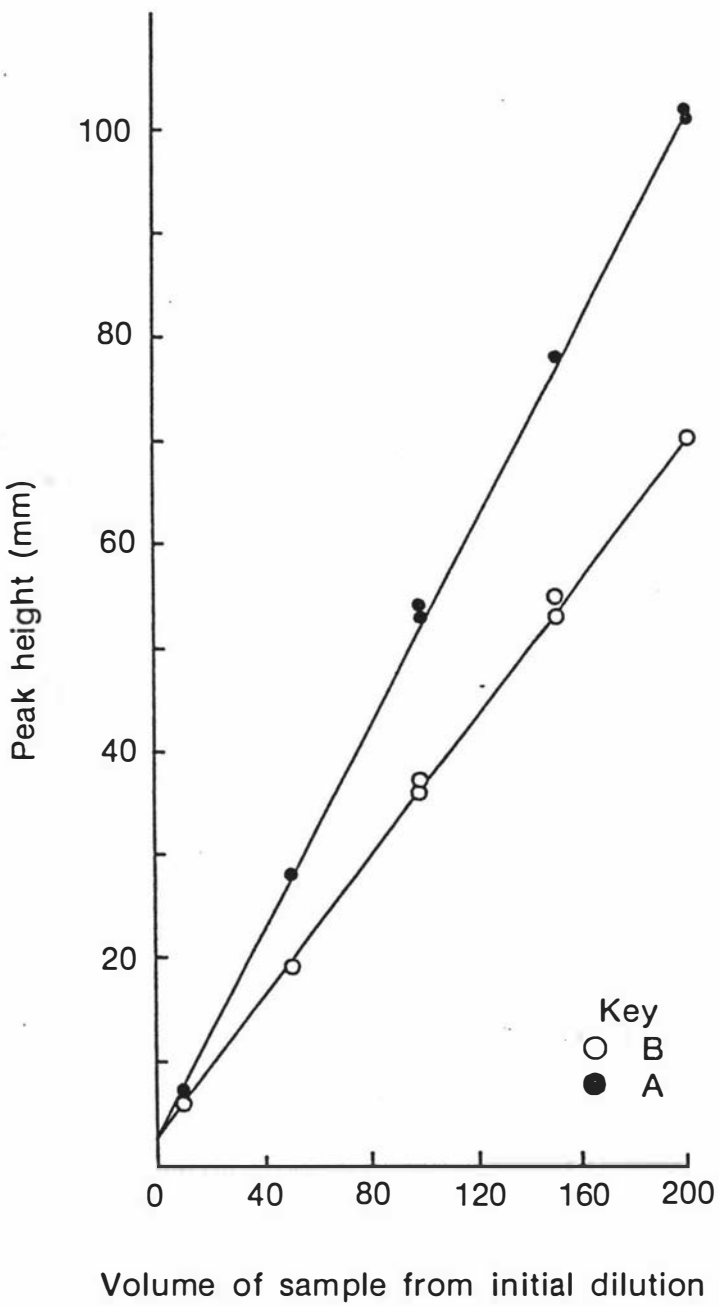


Figure 4.5 Plot of peak height against sample concentration to check for linearity in measurement of total peptide bonds available for acid hydrolysis of gluten.

since there are some free amino groups in gluten, that is, the N-terminal groups of the original proteins. This initial level can be seen in Figures 4.3 and 4.4. The initial values from all 15 data sets, Tables 4.7 to 4.21, were taken and reduced to a common concentration. The mean value was found to be 1.92×10^{-4} moles per gram with a standard deviation of 0.88×10^{-4} moles per gram. This value was substracted from the value obtained by total hydrolysis as finally shown in Table 4.26.

4.7.3 Determination of total available amide bonds

The method used was essentially that described for gluten hydrolysis in Section 4.4. The method is also the same as that described by Leach and Parkhill (1955) except that it was found to be more convenient to carry out the hydrolysis at 90°C instead of using a boiling water bath. Two separate experiments were conducted and the results from Run 6 (see Table 4.12) were also included. Each reaction bottle contained 0.0500 g of gluten with water and HCl added to make 5.0 ml of 2.0M HCl as described in Section 4.3 and was heated for 2, 4, or 6 hours. Duplicate bottles were reacted for each time. Ammonia analysis of samples from the bottles was done twice in separate determinations carried out as previously described. For the first determination duplicate samples were taken from each bottle, but for the second determination only single samples were taken. The samples were diluted with 4.5 ml buffer which was prepared from 0.5 ml of 0.2125 M phosphate buffer and 4.0 ml water. Data for preparation of the standard curve are included in the attached fiche as Appendix 4.6. The results of these determinations are shown in Table 4.27. The value used for further calculation was 2.0×10^{-3} moles of amide per gram of gluten.

4.7.4 Discussion of initial concentration determinations

The values for the number of peptide bonds are not absolute in the same way as the value for amide bond content of

Table 4.27: Absorbance (A_{340}) and concentration of ammonia resulting from complete deamidation of gluten

REACTION TIME (h)	BOTTLE A SAMPLE A ANALYSES a,b	BOTTLE A SAMPLE B ANALYSES a,b	BOTTLE B SAMPLE A ANALYSES a,b	BOTTLE B SAMPLE B ANALYSES a,b	MEAN (STANDARD DEVIATION)	AMMONIA IN SAMPLE (mM)
2	1.748 1.760	1.716 1.780	1.757 1.751	1.770 1.776	1.757 (0.020)	19.1
4	1.784 1.763	1.728 1.769	1.707 1.765	1.761 1.757	1.754 (0.025)	19.2
6	1.787 1.713	1.752 1.690	1.683 1.682	1.698 1.769	1.722 (0.041)	20.3
	BOTTLE A ANALYSIS a	BOTTLE A ANALYSIS b	BOTTLE B ANALYSIS a	BOTTLE B ANALYSIS b		
2	1.746	1.737	1.683	1.723	1.722 (0.020)	19.8
4	1.687	1.728	1.707	1.685	1.702 (0.020)	20.4
6	1.709	1.738	1.676	1.674	1.699 (0.030)	20.5

The value used for further calculation was 20 mM that is, 2.0×10^{-3} moles per gram

gluten. The factors affecting the TNBS and fluorescamine assays were discussed in Sections 2.6.1.1 and 2.6.2.2. The extent to which the values are correct can be checked by considering the ratio of amide to peptide bonds since amino acid analysis shows that about 30% of the protein is glutamine (MacRitchie, 1979). It follows that the proportion of amide to total peptide bonds should be about this figure.

The values determined are on an "as is" basis, that is, not corrected to a moisture free basis. To compare with other investigators the data can be stated on a total protein basis.

Now total protein = total nitrogen * 5.62 (1)

= 12.6(%) * 5.62 (2)

= 70.8%

therefore 100 g protein is contained in 141 g gluten

The value of 5.7 is usually used for conversion of total nitrogen (%) to protein (%) for wheat flour and gluten. A recent study (Mosse et al, 1985) suggested a value of 5.62 was more appropriate and this has been used here in equation (1) above. The total nitrogen value comes from Table 4.1.

Now the amide bond content of the gluten was 2.0×10^{-3} moles per gram of gluten from Table 4.27, it follows that amide bond content can be expressed as 282 mM per 100 g of protein. This is very similar to the value of 279 mM per 100 g of protein determined by Wu et al (1976).

Now total amide bonds are 2.0×10^{-3} moles per gram and total peptide bonds by analysis using fluorescamine are 7.93×10^{-3} moles per gram. That is, the proportion of amide bonds suggested by these figures is approximately 25%. It follows therefore, that this figure more nearly

represents the correct value than analysis using TNBS which indicates less than half the number of peptide bonds.

4.8 Analysis of experimental design and fitting of kinetic models

4.8.1 Initial rate determination

Of the various methods suitable for the analysis of kinetic data, the analysis of initial reaction rates was chosen (Levenspiel, 1972). The initial reaction rate is uncomplicated by factors such as product inhibition or changes of mechanism which may occur in the course of a reaction. Several authors have pointed out the possibility of changes in reaction rate coefficients for the hydrolysis of peptide bonds as the hydrolysis progresses. Much of this work was reviewed by Leach (1953).

Examination of reaction progress curves, e.g. Figures 4.3 and 4.4, showed that visual fitting of slopes was feasible. Visual fitting was used with the intention of using a calculation-based method if necessary.

4.8.1.1 Determination of initial rate of amide bond hydrolysis

Graphs were made of each set of ammonia vs time data, from Tables 4.7 to 4.21. The length of each scale was stretched or compressed as appropriate so that the initial rate of ammonia appearance could be conveniently measured. After the first set of measurements was tested for fit to the kinetic model (see Section 4.8.2.1) it was found that some measurements stood out as odd results. These were re-examined. The plots of ammonia against time for these data are shown in Figures 4.6, 4.7, and 4.8. The data for Run 7, Figure 4.6, are confused by the apparently high value for the initial concentration of ammonia.

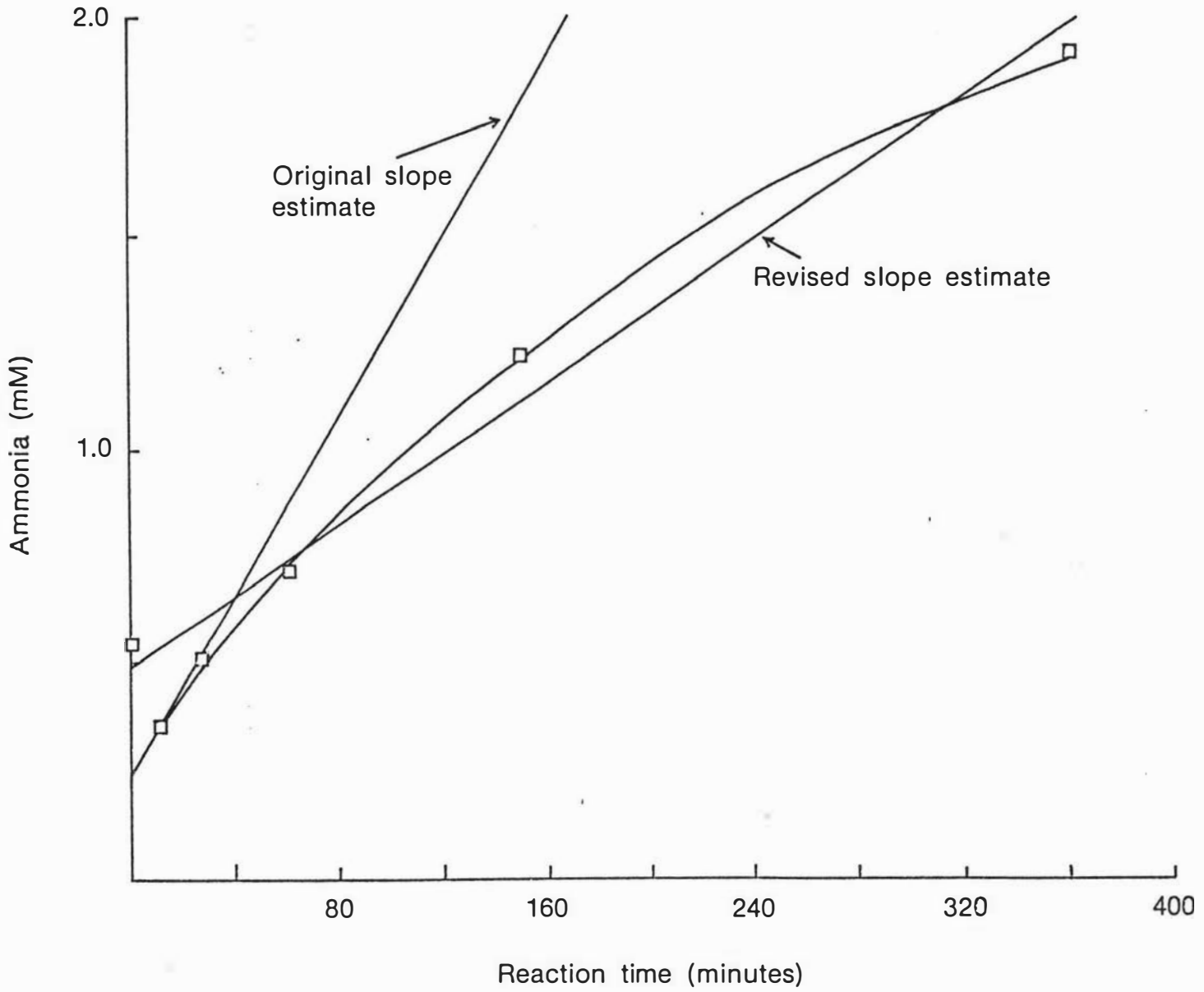


Figure 4.6 Ammonia evolution with time for initial rate estimation for Run 7.

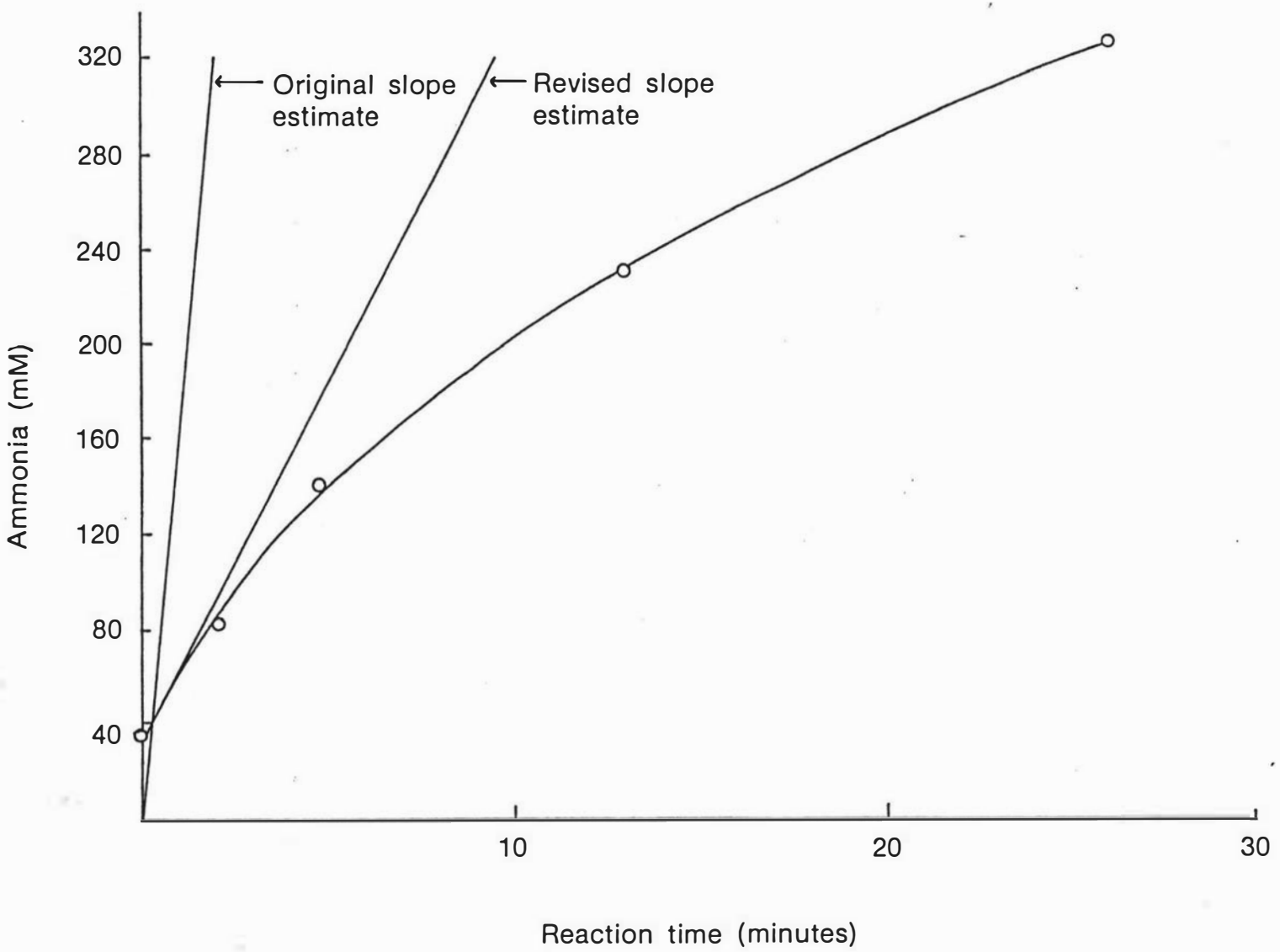


Figure 4.7 Ammonia evolution with time for initial rate estimation for Run 8.

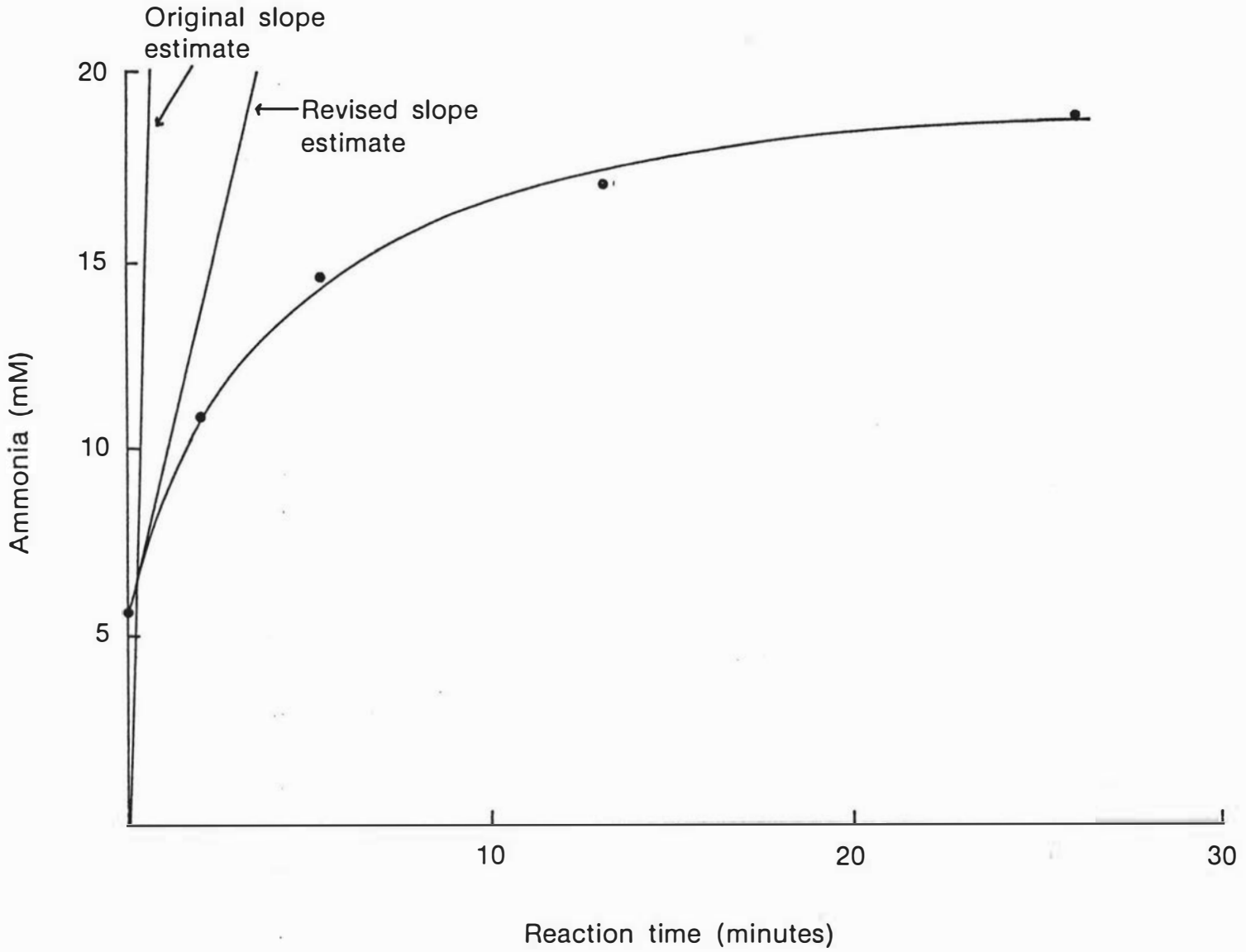


Figure 4.8 Ammonia evolution with time for initial rate estimation for Run 6.

The data for Run 8 and Run 6 gave high first estimates of reaction rate because the tangent was forced through the origin. It is likely that at these highest acid concentrations a substantial amount of "bench reaction" took place between acid addition and sampling. (See the calculations in Appendix 4.3). The rates were then revised as shown in Figures 4.7 and 4.8. The results of this reassessment are shown in Table 4.28.

It is assumed that the appearance of ammonia is equivalent to the hydrolysis of amide. There does not appear to be any measurable ammonia production from breakdown of N-terminal amino acids as was suggested by Leach and Parkhill (1955). This is shown by the data on total hydrolysis of amide groups in Section 4.7.3 and in Figure 4.3.

4.8.1.2 Determination of the initial rate of peptide bond hydrolysis

All the measurements were based on assessment of peptide bond hydrolysis determined using fluorescamine. A similar technique to that used for finding the initial rate of amide bond hydrolysis was used, that is plotting and manual fitting of tangents. The rate measurements obtained are shown in Table 4.29. After the initial measurements were tested for fit to the kinetic model it was found that some measurements stood out as odd results. These values were associated with either:

(i) runs with a high initial pH and/or reaction at 50°C

or (ii) rates that were guesses because of erratic data

In two cases, runs 2 and 11, the data suggest a decreasing level of free amino groups as discussed in Section 4.5.2. Here, two very low rates were inserted as guesses in the data of Table 4.29. High pH did not

Table 4.28: Revision of initial reaction rate estimates for some ammonia evolution data

EXPERIMENT	FIRST ESTIMATE (mM/min.)	REVISED ESTIMATE (mM/min.)
Experiment 7, Run 7	1.06×10^{-2}	4.12×10^{-3}
Experiment 8, Run 8	177	30.3
Experiment 5, Run 6	34.7	4.05

Table 4.29: Reaction rates and reaction rate constants calculated from initial rate measurements

RUN NO.	pH	a_{H^+} (g-mole/l)	AMIDE (g-mole/l)	RATE (g-mole/l.sec)	k (Amide)	1/T ($°K^{-1}$)	L/glu (Peptide)	RATE (g-mole/l.sec)	k (Peptide)	$\frac{d\ pH}{dt}$	$\frac{D[H^+]}{dt}$ (g-mole/l.sec)
	3.15	7.079×10^{-4}	0.150	7.63×10^{-9}	7.18×10^{-5}	3.096×10^{-3}	0.595	4.03×10^{-7}	9.58×10^{-4}	4.167×10^{-6}	6.812×10^{-9}
2	-0.179	0.1510	0.150	6.35×10^{-5}	2.80×10^{-4}	3.096×10^{-3}	0.595	1.67×10^{-7}	1.86×10^{-7}	6.944×10^{-7}	2.414×10^{-6}
3	3.2	6.310×10^{-6}	0.150	1.33×10^{-6}	1.41×10^{-2}	2.481×10^{-3}	0.595	1.07×10^{-5}	2.55×10^{-2}	$1.873.10^{-4}$	2.721×10^{-7}
4	-0.174	1.493	0.150	3.10×10^{-3}	1.38×10^{-2}	2.481×10^{-3}	0.595	3.15×10^{-4}	3.55×10^{-4}	1.111×10^{-4}	3.819×10^{-4}
5	1.84	0.0145	0.20	3.35×10^{-7}	1.16×10^{-3}	2.755×10^{-3}	0.0793	1.73×10^{-1}	1.50×10^{-4}	1.938×10^{-5}	5.470×10^{-2}
6	-0.229	1.694	0.020	5.78×10^{-4}	1.99×10^{-3}	2.755×10^{-3}	0.0793	1.23×10^{-5}	9.16×10^{-5}	4.630×10^{-7}	1.806×10^{-6}
7	3.9	1.259×10^{-4}	0.280	1.77×10^{-7}	1.94×10^{-3}	2.755×10^{-3}	1.110	1.60×10^{-6}	1.14×10^{-2}	3.704×10^{-6}	1.075×10^{-9}
8	-0.295	1.972	0.280	2.95×10^{-3}	9.16×10^{-4}	2.755×10^{-3}	1.110	1.49×10^{-4}	6.81×10^{-4}	5.000×10^{-5}	2.268×10^{-4}
9	0.866	0.136	0.020	1.88×10^{-7}	6.91×10^{-5}	3.096×10^{-3}	0.0793	1.08×10^{-6}	1.00×10^{-4}	2.315×10^{-7}	7.249×10^{-8}
10	0.871	0.135	0.020	2.78×10^{-5}	1.03×10^{-2}	2.481×10^{-3}	0.0793	1.61×10^{-5}	1.50×10^{-3}	6.150×10^{-5}	1.912×10^{-5}
11	0.905	0.124	0.280	7.67×10^{-7}	2.21×10^{-5}	3.096×10^{-3}	1.110	1.67×10^{-7}	1.21×10^{-6}	7.209×10^{-6}	2.058×10^{-6}
12	0.88	0.132	0.280	2.25×10^{-4}	6.09×10^{-3}	2.481×10^{-3}	1.110	1.32×10^{-4}	9.01×10^{-4}	1.357×10^{-3}	4.124×10^{-4}
13	0.90	0.126	0.150	2.95×10^{-5}	1.56×10^{-3}	2.755×10^{-3}	1.595	1.05×10^{-5}	1.40×10^{-4}	6.859×10^{-5}	1.990×10^{-5}
14	0.870	0.135	0.150	2.23×10^{-5}	1.10×10^{-3}	2.755×10^{-3}	0.595	4.75×10^{-6}	5.91×10^{-5}	5.417×10^{-5}	1.684×10^{-5}
15	0.897	0.127	0.150	1.52×10^{-5}	7.98×10^{-4}	2.755×10^{-3}	0.595	1.92×10^{-5}	2.54×10^{-4}	8.247×10^{-5}	2.412×10^{-5}

cause a problem with amide rate calculations for the same experiments.

An alternative analysis was then attempted. All of the data for N-terminal amino groups determined with fluorescamine, expressed as concentration of the determination standard L-glu, were divided by the concentration of gluten so that they were all reduced to the equivalent of experiments at 1.0% w/v gluten. These data were then plotted for each initial acid concentration as shown in Figures 4.9, 4.10 and 4.11. This technique enabled estimates of reaction rate to be made for each combination of initial acid concentration and reaction temperature, except for the condition 2M, 50°C where the data were judged to be too uncertain for a reasonable estimate to be made. The rate estimates obtained from this alternative technique are shown in Table 4.30.

For kinetic analysis of these data, an initial pH value was required. The value in each experiment varied because of the varying gluten concentrations. The values were taken from Table 4.29 and analysed as shown in Table 4.31. The mean value in each cell, as shown in Table 4.32, was used for the kinetic analysis.

4.8.1.3 Determination of initial rate of hydrogen ion consumption

The pH vs time data, from Tables 4.7 to 4.21, were plotted and an initial rate of d pH/dt determined.

Now $d\,pH/dt = d/dt \; -\log_{10} [H^+]$

and the rule for differentiation is

$$d/dx \log_a u = 1/u \log_a e \; du/dx$$

So d pH/dt was converted to $d[H^+]/dt$ using

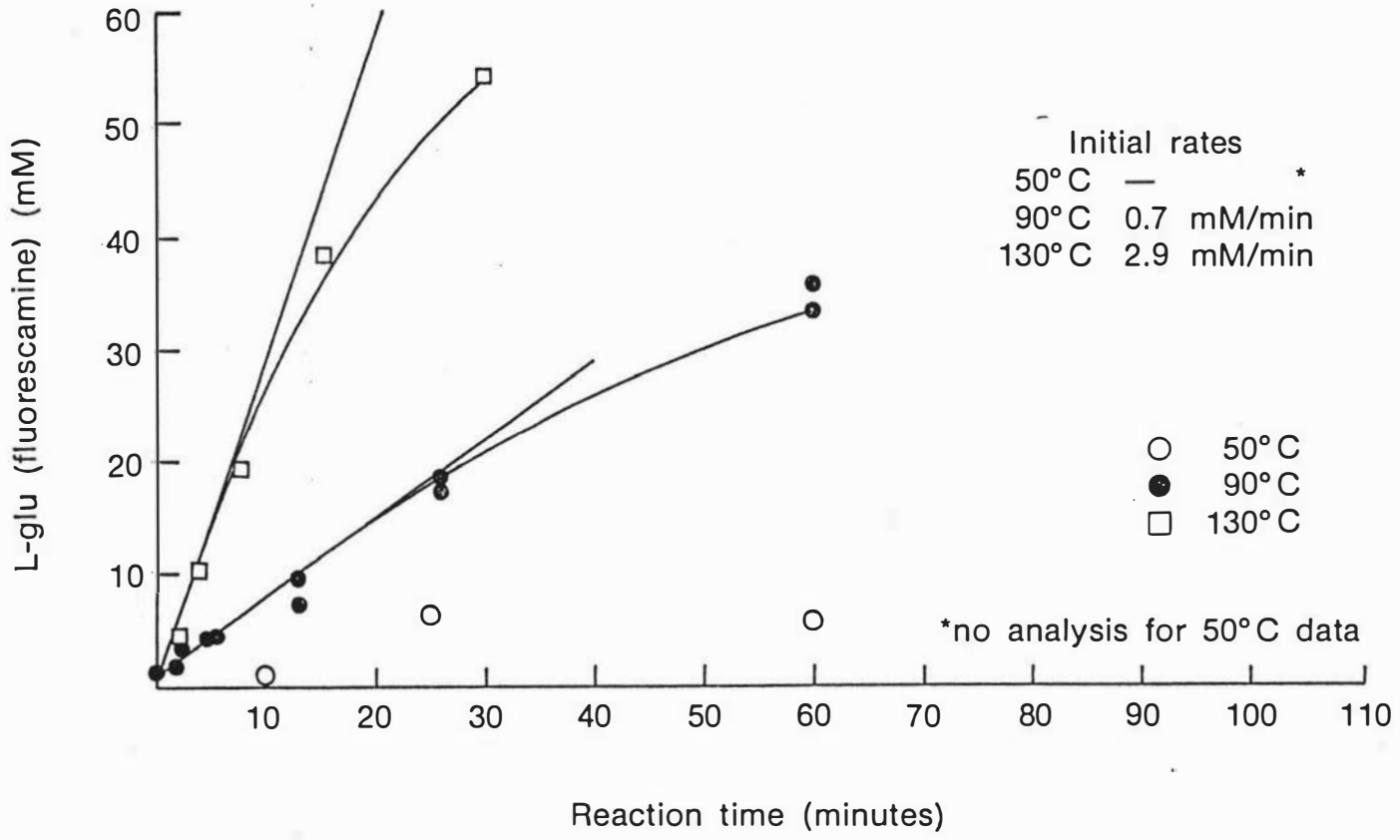


Figure 4.9 Peptide bond hydrolysis at reduced gluten concentration with 2M acid.

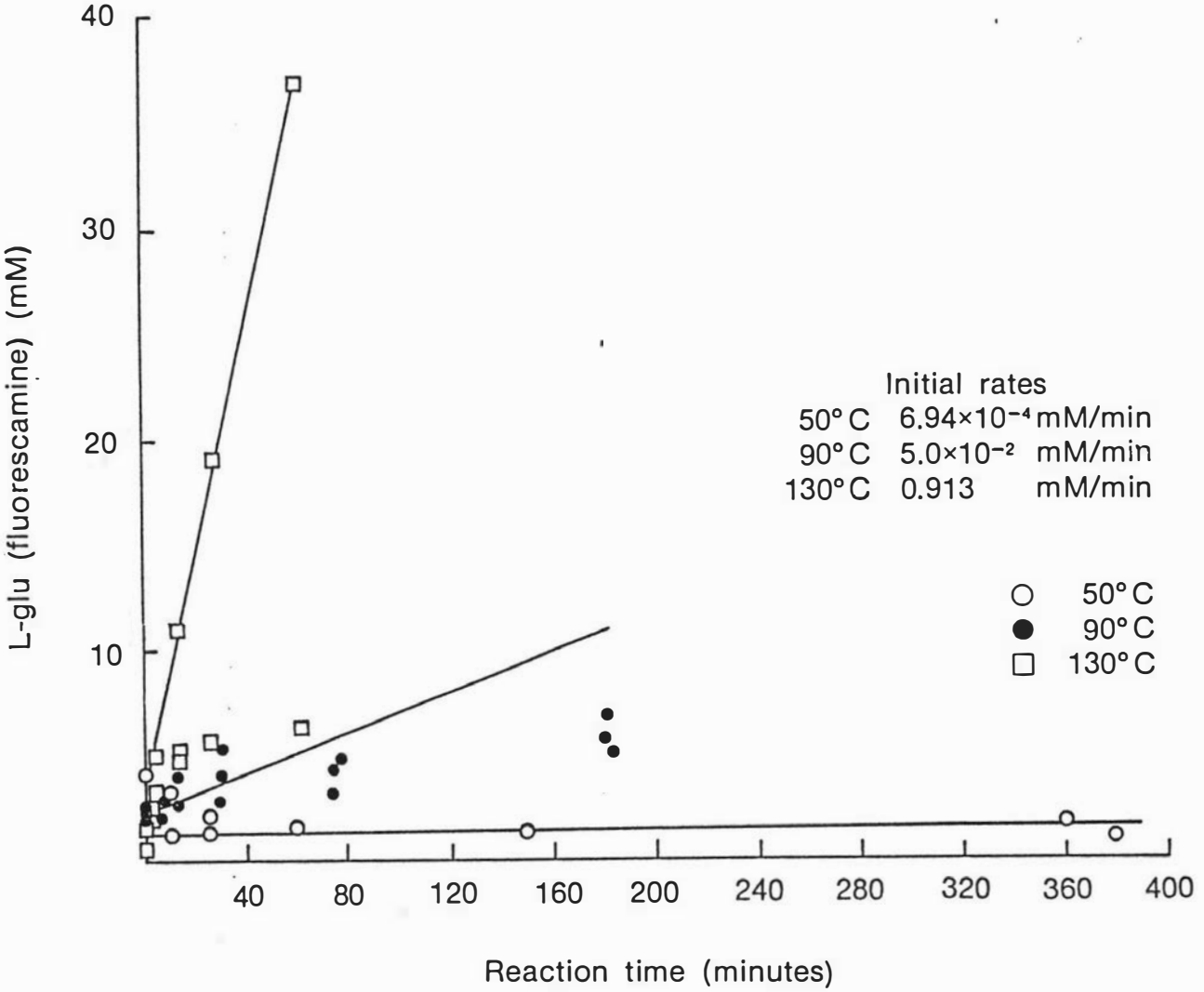
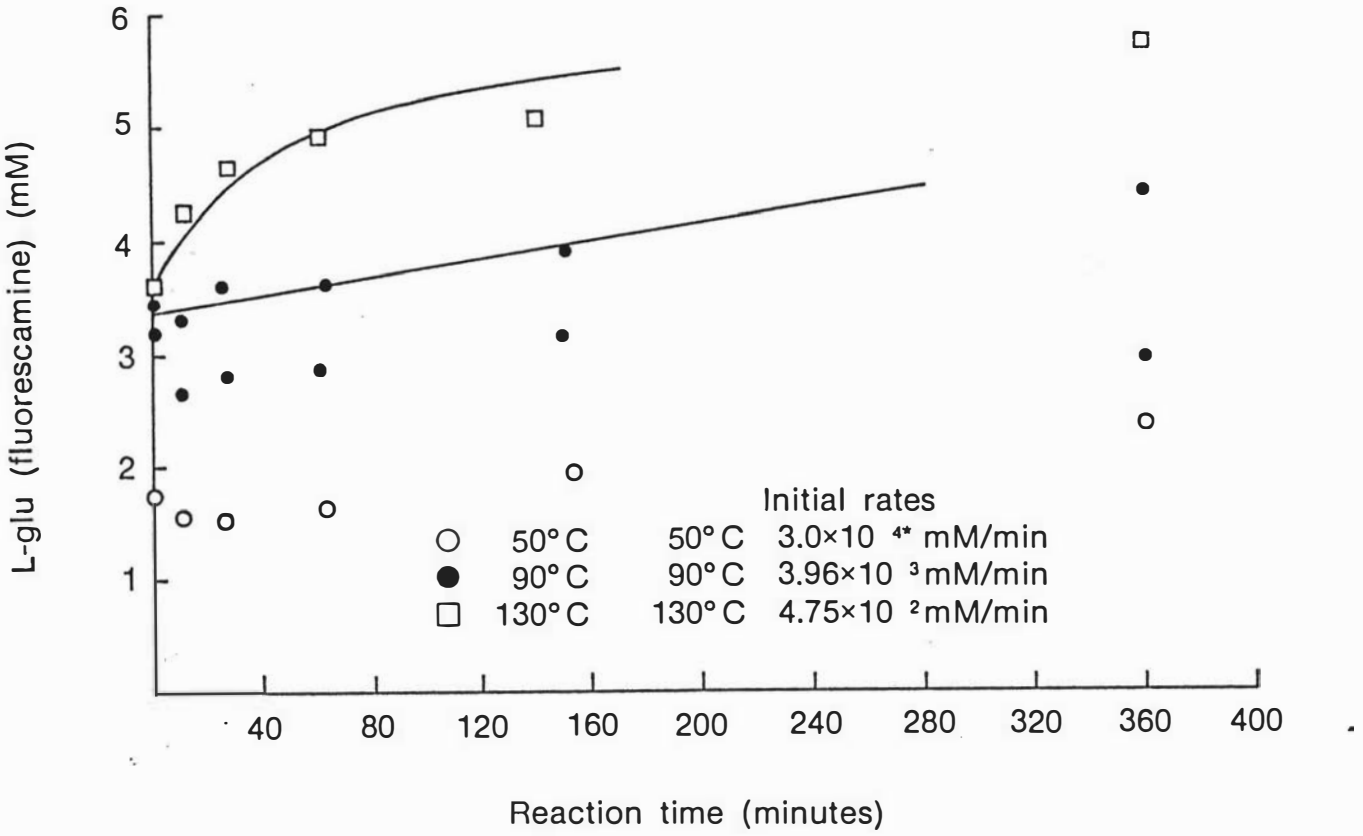


Figure 4.10 Peptide bond hydrolysis at reduced gluten concentration with 0.2M acid.



*Chosen as ½ rate for 50° C 0.2 M

Figure 4.11 Peptide bond hydrolysis at reduced gluten concentration with 0.02M acid.

Table 4.30: Peptide bond hydrolysis rates by alternative analysis

RATE (mM/min)

Temp (°C)	50	90	130
Acid Strength (M)			
2.0	-	0.7	2.9
0.2	6.94×10^{-4}	5.0×10^{-2}	0.913
0.02	3.0×10^{-4}	3.96×10^{-3}	4.75×10^{-2}

Table 4.31: Determination of the mean initial pH for alternative analysis

Temp (°C)	50	90	130
Acid Strength (N)			
2.0	-	-0.229	-0.229
		-0.295	
0.2	0.905	0.870	0.88
	0.866	0.90	0.871
		0.897	
0.02	3.15	1.84*	3.2
		3.9	

* Slope in Figure 4.11 taken through this set of data only

Table 4.32: Calculation of reaction rate coefficients for alternative analysis

REACTION RATE ($M.\ sec^{-1}$)	$[H^+]$ MEAN (M)	CONCENTRATION OF PEPTIDE (M)	k (CALCULATED) ($mole^{-1}\ sec^{-1}$)	1/T ($°K^{-1}$)
1.16×10^{-8}	0.130	7.93×10^{-2}	1.125×10^{-6}	3.096×10^{-3}
5.00×10^{-9}	7.08×10^{-4}(1)	7.93×10^{-2}	8.906×10^{-5}	3.096×10^{-3}
1.17×10^{-5}	1.83	7.93×10^{-2}	8.062×10^{-5}	2.755×10^{-3}
8.33×10^{-7}	0.129	7.93×10^{-2}	8.143×10^{-5}	2.755×10^{-3}
6.60×10^{-8}	0.014	7.93×10^{-2}	5.945×10^{-5}	2.755×10^{-3}
4.83×10^{-5}	1.69	7.93×10^{-2}	3.604×10^{-4}	2.481×10^{-3}
1.52×10^{-5}	0.133	7.93×10^{-2}	1.441×10^{-3}	2.481×10^{-3}
7.92×10^{-7}	6.31×10^{-4}(1)	7.93×10^{-2}	1.583×10^{-2}	2.48×10^{-3}

Note (1) High pH values omitted

$$d/dt \; -\log_{10} [H^+] = 1/[H^+] \; 0.4343 \; d\,[H^+]/dt$$

These data are shown in Table 4.29.

No correction was attempted for the apparently odd data of Runs 1 and 7, discussed in Section 4.5.2 or for the effects of changes in hydrogen ion activity or electrode error as disucssed in Section 4.2.5. The data in Table 4.29 therefore represent the rate of change of measured hydrogen ion activity.

4.8.2 Kinetic analysis of the experimental data

The rate data were fitted to the various models detailed below. Either stepwise or simple regression was used, and was accomplished using Minitab, a general purpose statistical package, available on the New Zealand Dairy Research Institute's computer system.

The models fitted were:

(i)

$$\text{Rate} = B_o + B_1a + B_2b + B_3c + B_4a^2 + B_5b^2 + B_6c^2 + B_7ab + B_8bc + B_9ac$$

i.e. a general first guess model which considers each variable, squares and two-way interactions

For these experiments, a, b, c take the value -1, 0, 1 as shown in the experimental design, Table 4.5.

(ii)

$\log_{10}$ Rate = B_o, B_1 ----------(as above)

(iii)

$$\log_{10} \text{Rate} = B_o + B_1 (\text{pH}) + B_2 \; 1000/T(^\circ K) + B_3 \log_{10} [\text{Amide}]$$

or

$$\log_{10} \text{Rate} = B_o + B_1 (\text{pH}) + B_2 \; 1000/t(^\circ K) + B_3 \log_{10} [\text{Peptide}]$$

i.e. fitting to the model

$$\text{Rate} = k_1[\text{Amide}][H^+]$$

or

$$\text{Rate} = k_2[\text{Peptide}][H^+]$$

i.e. the data are fitted to the model expected from a consideration of the hydrolysis ofamides and peptides as discussed in Sections 1.2.2, 1.2.3, 1.2.4, and 1.2.5.

where $k = k_o\, e^{-\frac{E}{R}\cdot\frac{1}{T}}$

That is, the effect of temperature on reaction rate is taken to be represented by Arrhenius' Law as described by this equation (Levenspiel, 1972).

(iv) $\log_e k = B_o + B_1\ 1000/T(°K)$

where k is calculated from

$$k = \text{Rate}/[\text{Amide}][H^+]$$

or $$k = \text{Rate}/[\text{Peptide}][H^+]$$

4.8.2.1 Analysis of the ammonia rate data

The rate data from Table 4.29 were fitted with the regression equations shown in Table 4.33. Also shown in Table 4.33 is the R^2, term by term, which indicates the proportion of the total variance explained by the inclusion of each term when entered in the order given. The total R^2 can also be corrected for the number of degrees of freedom and it is this corrected value which should be used to compare the fit obtained between equations with different numbers of terms. Similar data are also included in Tables 4.34 and 4.35.

Table 4.33: Regression equations for analysis of amide bond hydrolysis rate data. Ammonia evolution data is tested for fit to various models

MODEL DESCRIPTION		R^2 (%)	R^2 corrected for d.f. (%)	L.O.F. test
Fit to original design with first measurement of rates	Rate x 10^5 = 2.2 + 83.6 Acid + 41.1 Temp + 32.1 Gluten + $79.4A^2$ - $2.5T^2$ + $6.6G^2$ + 75.9 A*T + 59.3 A * G + 4.9 T * G	89.8	71.4	s (1%)
-type (ii) analysis				
R^2 for each term (%)	36.3 45.0 50.4 65.5 65.5 65.7 80.6 89.7 98.8			
Fit to model with first measurement of rates	Log_{10} (Rate x 10^5) = 11.8 - 1.04 pH - 3.50 (1000/T°K) + 0.869 $\log_{10}$ [Amide]	93.5	91.7	s (5%)
-type (iii) analysis	line A			
R^2 for each term (%)	63.0 87.7 93.5			
Fit to original design with revised measurement of rate	Log_{10} (Rate x 10^5) = 0.333 + 1.83 Acid + 1.06 Temp + 0.243 Gluten + $0.018A^2$ - $0.497T^2$ - $0.096G^2$ - 0.109 A * T + 0.246 A * G + 0.075 T * G	99.3	97.9	n.s.
-type (iii) analysis				
R^2 for each term (%)	71.0 94.7 95.9 96.0 98.3 98.4 98.5 99.2 99.3			
Fit to model with revised measurement of rates	Log_{10} (Rate x 10^5) = 11.5 - 0.985* pH - 3.45 (1000/T°K) + 0.899* $\log_{10}$ [Amide]	97.5	96.9	n.s.
-type (iii) analysis	line B			
R^2 for each term (%)	63.5 90.5 97.5			

k vs 1/T(°K) from revised rate data -type (ii) analysis in arithmetic form	$\log_{10} k = 15.5 - 8.07\ (1000/T°K)$	line C	91.7	91.0	n.s.

(* - coefficients tested for significant difference from 1.00 - both not significant)

The first attempt at analysis was to fit to a general model, type (i) above. The calculation showed that while each of the variables was highly significant there was significant lack of fit (LOF). (See Appendix 4.7 for an example of the information generated by the Minitab program and calculation of LOF). That is, all the variables affected the reaction rate but the model was inappropriate. A direct fit of the rate data to the expected kinetic model, type (iii) was attempted. This gave a much better, although far from perfect, fit. The Minitab program indicates data points which have a poor fit in relation to the rest of the data. At this point the original data were re-examined and the rates for rows 1, 3 and 4 revised as described in 4.8.1.1. With these revised rates the fitting procedure was repeated for type (iii) and an excellent fit was obtained. As a further check, a type (ii) analysis was made. Here too, the fit was excellent with most of the variance explained by the first three terms of the equation. This suggests that a multiplicative model, as in the type (iii) analysis is likely to be appropriate. A further test for fit to the proposed model is appropriate for type (iii) analysis. That is to test the coefficients in the equation which should be equal to one, for significant difference from one. This was done using the t test, details are given in Appendix 4.8 and, as noted in Table 4.33, no significant difference was found. This result shows that the equation resulting from the type (iii) analysis could be rewritten as

$$\text{Rate} = k\,[H^+][\text{Amide}]$$

where $k = k_o\, e^{-\frac{E}{R}\cdot\frac{1}{T}}$

describing the initial rate of hydrolysis of amide bonds in gluten. A first order dependence on hydrogen ion and amide concentration is demonstrated, with temperature dependence described by Arrhenius Law.

The data points were then used to calculate k for each run as shown in Table 4.29. A type (iv) analysis was made and an excellent fit obtained. The equations from the type (iii) and type (iv) analyses were plotted as shown in Figure 4.12. For the type (iii) analysis the procedure was to use the equation to calculate rate values at two temperatures. The reaction rate coefficient was then calculated by assuming

$$\text{Rate} = k[\text{Amide}][H^+]$$

Now Figure 4.12 shows that the effect of temperature on the reaction rate coefficient k can be described by Arrhenius Law

$$\text{i.e.} \quad k = k_o \, e^{-\frac{E}{R}\cdot\frac{1}{T}}$$

or, as plotted

$$\ln k = \ln k_o - \frac{E}{R}\cdot\frac{1}{T}$$

It follows that $-\frac{E}{R}$ is the slope of the line in Figure 4.12 and the activation energy E can be calculated since the value of R, the ideal gas law constant, is known. The calculated value was found to be:

for Line B in Figure 4.12

$$E = 15730 \text{ calories.mol}^{-1}$$

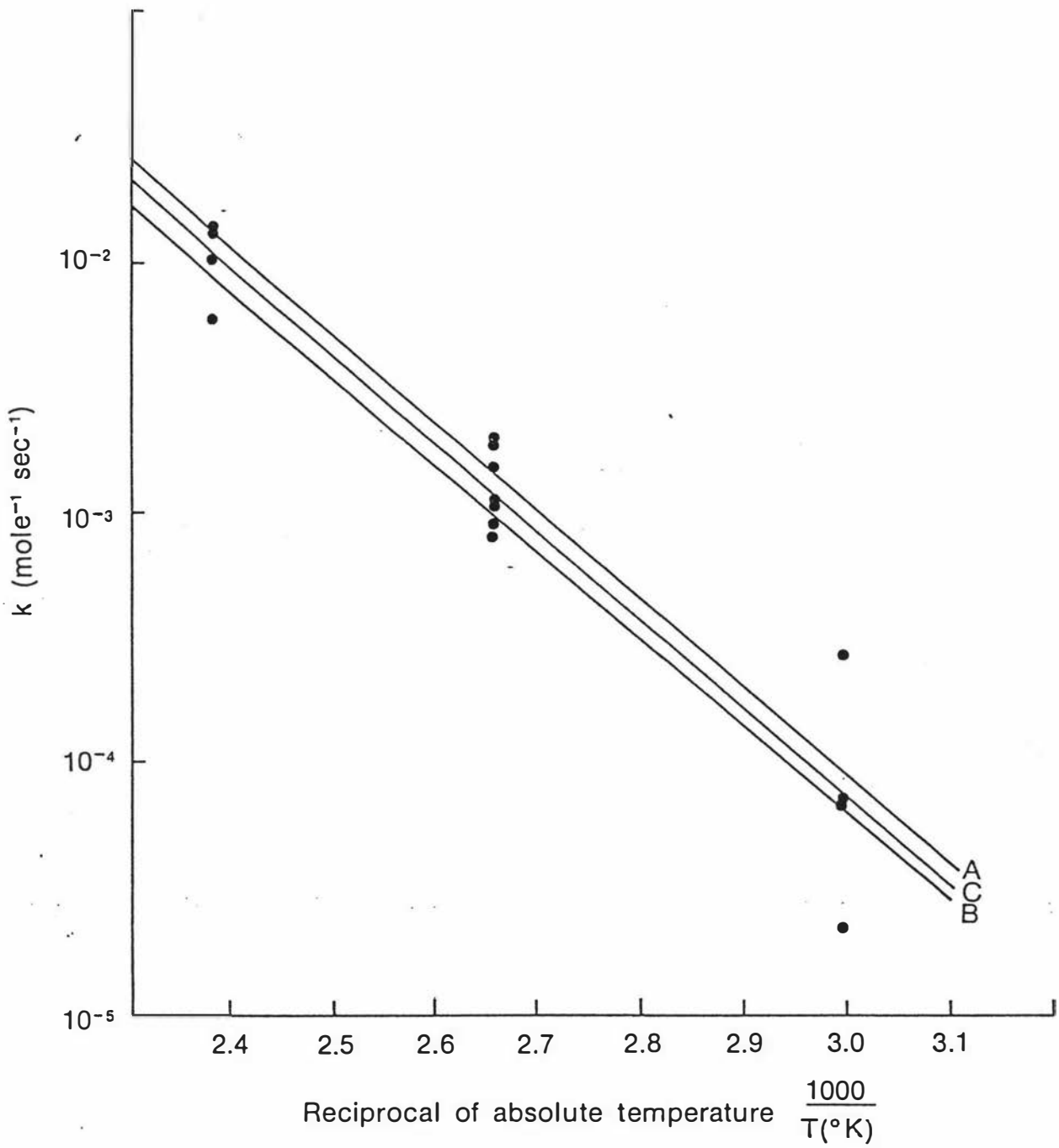


Figure 4.12 Reaction rate coefficients for amide bond hydrolysis plotted to show that the rate data can be represented by Arrhenius' Law.

All lines in Figure 4.12 have similar slope and Line B is the most reliable as shown in Table 4.33.

Details of the calculation of the activation energy are shown in Appendix 4.9.

4.8.2.2 Analysis of the N-terminal amino group rate data

The rate data from Table 4.29 were fitted to the various regression equations following the procedure developed for the amide rate data. The results are shown in Table 4.34. The fit, as measured by R^2 is poorer than that for the amide data. This is not unexpected because of the much greater error in the data as discussed in Section 4.6. There is, however, no significant LOF to the type (iii) equation which describes the expected kinetic model. The coefficients for the equation from the type (iii) analysis were tested for significant difference from one as described in Section 4.8.2.1 with details of the calculation given in Appendix 4.8. In this case the difference from one in the term -0.329 (pH) is significant, so that although the data fit the suggested equation, they do not fit the model very well. Some difficulties with the data were discussed in Section 4.5.2 and in Section 4.8.1.2 an alternative form of reaction rate analysis was described. The data from this analysis, Table 4.32, give an excellent fit when fitted to a type (iv) equation as shown in Table 4.34.

The equations from Table 4.34 (labelled A-D), were plotted in Figure 4.13 as described for the amide rate data. Examination of the data points on Figure 4.13 showed that the points with poor fit were associated with reactions which had a high initial pH and/or a low reaction temperature and therefore a very low reaction rate. In such cases, the reaction rate measurements, Table 4.29, are little more than guesses.

Table 4.34: Regression equations for analysis of peptide bond hydrolysis rate data. Data from the determination of N-terminal amino groups using fluorescamine is tested for fit to various models

MODEL DESCRIPTION

			R^2 (%)	R^2 corrected for d.f. (%)	L.O.F. test
Fit to original design	$(\text{Rate} \times 10^5) = 1.15 + 5.79\ \text{Acid} + 5.90\ \text{Temp} + 3.16\ \text{Gluten} + 3.68\ A^2 + 3.33T^2 -$				
-type (i) analysis	$0.75\ G^2 + 7.61\ A * T + 3.38\ A * G + 2.92\ T * G$		93.8	82.7	s (5%)
R^2 for each term (%)	24.5 49.9 61.3 65.2 65.4 86.6 90.7 93.8				
Log_{10} rate fitted to original design	$(\text{Log}_{10}\text{Rate} \times 10^5) = 0.006 + 0.613\ \text{Acid} + 1.0\ \text{Temp} + 0.269\ \text{Gluten} - 0.187A^2 -$				
-type (ii) analysis	$0.218T^2 - 0.130G^2 + 0.463\ A * T + 0.029\ A * G = 0.431\ T * G$		90.2	72.6	s (5%)
R^2 for each term (%)	17.9 75.3 78.7 80.3 80.6 85.7 85.8 90.2				
Log_{10} rate fitted to model	$(\text{Log}_{10}\ \text{Rate} \times 10^5) = 10.3 - 0.329^*\ \text{pH} - 3.58\ (1000/T°K) +$				
-type (iii) analysis	$0.605^*\ \text{Log}_{10}\ [\text{Peptide}]$	line A	77.0	70.8	n.s.
R^2 for each term (%)	12.6 70.7 77.0				
k from all data vs 1/T(°K)	$\log_e k = 14.3 - 8.25\ 1000/T(°K)$	line B	40.2	35.6	s (5%)
- in arithmetic form	i.e. $k = k_o e^{-\frac{E}{R}\cdot\frac{1}{T}}$ $k = (e^{14.3})(e^{-8250.1/T})$				
k from forced data vs 1/T(°K)	$\log_e k = 15.4 - 8.84\ 1000/T(°K)$	line C	64.6	58.7	-
- in arithmetic form	$k = (e^{15.4})\ (e^{-8840\ 1/T})$				

from forced data vs $1/T(^{o}K)$	$Log_e\ k = 18.5 - 10.3\ 1000/T(^{\circ}K)$	line D	94.7	93.4	-
with high pH values omitted (Table 4.32)					
in arithmetic form	$k = (e^{18.5})\ (e^{-10300\ 1/T})$				

* coefficients tested for significant difference from one, 0.605 - ns, - 0.329 s)

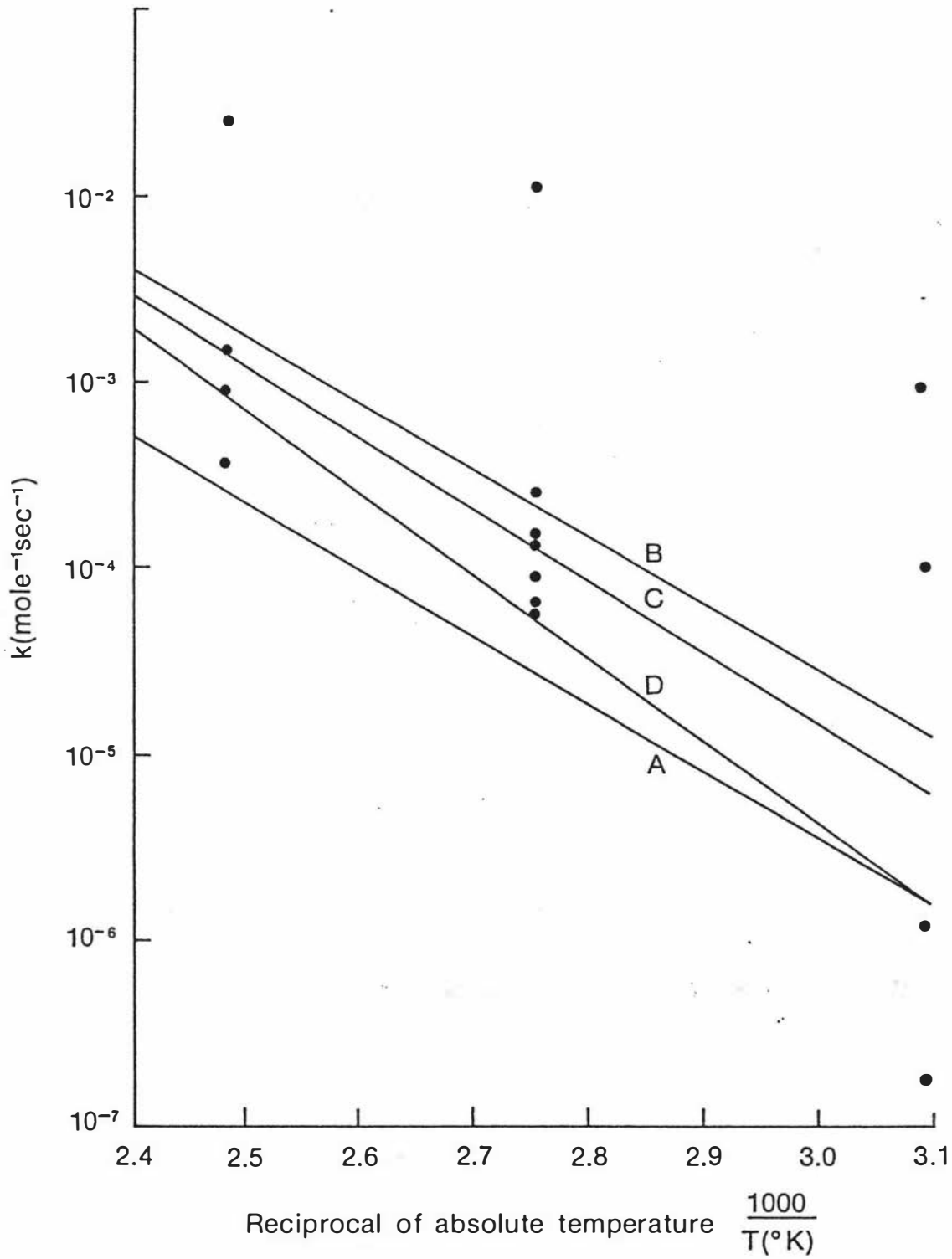


Figure 4.13 Reaction rate coefficients for peptide bond hydrolysis plotted to show that the rate data can be represented by Arrhenius' Law.

Although the data quality is not as good as that for amide hydrolysis the results show that the initial rate of acid hydrolysis of peptide bonds in gluten can be described by equations of the form

$$\text{Rate} = k\,[H^+][\text{Peptide}]$$

$$\text{where } k = k_o e^{-\frac{E}{R}\cdot\frac{1}{T}}$$

From the slope of the lines in Figure 4.13 the activation energy can be calculated. The calculated values were found to be:

for Line A in Figure 4.13
$E = 16462$ calories mol^{-1}

for line D in Figure 4.13
$E = 20411$ calories mol^{-1}

These two lines represent the most reliable calculations as shown in Table 4.34.

Details of the calculation of the activation energy are shown in Appendix 4.9.

4.8.2.3 Analysis of the hydrogen ion consumption rate data

The rate data from Table 4.29 were fitted to regression equations using type (i) and type (ii) analysis. The equations are shown in Table 4.35. Although the R^2 values obtained are quite high there is still a significant lack of fit. This is not surprising because of the difficulties with some of the pH measurements as discussed in Section 4.5.2. Next, regression analyses were made of the rate of hydrogen ion consumption and the rate of peptide bond hydrolysis and, the rate of

able 4.35: Regression equations for analysis of hydrogen ion depletion rate data. The rate data is tested for fit to various models and regression against other rate data

IODEL DESCRIPTION		R^2 (%)	R^2 adjusted for d.f.	L.O.F. test
it to original design type (i) analysis	(Rate x 10^5) = 2.02 + 7.64 Acid + 10.1 Temp + 7.75 Gluten + 1.23 A^2 + 6.34 T^2 + 2.48 G^2 + 9.46 A * T + 5.64 A * G + 9.78 T * G	97.6	9.32	s (1%)
^{2} for each term (%)	16.3 44.8 61.5 6.6 66.5 67.3 79.8 84.2 97.6			
$\log_{10}$ rate fitted to riginal design type (ii) analysis	Log_{10} (Rate x 10^5) = 6.301 + 1.44 Acid + 1.07 Temp + 0.265 Gluten - 1.08 A^2 - 0.168 T^2 – 0.366 G^2 + 0.149 A * T + 1.22 A * G - 0.030 T * G	95.8	88.7	s (1%)
^{2} for each term	43.0 66.7 68.2 78.6 78.7 80.1 80.4 95.8 95.8			
$\log_{10}$ rate vs Log_{10} eptide hydrolysis rate	Log_{10} (Rate x 10^5) = -0.254 + 1.04 ($\log_{10}$ (Peptide Hydrolysis rate x 10^5) (both rates expressed as positive)	47.1	43.1	-
nd in arithmetic form	Hydrogen ion consumption rate x 10^5 = -.557 (Peptide Hydrolysate Rate x 10^5) $^{1.04}$			
$\log_{10}$ rate vs $\log_{10}$ amide ydrolysate	Log_{10} (Rate x 10^5) = 0.580 + 0.859 ($\log_{10}$ (Amide Hydrolysis Rate x 10^{-5}) (both rates expressed as positive)	72.4	70.3	-
nd in arithmetic form	Hydrogen ion consumption rate x 10^5 = 0.263 (Amide Hydrolysis Rate x 10^5)$^{0.859}$			

Log_{10} Rate vs log_{10} (Amide + Peptide Hydrolysis Rate)	Log_{10} (Rate x 10^5) = -0.963* + 0.998 (log_{10} (Amide + Peptide Hydrolysis Rate) x 10^5)	61.3	58.3	-

and in arithmetic form Hydrogen ion consumption rate x 10^5 = 0.109((Amide + Peptide Hydrolysis Rate) x 10^5)$^{0.998}$

* coefficient tested for significant difference from expected value of -0.963, n.s. see Appendix 4.8

hydrogen ion consumption and the rate of amide bond plus peptide bond hydrolysis. This procedure was carried out to test the stoichiometry of the reaction which was expected to be that one hydrogen ion is consumed for each amide bond hydrolysed, see Section 1.2.2 and 1.2.7, and each peptide bond hydrolysed as discussed in Section 1.2.5. The regression equations are shown in Table 4.35. The highest R^2 value is that for the amide bond hydrolysis data. This is the expected result because of the greater errors in the peptide bond hydrolysis data discussed in Sections 4.5.2 and 4.6.

The hydrogen ion consumption rate data used are not corrected for the effects of the electrode system used i.e. the system measures activity, not concentration and is subject to the acid error of glass electrodes as discussed in Secton 4.2.5. The relationship between the measured activity and concentration is not a constant, as assumed in the regression analysis, but a function of acid concentration. The relationship is shown in Figure 4.14 which uses data from Table 4.3.

The figure does show, however, that a reasonable first assumption could be that the ratio of measured hydrogen ion activity to true concentration is constant over the range of concentration used in these experiments i.e. 0.02-2.0M. A value of 0.65 could be assumed. The last equation in Table 4.35 has, however, a coefficient of 0.109.

This value is derived from data subject to error and using the t-test it can be shown that the difference between the derived value of 0.109 and the expected value of approximately 0.65 is not significant. Details of the t-test are shown in Appendix 4.8.

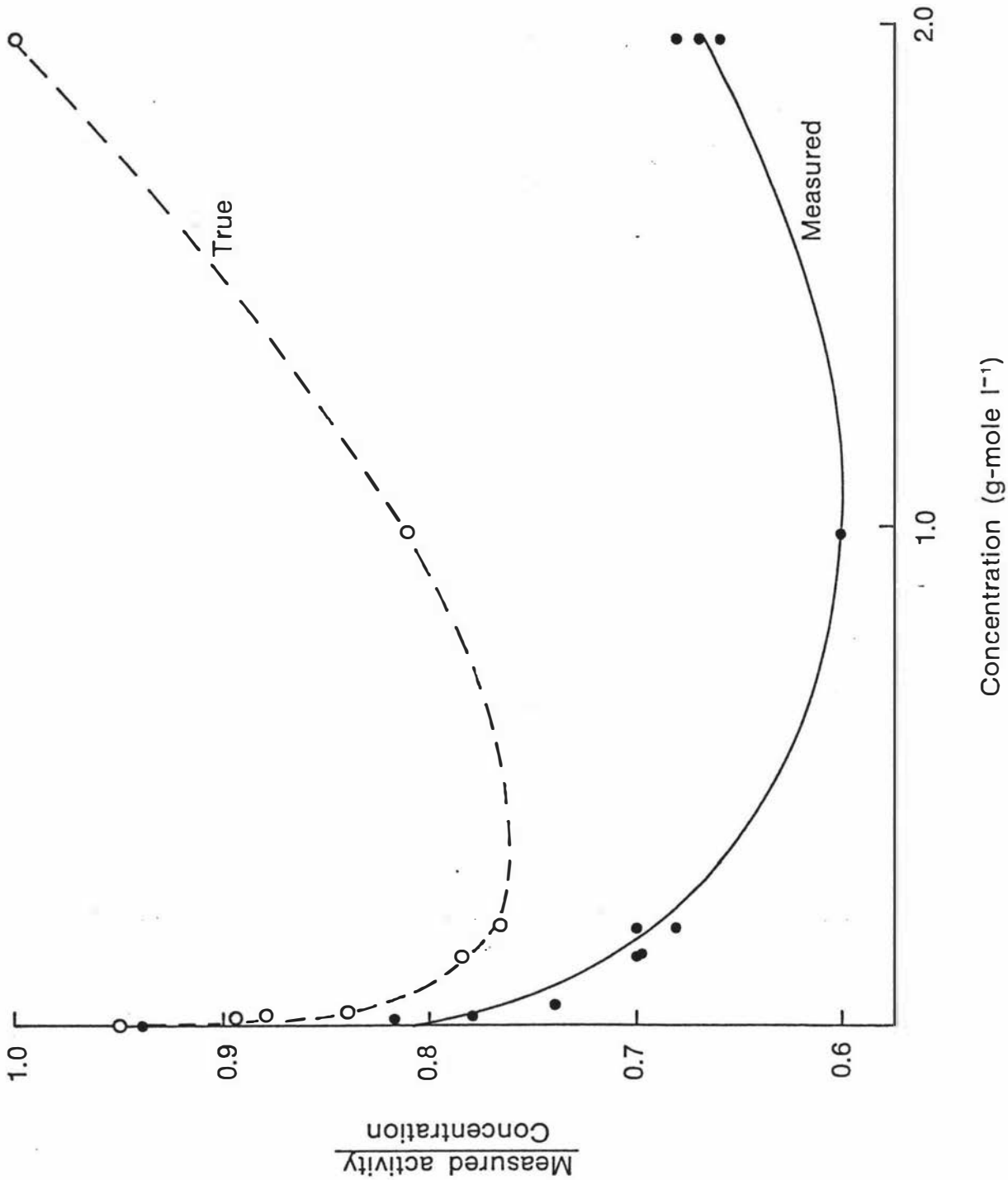


Figure 4.14 Measured hydrogen ion activity.

Another approach to determination of the stoichiometry was to consider only the data from the repetition experiments, runs 13, 14 and 15 (Table 4.5).

The mean rate of hydrogen ion consumption and the mean rate of amide bond hydrolysis plus peptide bond hydrolysis were determined and the ratio of these rates shown to be 0.60 (σ = 0.158) (Appendix 4.10). A further approach was to consider changes in pH, ammonia and peptide bond hydrolysis after a substantial part of the reaction was completed. Doubtful data, as discussed in Section 4.5.2, were omitted. From a total of 10 runs the ratio of the mean changes in hydrogen consumption and amide bond plus peptide bond hydrolysis was found to be 0.46 (σ_{n-1} = 0.27). (Calculations are shown in Appendix 4.11).

The calculations above support the expected stoichiometry but are subject to four sources of error, after allowing for the systematic error of the electrode discussed in Section 4.2.5.

First there is the error of the pH determinations where, whether due to fouling of the electrode by protein or other reasons, large variations are evident. Second there is error in the ammonia determinations and third, error in the peptide bond hydrolysis determinations. For all of these there is additional error in the determination of the rates of change used in the calculations. A fourth source of error is in the absolute value of the peptide bond hydrolysis measurements as discussed in Section 2.6.1 and 4.7.4. The data for the ratio of amide to peptide bonds Section 4.7.3 suggest that the peptide bond measurements determined using fluorescamine are a little high, i.e. the ratio is 25% when expected to be nearer to 30%. If this is so, then the effect would be to make the ratio

of hydrogen ions consumed to amide bond plus peptide bond hydrolysis a little low, which appears to be so.

All of the above leads to the conclusion that

(i) the assumed stoichiometry where one hydrogen ion is consumed for each peptide or amide bond hydrolysed is supported by the experimental data

(ii) that the use of a factor or activity coefficient of about 0.65 to correct activity measurements to concentration is reasonable.

4.8.3 An alternative form of analysis for some data

The analysis of kinetic data described in Section 4.8.2 is a differential form of analysis based on measurement of initial rates of reaction. The usual alternative form of analysis is an integral form of analysis which considers the reaction data over the time course of the reaction and requires integration of the rate expression to be tested (Levenspiel, 1972). A difficulty with the present work is that one of the reaction substrates, hydrogen ions, is consumed in both the amide bond and peptide bond hydrolysis reactions. There is, however, one special circumstance which avoids taking account of the dual consumption of hydrogen ions, that is when the hydrogen ions are present in great excess and can be regarded as effectively constant throughout the reaction. In the present work this occurs for Run 6 which has a high acid concentration and low gluten concentration and Runs 2 and 9 which have litte acid consumption because of the low reaction temperature.

Now the expected rate equation is

$$- \text{rate} = k\,[\text{Amide}][H^+]$$

but if $[H^+]$ is effectively constant then

$$- \text{rate} = k' [\text{Amide}] \quad \text{where } k' = k[H^+]$$

or in more general terms

$$- rA = - d \frac{C_A}{dt} = k' C_A$$

and defining fractional conversion XA of a given reactant A as the fraction of reactant converted into product

$$\text{i.e.} \quad X_A = \frac{N_{Ao} - N_A}{N_{Ao}}$$

where N is the number of moles of reactant then it can be shown that

$$\frac{dX_A}{dt} = k' (1 - X_A)$$

and in integrated form

$$- \ln (1 - X_A) = k't$$

(Levenspiel, 1972)

It follows that for a reaction described by this type of kinetics a plot of $\ln (1-X_A)$ against reaction time will give a straight line of slope -k'.

This type of plot was made for the amide bond hydrolysis data of Runs 2, 6 and 9 and for the peptide bond hydrolysis data of Run 6. The calculations of fractional concentrations are shown in Appendix 4.12 and the data are plotted in Figures 4.15, 4.16 and 4.17.

The peptide bond hydrolysis data for Run 6, shown in Figure 4.15, clearly show a straight line fit over the initial 40% hydrolysis of peptide bonds thus confirming the kinetic equation demonstrated in Section 4.8.2.2. A similar

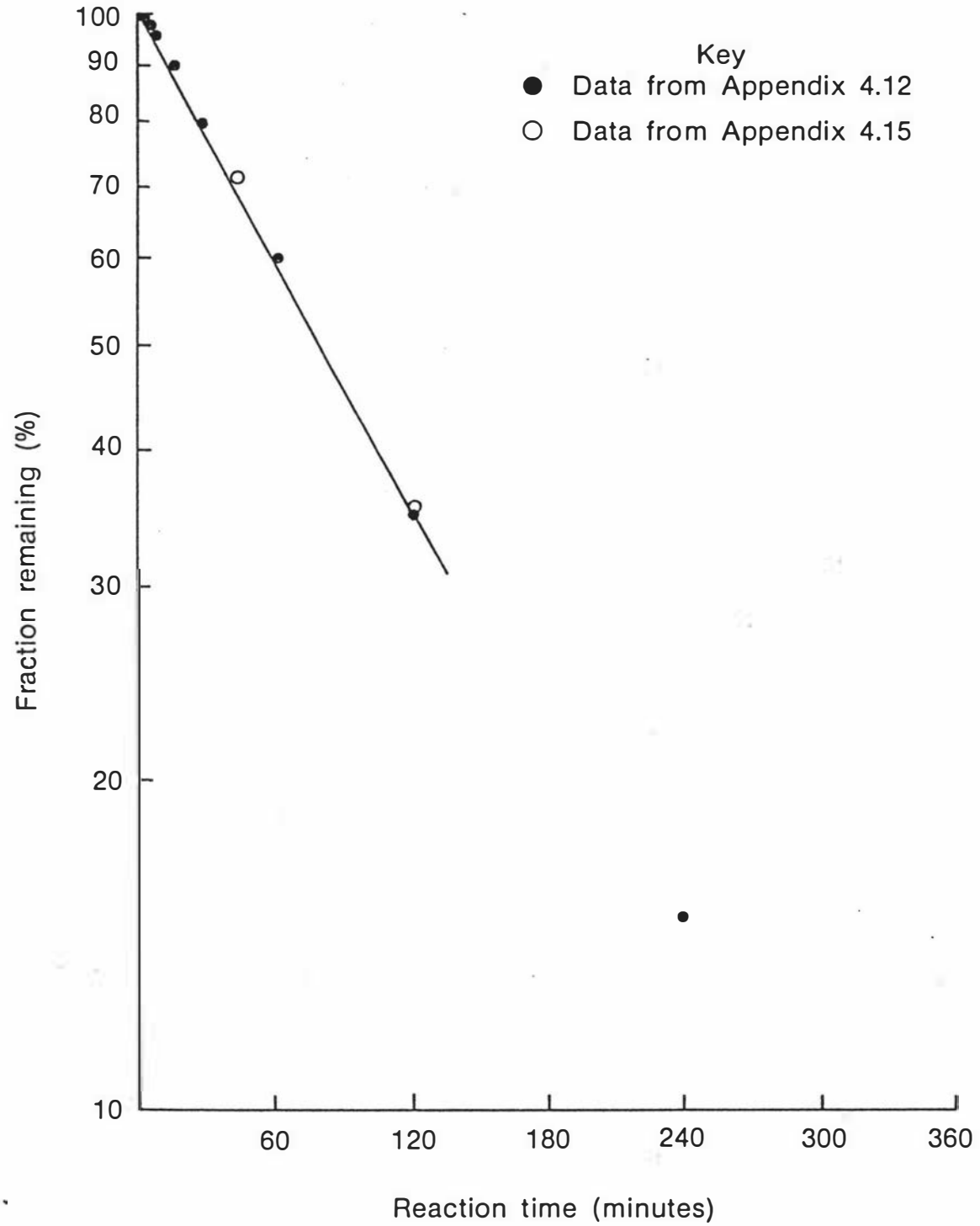


Figure 4.15 Peptide bond hydrolysis for Run 6.

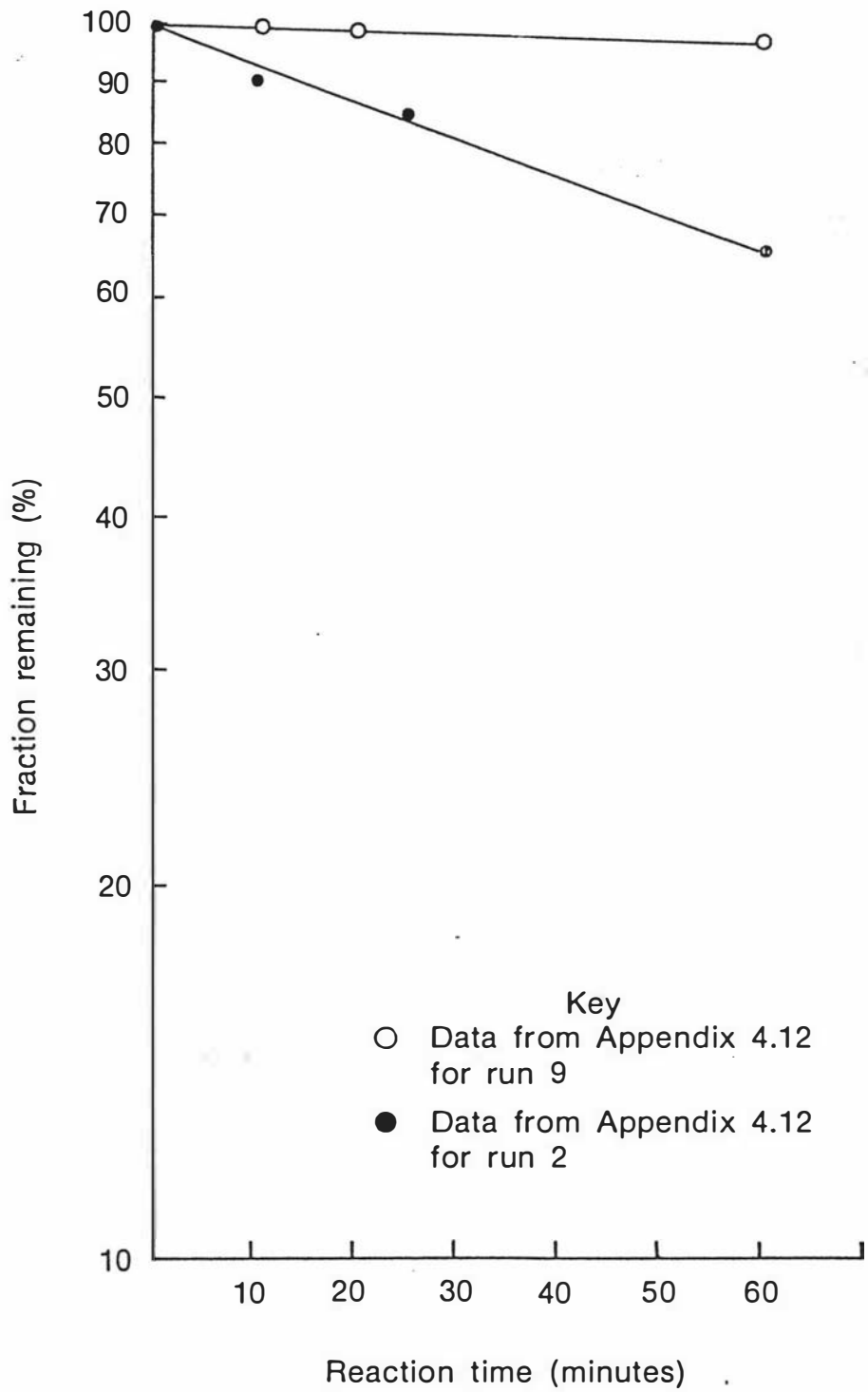


Figure 4.16 Amide bond hydrolysis for Runs 2 and 9.

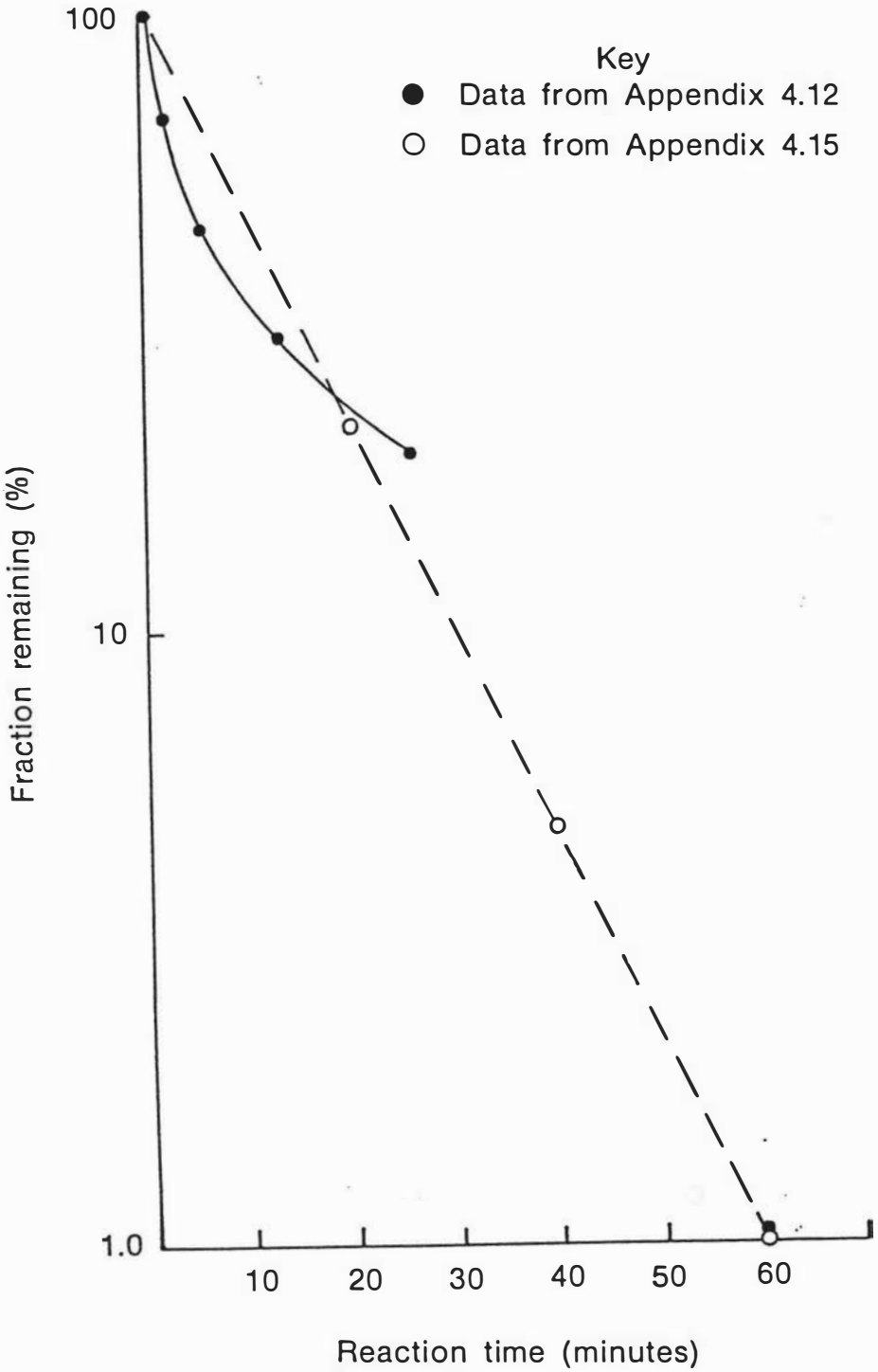


Figure 4.17 Amide bond hydrolysis for Run 6.

confirmation is given by the amide bond hydrolysis data from Runs 2 and 9 shown in Figure 4.16. These data, however, cover only a small fraction of total deamidation. For Run 6, if data only up to 26 minutes of reaction are considered then a curve is indicted (Figure 4.17) but if a further point, at 60 minutes of reaction, is considered then a straight line is indicated, also shown on Figure 4.17.

Because of the high reaction temperature the data for short reaction times may be in error because of the time required for bottles to cool and the amount of 'bench reaction' due to the high acid concentraion. For these reasons the straight line, Figure 4.17, would seem to be the valid conclusion and in line with the kinetic equation demonstrated in Section 4.8.2.1.

4.8.4 Conclusions from results of kinetic analysis

Measurement of the initial rates of amide bond hydrolysis, peptide bond hydrolysis and hydrogen ion consumption rate were subjected to regression analysis. The results showed that the reaction kinetics could be described by equations:

$$\text{Rate of amide bond hydrolysis} = k_1\,[\text{Amide}][H^+] \qquad (1)$$

$$\text{Rate of peptide bond hydrolysis} = k_2\,[\text{Peptide}][H^+] \qquad (2)$$

Both reactions are catalysed by hydrogen ions and in both reactions hydrogen ions are consumed in stoichiometric ratio to the hydrolysis of amide or peptide bonds.

4.9 Simulation of reaction using kinetic equations

The equations (1) and (2), Section 4.8.4, have been shown to describe the initial rate hydrolysis but have not been tested for description of the time course of the whole reaction except as described in Section 4.8.3. One method of making such a test is to use the equations in a simulation of reaction progress

which is also useful for prediction of the reaction under conditions other than those of the experiments.

The equations, (1) and (2), describe reactions which occur in parallel. The substrate [H^+] is common to both reactions. An analytical solution is available for the individual reactions (Laidler, 1965) but not for the case of reactions in parallel. Some special cases do have analytical solutions. Competitive parallel reactions were discussed by Hill (1977) and a technique for finding relative values of two rate constants described.

Various cases of parallel reaction are also described by Capellos and Bielski (1972). An appropriate specific case is not described, except for that of hydrogen ions present in gross excess, so that the reactions become psuedo first order. Since an analytical solution of the equations was not available, a numerical solution was attempted.

First, initial values were found for the components of equations (1) and (2), that is amide, peptide and H^+ concentrations. Then the reaction rate equations were integrated over a short time interval. New values of amide and peptide concentrations were calculated by subtracting the result of the integration from the initial values. A new value of the H^+ concentration was also calculated but included a correction factor to allow for the initial value of H^+ being an activity whereas the stoichiometry of the reaction depends on concentration.

A computer programme using the BASIC computer language was written. Integration was performed using a sub-routine from the NAG Fortran Library which is accessible on the New Zealand Dairy Research Institute's computer system (Numerical Algorithms Group, Oxford OX2 7DE, England). The routine used, D02 BAF, integrates a system of ordinary differential equations over a wide range with suitable initial conditions using a Runge-Kutta-Merson method (Rose, 1974). The programme is arranged so that the results for every 10 seconds of reaction time are recorded. The programme is shown in Appendix 4.13.

A first test of the operation of the programme was made using the data of Run 6 as discussed in Section 4.8.3 This test was chosen because the results do not depend on a choice of correction for the effect of hydrogen ion activity since the hydrogen ion concentration is almost constant. To perform this test the slopes of the lines, k $[H^+]$, in Figures 4.15 and 4.17 were measured, the calculation is given in Appendix 4.14, and the values for k and the initial conditions specified in Table 4.36 were used in the simulation. The calculated values obtained are shown in Appendix 4.15 and on Figures 4.15 and 4.17. The excellent fit obtained showed that the simulation programme was working correctly and that choice of simulation time interval and tolerance of the solution, Appendix 4.13, were adequate.

To test further the operation of the programme a table was constructed of the ammonia determination, N-terminal amino group determination, and pH data from the repetition runs, runs 13, 14, and 15 (Table 4.5). Values for each measurement at selected reaction times were taken of data smoothed by plotting and means and standard deviations were calculated. The data are shown in Appendix 4.16. The data are plotted ± 2 s.d. in Figures 4.18, 4.19 and 4.20. That the simulated data should fit within the standard deviation limits was chosen as a suitable, although arbitrary criterion for satisfactory modelling. There is still some choice of values for reaction rate coefficients from Figures 4.12 and 4.13 and of correction factor, Figure 4.14. An alternative approach might be to calculate confidence limits for the regression lines in Figures 4.12 and 4.13 and thus show a band of possible solutions to the reaction equations within which most of the reaction data should fall.

Various combinations of constants, shown in Table 4.37 were chosen for calculation. For amide bond hydrolysis only one reaction rate constant was chosen, from Line B in Figure 4.12, because that was considered to be the most reliable as shown from the regression data in Table 4.33. Several values of reaction rate constant were selected from Figure 4.13 because of

Table 4.36: Initial concentrations and reaction rate constants for simulation of Run 6

Am (0) = 0.02	(M)	
Pep (0) = 0.079	(M)	
H^+ (0) = 1.702	(M)	(pH = 0.231)
$k_1 [H^+]$ = 1.279×10^{-3}		(sec^{-1})
$k_2 [H^+]$ = 1.458×10^{-4}		(sec^{-1})
correction factor = 0.65		
k_1 = 7.515×10^{-4}		($mole^{-1}.sec^{-1}$)
k_2 = 8.566×10^{-5}		($mole^{-1}.sec^{-1}$)

the greater uncertainty of that data. Similarly, a range of possible values was used for correction of concentration-based stoichiometry to activity measurements based on the calculations discussed in Section 4.8.2.3. The mean initial conditions for Runs 13, 14 and 15, shown in Table 4.37 were used to simulate the reaction using the variables also shown in Table 4.37 in the various cells. First the data for cells 2, 5 and 8 were produced. The results are summarised in Appendix 4.17 and shown on Figures 4.18, 4.19 and 4.20. Then the data for cells 4, 3, 6 and 9 were produced and similarly summarised in Appendix 4.17. After examination of these data two further combinations, cells 10 and 11, using the variables shown in Table 4.37 were used. These data were also recorded in Appendix 4.17 and the data for cell 10 are shown on Figures 4.18, 4.19 and 4.20.

The data show that the lower values of reaction rate coefficient for peptide bond hydrolysis, k_2 and correction factor are more suitable in this instance. This conclusion, however, is valid only for the one value of amide hydrolysis rate constant, k_1. Examination of the data in Figures 4.18, 4.19 and 4.20 indicates that an even better fit could be obtained if a slightly lower value of k_1 were used with the conditions of cell 2 of Table 4.37.

The curvature of the lines in Figures 4.18, 4.19 and 4.20 suggests, however, that higher values of k_1, would give a better fit in the initial stages of the reaction. It may be that the values of k_1 are decreasing, as discussed by Leach (1953), and this is particularly likely for peptide bond hydrolysis because of the marked variation in lability of different peptide bonds.

In conclusion it can be seen that a good simulation of the reaction can be achieved. This implies that the kinetics, as determined by initial rate analysis are a good model of the process and correctly state the reaction.

Table 4.37: Combination of reaction rate constants and activity correction factors for test of reaction simulation

For initial conditions

Amide = 0.150 M
Peptide = 0.595 M
Hydrogen ion activity = 0.128 M
Reaction temperature = 90°C

and Reaction time = 10,800 secs.

If $k_1 = 1.02 \times 10^{-3}$ (from curve B, Figure 4.12)

and:

		Activity Correction Factor		
		0.11	0.60	0.65
	5.2×10^{-5}	(1)	(2)	(3)
k_2	1.3×10^{-4}	(4)	(5)	(6)
(from Figure 4.13)	2.2×10^{-4}	(7)	(8)	(9)

And after evaluation of the above two further trials were made with conditions

Cell Number	Correction Factor	k_1	k_2
(10)	0.57	1.02×10^{-3}	7×10^{-5}
(11)	0.55	1.02×10^{-3}	8×10^{-5}

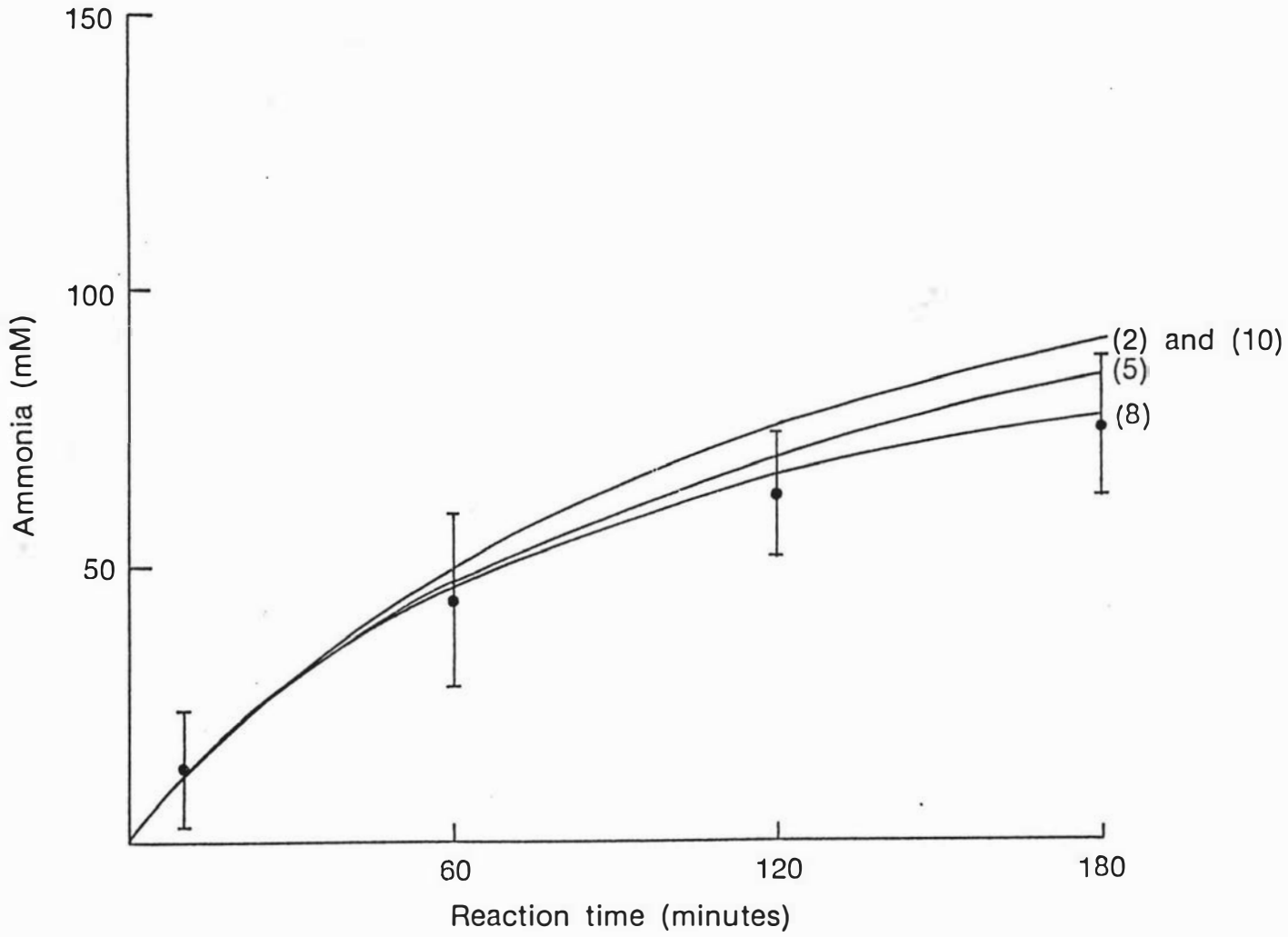


Figure 4.18 Ammonia data from repetition runs of experimental design used for test of reaction simulation.

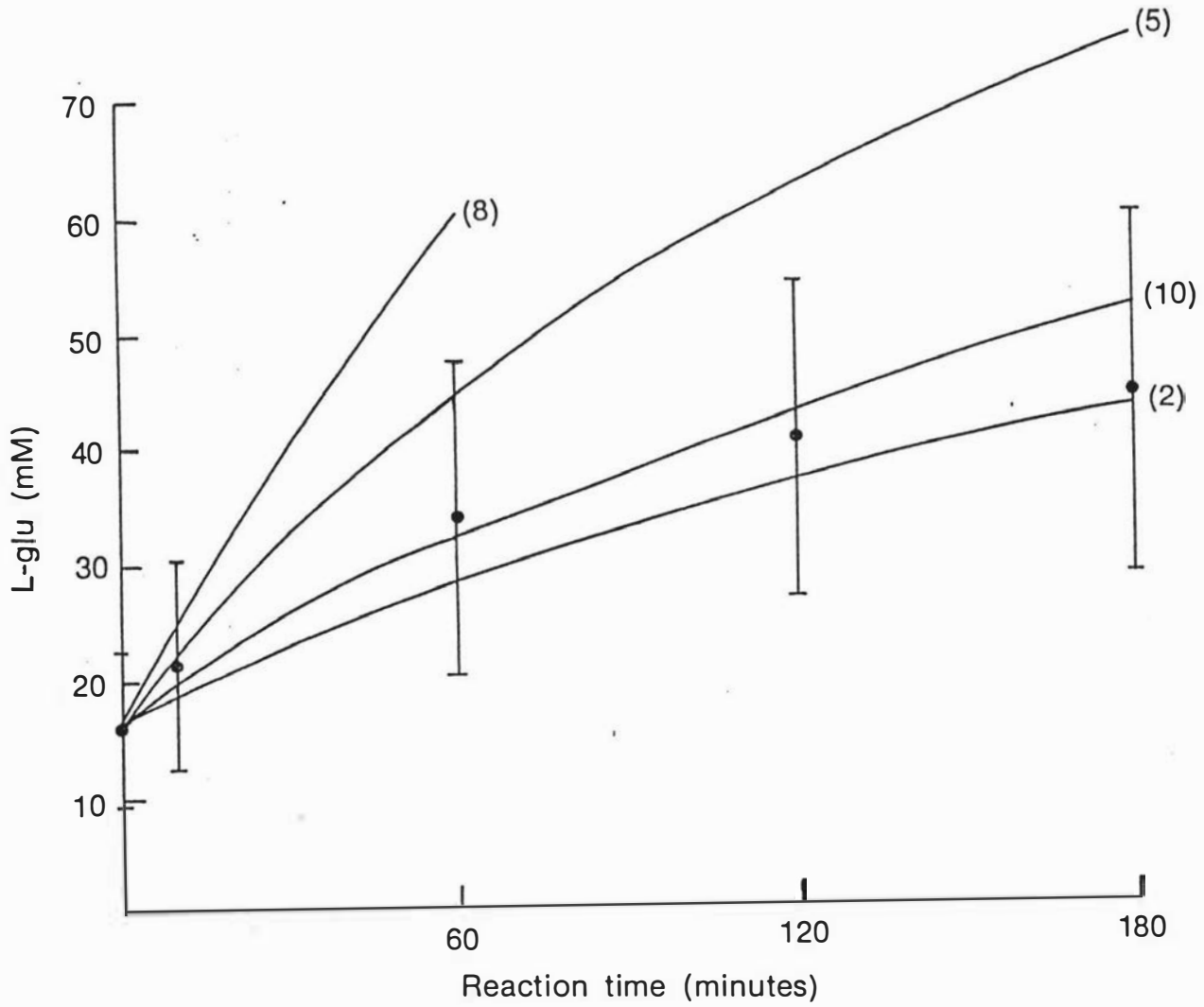


Figure 4.19 N-terminal amino group determination data from repetition runs of experimental design used for test of reaction simulation.

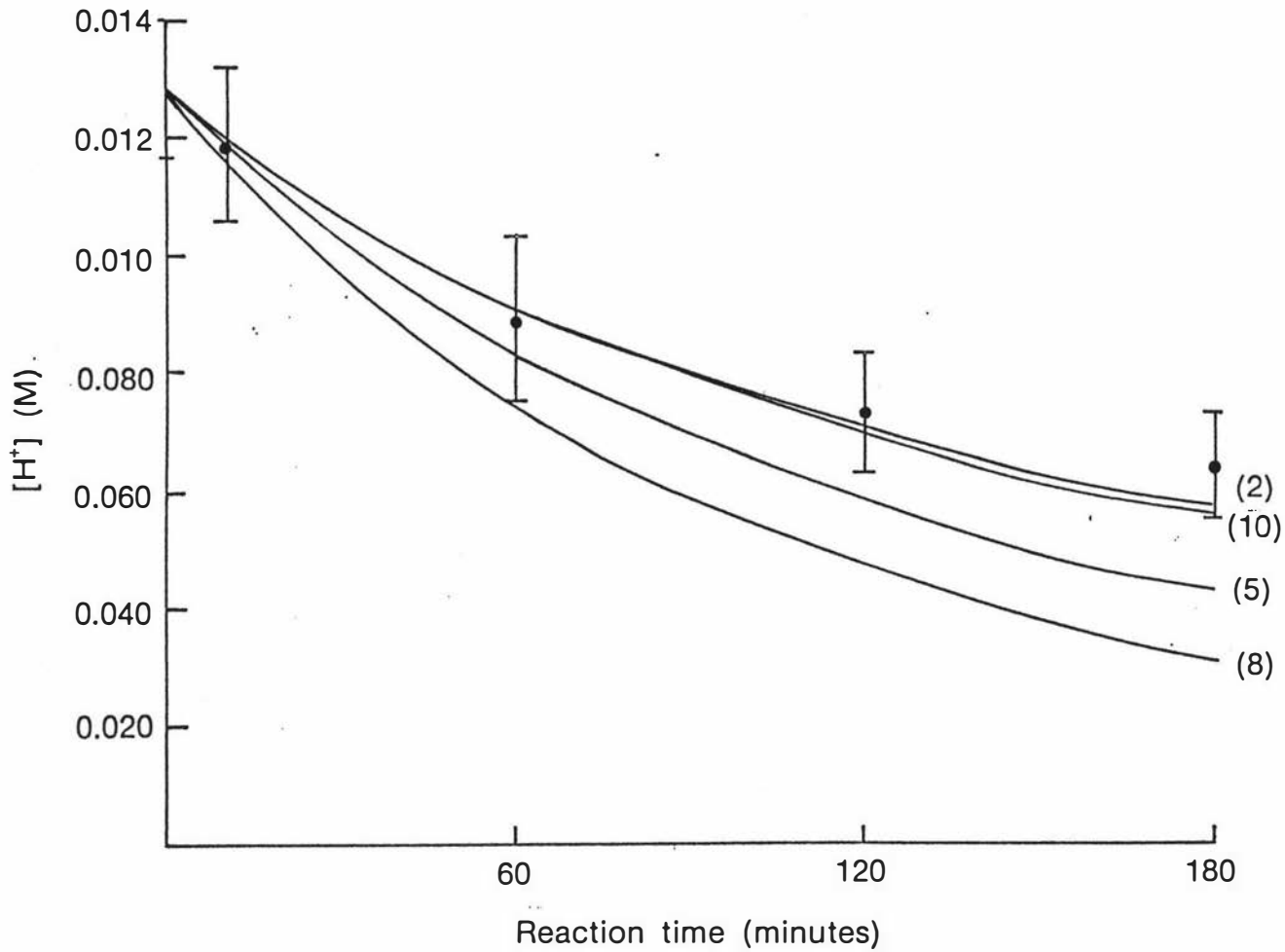


Figure 4.20 Hydrogen ion activity data from repetition runs of experimental design used for test of reaction simulation.

4.10 An analysis of the data of Vickery (1922)

The work of Vickery (1922) provided comprehensive data for the hydrolysis of gliadin. The temperature and gliadin concentration were held constant and the acid concentration was varied. The data are shown in Table 4.38.

The data are expressed as ammonia N (nitrogen) and amino N as a fraction of the total N. The total ammonia N, i.e. amide groups, was taken to be 25%. The fraction of unreacted amide groups can then be calculated from the ammonia data as shown in Table 4.38. The fraction of unreacted amino groups is similarly calculated from the amino N data and the data are also shown in Table 4.38. These data are plotted on semi-logarithmic coordinates for ammonia in Figure 4.21 and for amino N in Figure 4.22.

In general, the kinetics of the early parts of each reaction can be described by a straight line through the origin. There is sufficient uncertainty in the data for there to be some choice in placing lines and these were fitted by visual inspection. The slopes of the lines were calculated; the calculations are shown in Appendix 4.18, and recorded in Table 4.39. Now the slope of the line is equal to $k[H^+]$ as discussed in Section 4.8.3. Earlier discussion, Section 1.2.7, suggested that the kinetics of gliadin hydrolysis are similar to those of gluten. To calculate k there is a choice of using either the acid concentration or activity. These options are explored in Table 4.39.

Comparison of the calculated values of k with the data of this present work in Figures 4.12 and 4.13 at

$$\frac{1}{T} = 2.72 \times 10^{-3} \ {}^{o}K^{-1} \quad (94^{o}C)$$

show that the values of k calculated using activity are closer to the values calculated for this present work. In this work

Table 4.38: The data of Vickery (1922)

0.027M HCl at 93-94°C			
TIME (hrs)	TOTAL N AS AMMONIA A (%)	FRACTION OF AMIDE REMAINING(1)	FRACTION OF AMINO IN REMAINING (2)
5	7.7	0.69	
15.5	13.2	0.48	
20	14.3	0.44	
28	15.5	0.39	
44	16.8	0.34	
70	17.7	0.30	
118	19.5	0.24	

0.1M HCl at 93-94°C				
TIME (hrs)	TOTAL N AS AMMONIA A (%)	TOTAL AMINO N (%)		
1	8.5		0.67	-
2	14.4	3.7	0.43	0.963
3	17.4		0.32	-
4	18.8	3.5	0.26	0.965
8	21.7	5.3	0.15	0.947
11	22.9	6.3	0.10	0.937
17	23.4	7.6	0.08	0.924
26	24.1	13.6	0.05	0.864
40	24.2	17.3	0.05	0.827

0.2M HCl at 93-94°C

TIME (hrs)	TOTAL N AS AMMONIA N (%)	TOTAL AMINO N (%)		
0.5	10.7		0.58	-
1	14.0	2.5	0.45	0.975
3.5	21.8	4.6	0.15	0.954
6	23.8		0.07	-
16	23.6	15.6	0.07	0.844
24	23.9	23.3	0.06	0.767
40	24.4	30.0	0.04	0.700

0.5M HCl at 93-94°C

TIME (hrs)	TOTAL N AS AMMONIA N (%)	TOTAL AMINO N (%)	FRACTION OF AMIDE REMAINING	FRACTION OF AMINO N REMAINING
0.5	17.4		0.32	-
1	21.0	4.3	0.18	0.957
2	23.4	6.5	0.08	0.935
4	24.5	12.3	0.04	0.877
16	24.6	29.7		0.703
22	24.9	37.9		0.621
40	25.0	56.8		0.598
				0.432

1.0M HCl at 94-95°C

TIME (hrs)	TOTAL N AS AMMONIA A (%)	TOTAL AMINO N (%)	
0.5	22.2		-
1	23.6	9.6	
2	24.2	11.5	0.904
7	25.0	31.2	0.885
12	24.8	42.8	0.688
14	24.9	46.7	0.572
16	25.0	49.2	0.533
18	24.9	51.1	0.508
22	25.0	57.5	0.489
27	25.0	59.9	0.425
40	25.1	69.1	0.401
49	25.3	76.9	0.309
69		83.7	0.231

2.0M HCl at 94-96°C

TIME (hrs)	TOTAL N AS AMMONIA A (%)	TOTAL AMINO N (%)	
1	24.7	21.7	0.783
3.3	24.9	39.5	0.605
5	25.1	43.7	0.563
7	24.9	50.2	0.498
9	25.0	55.5	0.445
12	25.2	62.1	0.379
17	25.5	74.7	0.253
24	25.4	79.5	0.205
45	25.2	84.0	0.160
65	25.5	88.5	0.115

Notes (1) The maximum value of total N as ammonia N was taken as 25.5

(2) The data are treated as if the amino N is due solely to peptide

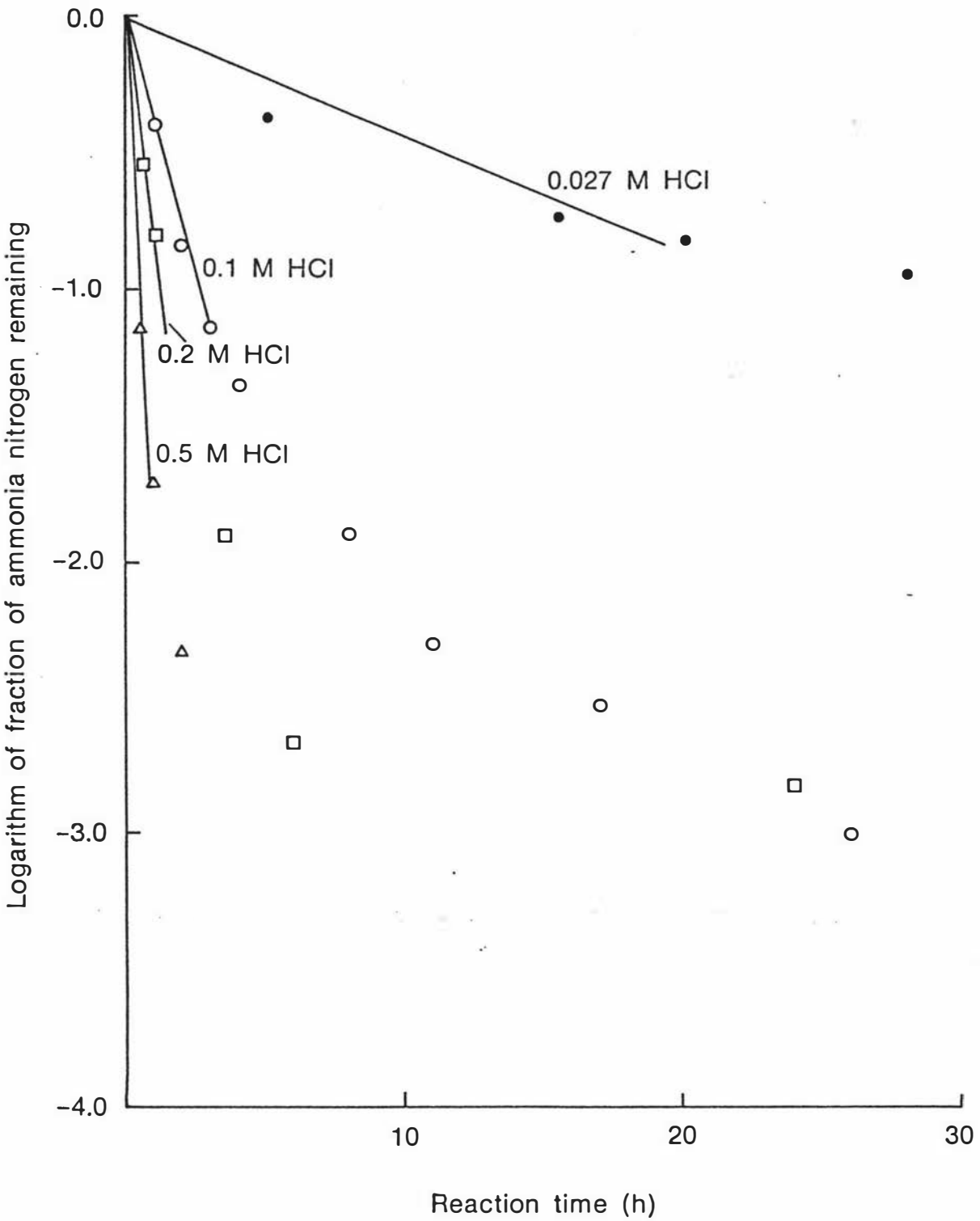


Figure 4.21 The amide bond hydrolysis data of Vickery (1922).

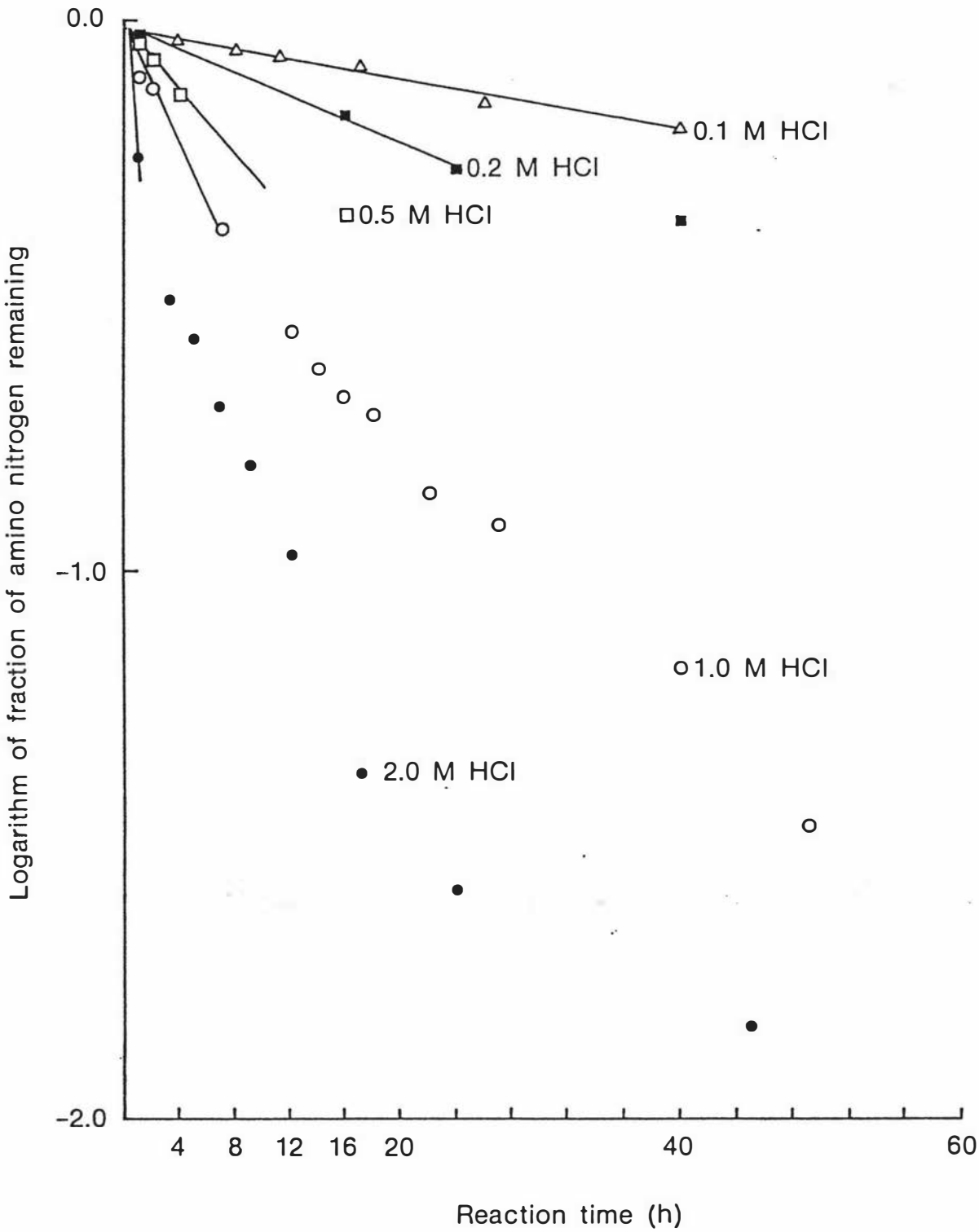


Figure 4.22 The peptide bond hydrolysis data of Vickery (1922).

Table 4.39: Reaction rate data coefficients calculated from the data of Vickery (1922)

ACID CONCENTRATION (M)	SLOPE[1]	k hr^{-1}	k hr^{-1}
A:Amino N			
0.1	-4.5×10^{-3}	0.045	1.25×10^{-5}
0.2	-0.010	0.0.050	1.39×10^{-5}
0.5	-0.028	0.056	1.56×10^{-5}
1.0	-0.053	0.053	1.47×10^{-5}
2.0	-0.233	0.117	3.25×10^{-5}
B:Ammonia N			
0.027	0.043	1.59	4.42×10^{-4}
0.1	0.4	4.0	1.11×10^{-3}
0.2	0.83	4.15	1.15×10^{-3}
0.5	2.13	4.26	1.18×10^{-3}

Note (1) - as calculated in Appendix 4.18
Now slope = $k[H^+]$

see Section 4.8.3

ACID CONCENTRATION (M)	ACID ACTIVITY[1] (M)	SLOPE[2]	k hr^{-1}	k s^{-1}
A:Amino N				
0.1	0.0798	-4.5×10^{-3}	0.056	1.56×10^{-5}
0.2	0.154	-0.010	0.065	1.81×10^{-5}
0.5	0.385	-0.028	0.073	2.03×10^{-5}
1.0	0.81	-0.053	0.065	1.81×10^{-5}
2.0	2.0	-0.235	0.178	4.94×10^{-5}

B:Amino N				
0.027	0.0227	-0.043	1.89	5.25×10^{-4}
0.1	0.0798	-0.4	5.01	1.39×10^{-3}
0.2	0.154	-0.83	5.39	1.50×10^{-3}
0.5	0.385	-2.13	5.53	1.54×10^{-3}

NOTES: (1) Calculated by using the true activity coefficient from Figure 4.14
i.e. Activity = Concentration times activity coefficient

(2) Rate = slope = $k A_{H^+}$

the activity is actually slightly less than true, as discussed in Section 4.8.2.3, thus giving slightly higher apparent values of the reaction rate coefficient. It can be concluded therefore that excellent agreement with the data of Vickery (1922) was achieved.

4.11 Further discussion of kinetics experiments

The approach and technique used in this work have proven generally satisfactory with some difficulties highlighted. The kinetics of acid hydrolysis of amide and peptide bonds of gluten have been shown to:

(a) follow the kinetics of hydrolysis of amides established by several authors as discussed in Section 1.2.2

(b) follow the kinetics of peptide bond hydrolysis established with model systems as discussed in Section 1.2.5

Two problems complicated the kinetic analysis. The first was the 'bench reaction' which took place for the experiments with higher acid concentrations. It is suggested that this could be avoided by sampling directly from the reaction and quenching the reaction, by neutralization.

The second and major problem was that of the complications due to the pH electrode. These could be avoided by using an excess of acid and determination of the acid concentration or activity by calculation. In the present work drift in the electrode readings, whether due to high acid concentrations or the presence of protein added to the error of the determinations. The lack of fit (LOF) calculations discussed in Section 4.8 are based on repetition experiments with conditions that fall in the middle of the range of experimental conditions. The difficulties in pH measurement occurred at the lowest and highest pH values. This may mean that the LOF calculations shown in Tables 4.33-4.35 overestimate the lack of fit. This type of problem was discussed by Shelton et al (1983). Such

considerations do not, however, affect the conclusions drawn from the experiments in the present work.

The choice of the initial rate form of analysis for the kinetic data (Levenspiel, 1972) is particularly appropriate for this present work. Most methods of kinetic analysis depend upon the reaction rate coefficient, usually using the symbol k, being a constant. Where the nature of the reaction changes, in this case peptide bonds between different amino acids are being hydrolysed and amide bonds may vary in their exposure to solvent due to the orientation of the protein as well as there being some amide groups on asparagine rather than glutamine molecules, it is inevitable that the reaction rate coefficients, k's, are a function of the extent and possibly the conditions of the reaction. Analysis of such reactions using an integral form of analysis (Levenspiel, 1972) may lead to incorrect conclusions about the kinetics if the reaction rate coefficients are assumed constant. Such difficulties are avoided with the initial rate form of analysis.

A visual technique was used to determine initial reaction rates as described in Section 4.8. Accurate determination of initial reaction rates is often difficult and various techniques have been applied to assist in their measurement. One such technique is that described by Cornish-Bowden (1975), which appears to be suitable for this application.

Other studies of hydrolysis catalyzed by acids have investigated the problem of how to express the effect of acid concentration. Should it be concentration, activity or some form of acidity function, as discussed in Section 1.2.2?

Duboux and Mermoud (1928) studied the inversion of sucrose catalysed by HCl between the concentrations of 0.091 and 3.60M, i.e. very similar conditions to those reported in this present work. It was concluded that the reaction rate was proportional to the activity of hydrogen ions and not the concentration. In similar studies both Barnett and O'Connor (1971) and Zhong et al

(1984) both found little difference between using activity and acidity function where the acid concentrations were in the range shown in this study. It is therefore concluded that a more complex analysis of the kinetic data of the present work using the acidity functions described in Section 1.2.2 is not warranted. This conclusion is supported by the data obtained i.e. good correlation between activity measurements and reaction rates.

It follows that over the range of acid concentration studied neither the level of protonation of the amide nor the activity of water in the reaction, which are the factors considered in various acidity functions, have any substantial effect.

A corollary to the above is that pH determination is a practical and meaningful method for control and analysis of hydrolysis reactions.

Knowing that hydrogen ion activity is a controlling factor in the reactions leads to an explanation of the results of some other authors. Both Aranyi and Hawrylewicz 1972) and Wu et al (1976) used acetic acid and hydrochloric acid as hydrolysis catalysts. Much greater concentrations of acetic acids were needed to achieve the same extent of hydrolysis as for hydrochloric acid using otherwise similar conditions. The effect can be explained by the partial dissociation of acetic acid in solution in contrast to the complete dissociation of hydrochloric acid so that similar results are obtained with similar concentrations of hydrogen ion in solution. A detailed explanation was given by Moore and Pearson (1981), (Section 1.2.2) and also demonstrated in the experiments of Mittal et al (1981).

The activity referred to is the measured activity which includes the errors discussed in Section 4.2.5, 4.5.2 and 4.8.1.3.

The activation energies reported in Sections 4.8.2.1 and 4.8.2.2 are similar to the values reported by Leach (1953).

The simulation data, discussed in Section 4.9 show that a good prediction of amide bond hydrolysis can be obtained up to a level of about 40% amide bond hydrolysis, the first 60 minutes of reaction in Figure 4.15. Beyond this level of hydrolysis the prediction is still useful but complications such as possible changes in reaction rate coefficient become apparent. The predictions for peptide bond hydrolysis are less certain but still useful up to a level of about 40% amide bond hydrolysis. The level of peptide bond hydrolysis after 60 minutes reaction in Figure 4.19 is only about 4%.

For peptide bond hydrolysis changes in k would be expected because of the many different types of peptide bond. The trend to overestimate the hydrolysis shown in Figure 4.19, and consequentially the hydrogen ion consumption in Figure 4.20 is the expected result.

Conventional kinetic analysis treats reaction rate coefficients as constant. Where it is likely that the coefficients change, as in this present work, initial rate analysis is a safe first approach.

For amide bond hydrolysis changes in k might be due to a difference in rate of hydrolysis of amide groups on asparagine as compared to glutamine. For gluten the proportion of asparagine compared to glutamine is small, Table 1.1, so the effect should also be small.

There may also be some effect of neighbouring groups on the hydrolysis of amide groups in glutamine.

5. PREPARATION OF TEST SAMPLES FOR FUNCTIONAL ANALYSIS

5.1 Choice of sample preparation conditions

The work of Wu (1976) showed that large changes in functional properties could be expected over a small initial range of deamidation, shown in Figure 1.3. In the present work it was desired to both effectively examine functional property changes over this initial range and also at higher levels of deamidation. This was done by first choosing a set of initial hydrolysis conditions. The criteria for choice were:

(i) That time of reaction should be the only hydrolysis variable. Hydrolysis at, for example, different temperatures might have effects other than that due to the deamidation.

(ii) That the reaction should proceed to reasonably high levels of deamidation within a reasonable length of time.

(iii) That the reaction should be at a moderate temperature.

The conditions chosen to meet these criteria were:

gluten at 10% w/v
HCl to give $[H^+] = 0.5$ g-mol l^{-1}
and heating at 70°C for periods of up to five hours.

The reaction times were chosen so that the majority of the samples would have relatively low levels of deamidation. Repetition of two runs was included so that an estimate of variation in the preparation technique could be made. The reaction times chosen are shown in Table 5.1.

5.2 Choice of sample recovery conditions

Most earlier studies of the deamidation of gluten by acid hydrolysis used precipitation to recover the protein.

Table 5.1: Design and actual reaction conditions for the preparation of hydrolysed gluten powders

PREPARATION NUMBER	DESIGN REACTION TIME AT 70°C (s)	ACTUAL REACTION TIME (s)	MEAN REACTION TEMPERATURE (°C)
(1)	(1)	-	-
(2)	nil(2)	-	-
(3)	80	90	65
(4)	120	135	64
(5)	120	140	72.5
(6)	270	174	67
(7)	600	660	71
(8)	1380	1380	75
(9)	3600	3900	72
(10)	3600	3660	64
(11)	18000	18000	71

Note: (1) Gluten suspension - no acid added
(2) Acid added but no heating

The data of Wu (1976) indicate that losses of protein, when recovered by precipitation, would increase as protein soubility increased. When dialysis was used for protein recovery, losses increased with increasing levels of deamidation.

It was thought that a study of the functional properties of only the precipitated protein, particularly when the recovered proportion was low, might lead to results not representative of the functional changes occuring. For this reason it was decided to use some form of dialysis. Ultrafiltration was considered. Contact with hydrochloric acid is not desirable for ultrafiltration membranes so neutralization of the hydrolysate was investigated. In one preliminary product preparation trial it was found that addition of concentrated NaOH resulted in the hydrolysed gluten suspension turning to a grey-green colour. Laboratory experiments showed that the colour development was pH dependent. Colour development began at about pH 7 and increased with increasing pH. Localised high pH due to poor mixing needed to be avoided in order to avoid colour development even at moderate pH. This colour development phenomenon is thought to be due to a reaction associated with iron (Southward, 1985). The iron content of the gluten was found to be about 65 ppm (analysis performed by NZ Ministry of Agriculture and Fisheries, Regional Dairy Laboratory, Mt Maunganui, New Zealand). It was decided to use dialysis of the hydrolysate without neutralization.

After dialysis the gluten suspension was to be spray dried. Vital wheat gluten is prepared using pneumatic conveying driers and retains satisfactory functional properties. The effect of drying, pH at drying, and other factors were investigated by Pfeifer et al (1958). It was thought that the spray drying would have little effect but a small portion of the dialysed hydrolysate was freeze dried as a control sample.

5.3 Sample preparation details

Three kg of gluten were added to 25.8 kg of tap water by shaking gently through a coarse mesh sieve with stirring to prevent lumps forming. Then 1.98 l of concentrated HCl were added. It was important not to delay too long before processing after adding the gluten or the acid because the gluten formed a large tough lump of dough.

The acid gluten mixture was then poured into a small feed tank situated immediately above the suction intake of a twin lobe positive pump (Howard, Eastbourne, East Sussex BN227SE, England) which was connected to a parallel plate spiral heat exchanger (W Schmidt KG D-7518 Bretten, West Germany). The heat exchanger was heated with hot water. The emerging gluten-acid mixture was held for the required time by either pumping through tubing of the required length and cooling through an Alfa Laval A3HRB plate heat exchanger (Alfa Laval, Hamilton, New Zealand) or, for holding times of more than a few minutes, by passing the heated mixture into a container which was held in a water bath to maintain the required temperature. After the required holding time the acid-gluten mixture was passed back through the Schmidt heat exchanger, operating wth cold water to cool.

The gluten was present as a heavily flocculated suspension, the extent of which varied somewhat depending on how long it was held before treatment. Several heat exchangers were tested but only the Schmidt heat exchanger could process the suspension without blocking. The pump and heat exchanger combination was run on water to obtain stable flow and temperature before the gluten-acid mixture was added to the feed tank. A small quantity of acid was added to the water in the feed tank as it was drawn down to avoid lumps of gluten being drawn into the system ahead of the main flow and being heated in a no-acid environment. The residence time in the heat exchanger and tubing systems was observed by injection of a dye marker into the bottom of the feed tank when the system was operating on water. Dye injection was complete within one second. Residence time

was reckoned from the first appearance of the dye and the residence time distribution as the length of time the dye was visible in the heat exchanger or tubing exit.

There were some difficulties in controlling the system temperature because of variations in the temperature of the hot water feeding the system. In addition when the change from water to acid gluten mixture was made the temperature sometimes, but not always, dropped 2-3°C. The temperature of the flow from the heat exchanger was recorded throughout each run with approximately six readings for each run. The arithmetic mean temperature for each run is recorded in Table 5.1 and the data represent a range of about 4°C for each run.

The dye appeared at the exit of the Schmidt heat exchanger after about 30 seconds with a residence time distribution of about 15 seconds which increased to about 30 seconds over the whole system. With the gluten-acid mixture in the system the residence time distribution appeared to be even greater. After about 2 minutes treatment the gluten-acid suspension was smooth rather than flocculated. The first two litres of suspension to come through the system were obviously diluted with water and discarded. Similarly, the final few litres were discarded. The residence time distributions and the time required to pump a batch through the cooling system, 2-3 minutes, for the longer holding times, made estimation of a batch holding time difficult. The times recorded in Table 5.1 are estimates and can probably be regarded as having an accuracy of $\pm$ 1 minute.

The cooled gluten suspensions were pumped using a small peristaltic pump into dialysis tubing cut into approximately 0.6 m lengths (size 36 dm, Union Carbide Corporation, Chicago, Illinois, USA). The progress of the dialysis was monitored by pH measurement of the dialysate. After dialysis the hydrolysed gluten suspension was removed from the tubes and stored frozen until required for spray drying or freeze drying.

For spray drying the frozen suspensions were thawed at room temperature. Immediately before drying the suspensions were passed through a colloid mill (Model JV 10, Probst and Class, Rastatt, Baden, Germany) in order to break up the heavy flocs which formed during dialysis and freezing.

Spray drying was carried out using a pilot scale spray drier with an atomizer disc (nominal capacity 80 kg/hr) (Anhydro, Copenhagen, Denmark). The drier was operated so that the inlet temperature was 190°C outlet temperature 68°C and atomizer speed was 13,500 rpm.

Some of the gluten stuck to the walls of the drier in a ring around the atomizer. Some powder was removed by the powder collection system and kept separate from powder which collected on the bottom cone of the drier which was removed separately as a 'sweepings' fraction.

The powder was stored in sealed polythene bags. An exception was the powder samples used for tasting. These were stored in heat sealed laminated foil sachets until required.

Samples of dialysed gluten suspension for freeze drying were frozen in small loaf tins and dried over a period of about 38 hours using a tray temperature of 30°C.

Samples were taken from various points in the process. Liquid samples were taken of the gluten-acid suspension before and after treatment. The pH was measured and the after treatment samples were stored frozen. A sample of the suspension after dialysis was taken, the pH measured, and then stored frozen for later analysis. Samples of spray dried powder and sweepings from each run were taken, and similarly of the freeze dried powder.

Three kg of gluten were dispersed in water and spray dried to test the spray drying system. The powder produced was included as an additonal control sample. Two further samples were

prepared with the objective of allowing the hydrolysis to proceed to completion, that is, most of the hydrogen ion consumed and then drying without dialysis. Twenty-five grams of gluten were weighed into a 250 ml pyrex glass flask with a screw cap.

Sample A was dispersed with 229.1 ml water and 2.083 ml 12M HCl (total volume 250 ml)

Sample B was dispersed with 230.9 ml water and 0.417 ml 12M HCl (total volume 250 ml).

The flasks were placed in a water bath controlled at 70°C.

After 5 hours the flasks were removed and samples taken for ammonia analysis. Heating of the flasks continued the next day and on several subsequent days, with the flasks standing at room temperature, between heating periods. Finally, after 70.5 hours at 70°C over a period of 24 days, further samples were taken for ammonia analysis and the contents of the flasks were poured into shallow trays, frozen and then freeze dried a few days later.

5.4 Analysis of process samples

Chemical analyses of liquid and powder samples were performed by the Analytical Chemistry Section of the New Zealand Dairy Research Institute using the methods described in Ministry of Agriculture and Fisheries (1979).

Sample numbers are linked to specific treatments in Table 5.1. The analysis of liquid samples is shown in Table 5.2 (in this and other tables values given as percentages are percent w/w). The data show the consumption of hydrogen ions increasing with increasing treatment time. The non protein nitrogen, that is peptides not precipitated by trichloroacetic acid, also increases indicating peptide bond hydrolysis. In dialysis there appears to be a substantial loss of solids, total nitrogen (protein) and a high loss of non protein nitrogen (lower

Table 5.2: Analysis of liquid samples during hydrolysate preparation

PREPARATION NUMBER	SUSPENSION pH BEFORE HEATING	SUSPENSION pH AFTER HEATING	SUSPENSION AFTER HEATING			SUSPENSION pH AFTER DIALYSIS	SUSPENSION AFTER DIALYSIS		DIALYSIS
			TOTAL SOLIDS (%)	TOTAL NITROGEN (%)	NON PROTEIN NITROGEN (%)		TOTAL SOLIDS (%)	TOTAL NITROGEN (%)	NON PROTEIN NITROGEN (%)
(1)	-	-	-	-	-	-	-	-	-
(2)	0.302	0.302	11.85	1.12	0.054	4.061	n.d.	n.d.	n.d.
(3)	0.331	0.334	11.18	0.99	0.066	3.8	6.19	0.77	6.012
(4)	n.d.(1)	0.354	10.9	1.12	0.055	3.904	5.65	0.72	0.010
(5)	0.328	0.344	10.8	1.14	0.069	3.930	7.71	0.95	0.012
(6)	0.325	0.367	9.0	0.94	0.054	3.853	5.23	0.66	0.010
(7)	n.d.	0.371	11.1	1.17	0.091	3.683	6.63	0.84	0.020
(8)	0.325	0.374	11.1	1.21	0.159	5.086	6.05	0.79	0.033
(9)	0.315	0.390	11.4	1.26	0.217	4.747	4.24	0.71	0.045
(10)	0.324	0.415	10.0	0.95	0.149	3.897	5.02	0.67	0.030
(11)	0.330	0.426	11.6	1.24	0.280	4.473	5.18	0.59	0.052

Note (1) n.d. = not determined

molecular weight) fractions. The loss of almost half of the protein in dialysis was surprising. Dialysis with Visking (Union Carbide) tubing is common. A search of Chemical Abstracts (available on Dialog, a computerised database) found no measurements of the molecular weight selectivity of the tubing and no information could be supplied by the manufacturers. Incomplete information is given by Stewart (1977).

Analysis of the gluten used, Table 5.3, shows little variation as might be expected. The chloride levels reflect the use of sodium chloride in the preparation of gluten.

The analysis of the hydrolysed gluten powders, Table 5.4, is less consistent but some trends can be seen. The level of non-protein nitrogen is high at higher levels of hydrolysis. The chloride levels indicate that most of the acid was removed by dialysis. No pattern can be seen in the weights of spray dried powder recovered but the total amounts recovered are consistent with some processing losses and approximately half lost in dialysis. It follows that the amount caught up in the spray drier was small.

The degree of deamidation of the sample preparations was determined by various means. First, the spray dried powders were subject to complete deamidation for 2, 4 and 6 hours as described in Section 4.7.3. The analysis details are shown in Appendices 5.1 and 5.2 in the attached fiche and the results in Table 5.5. The results have not been corrected for the moisture differences between the gluten and the spray dried powder. The calculation example in Table 5.5 shows that the error is small. Next, the liquid hydrolysates were retrieved from frozen storage and the level of ammonia determined. These values do not require correction for moisture differences but clearly the hydrolysis has continued, albeit at a low rate, during storage. All the values are therefore rather high but confirm the values obtained by deamidation of the powders. The analysis details are shown in Appendices 5.3 and 5.4 in the attached fiche. As a

Table 5.3 Analysis of gluten before hydrolysis

PREPARATION NUMBER	MOISTURE (%)	TOTAL NITROGEN (%)	NON PROTEIN NITROGEN (%)	FAT (%)	CHLORIDE $mM.kg^{-1}$
(2)	7.48	12.85	0.13	4.8	34
(3)	7.99	12.65	0.15	5.2	51
(4)	7.56	12.65	n.d.(1)	n.d.	n.d.
(5)	7.48	12.78	0.13	5.7	58
(6)	7.88	12.71	n.d.	n.d.	n.d.
(7)	7.78	12.71	n.d.	n.d.	n.d.
(8)	7.81	12.81	0.17	6.2	64
(9)	7.87	12.82	0.17	4.5	71
(10)	7.39	12.85	n.d.	n.d.	n.d.
(11)	7.70	12.91	n.d.	n.d.	n.d.
Mean:	7.69	12.77			
Standard Deviation	0.20	0.09			

Note (1) n.d. = not determined

Table 5.4: Analysis of hydrolysed gluten powders

PREPARATION NUMBER	MOISTURE (%)	TOTAL NITROGEN (%)	NON PROTEIN NITROGEN (%)	FAT (%)	CHLORIDE ($mM.kg^{-1}$)	MOISTURE OF SWEEPINGS (%)	MOISTURE OF FREEZE DRIED POWDER (%)	WEIGHT OF SPRAY DRIED POWDER (g)	WEIGHT OF SPRAY DRIED SWEEPINGS (g)
(1)	4.8	n.d.[1]	n.d.	n.d.	n.d.	n.d.	n.d.	840	n.d.
(2)	3.68	13.93	0.12	4.3	227	Note (1)	0.76	560	n.d.
(3)	3.72	13.78	0.19	3.6	147	1.55	0.68	1360	400
(4)	4.15	14.08	0.15	3.4	169	2.10	1.66	935	760
(5)	3.19	13.73	0.19	4.3	124	2.18	1.98	1350	410
(6)	4.22	13.70	0.24	3.9	147	1.74	2.03	1080	230
(7)	3.78	13.41	0.29	4.6	129	3.18	1.45	1240	387
(8)	5.57	10.80	1.23	5.6	72	2.63	14.28	376	1030
(9)	4.15	12.59	0.89	5.6 7.0	89	1.14	0.31	836	873
(10)	3.62	12.22	0.64	5.7 7.2	95	1.13	3.40	700	400
(11)	4.75	12.60	0.87	6.4	51	1.60	6.21	985	615
(12)[2]	1.81	13.04	1.88	5.97	505				
(13)[2]	1.79	13.43	0.55	6.10	263				

Note (1) n.d. = not determined

Notes: (1) For this sample a powder was obtained as for the other samples but the inside of the drier was coated with a sticky mess. That is, no sweepings could be recovered.

(2) These are the additional samples prepared without dialysis with freeze drying.

Table 5.5: Determination of the degree of amide bond hydrolysis of spray dried samples determined by complete deamidation and also by ammonia determination of stored liquid hydrolysates

PREPARATION NUMBER	AMMONIA FROM TOTAL DEAMIDATION (mM)	AMMONIA ±2 s.d. (mM)	DEGREE OF DEAMIDATION (%)	DEGREE OF DEAMIDATION ±2 s.d. (%)	AMMONIA IN STORED LIQUID HYDROLYSATE (mM)	AMMONIA ±2 s.d. (mM)	DEGREE OF DEAMIDATION (%)	DEGREE OF DEAMIDATION ±2 s.d. (%)
(1)	20	17.2-21.6	0	- 8 - 14	-	-	-	-
(2)	21	18.2-23.7	-5	-19 - 9	29	19.6-35.2	14	10-18
(3)	20.2	18.3-22	-1	-10 - 9	27	24.4-29.6	14	12-15
(4)	21	19.4-23.6	-5	-18 - 3	24	18.5-30.8	12	9-15
(5)	21	19.6-22.4	-5	-12 - 2	29	27.2-29.6	15	14-14
(6)	20.2	17.4-23.2	-1	-16 - 13	20	18.4-21.6	10	9-11
(7)	17.7	16.7-18.7	11.5	6.5 - 17	40	35.6-45.2	20	18.23
(8)	13.2	12.2-14.2	34	30 - 39	70	60.4-80.0	35	30-40
(9)	12.7	12.3-13.1	37	35 - 39	99	78.0-119.4	51	39.60
(10)	14.3	13.4-15.1	29	25 - 33	65	58.0-72.0	33	29-36
(11)	9	7.8-10.3	55	49 - 61	141	127-157.0	70	64.79

Example moisture correction calculation

Mean initial gluten moisture = 7.69% (Table 5.3)
Therefore, expected ammonia for complete deamidation (mfb) = 21.54mM
For Sample (8) ammonia = 13.2 mM at 5.57% moisture (Table 5.4)

On a moisture free basis ammonia = 13.9%

Therefore, Degree deamidation mfb = 35.3%
Degree deamidation uncorrected = 34%

further check the level of deamidation of the freeze dried samples was determined. Details of the analysis are shown in Appendices 5.5 and 5.6 in the attached fiche. The results are shown in Table 5.6. Again, no corrections have been made for moisture content. There appears to be no significant difference in the level of deamidation compared to the spray dried samples and none was expected. The level of deamidation of the freeze dried samples prepared without dialysis was determined in the same experiment. The results, shown in Table 5.7 indicate that no deamidation has taken place. This is a false result because the ammonia released by the hydrolysis would have been retained with the sample during drying since there was no dialysis for its removal. This is confirmed by analysis of the corresponding samples of hydrolysate. The details of these analyses are shown in Appendices 5.5 and 5.7. The results in Table 5.7 show the high and low levels of deamidation expected due to the original acid additions described in Section 5.3.

The level of peptide bond hydrolysis was estimated by determination of N-terminal amino groups using fluorescamine. Spray dried powder samples, 0.05g were weighed into reaction bottles and water and acid were added so that the final volume was 5.0 ml and the acid concentration was 1.0M. The purpose of this procedure was to assist in dispersion of the samples. The suspensions were then sampled and the determinations made as previously described in Section 2.6.1. The details of the determination are shown in Appendices 5.8 and 5.9 in the attached fiche. The results of the analysis are shown in Table 5.8. The data show that the level of hydrolysis increases with increasing treatment time as expected.

5.5 Comparison of experimental and simulated hydrolysis

Two possible approaches for comparing simulated and experimental data would be appropriate here. The first would be to take the measured conditions for each product preparation, Table 5.1, and run the simulation for the appropriate reaction time. A second approach would be to run the simulation using the average

Table 5.6: Determination of the degree of amide bond hydrolysis of freeze dried samples determined by complete deamidation

PREPARATION NUMBER	AMMONIA HYDROLYSIS	AMMONIA ± 2 s.d.	DEGREE OF DEAMIDATION	DEGREE OF DEAMIDATION ± 2 s.d.
(1)	n.d.(1)	n.d.	n.d.	n.d.
(2)	21.6	18.2-24.8	-8	-24-9
(3)	21.2	18.8-23.6	-6	-18-6
(4)	21.6	19.0-24.2	-8	-21-5
(5)	21.4	17.8-25.0	-7	-25-11
(6)	21.6	18.0-25.0	-8	-25-10
(7)	21.8	15.7-23.6	-9	-18-22
(8)	16.9	14.2-19.6	16	2-24
(9)	14.2	12.2-15.5	29	23-49
(10)	16.6	14.6-18.8	17	6-27
(11)	8.6	6.2-10.6	57	47-69

Note (1) n.d. = not determined

Table 5.7: Determination of the degree of amide bond hydrolysis of samples prepared without dialysis

PREPARATION NUMBER	AMMONIA IN HYDROLYSATE AFTER: INITIAL (mM)	5 HRS (mM)	75 HRS (mM)	DEGREE OF DEAMIDATION (%)	AMMONIA FROM COMPLETE DEAMIDATION OF DRY SAMPLE (mM)	APPARENT DEGREE OF DEAMIDATION (%)
(12)	3	7	59	30	21.4	nil
			52	26		
(13)	2.6	0.8	3.4	2	21.6	nil
			4.4	2		

Table 5.8: Determination of the degree of peptide bond hydrolysis of spray dried samples by determination of N-terminal amino groups

PREPARATION NUMBER	L-Glu IN SOLUTION (mM)	L-Glu ±2 s.d. (mM)	DEGREE OF HYDROLYSIS (%)	DEGREE OF HYDROLYSIS ±2 s.d.
Gluten	3.0	-6-10.2	1.4	-10-10.4
(1)	2.0	2.0-2.0	0.1	0.1
(2)	2.3	2.1-2.5	0.5	0.2-0.7
(3)	1.9	0.8-3.0	0.0	-1.4-1.4
(4)	2.0	1.6-2.4	0.1	-0.4-0.6
(5)	2.5	1.8-3.2	0.7	-0.2-1.6
(6)	2.5	1.7-3.3	0.7	-0.3-1.7
(7)	2.9	2.6-3.3	1.2	0.9-1.7
(8)	3.4	2.7-4.2	1.9	1.0-2.9
(9)	3.7	3.3-4.0	2.2	1.7-2.6
(10)	3.9	3.2-4.7	2.5	1.6-3.5
(11)	4.7	3.8-5.6	3.5	2.4-4.6

conditions and attempt to explain deviations from the simulated result in terms of the actual experimental conditions, e.g. in this instance, variations in temperature. Because there is some degree of choice in the rate coefficients used in the simulation, as discussed in Section 4.9 it was simpler to use this second approach since fewer simulation runs are necessary.

The initial conditions chosen for the simulation are shown in Table 5.9. The amide and peptide concentrations were calculated from the gluten concentration used and the measurements described in Section 4.7. The initial hydrogen ion concentration is a mean value calculated from the measurements.

The reaction rate coefficient for amide bond hydrolysis k, was taken from line B in Figure 4.12. The reaction rate coeffficients for peptide bond hydrolysis were taken from lines D and C in Figure 4.13. The choices were based on the more extensive investigation reported in Section 4.9.

The data from the simulation are recorded in Appendix 5.10. The same data are shown in Figures 5.1, 5.2 and 5.3

The data from Tables 5.2, 5.5 and 5.8 were also plotted in Figures 5.1, 5.2 and 5.3. Each measurement was recorded at $\pm$ 2 standard deviations. A log-linear scale was chosen in order to conveniently represent the data.

By careful selection of the possible reaction rate constants a reasonable fit can be obtained over the early or, initial rate part of the reaction. It is clear, however, as suggested in Chapter 4, that the simulation does not adequately describe the whole of the reaction.

It can also be seen that an empirical model of the form

degree of hydrolysis or, pH = k'. ln (reaction time)

Table 5.9: Initial conditions and reaction rate coefficients for simulation of product preparation reactions

Initial conditions:

Amide	=	0.20M
Peptide	=	0.793M
H^+	=	0.476M
Temperature	=	70°C

Reaction rate coefficients:

for line A $k_1 = 2.85 \times 10^{-4}$ (from line B, Figure 4.12)
(Figures 5.1-5.3)
$k_2 = 9.3 \times 10^{-6}$ (from line D, Figure 4.13)

Activity correction factor = 0.60

and for line B as above exceptthat
(Figures 5.1-5.3) $k_2 = 3.0 \times 10^{-5}$ (from line C, Figure 4.13)

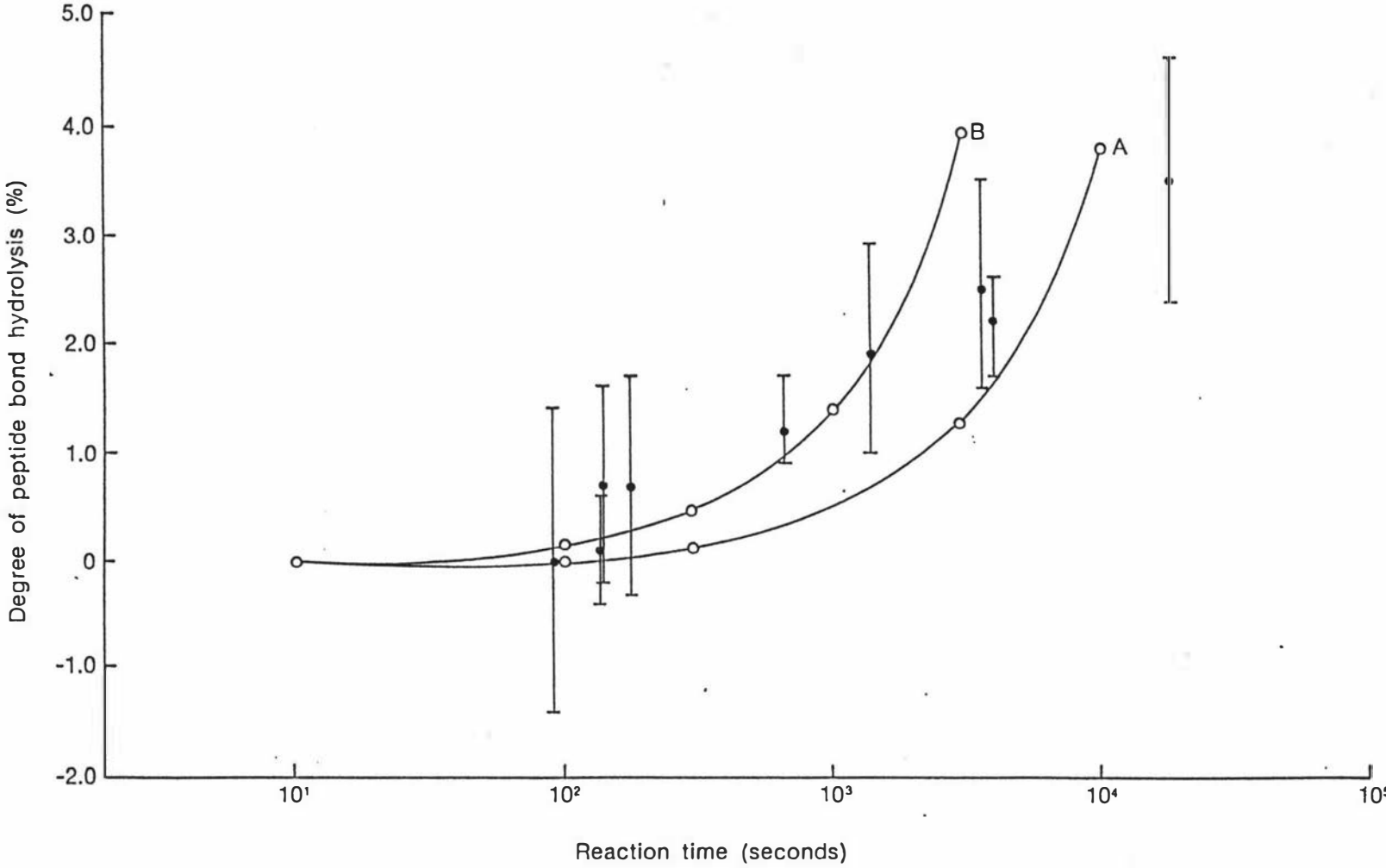


Figure 5.1 Predicted and experimental levels of peptide bond hydrolysis for product preparations.

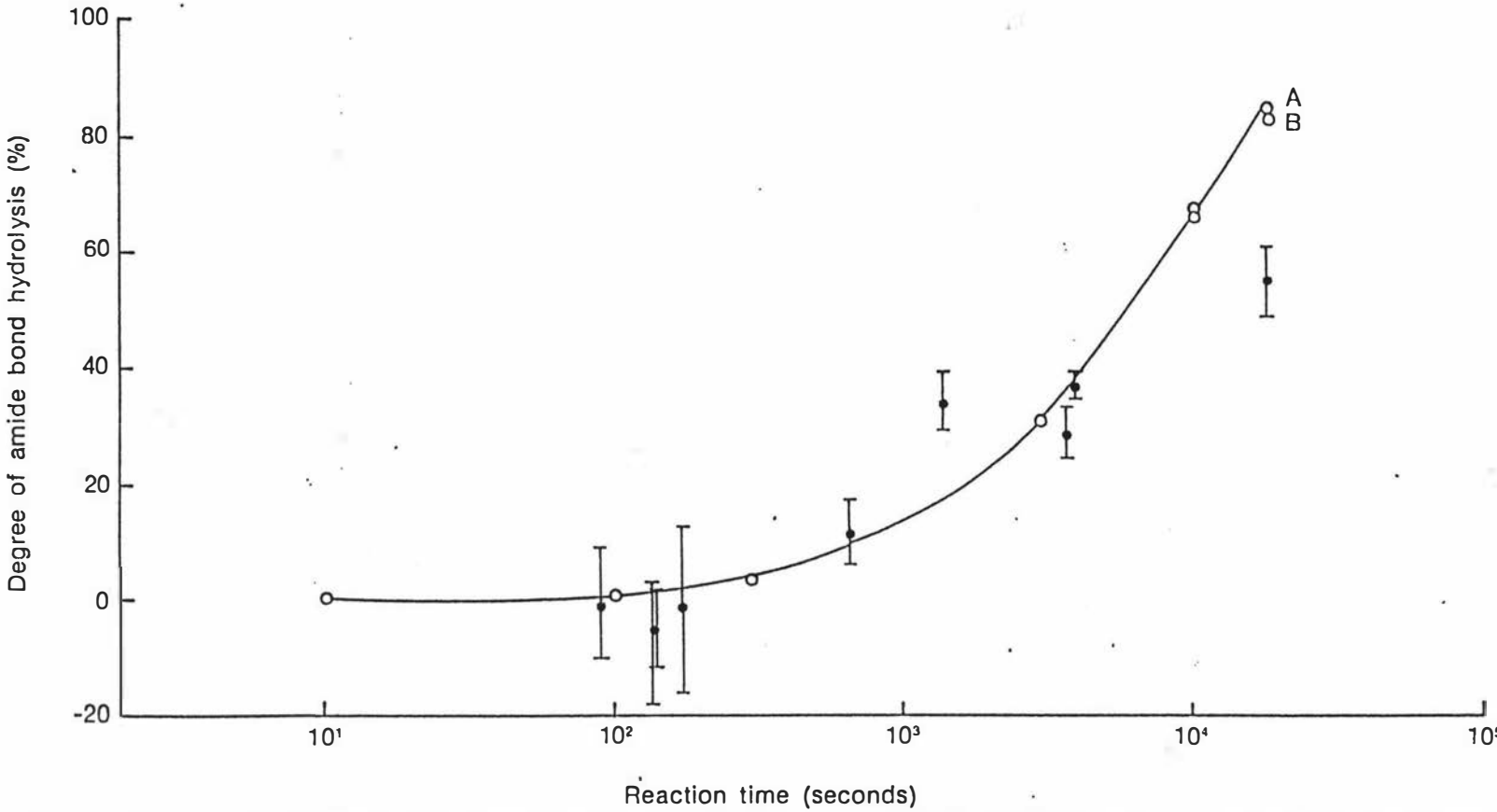


Figure 5.2 Predicted and experimental levels of amide bond hydrolysis for product preparations

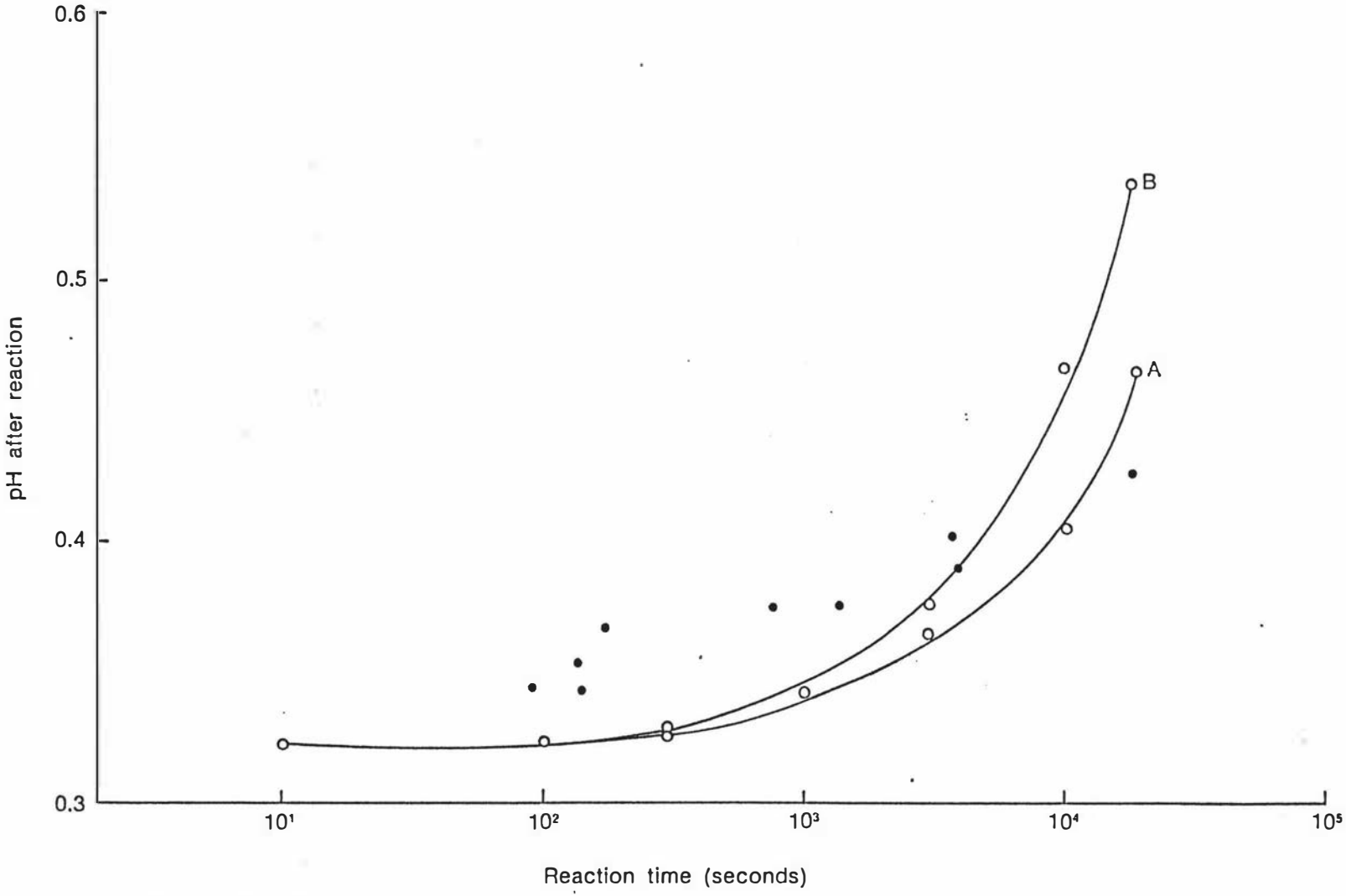


Figure 5.3 Predicted and experimental levels of peptide bond hydrolysis for product preparations.

where k' is a function of the initial conditions

would fit the data quite well.

5.6 Discussion

A wide range of deamidation levels was achieved to provide a basis for further functional testing.

There must be some doubt, however, as to whether the samples are representative of likely commercial production. Some fractionation may have taken place when material, probably insoluble aggregates, stuck to the drier wall, although not a large proportion was lost in this way. Commercial production in a drier with a much greater diameter would probably avoid the problem.

The material lost in the dialysis was probably mostly smaller, soluble molecules which might be expected to make a substantial contribution to functional properties. Other authors, as described in Sections 1.2.7 and 1.4.2 make no mention of the effects of fractionation by either dialysis or precipitation but attribute functional changes solely to protein modification.

Recovery in high yield would be an important factor in any commercial production of acid hydrolysed gluten.

Dispersion of the gluten in flocs or smooth dispersion does not appear to affect the rate of hydrolysis but is obviously important from a materials handling point of view. This should be a factor in choosing the reaction pH.

The data shown in Figures 5.1 to Figure 5.3 confirm the data of Section 4.9. That is that the simulation tends to overestimate the extent of reaction probably because of the apparent change in reaction rate coefficients.

6. FUNCTIONAL PROPERTIES OF TEST SAMPLES

6.1 Determination of the alkali requirement of hydrolysed glutens

6.1.1 Introduction

Alkali requirement is commonly used as a convenient measurement of the amount of alkali required to dissolve casein in the preparation of caseinates. The value is a function of both the inherent properties of the protein and the ions present which in turn depend on the casein preparation process (Roeper, 1974). For casein the alkali requirement is defined as the volume of 0.1M NaOH required, in ml/gm of casein, to convert an aqueous dispersion of acid casein into a 50 g/litre sodium caseinate solution having a pH of 6.7 at a temperature of 20°C (New Zealand Ministry of Agriculture and Fisheries, 1983).

A similar measurement can be made for hydrolysed glutens where the alkali required would be expected to vary according to the number of carboxyl groups to be neutralized, that is, depending on the extent of deamidation and the residual acid.

After some initial trial and error the alkali requirement method for casein was adapted for use with hydrolysed glutens.

6.1.2 Method

A two litre batch of 0.5M NaOH was prepared from a concentrated solution designed to give one litre of 1.0M NaOH (BDH Chemicals Ltd., Poole, England).

Forty-five ml of water was added to a tall form 300 ml stainless steel beaker containing 2.50 ($\pm$0.01) g of gluten sample. A measured amount of 0.5M NaOH was added from a 10 ml burette.

The beaker was then placed in a water bath controlled at 60°C and the contents gently mixed with a turbine mixer. The beaker was covered with an aluminium foil cap to minimize evaporation. After twenty minutes the beaker was removed from the bath and part of the contents transferred to a McCartney bottle in a waterbath controlled at 25°C. After a suitable time for temperature equilibration the pH was measured. By adding varying amounts of alkali a graph of alkali requirement vs pH could be constructed.

6.1.2.1 Method Test

For some samples, particularly the sparingly treated glutens, the dispersion was poor. After twenty minutes of mixing the undissolved solid consisted of one or more thoroughly wetted dough lumps. It was thought that 60°C may have been too high a temperature and was causing heat gelation of the gluten. Untreated gluten, the most difficult material to disperse, was mixed at different temperatures with the result shown in Table 6.1. It was concluded that 60°C was satisfactory, because when the data were compared with that obtained later, shown in Figure 6.1, it was seen that the reaction was incomplete at 20°C i.e. the pH was higher than expected but at the expected level for reactions at 40°C and 60°C.

To check that a mixing time of 20 minutes was adequate a mixture of double quantity was prepared. Five gm of sample (10) was prepared in 90 ml of water and 6.17 ml of 0.5M NaOH was added. After 20 minutes mixing a sample was removed for pH determination. Mixing was continued and a second sample for pH determination was removed one hour later. The pH values were 6.439 and 6.398 respectively. The measurements indicated that a slight further reaction had occurred during the additional mixing time.

Table 6.1: Determination of the alkali requirement of gluten at various reaction temperatures

MIXING TEMPERATURE (°C)	ALKALI ADDITION (ml)	FINAL pH[1]	NATURE OF DISPERSION
ca 20	0.41	7.166	single lump
40	0.64	7.595	single lump
60	0.51	7.113	single lump

Note [1] A high pH, off the line in Figure 6.1 would indicate unreacted material

6.1.2.2 Variation of method for two samples

Only a limited quantity of samples (12) and (13) was available. The alkali requirement determination was made as previously described except that successive additions of alkali were made to one 2.5 gm sample. The procedure is shown in Table 6.2.

6.1.3 Results

A series of alkali additions and pH measurements was made for all spray-dried samples (see Table 5.1). The results are shown in Figure 6.1.

6.1.4 Discussion of results

The values found in these experiments have not been conclusively shown to represent an equilibrium value although the samples were not still rapidly dissolving when the measurements were made. The solubility of a protein found under any particular set of conditions may depend on the dissolving conditions as well as the inherent properties of the protein (Shen, 1976).

The data show an increasing alkali requirement consistent with the neutralization of glutamic acid carboxyl groups resulting from hydrolysis of the amide groups of glutamine. At pH 9-11 there is a discontinuity in the curves of Figure 6.1, probably representing the pK value of tyrosine and/or lysine (Edsall, 1943).

6.2 Flavour analysis of sample preparations

Samples, prepared as previously described were tasted as 5% total solids suspensions at pH 7.5 by a trained panel under controlled conditions. A detailed description of the methodology and results is given in Appendix 6.1.

The most noticeable feature of the taste was the burning/bitter sensation of some of the samples. This was mentioned by Wu et al (1976) and also by Fung et al (1977). Bitterness is a well

Table 6.2: Variation in alkali requirement determination procedure for samples (12) and (13)

(12)		(13)	
VOLUME 0.5M NaOH (ml)	pH	VOLUME 0.5M NaOH (ml)	pH
1.0	3.82	1.0	5.884
plus 5.05	8.891	plus 0.52	7.999
add 0.5g sample	7.015	add 0.5g sample	6.693

The data were then recalculated as follows:

(12)			(13)		
VOL NaOH (ml/gm)	pH	VOL NaOH (ml/2.5 gm)	VOL NaOH (ml/gm)	pH	VOL NaOH (ml/2.5 gm)
0.40	3.82	1.0	0.40	5.884	1.0
2.42	8.891	6.05	0.608	7.999	1.52
2.02	7.015	5.04	0.507	6.693	1.27

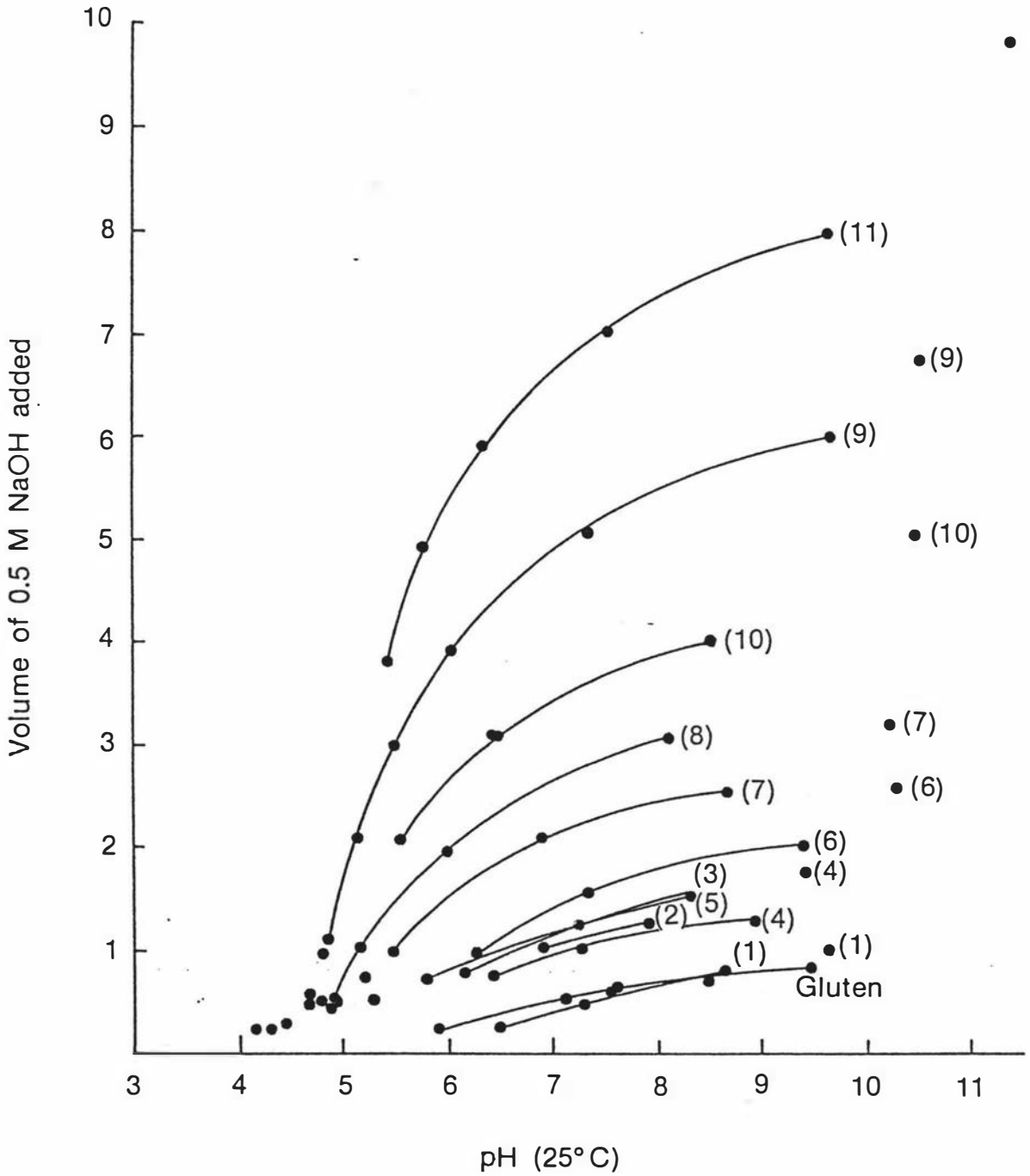


Figure 6.1 Alkali requirement versus pH for acid hydrolysed gluten samples.

known phenomenon when proteins are hydrolysed and its intensity depends on the level of peptide bond hydrolysis (Adler-Nissen and Sejr Olsen, 1979).

Figure 3 from the report is shown here as Figure 6.2 and clearly shows the increase in burning/bitter flavour as hydrolysis proceeds. For the flavour analysis sample numbers were assigned in ascending order of alkali requirement of pH 7.5 using the data from Figure 6.1. These numbers differ slightly from those assigned in Table 5.1 and the differences are shown in Table 6.3 and Figure 6.2.

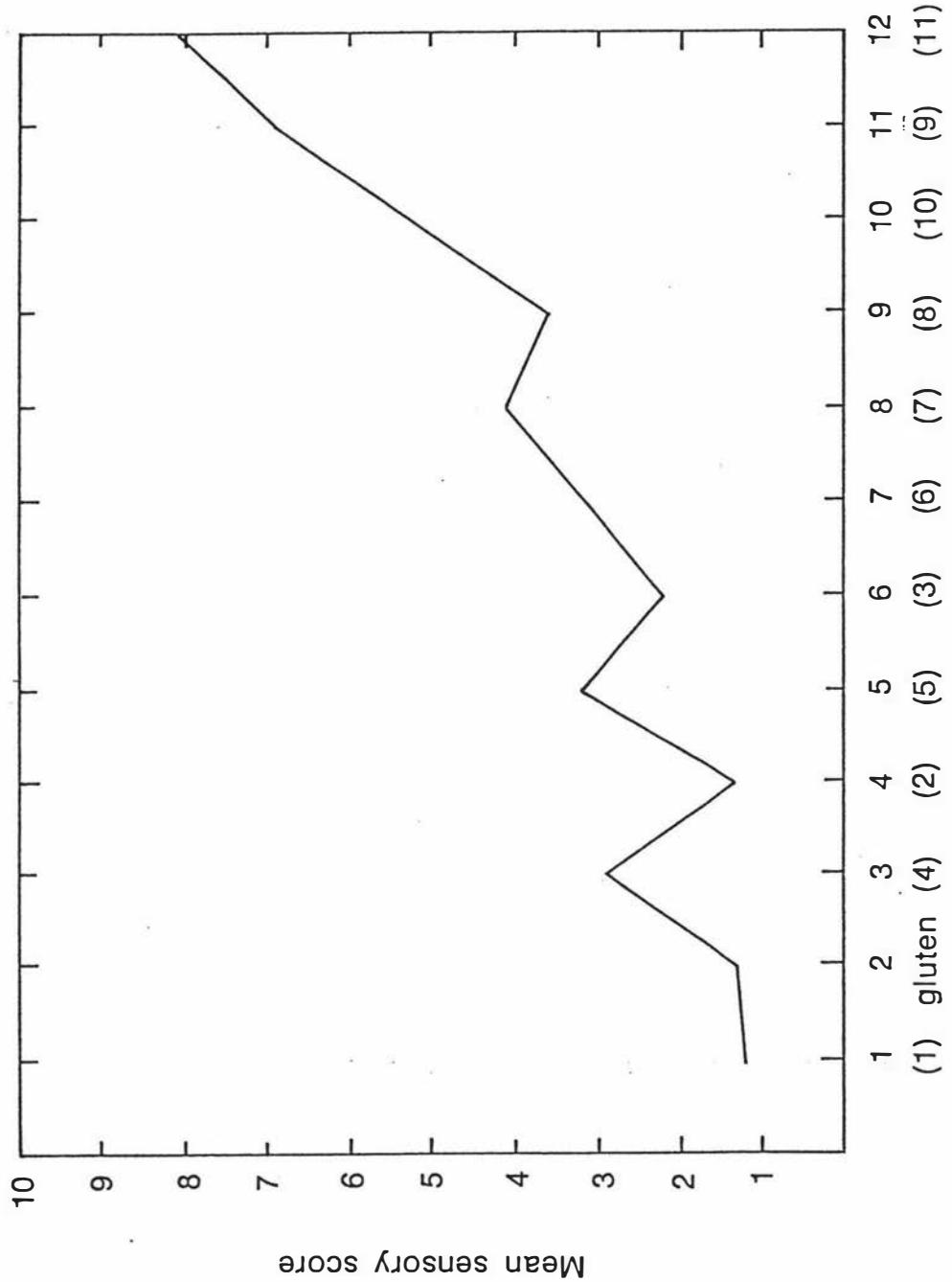
A decrease in wheaty cereal flavour and increase in rancid/nut flavour as hydrolysis proceeded were also noted.

6.3 Determination of solubility of sample preparations

Protein solubility is a function of the pH and concentration at which it is determined. The values obtained also depend on the method of dispersion (Shen, 1976). A distinction has been made between dispersibility and solubility depending on the centrifugal force used in separating dispersed (soluble) material from insoluble material, (Li-Chan et al, 1985). The method of determination of soluble material e.g. total solids, Folin-Lowry, absorbance (A280) or Biuret for protein, or, as in this case total nitrogen by Kjeldahl analysis, also has a considerable effect on the values obtained.

6.3.1 Determination method

The sample to be tested was weighed, 1.5 g, and transferred to a tall form 300 ml stainless steel beaker. Then, 47 ml of 0.2125M phosphate buffer, pH 7.6, 0.5M NaOH as required (Table 6.4) and water to make a total volume of 100 ml were added to the beaker with the sample. Dispersion was achieved by mixing with an Ultra turrax blender at full speed for 30 seconds. (Type 18/10, Janke and Kunkel GMbH and Co K.G., IKA-Werk D 7813 Staufen, West Germany). There was some slight loss of sample sticking to the mixing head



*Sample numbers in brackets are those assigned in Table 5.1

Figure 6.2 Effect of increasing levels of acid hydrolysis on the burning/bitter attribute of gluten.

Table 6.3: Sample codes of sample preparations for flavour analysis

TASTE PANEL EVALUATION NUMBERS	EQUIVALENT NUMBERS FROM TABLE 5.1
Sample 1	(1)
Sample 2	Untreated gluten
Sample 3	(4)
Sample 4	(2)
Sample 5	(5)
Sample 6	(3)
Sample 7	(6)
Sample 8	(7)
Sample 9	(8)
Sample 10	(10)
Sample 11	(9)
Sample 12	(11)

Table 6.4: Alkali additions, meausred pH and nitrogen solubility of sample preparations in 0.1M phosphate buffer

PREPARATION NUMBER	ALKALI[1] ADDITION 0.5M NaOH	pH[2] OF SUPER-NATANT (SPRAY DRIED SAMPLE)	pH[3] OF SUPER-NATANT (FREEZE DRIED SAMPLE)	VOLUME OF SEDIMENT IN 15ml (SPRAY DRIED SAMPLE) (ml)	VOLUME OF SEDIMENT IN 15ml (FREEZE DRIED SAMPLE (ml)	TN OF SUPER-NATANT (SPRAY DRIED SAMPLE) (% w/v)	TN OF SUPER-NATANT (FREEZE DRIED SAMPLE) (% w/v)	NITROGEN SOLUBILITY (SPRAY DRIED SAMPLE) (%)	NITROGEN SOLUBILITY (FREEZE DRIED SAMPLE (%)
Gluten	0.36	7.754	-	<0.1	-	0.008	-	4.2	-
(1)	0.34	7.747	-	<0.1	-	0.008	-	4.1	-
(2)	0.74	7.732	7.762	<0.1	<0.1	0.012	0.008	5.8	3.8
(3)	0.82	7.720	7.723	<0.1	<0.1	0.047	0.043	23.0	20.4
(4)	0.68	7.725	7.732	<0.1	<0.1	0.022	0.018	10.5	8.4
(5)	0.80	7.745	7.752	<0.1	<0.1	0.037	0.033	18.1	16.0
(6)	0.98	7.764	7.806	0.1	<0.1	0.051	0.033	25.1	15.9
(7)	1.42	7.746	7.783	0.2	0.3	0.120	0.112	60.2	55.9
(8)	1.76	7.751	7.736	1.0	0.95	0.117	0.118	72.9	79.6
(9)	3.12	7.741	7.698	0.85	0.75	0.167	0.180	89.3	92.7
(10)	2.28	7.773	7.796	1.1	1.2	0.135	0.145	74.4	79.7
(11)	4.26	7.751	7.707	0.15	1.12	0.135	0.178	90.3	96.4
(12)	3.42	-	7.786	-	0.72	-	0.174	-	89.8
(13)	0.89	-	7.745	-	0.1	-	0.023	-	11.5

Notes (1) From Figure 6.1 but adjusted for 1.5 g sample
(2) Buffer pH 7.700
(3) Buffer pH 7.701

and on the sides of the beaker. After dispersion, insoluble floating material was removed from the tubes which were then spun at 3000 rpm for 25 min in the MSE centrifuge. The volume of sediment was noted and the supernatant transferred to a sample bottle. The pH of each solution was measured and then the sample was submitted for determination of total nitrogen by Kjeldahl analysis.

6.3.2 Results

The results of the sediment and total nitrogen determinations are shown in Table 6.4. For the samples with less treatment much of the insoluble material floated on the surface. The sediment results, therefore, are not meaningful except for samples 8 to 12.

The calculations of percentage nitrogen solubility which are shown in Table 6.4, take account of the nitrogen added, from Tables 5.3 and 5.4, and the specific volume of the samples. It is assumed that the total nitrogen content of the freeze dried samples (not determined) is the same as that of the spray dried samples but correction has been made for the different moisture content. The data show good agreement between solubility determinations on corresponding spray dried and freeze dried samples. Regression analysis (not shown) gives R^2 = 99.0%. The data show no difference between spray dried and freeze dried samples, thus indicating that the spray drying is not detrimental. Similarly, there is good agreement between the alkali requirement and solubility data, that is, solubility increase is associated with an increase in free carboxyl groups due to deamidation. These features are illustrated in Figure 6.3 with data from Table 6.4.

6.4 Further analysis of alkali requirement and degree of amide bond hydrolysis results

The relationship between alkali requirement and degree of hydrolysis was examined by taking the alkali requirement data

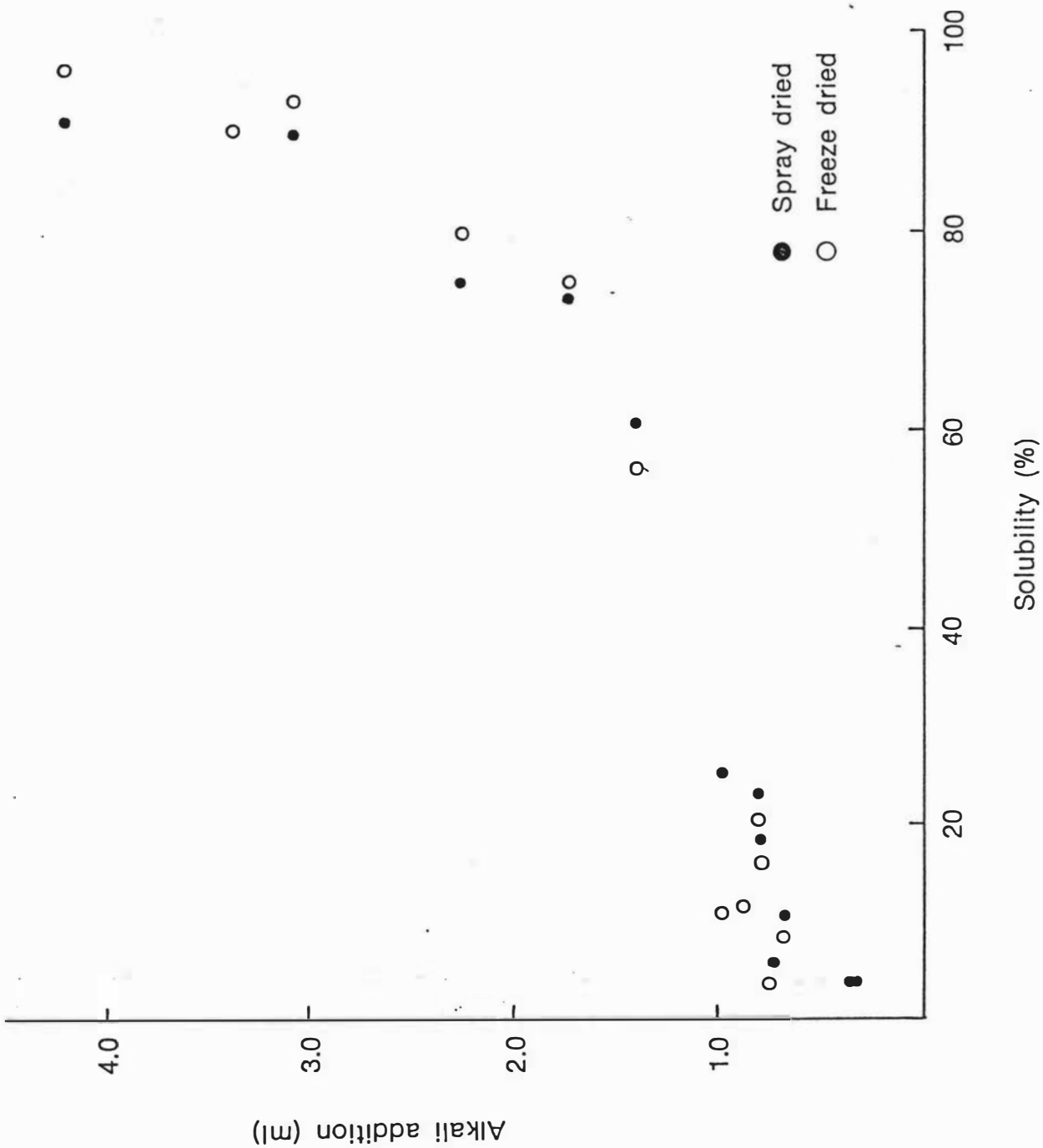


Figure 6.3 The relationship between alkali requirement and solubility for acid hydrolysed gluten samples.

from Table 6.4 and the degree of amide bond hydrolysis data (Tables 5.5 and 5.6) for the more extensively hydrolysed samples; 7, 9, 10 and 11, and gluten. Linear regression analysis was carried out on the data. The results are recorded in Table 6.5. The data for the spray dried samples are plotted in Figure 6.4 and all the regression lines are shown. The information can be used to estimate the degree of amide bond hydrolysis for slightly hydrolysed samples which cannot be distinguished by direct measurement of ammonia evolved in total deamidation. The alkali requirement data appear to give an accurate estimate of the level of deamidation.

6.5 Determination of emulsification properties

The emulsification properties of the sample preparations were examined with a view to testing the hypothesis proposed in Section 1.5.6, i.e. that the analogy between protein hydrophobicity and emulsifier HLB values is valid. It was decided that the test system should have the following features.

(1) A realistic ionic strength.

(2) A pH that gave a moderate solubility to the proteins.

(3) A high oil content so that the proteins were severely tested.

(4) Oils that remained liquid at room temperature so that the results were not complicated by phase changes.

Castor and soyabean oils were selected because their reported required HLB's were quite different, that is, 14 and 6 respectively (ICI Americas Inc., 1980).

6.5.1 Experimental methods

Emulsifiers used for comparison were Span 80 and Tween 80 manufactured by ICI Americas Inc (Wilmington DE 19897 USA). Their HLB values were 4.3 and 15.0 respectively. The

Table 6.5: Regression analysis of alkali requirement and degree of amide bond hydrolysis

DATA SOURCE	REGRESSION EQUATION(1)	R^2 (%) (ADJUSTED) (FOR DF)	LINE ON FIGURE 6.4
Spray dried samples including Gluten Table 5.5	AR = 0.366 + 0.0661 DHSD	89.5 (86.9)	A
Liquid hydrolysate samples Table 5.5	AR = 0.141 + 0.0581 DHLIQ	95.4 (93.9)	B
Freeze dried samples Table 5.6	AR = 1.56 + 0.0457 DHFD	91.9 (89.2)	C

Note (1): The abbreviations DHSD, DHLIQ and DHFD refer to degree of amide bond hydrolysis values measured on spray dried, liquid and freeze dried samples respectively.

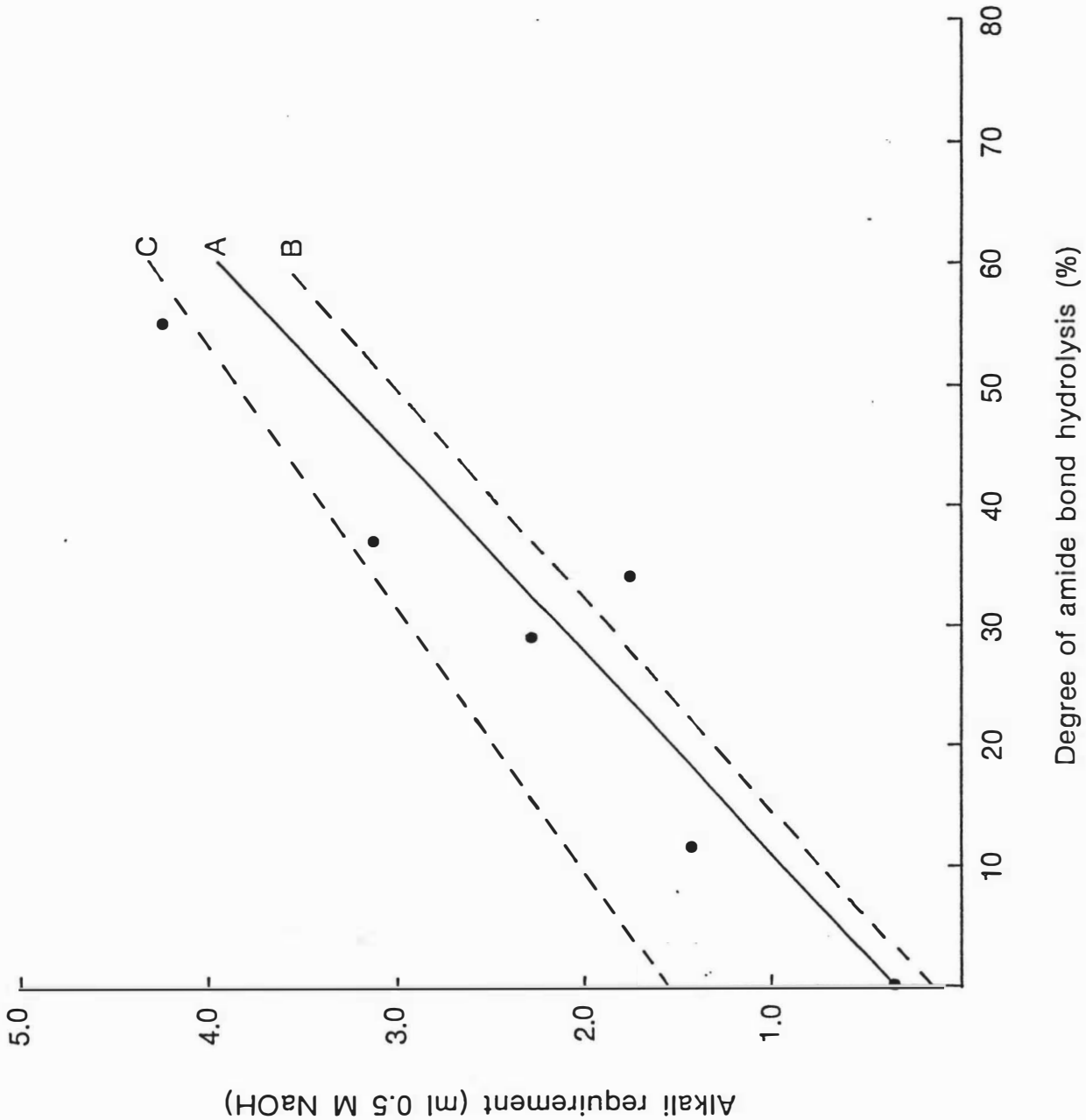


Figure 6.4 The relationship between alkali requirement and degree of amide bond hydrolysis for sample preparations.

emulsifiers were mixed in arithmetic proportions to give the desired emulsifier HLB (ICI Americas Inc., 1980). The total weight of emulsifier in each case was 0.75 g per 100 ml of emulsion. They were used at levels of 0.75 g per 100 ml of emulsion. This level was found to give some emulsion and some free oil when tested in the micro-haematocrit test described below. The same level, 0.75 g per 100 ml of emulsion, was used for the sample preparations.

Emulsions were prepared in a manner similar to that described for the solubility test (Section 6.3).

Sample preparation was weighed into a tall form 300 ml stainless steel beaker. The appropriate additions of 0.5M NaOH, 23.5 ml 0.2125M phosphate buffer pH 7.6, and water were made to give a total volume of 50 ml including an allowance for the specific volume of the sample. The sample concentration pH and ionic strength were therefore the same for each sample as used in the solubility test (Section 6.3). When emulsifiers were used in place of sample preparations they were weighed into the oil. Buffer was prepared as above but no NaOH was required.

Next, 50 ml of either soyabean or castor oil was added to the buffer solution in the beaker and an emulsion was formed by Mixing with an Ultra Turrax blender at full speed for 100 seconds. The emulsions were allowed to stand overnight after which all foam was found to have disappeared.

The emulsions were then mixed by hand to redisperse the cream layer which had formed. The emulsion was then poured off, 50 ml into a 50 ml measuring cylinder and about 5 ml to a 10 ml beaker for further sampling.

Three methods were used for analysis of the emulsions. The height of the serum/cream layer in the measuring cylinders was recorded at various time intervals. After 24 hours

there was no further separation and the cylinders were photographed at this point in some experiments.

The test proposed by Pearce and Kinsella (1978) was used in a slightly modified form. Emulsion samples were serially diluted to give a final dilution of one in a thousand. Dilutions were made into both 0.1% SDS and 1% SDS. The 1% SDS was included because of the reported effect of SDS concentration as a measure of the flocculation strength of an emulsion (Kamata et al, 1984). The dilutions were prepared by making a series of one in ten dilutions. The dilution procedure was to add 0.5 ml sample to 4.5 ml of 1% SDS solution and to add 0.5 ml sample to 0.5 ml 1% SDS and 4.0 ml water. Each dilution was thoroughly mixed before making the next transfer. The diluted samples were mixed again just before transfer to a quartz cuvette. The absorbance at 500 nm was determined against a 1% SDS standard solution using a Perkin Elmer spectrophotometer. The results are reported directly as measured absorbance as an alternative to further calculations and assumption.

The centrifugal method of emulsion characterization developed by McDermott et al (1981) was adapted as follows. One ml of the emulsion was placed in a multiwell tissue culture plate where the wells have a 1 ml working volume. (Falcon Plastics, Rutherford NJ, USA). Then 5 µl of 2% methylene blue (in 21% ethanol) and 5 µl of 4% oil red 'O' (in 70% ethanol and acetone (1:1)) were added. The dyes were thoroughly mixed into the emulsion and then the emulsion was drawn into duplicate 75 mm micro-haematocrit capillary tubes (Lancer, Sherwood Medical, St Louis Mo., 63103 USA). The tubes were sealed with a vinyl plastic putty, Critoseal (Monoject Scientific, Sherwood Medical, St Louis Mo., 63103 USA). The samples were centrifuged using a micro-haematocrit centrifuge which operates at 12000 rpm (14000 g) (Heraeus-Christ GmbH, D3360 Osterode am Harz, Postbox 1220, West Germany).

The samples were spun for various times from 10 seconds to 20 minutes. After each time interval the tubes were removed and the height of each layer, i.e. free oil, emulsion and serum was read as a percentage of the total height using a micro-haematocrit reader. (MSE Scientific Instruments, Manor Royal, Crawley, Sussex, England).

6.5.2 Confirmation of the required HLB value of oils

Castor and soyabean oils were tested and the results of the Pearce-Kinsella test and the micro-haematocrit test are shown in Tables 6.6 and 6.7 respectively. The best emulsions are those with maximum dispersion of the oil i.e. the highest absorbance in Table 6.6 or greatest emulsion layer after centrifugation. Both tests indicate the best emulsification for soyabean oil at HLB = 8 and for castor oil when HLB = 14. These values are similar to the values of 6 and 14 quoted by ICI (ICI Americas Inc., 1976). Similar results were obtained in an earlier test, results not shown, using Span 80 and Tween 20. Both emulsion evaluation tests should give similar results since the Pearce-Kinsella test measures the oil droplet surface area which is indicative of droplet size, and the micro-haematocrit test measures the rate at which the cream layer compresses.

After prolonged centrifugation two cream layers could be seen, which may indicate some separation of the emulsifiers. When preparing these emulsions the emulsifiers were weighed into separate portions of the oil which were then mixed in the required proportions. This practice might contribute to the phenomenon. Free oil layers were noted to be present in inverse proportion to the cream layer height.

6.5.3 Emulsification properties of sample preparations

Emulsions were prepared with each sample preparation using both castor and soyabean oils. The results of evaluation using the Pearce-Kinsella and micro-haematocrit test

Table 6.6: Results of Pearce-Kinsella test for determination of required HLB of oils

OIL	EMULSIFIER HLB	A_{500} 1% SDS	OIL	EMULSIFIER HLB	A_{500} 1% SDS
Soy	5	0.035	Castor	10	0.365
Soy	6	0.541	Castor	11	0.323
Soy	7	0.466	Castor	12	0.339
Soy	8	0.726	Castor	13	0.304
Soy	9	0.651	Castor	14	0.445
Soy	10	0.582	Castor	15	0.314

Table 6.7: Results of micro-haematocrit test for determination of required HLB of oils

OIL	EMULSIFIER HLB	EMULSION LAYER[1] HEIGHT (%)	OIL	EMULSIFIER HLB	EMULSION LAYER[2] HEIGHT (%)
Soy	5	0,0	Castor	10	33,34
Soy	6	15,15	Castor	11	28
Soy	7	24,28	Castor	12	27,28
Soy	8	40,41	Castor	13	36,37
Soy	9	33,34	Castor	14	35,39
Soy	10	30,37	Castor	15	37,30

Notes (1) After 30 minutes centrifugation
(2) After 1 minute centrifugation

methods are shown in Tables 6.8 and 6.9. The data indicate a clear difference between samples and an apparent difference in optimum level of deamidation for emulsification with each oil. This would be expected in terms of the hypothesis stated in Section 1.5.6. To illustrate these effects the data were plotted as shown in Figures 6.5 and 6.6. The level of deamidation for each sample was estimated from the alkali requirement data of Table 6.4 and the regression line in Figure 6.4. A further illustration is given in Figure 6.7 which shows the emulsion series with castor oil after the equilibrium had been reached. The change in solubility of the preparations is evident.

An alternative explanation of the results is that the changes in emulsifying power are largely due to the changes in solubility of the protein. With regard to the hypothesis, the HLB theory does not depend on solubility of the emulsifying agent in either phase but dispersion is assumed.

To test this alternative a further experiment was carried out. In this experiment the weight of the sample was varied according to the solubility, from Table 6.4, with the aim of achieving a constant level of total nitrogen in solution (TN = 0.169 as for sample 11, Table 6.4). The samples were prepared as previously described except that 150 ml of aqueous phase was prepared. The quantities of sample and alkali added are shown in Table 6.10. Buffer and water were added as previously described, the volume of water being adjusted to allow for the volume occupied by the sample. Mixing of the larger quantities of sample was quite difficult because a strong dough was formed. The prepared solutions were allowed to stand overnight, then the insoluble gluten was removed and the residue centrifuged (MSE, 1000 rpm, 20 minutes) and the supernatant decanted. Samples were set aside for pH determination and total nitrogen analysis. There was sufficient supernatant

Table 6.8: Results of Pearce-Kinsella test for determination of emulsification properties of sample preparations

PREPARATION NUMBER	CASTOR OIL		SOYABEAN OIL		DEAMIDATION[1] BY REGRESSION ANALYSIS (%)
	A_{500} 0.1% SDS	A_{500} 0.1% SDS	A_{500} 0.1% SDS	A_{500} 0.1% SDS	
Gluten	0.042	0.097	0.041	0.010	0
(1)	0.090	0.166	0.049	0.028	0
(2)	0.040	0.130	0.315	0.328	7
(3)	0.496	0.600	0.593	0.791	8
(4)	0.242	0.329	0.470	0.464	6
(5)	0.427	0.581	0.520	0.550	8
(6)	0.371	0.381	0.391	0.406	11
(7)	0.632	0.706	0.780	1.015	18
(8)	0.720	0.815	0.949	0.865	25
(9)	0.614	0.619	1.113	1.140	46
(10)	0.705	0.697	0.975	1.189	33
(11)	0.311	0.296	0.658	0.832	65

Note (1) From Alkali requirement data
Table 6.2 and Line A in Figure 6.4

Table 6.9: Results of micro-haematocrit test for determination of emulsification properties of sample preparations

PREPARATION NUMBER	CASTOR OIL EMULSION LAYER AFTER 30 SECONDS CENTRIFUGATION (%)	SOYABEAN OIL MULSION LAYER AFTER 2 MINUTES CENTRIFUGATION (%)	DEAMIDATION BY REGRESSION ANALYSIS (%)
Gluten	none	none	0
(1)	none	none	0
(2)	none	-,52	7
(3)	63,65	58,56	8
(4)	56,52	54,53	6
(5)	62,62	57,57	8
(6)	60,60	51,51	11
(7)	65,64	56,56	18
(8)	84,85	69,69	25
(9)	87,87	89,89	46
(10)	89,89	76,73	33
(11)	72,72	74,74	65

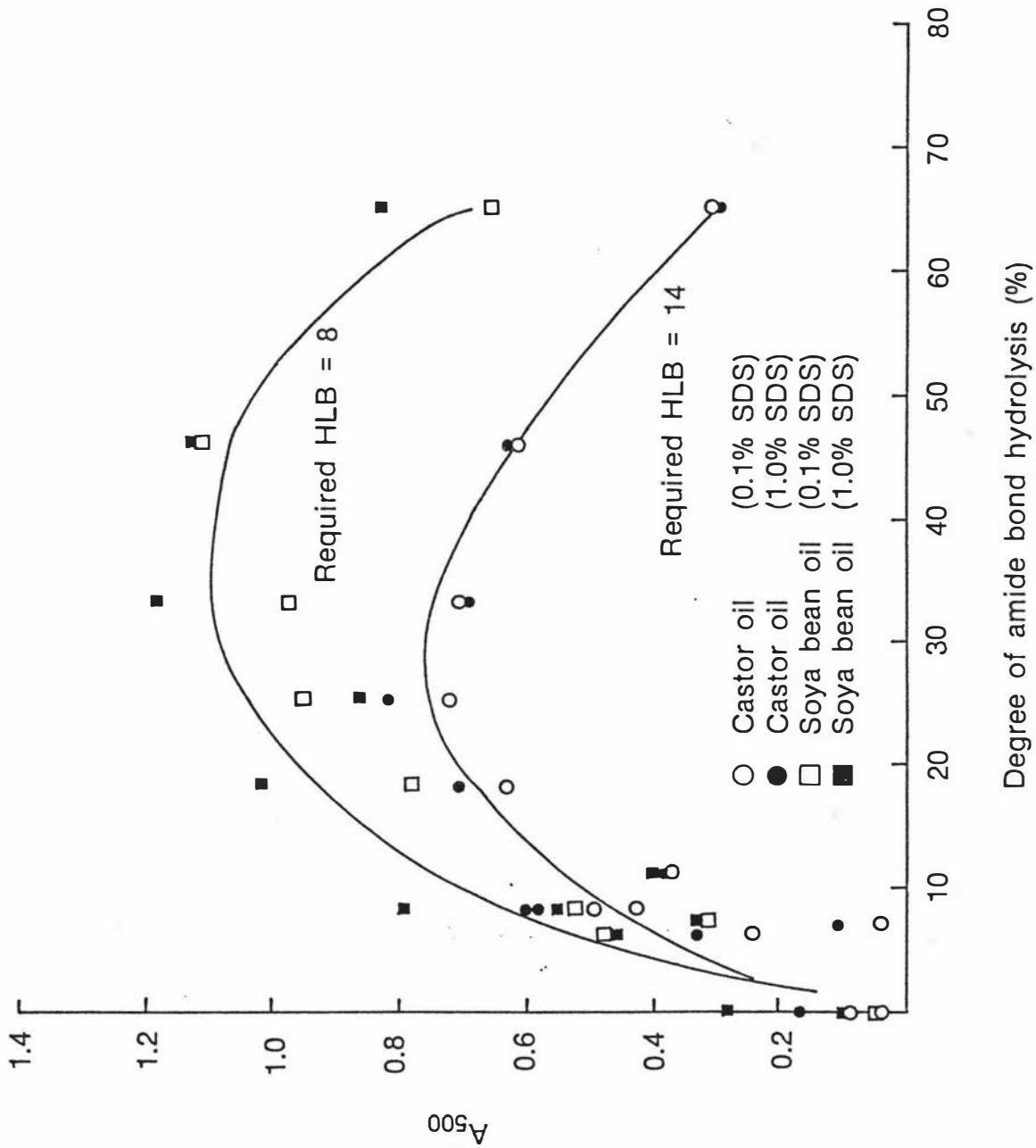


Figure 6.5 Differences in apparent optimum degree of amide bond hydrolysis for emulsification with different oils using the Pearce-Kinsella test.

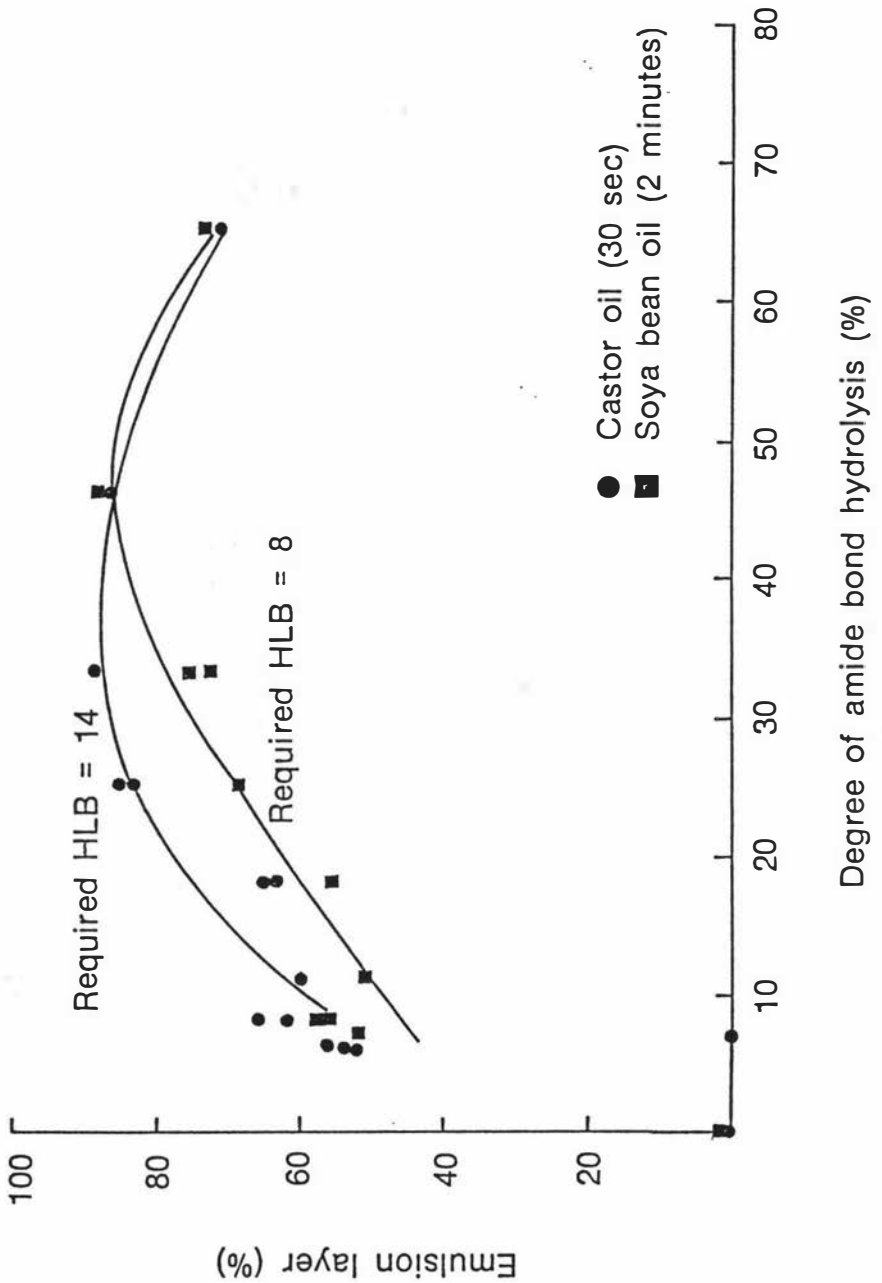


Figure 6.6 Differences in apparent optimum degree of amide bond hydrolysis for emulsification with different oils using the microhaematocrit test.

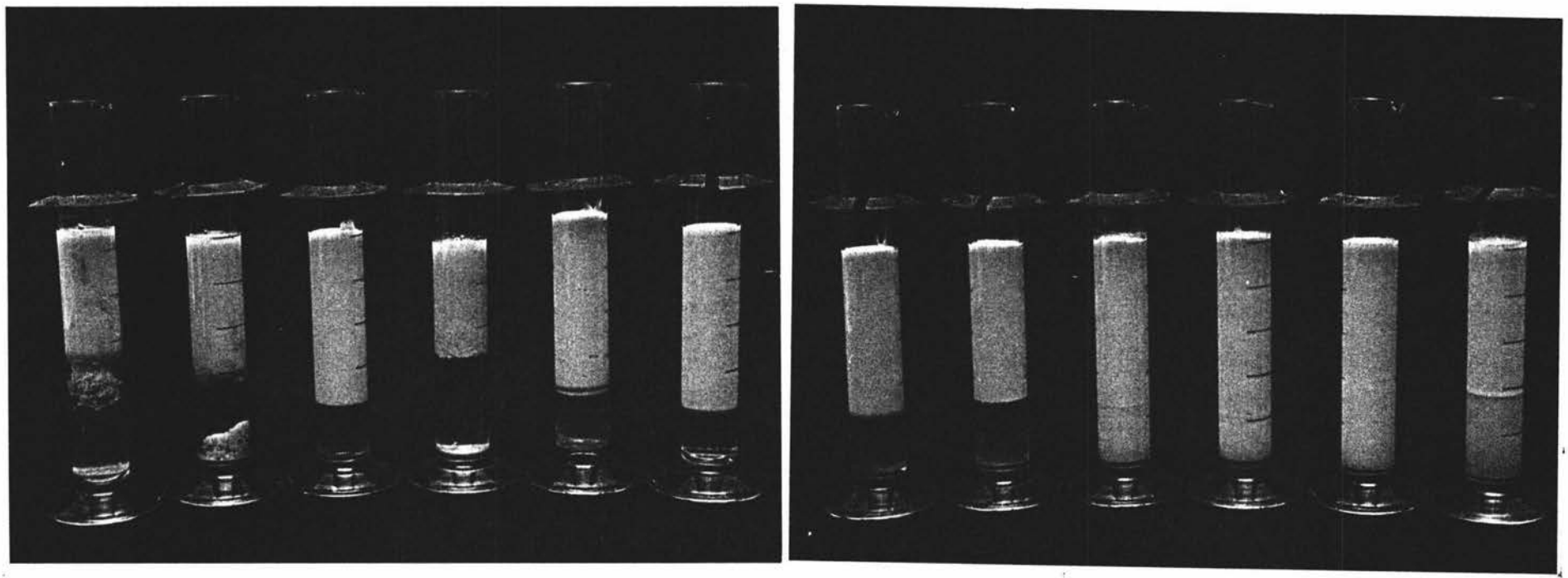


Figure 6.7 Separation under gravity of castor oil emulsified with sample preparations.

Table 6.10: Analysis of samples prepared for emulsions with constant soluble total nitrogen

PREPARATION NUMBER	QUANTITY OF SAMPLE (g)	VOLUME OF 0.5M NaOH (ml)	pH OF SUPERNATANT	TOTAL NITROGEN OF SUPERNATANT (%)
Gluten	47.53	-	-	-
(1)	47.53	-	-	-
			7.053	0.106
(2)	31.69	10.42	7.033	0.108
(3)	8.09	2.95	7.511	0.106
(4)	17.28	5.22	7.325	0.118
			7.504	0.100
(5)	10.28	3.66	7.488	0.102
(6)	7.46	3.25	7.586	0.118
(7)	3.17	2.00	7.553	0.163
(8)	3.20	2.50	7.524	0.173
(9)	2.28	3.16	7.491	0.175
(10)	2.82	2.86	7.515	0.176
(11)	2.25	4.26	7.436	0.173

Note (1) Duplicate preparations were made of samples 2 and 5

for only 40 ml per emulsion so the quantity of oil was also reduced to 40 ml. The emulsions were analysed as previously described.

The results of the pH and total nitrogen determinations are included in Table 6.10. Results of the Pearce-Kinsella test and micro-haematocrit test are given in Tables 6.11 and 6.12. Because the total nitrogen in each solution was not exactly the same, these data are plotted in Figure 6.8 as absorbance divided by total nitrogen. It is clear from these results that emulsion-forming properties of the proteins are governed by other factors in addition to the level of total nitrogen in solution. The samples with low levels of hydrolysis are poorer emulsifiers. The difference in performance with different oils is apparent but the data are otherwise rather confusing.

The effect of protein concentration on the formation of emulsions has been reported by various authors each giving very different results. Pearce and Kinsella (1978) showed a near linear increase in emulsifying activity with concentration whereas Sabharwal and Vakaleris (1972) showed first an increase then a plateau followed by a decrease at higher concentration.

To determine the effect of concentration in the test system and to offer further explanation of the data shown in Figure 6.8, two of the sample preparations, 5 and 11 were tested at various concentrations in the emulsion system.

Emulsions were prepared using castor oil, as previously described, except that the concentration of the sample preparation was varied. The concentrations used and the results of the Pearce-Kinsella test are shown in Table 6.13. The data show an increase in emulsifying power as the concentration increases. The same result was found with the micro-haematocrit test, results not shown, and by

Table 6.11: Results of Pearce-Kinsella test for determination of emulsification properties of sample preparations with constant soluble total nitrogen

PREPARATION NUMBER	CASTOR OIL		SOYABEAN OIL		DEAMIDATION BY REGRESSION ANALYSIS (%)
	A_{500} 0.1% SDS	A_{500} 0.1% SDS	A_{500} 0.1% SDS	A_{500} 0.1% SDS	
Gluten	-	-	-	-	0
(1)	-	-	-	-	0
(2)	0.080	0.060	0.108	0.151	7
(3)	0.518	0.450	0.744	0.862	8
(4)	0.103	0.070	0.114	0.252	6
	0.503	0.392	0.480	0.662	
(5)	0.473	0.465	0.581	0.773	8
(6)	0.646	0.695	0.879	0.885	11
(7)	0.663	0.623	1.029	0.980	18
(8)	0.624	0.688	1.183	1.154	25
(9)	0.735	0.827	0.909	0.921	46
(10)	0.643	0.723	1.192	1.182	33
(11)	0.401	0.430	1.061	1.024	65

Table 6.12: Results of micro-haematocrit test for determination of emulsification properties of sample preparations with constant soluble total nitrogen

PREPARATION NUMBER	CASTOR OIL EMULSION LAYER AFTER 30 SECONDS CENTRIFUGATION (%)	SOYABEAN OIL EMULSION LAYER AFTER 30 SECONDS CENTRIFUGATION (%)
Gluten	-	-
(1)	-	-
(2)	nil	nil
(3)	60,60	62,64
(4)	nil	nil
	59,59	63,63
(5)	60,60	62,62
(6)	79,80	79,78
(7)	91,92	92,91
(8)	90,90	98,99
(9)	93,94	97,98
(10)	88,90	100,100
(11)	88,80	100,100

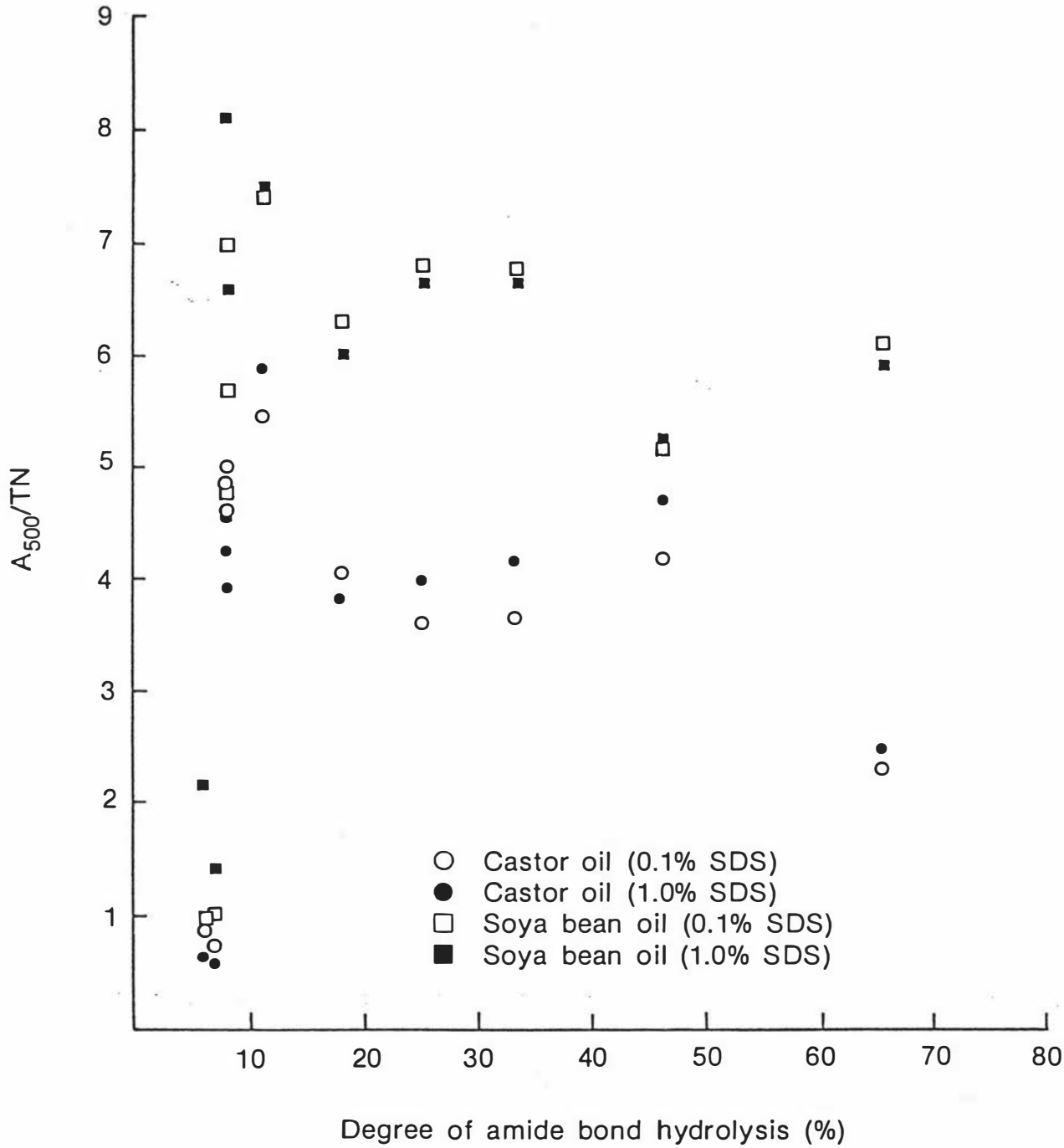


Figure 6.8 The effect of the degree of amide bond hydrolysis on emulsification properties of sample preparations with constant soluble total nitrogen as determined by the Pearce-Kinsella test.

Table 6.13: Results of Pearce-Kinsella test for determination of emulsification properties of sample preparations at various levels of sample.

CONCENTRATION[1] OF SAMPLE 5 (%)	ESTIMATED[2] SOLUBLE TOTAL NITROGEN IN AQUEOUS PHASE (%)	CASTOR A_{500} 0.1% SDS	OIL A_{500} 1.0% SDS	CONCENTRATION OF SAMPLE 11 (%)	ESTIMATED SOLUBLE TOTAL NITROGEN IN AQUEOUS PHASE (%)	CASTOR A_{500} 0.1% SDS	OIL A_{500} 1.0% SDS
0.05	0.002	0.031	0.113	0.05	0.011	0.170	0.173
0.10	0.005	0.057	0.125	0.10	0.022	0.316	0.312
0.20	0.010	0.046	0.105	0.20	0.045	0.318	0.334
0.50	0.025	0.254	0.272	0.50	0.113	0.300	0.279
0.75	0.037	0.377	0.420	0.75	0.169	0.298	0.304
1.50	0.074	0.473	0.483	1.50	0.338	0.374	0.364
2.0	0.099	0.542	0.624				
3.0	0.148	0.736	0.631				
5.0	0.241	0.695	0.984				

Notes (1) Concentration is weight percent in total emulsion (100 ml)

(2) Estimated assuming all Total Nitrogen dissolved in aqueous phase, from data of table 6.4 (50 ml)

standing under gravity. These results are shown in the photographs of Figure 6.9.

When the Pearce-Kinsella test data from Table 6.13 are plotted against sample concentration as in Figure 6.10 the samples appear very different. When, however, they are plotted against concentration of total nitrogen in solution, calculated from the data of Table 6.4, the initial slopes of the lines in Figure 6.11 suggest that the effect of each sample preparation might be the same at lower concentrations.

Two further sample preparations, 7 and 10 were tested in the same manner. In addition, 100 ml solutions of each concentration were prepared. These were clarified by centrifugation (MSE, 50 ml tubes, 20 mins at 1000 rpm). The volume of sediment was noted, small quantities of floating material were removed by suction and the supernatant decanted. Sample (10) contained a fine sediment only partially removed by the centrifugation, which was carried over in the decantation. A portion of each supernatant was set aside for pH determination and total nitrogen determination. The weights of sample, pH and total nitrogen of the supernatant and the results of the Pearce-Kinsella test are given in Table 6.14. Photographs of the emulsions after standing under gravity are shown in Figure 6.12.

The Pearce-Kinsella test data from Table 6.14 are plotted in Figures 6.13 and 6.14 in the same way as in Figures 6.10 and 6.11. It is clear that the emulsion effect is due to the soluble portion of the sample preparations, the solid material having no discernible effect. A plot of total nitrogen against concentration, Figure 6.14 shows that the concentration of total nitrogen is directly proportional to the sample concentration, that is, no solubility limit is involved. The data for sample 10 show the effect of the poor sedimentation and carry-over of solid material,

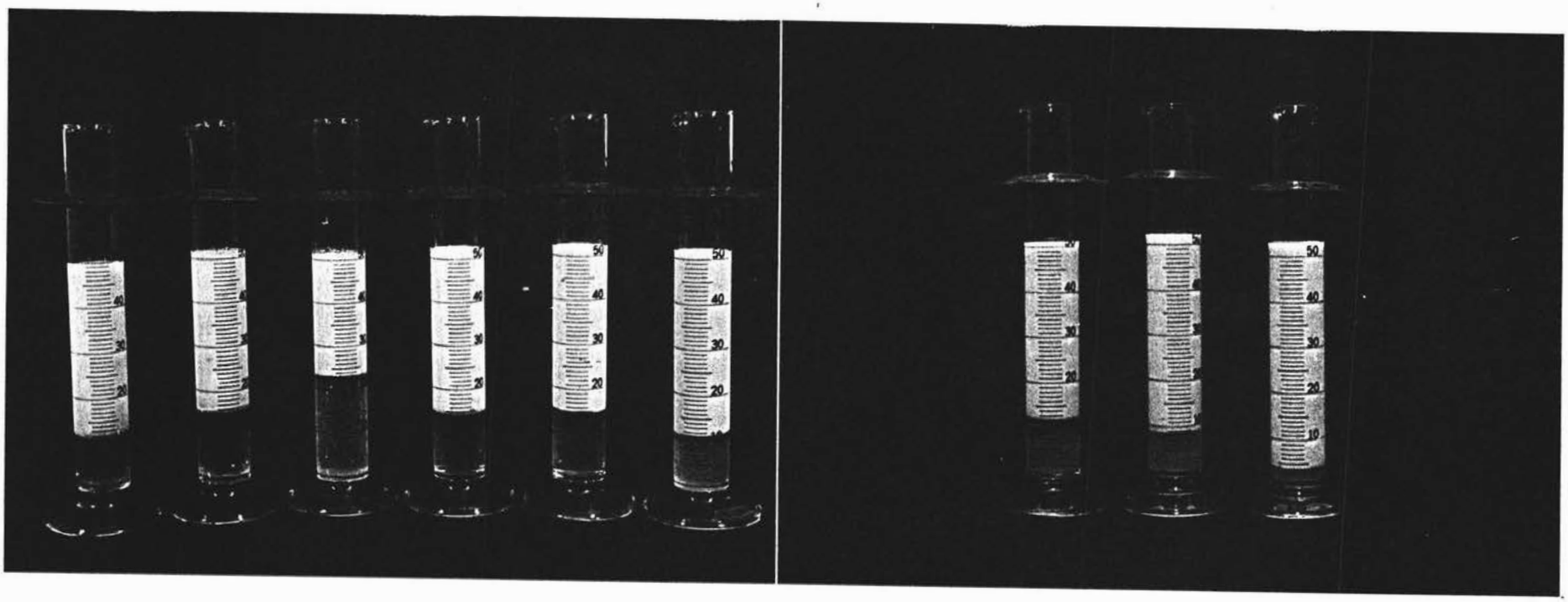


Figure 6.9 Separation under gravity of castor oil emulsified with different levels of sample.

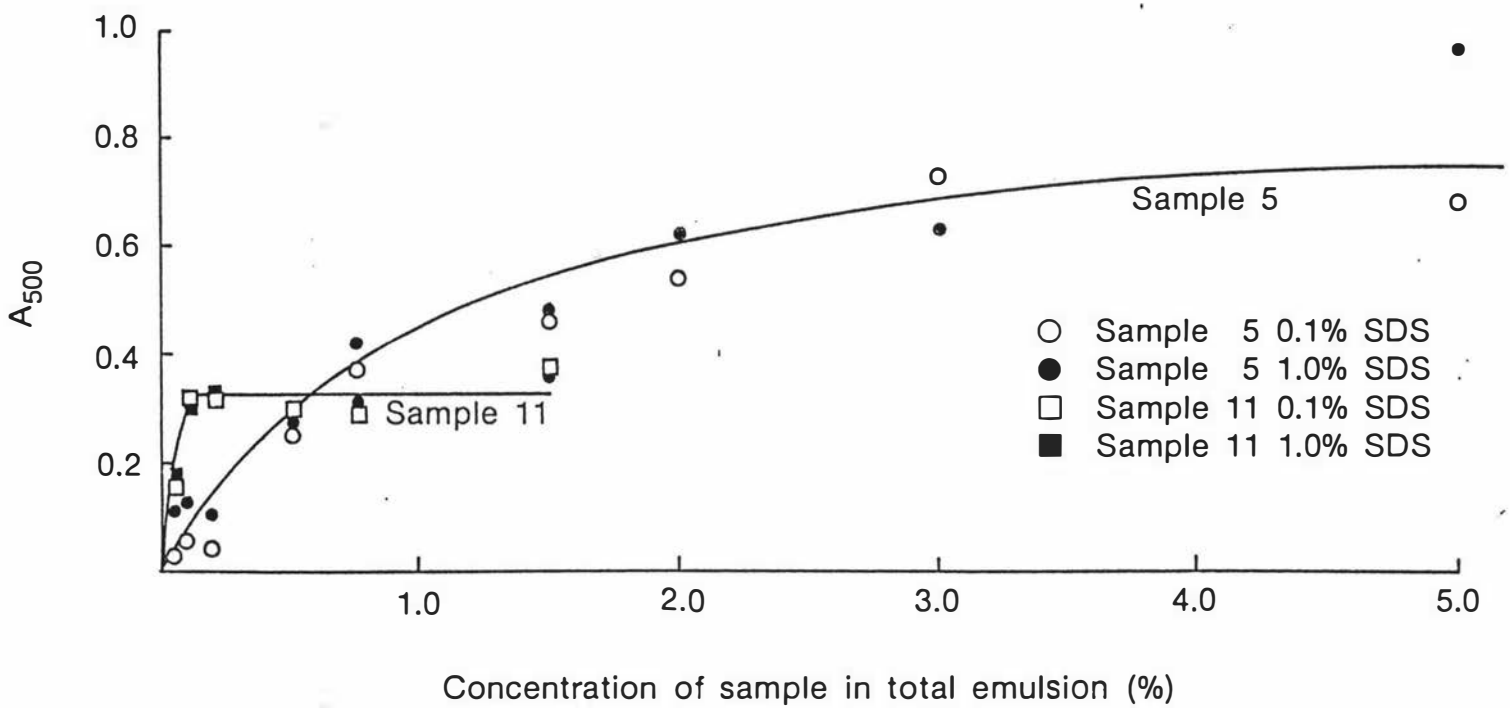


Figure 6.10 Results of Pearce-Kinsella test for determination of emulsification properties of sample preparation at various levels of sample.

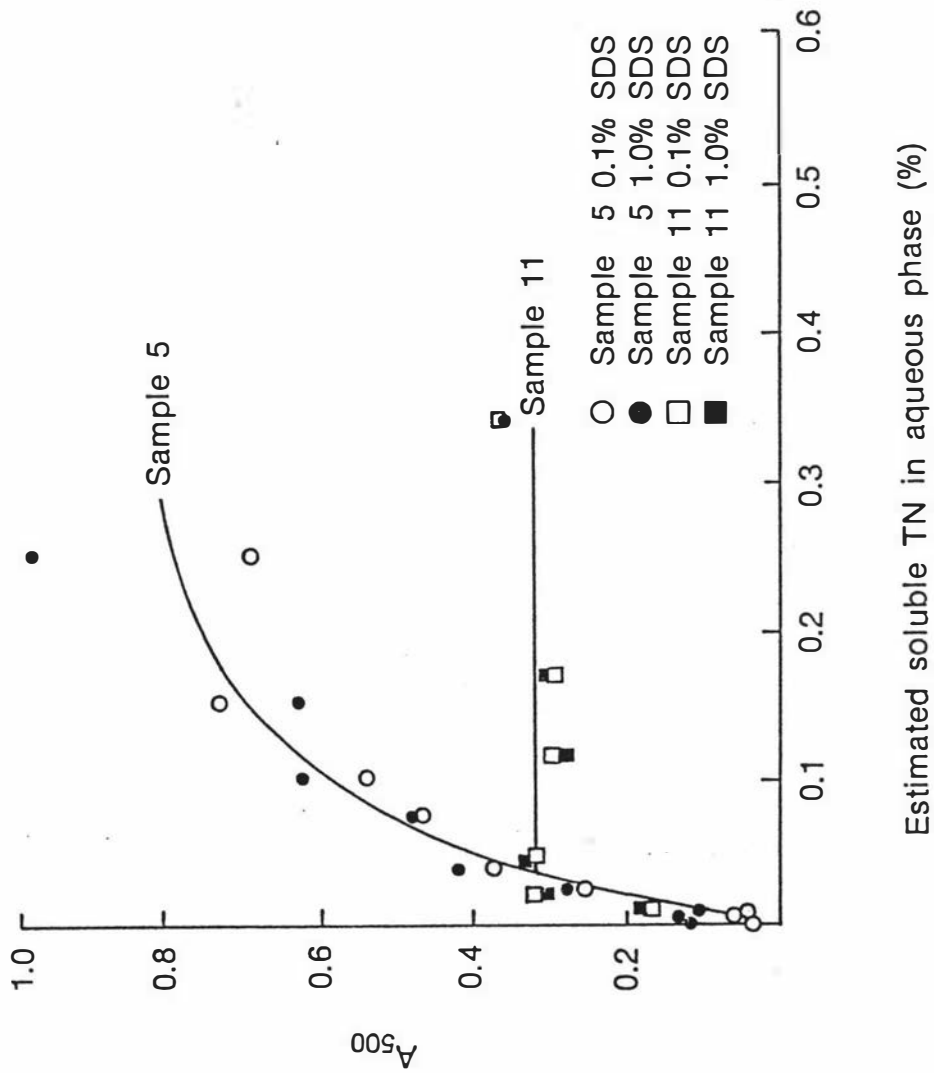


Figure 6.11 Results of Pearce-Kinsella test for determination of emulsification properties of sample preparation at various levels of soluble total nitrogen.

Table 6.14: Results of Pearce-Kinsella test, pH and soluble total nitrogen determinations for determination of emulsification properties of sample preparations at various levels of sample

SAMPLE NUMBER	WEIGHT OF SAMPLE IN 100 MLS OF AQUEOUS PHASE (g)	SEDIMENT AFTER CENTRIFUGATION (ml)	pH OF SUPERNATANT	MEASURED TOTAL NITROGEN IN SUPERNATANT (ml)	WHOLE SAMPLE A_{500} 0.1% SDS	WHOLE SAMPLE A_{500} 1.0% SDS	SUPERNATANT A_{500} 0.1% SDS	SUPERNATANT A_{500} 1.0% SDS	CALCULATED TOTAL NITROGEN SUPERNATANT FROM FIG. 6.15 (%)
(7)	0.1	<0.5	7.64	0.008	0.068	0.051	0.046	0.076	-
	0.2	<0.5	7.66	0.016	0.068	0.070	0.094	0.190	-
	0.6	0.5,0.75	7.69	0.047	0.390	0.437	0.399	0.497	-
	1.5	1.3,2.0	7.64	0.114	0.660	0.680	0.608	0.808	-
	3.0	3.2,3.6	7.61	0.226	0.656	0.852	0.705	0.957	-
	6.0	8.5,7.5	7.61	0.447	0.850	0.931	0.785	0.836	-
(10)	0.1	<0.5	7.74	0.01	0.059	0.130	0.068	0.106	0.01
	0.2	<0.5	7.64	0.02	0.291	0.356	0.231	0.235	0.02
	0.06	1.7,1.6	7.61	0.05	0.408	0.458	0.620	0.628	0.05
	1.5	4.6,4.8	7.65	0.13	0.568	0.741	0.723	0.784	
	0.135								
	3.0	10.8,10.0	7.69	0.27	0.644	0.851	0.753	0.913	0.272
	6.0	approx 20	7.69		0.773	0.883	0.919	1.217	0.49

Note (1) Concentration is weight percent in aqueous phase of data of table 6.13

(2) The sediment for sample 10 was a diffuse layer and not really a compact sediment as for 7.

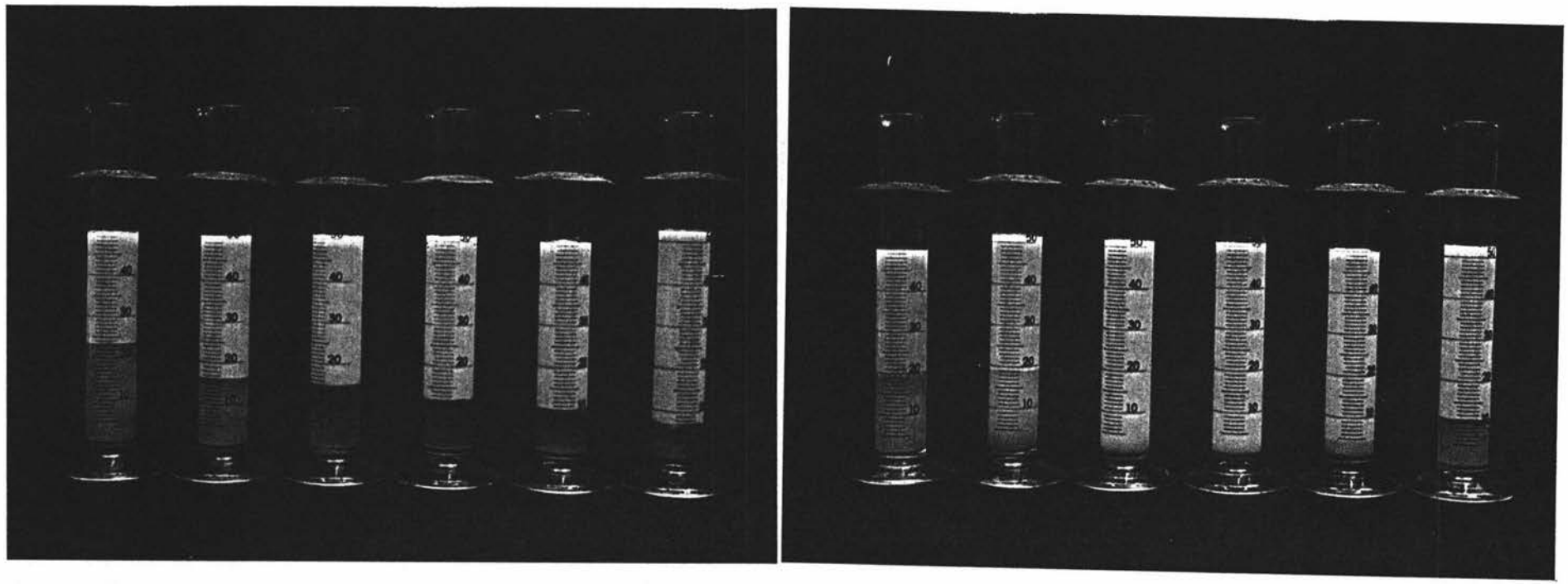


Figure 6.12 Separation under gravity of castor oil emulsified with different levels of sample with and without insoluble material removed by centrifugation.

Figure 6.12 (Continued) Separation under gravity of castor oil emulsified with different levels of sample with and without insoluble material removed by centrifugation.

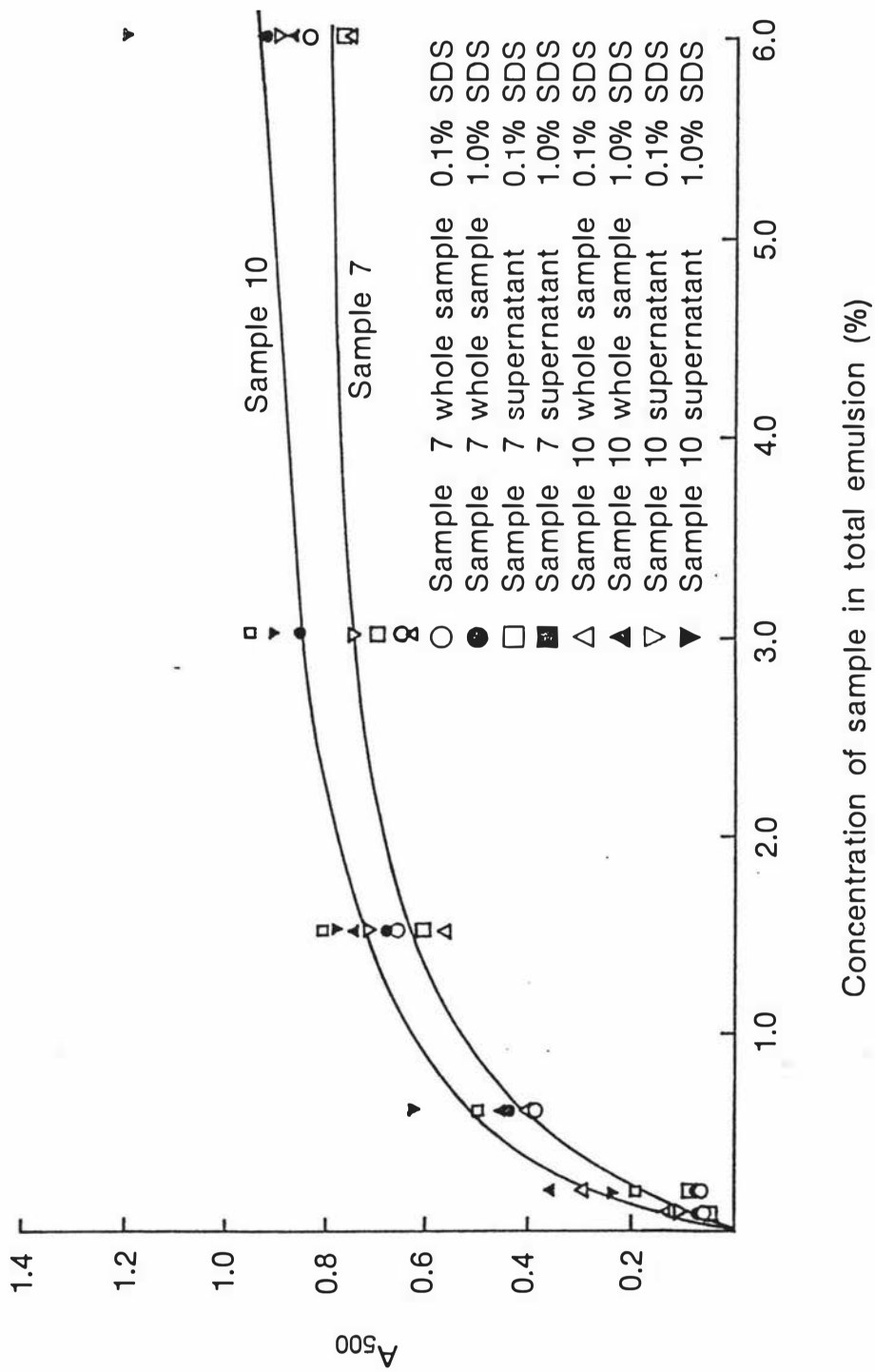


Figure 6.13 Results of Pearce-Kinsella test for determination of emulsification properties of sample preparations at various levels of sample with and without insoluble material removed by centrifugation.

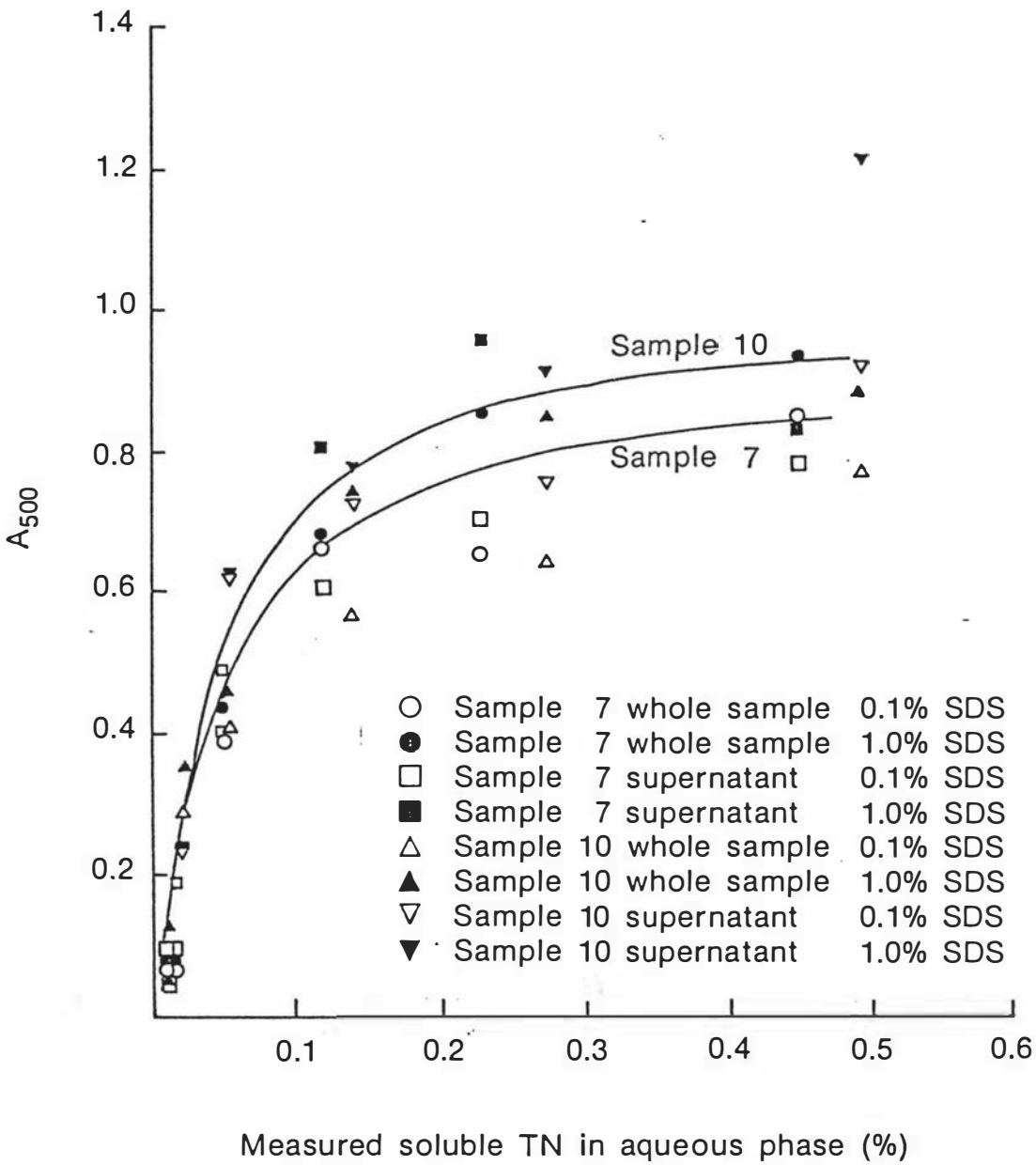


Figure 6.14 Results of Pearce-Kinsella test for determination of emulsification properties of sample preparations at various levels of soluble nitrogen with and without insoluble materials removed by centrifugation.

particularly for the highest concentration sample. Figure 6.15 shows how the TN was recalculated in Table 6.14 and used in Figure 6.14.

A further consequence of the original hypothesis is that if the sample preparations are acting similarly to emulsifiers which can be described by HLB numbers, they should show additivity, that is, the HLB of any mixture of sample preparations should fall between the separate values. To test this aspect of the hypothesis samples 5 and 11 were chosen. Reference to Table 6.9 for levels of deamidation, and to Figure 6.5 shows that mixtures of the two sample preparations should give enhanced emulsifying power compared to either alone. Emulsions were prepared using castor oil with quantities of each sample preparation as shown in Table 6.15. The results of the Pearce-Kinsella test are also shown in the table. A similar result, not shown, was obtained by using the micro-haematocrit test. There is no enhancement effect and this is also demonstrated by the emulsions standing under gravity shown in the photograph in Figure 6.16.

6.6 Determination of hydrophobicity of sample preparations

6.6.1 Introduction

Various measures of protein hydrophobicity and their means of determination were discussed in Section 1.5.2. The available techniques have been reviewed by Nakai (1983) who discussed the particular relevance of surface hydrophobicity to functional properties. For this work, it was desired to determine the surface hydrophobicity of the sample preparations. These data could then be used, first, to compare with the emulsions data of Section 6.5 as a contribution toward proving the hypothesis stated in Section 1.3.6. A second objective was to repeat the measurements made by Matsudomi et al (1982). A further objective was to examine the suggestion of Hayakawa and Nakai (1985) that the different probes 8-anilino-1-

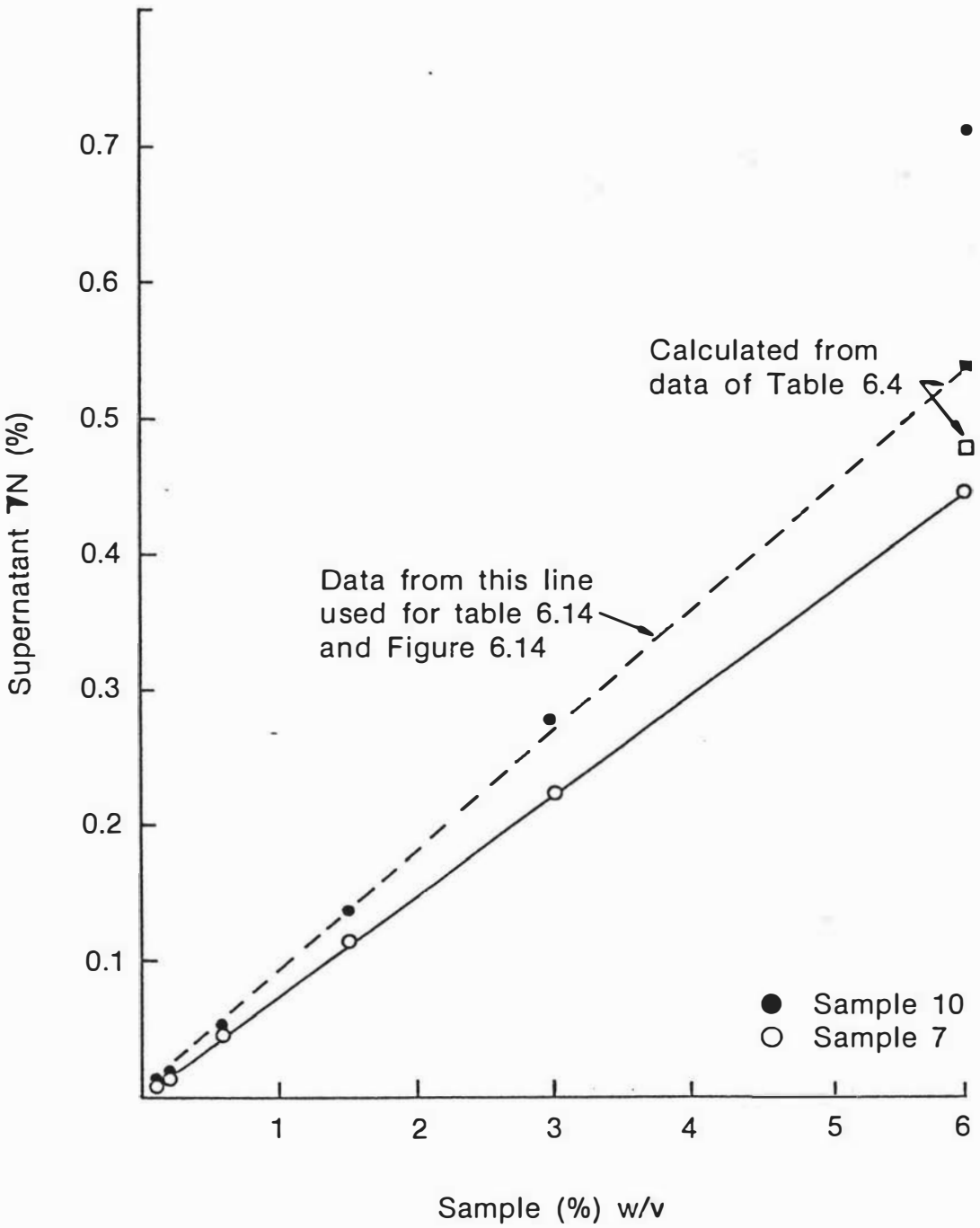


Figure 6.15 Total nitrogen in solution at various levels of sample concentration.

Table 6.15: Results of Pearce-Kinsella test for determination of emulsification properties of mixtures of sample preparations

WEIGHT OF SAMPLE 5 IN 100 ML OF EMULSION (g)	WEIGHT OF SAMPLE 11 IN 100 ML OF EMULSION (g)	CASTOR OIL A_{500} 0.1% SDS	CASTOR OIL A_{500} 0.1% SDS	CASTOR OIL A_{500} 1.0% SDS	MEAN VALUE A_{500}
0.75	-	0.315	0.268	0.316	0.300
0.56	0.19	0.297	0.311	0.280	0.296
0.375	0.375	0.312	0.287	0.376	0.325
0.19	0.56	0.323	0.305	0.282	0.303
-	0.75	0.312	0.339	0.345	0.332
0.375	0.375	0.285	0.314	0.305	0.302

Figure 6.16 Separation under gravity of castor oil emulsified with mixtures of product preparations.

naphthalenesulfonate (ANS) and 9, 11, 13, 15-cis trans trans cis octadecatetraenoic acid (cis parinaric acid, CPA) were measuring aromatic and aliphatic hydrophobicities respectively, and that these made separate contributions to protein functionality.

To achieve these objectives, hydrophobicity was determined for the soluble portion of each sample and the total sample solubilized in SDS. Both ANS and CPA were used for the determinations.

6.6.2 Test methods

The samples were dispersed and tested using the same conditions as for the solubility and emulsifying power determinations already described. Two dispersions of each sample, 0.100 ± 0.005 g, were made. The first was dissolved in 50 ml of 0.1M phosphate buffer, pH 7.6, which contained 1% SDS. The second was dissolved in the same buffer but without SDS. During the dispersion an unstable foam was formed. The dispersions were allowed to stand overnight after which time the foam had entirely collapsed. The dispersions were then centrifuged (MSE 1000 rpm, 20 min in 50 ml tubes). The volume of the sediment layer in each tube was recorded and a sample of the supernatant taken for total nitrogen determination. The supernatants were further diluted as required using buffer, or buffer containing SDS, as appropriate.

ANS was dissolved in 0.1M phosphate buffer, pH 7.6, to give a concentration of 10 mM. CPA was dissolved in absolute ethanol to give a concentration of 3.6 mM with equimolar BHT (butylated hydroxy toluene). (An ampoule of CPA contains 10 mg which is dissolved in 10 ml absolute ethanol).

The hydrophobicity determination was carried out as described by Creamer et al (1982). For each sample preparation 2 ml of buffer and 10 ul of CPA or ANS solution

were added to a quartz cuvette. The fluorescence was determined and then suitably diluted protein solutions were added in small increments, between 20 and 200 µl depending on the fluorescence response, up to a total addition of 1000 µl.

Fluorescence intensity was measured using a Perkin Elmer MPF-2A fluorescence spectrometer fitted with a 150 W Xenon lamp and a temperature controlled cuvette holder. The fluorescence of each sample with ANS was read with an excitation wavelength of 420 nm and slitwidth of 20 nm, according to Hayakawa and Nakai (1985). The results were recorded as peak heights on a recorder chart. The peak height data at each sample concentration, calculated as TN in the cuvette, were divided by the peak height without sample addition. A graph was constructed of this relative fluorescence against concentration. A straight line was drawn through the points and the slope measured.

Surface hydrophobicity is a relative measurement without units. Most authors report that the initial slope of the fluorescence intensity versus concentration line is taken. In this instance it was found that the plot was an initial curve and most of the data fell on a subsequent straight line. It was the slope of this line which was used in these calculations.

6.6.3 Results

The results of the sediment and TN determinations for samples dispersed with and without SDS are shown in Table 6.16. Some of the TN data, for example that for sample 6, appear to be wrong. Thus, values calculated from the data of Table 6.4 are included for comparison and in the case of samples 6 and 9 were used in the calculation of relative hydrophobicity. The TN data for the samples dispersed with SDS show its effectiveness in dispersion of the protein as shown in Section 3.1

Table 6.16: Sediment and total nitrogen (TN) values of supernatants of sample preparations dispersed with and without SDS

PREPARATION NUMBER	VOLUME OF[1] SEDIMENT IN 50 ML (WITH SDS)	VOLUME OF[2] SEDIMENT IN 50 ML (WITHOUT SDS)	TOTAL NITROGEN OF SUPERNATANT (WITH SDS)	TOTAL NITROGEN OF SUPERNATANT (WITHOUT SDS)	TOTAL NITROGEN OF SUPERNATANT CALCULATED FROM DATA OF TABLE 6.4 (WITHOUT SDS)
	(ml)	(ml)	(%)	(%)	(%)
Gluten	1.0	-	0.021	-	-
(1)	1.0	-	0.020	-	-
(2)	0.25	tr,s	0.025	0.002	0.002
(3)	0.25	tr,s	0.024	0.005	0.006
(4)	0.25	tr,s	0.025	0.004	0.003
(5)	0.25	tr,s	0.025	0.005	0.005
(6)	0.25	0.25,s	0.024	<0.0005	0.007
(7)	0.25	0.25,s	0.027	0.014	0.016
(8)	0.25	0.5	0.022	0.015	0.016
(9)	0.25	0.25	0.025	0.042	0.022
(10)	0.25	0.5	0.026	0.017	0.018
(11)	0.25	tr	0.025	0.024	0.022
(12)	-	0.25	-	0.024	0.023
(13)	-	tr,s	-	0.005	0.003

Notes (1) Lowest calibration mark on centrifuge tube is 0.5 ml

(2) tr = trace only

s = material floating on surface, removed before centrifugation.

The calculated slope values, that is, surface hydrophobicity for each sample preparation without SDS and determined with both ANS and CPA are shown in Table 6.17. These data are also plotted in Figure 6.17 and 6.18 as a function of the degree of amide bond hydrolysis. The linear relationship found by Matsudomi et al (1982) is clearly demonstrated.

It was found that the surface hydrophobicity of samples solubilized with SDS could not be determined with either ANS or CPA. Creamer and Richardson (1984) found that ANS gave a fluorescence response with SDS and a similar effect was found with CPA in this work.

When the analysis was carried out with CPA and the initial 2 ml of buffer in the cuvette contained 1% SDS, a very high response was obtained. As protein was added, the response decreased. When the initial 2 ml of buffer did not contain SDS then a response to the addition of dilution buffer containing SDS was found which could not be distinguished from addition of buffer containing both sample preparation and SDS. This problem could be avoided by further dilution of the samples and increasing the fluorimeter sensitivity so that the SDS added was at a level that did not give a discernible effect. In these circumstances it was found that the response was the same for all the sample preparations. In view of the finding, in Section 6.5, that the emulsion properties depend only on the soluble portion of the sample preparations, the surface hydrophobicity of which have been satisfactorily determined, this interesting phenomenon was not pursued further.

6.7 Discussion of results of functional property testing

The alkali requirement of proteins is usually associated with the solubility of casein. Cohn and Ferry (1943), in a discussion of protein solubility, described the mechanism of casein solubilization as each mole of base carried an equivalent

Table 6.17: Surface hydrophobicity of supernatants of sample preparations dispersed without SDS

PREPARATION NUMBER	So ANS DATA SLOPE x 10^{-4}	So CPA DATA SLOPE x 10^{-4}	DEAMIDATION BY REGRESSION ANALYSIS (%)
Gluten	-	-	0
(1)	-	-	0
(2)	0.164	-	7
(3)	0.163	0.009	8
(4)	0.150	0.006	6
(5)	0.150	0.061	8
(6)	0.154	0.036	11
		0.072	
(7)	0.280	0.088	18
(8)	0.227	0.064	25
(9)	0.367	0.136	46
(10)	0.299	0.079	33
		0.140	
(11)	0.338	0.178,0.198	65
(12)	0.420	0.143	51
(13)	0.154	0.003	9

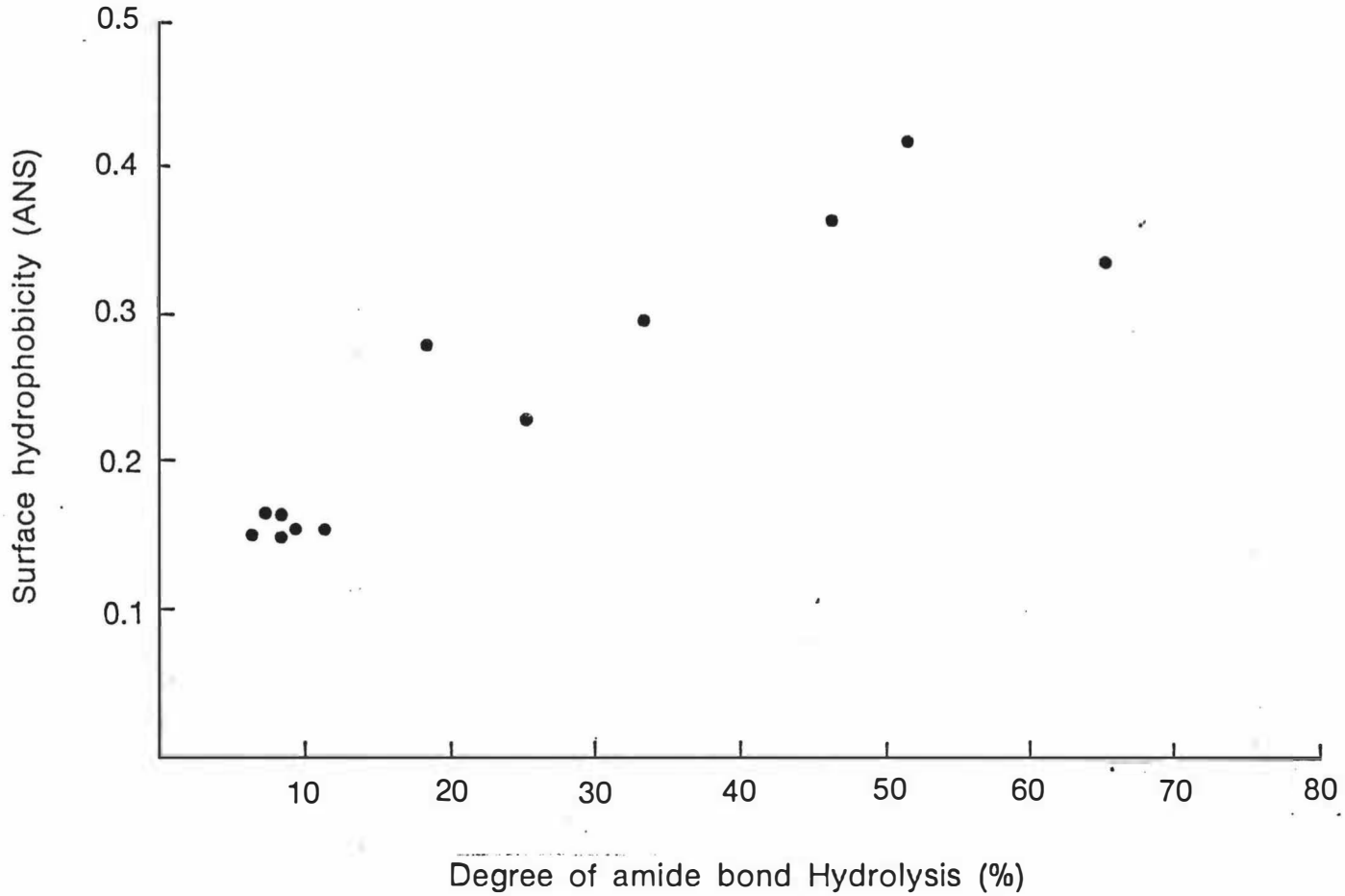


Figure 6.17 Surface hydrophobicity of the soluble portion of sample preparations measured with 8-anilino-1-naphthalenesulfonate (ANS).

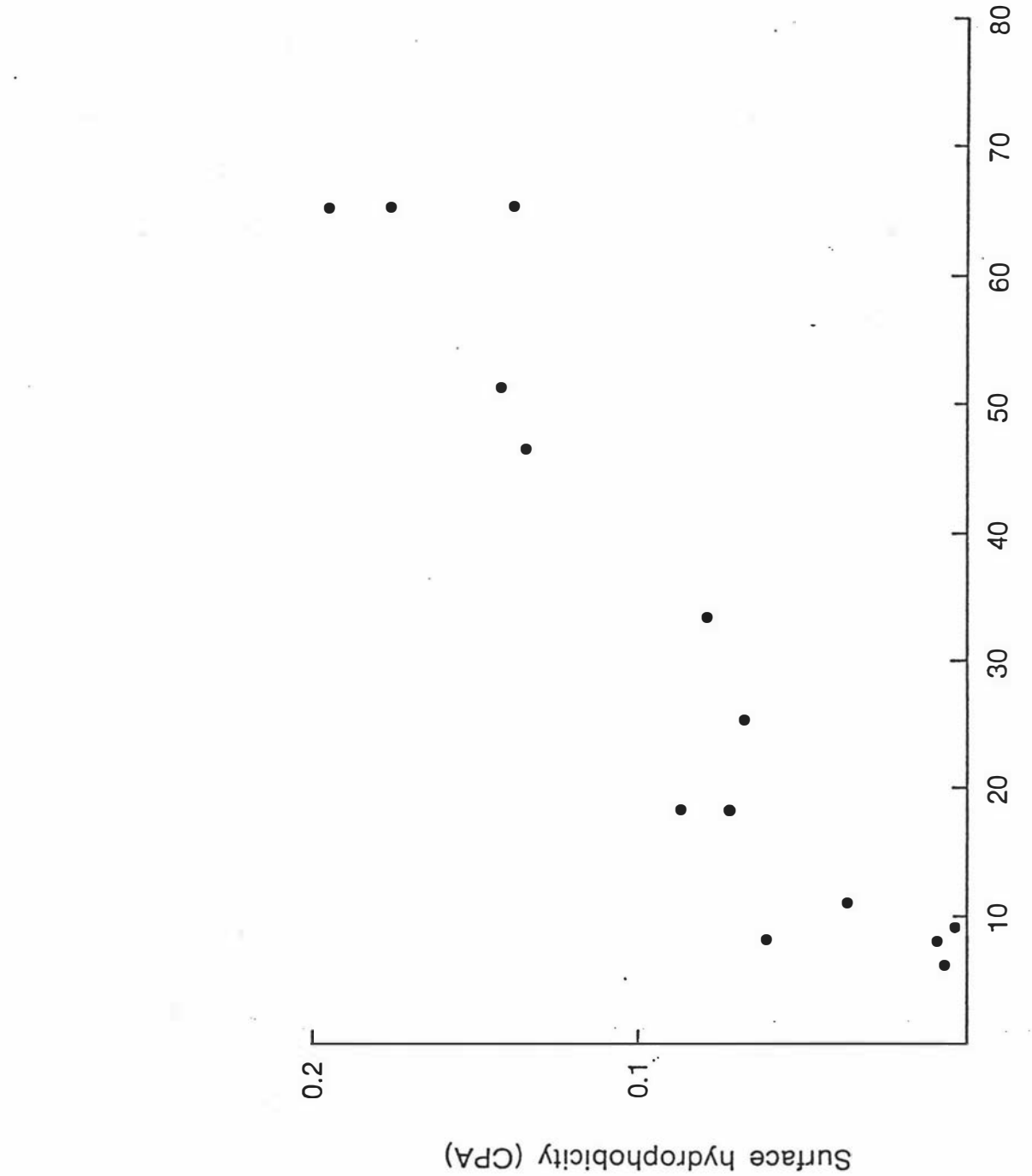


Figure 6.18 Surface hydrophobicity of the soluble portion of sample preparations measured with cis parinaric acid (CPA).

weight of casein into solution. A similar mechanism of charge neutralization appears to operate for gluten where the extent of solubilization is limited by the availability of carboxyl groups for reaction with the alkali.

In addition, the varying level of alkali requirement has been shown to be a simple indicator of the extent of protein deamidation as illustrated in Figure 6.4. The source and extent of errors in determination of the degree of deamidation were discussed in Section 4.6.

The solubility of deamidated gluten is also affected by the pH and ionic strength at which it is determined. The effect of pH and extent of deamidation was described by Matsudomi et al (1981) and by Finley (1975). The extreme sensitivity of gliadin dispersion characteristics to ionic strength was described by Holme and Briggs (1959) and a general discussion of the effect of other ions on the solubility of proteins was given by Cohn and Ferry (1943). It follows, therefore, that the solubility values for deamidated gluten determined in this present work are a function not only of the specific treatment for each sample but also of the pH and ionic strength at which the determinations were made. For these reasons care was taken to ensure that subsequent emulsion tests were made with gluten in the aqueous phase at a pH and ionic strength at which solubility determination had been made.

Although the data are portrayed as a function of the extent of deamidation the results cannot be separated from the concomitant effect of peptide bond hydrolysis. The results of the flavour studies illustrated in Figure 6.2 and Appendix 6.1 show clearly that the flavour characteristics are a function of the extent of treatment of gluten. Most previous studies of the effect of hydrolysis of proteins on flavour refer to enzyme hydrolysis of peptide bonds. While changes with the extent of hydrolysis are noted there is little quantitative data (Adler-Nissen and Sejr Olsen 1979, 1982). Bitterness, in particular, has been linked to hydrophobicity, (Adler-Nissen and Sejr Olsen 1982) and

measured hydrophobicity increases were shown in this present work. A definite link between bitter peptides and hydrophobicity has not, however, been demonstrated.

The possiblity of bitterness being associated with the formation of pyrrolidone carboxylic acid was noted in Section 1.2.6. The formation of PCA was not measured in this work but would be a useful measurement in further studies of the bitter flavour development. Methods for the determination of PCA have been described by Orlowski et al (1969), Ramakrishna and Krishnaswamy (1967) and Blombach (1967).

The studies described in Section 6.5 give a qualified confirmation of the hypothesis proposed in Section 1.5.6. As with all studies of functional properties the results obtained are as much characterisations of the methodology as descriptions of the properties of the test samples.

An unexpected observation was the visible separation into two cream or emulsion layers when using mixtures of Span 80 and Tween 20. The HLB theory assumes that molecules of each emulsifier are evenly distributed on the surface of oil droplets. If this is not so it could be due to the emulsion preparation technique or alternatively suggests some re-evaluation of the mode of action of emulsifier mixtures. In this present work the relative size of the two layers was not measured and so could not be related to the proportions of each emulsifier present. Future studies, however, could include such measurements. The behaviour of an emulsion under centrifugation conditions is not necessariy an indication of its long term behaviour but is a useful indicator analogous to accelerated storage trials generally conducted at above ambient temperatures.

The data for study of the emulsification properties of the sample preparations illustrate clearly the difference in results obtained with different oils. Many previous studies, as discussed in Section 1.5.5, have drawn general conclusions about

the value of particular proteins as emulsifiers based on studies with a single oil. Such a conclusion for a surfactant such as the Tween or Span series used in this study would not be regarded as acceptable, but has previously been so regarded in the field of evaluation of functional properties of proteins.

The different optima for each oil shown in Figures 6.5 and 6.6 validate the hypothesis of an analogy between HLB and hydrophobicity of proteins. There is, however, no additive effect, as shown in Table 6.15. This might be explained in terms of the size of protein molecules compared to most emulsifier molecules. A protein molecule might occupy a relatively large area of oil droplet surface, presumably as a monolayer, so that the charge environment at the surface of an oil droplet is determined by the protein alone. If mixtures of proteins adsorb to the surface then the environment is similarly mixed. If however mixtures of emulsifiers of differing chain lengths are oriented on the surface of an oil droplet with hydrophobic tails immersed in the oil surface and hydrophilic heads in the aqueous environment then a close packing effect is possible and a uniform charge environment can result from the mixture of emulsifiers. Such a picture is the basis of the HLB theory. If this model is correct then the fact that proteins do not show an additive effect does not invalidate the analogy between HLB and hydrophobicity of proteins.

The experiments with differing levels of protein giving the data shown in Figures 6.10, 6.11, 6.13 and 6.14 suggest that the protein acts by absorbing to the oil surface in a finite layer, thus lending support to the mechanism suggested above. Further studies could examine the oil droplet surface area and thus calculate the thickness of the adsorbed protein layer. The data also suggest, especially as shown in Figure 6.11, that only soluble protein takes part in stabilizing the emulsion.

A useful means of testing the emulsion stabilizing properties of proteins is thus suggested, i.e. a test with a fixed quantity of several oils at varying levels of soluble protein.

It is suggested that real food systems, which generally have mixtures of emulsifiers and proteins could also be understood in these terms i.e. formation of a surface adsorbed layer on the oil droplets. Phenomena such as competition for the surface could then be examined by varying order of addition of protein emulsifier etc.

The hydrophobicity results shown in Figures 6.17 and 6.18 confirm the data of Matsudomi et al (1982) and also the analogy between HLB and hydrophobicity since as hydrophobicity increases the optimum sample preparation for a particular oil relates to a decreasing required HLB of that oil.

Further definitive confirmation could be made using a pure protein deamidated under very mild conditions to avoid peptide bond hydrolysis and totally soluble at all levels of deamidation.

6.8 Conclusions

The analogy between HLB and hydrophobicity suggested by Nakai (1983) has been confirmed within the limits of the test system. The validity of criticism of previous work and conclusions regarding the emulsification properties of proteins has been confirmed by the experimental data of this present work.

7. AN ASSESSMENT OF THE POTENTIAL OF PEPTIDOGLUTAMINASES I AND II IN DEAMIDATION OF GLUTEN

7.1 Basis of investigation

The studies of Kikuchi and Sakaguchi (1973) indicated the possibility of enzymatic deamidation of proteins. Two enzymes, peptidoglutaminases I and II (E.C. 3.5.1.43 and E.C. 3.5.1.44), were shown to hydrolyse peptide-bound glutamine, a property not found in previously described glutaminase enzymes. It was found that L-glutamine presented in polypeptide chains composed of more than four amino acid residues was a poor substrate for peptidoglutaminase II. Two L-glutamine residues in oxidsed insulin A chain were, however, attacked by the enzyme. It was thought that enzymatic deamidation of gluten could be a useful alternative to acid catalysed deamidation and a preliminary investigation was undertaken.

7.2 Method of investigation

Freeze dried enzyme preparations, about 3 mg protein of purified peptidoglutaminase I and about 8 mg protein of purified peptidoglutaminase II with specific activities of 250 and 50 μ moles of ammonia formed per minute per mg of protein from glutaminyl peptide at 30°C respectively were kindly supplied by Dr M. Kikuchi of Kikkoman Corporation, Japan.

Methods of test for specific activity using suitable substrates have been described (Kikuchi and Sakaguchi, 1976). Carbobenzoxy-L-glutamine (CBZ-L-gln) was supplied by Dr D R K Harding of Massey University, New Zealand. N-acetyl-L-glutamine (Sigma Chemical company, St Louis, Mo 63178, USA) was also used as a substrate for peptidoglutaminase I (Kikuchi and Sakaguchi, 1973). Neither tert-amyloxycarbonyl-L-glutaminyl-L-proline) nor a suitable substitute was available.

N-acetyl-L-glutamine (N-acetyl-L-glu) and carbobenzoxy-L-gln (CBZ-L-gln) were prepared as 0.2M solutions in water adjusted to

pH 6.0 with NaOH. This solution was used at a rate of 50 ul in a total reaction volume of 1.0 ml, that is, 1×10^{-5} moles of amide. Now gluten contains 2×10^{-3} moles amide per gram and was used at a rate of 0.1 gm in a total reaction volume of 1.0 ml, that is, 2×10^{-4} moles of amide. The most hydrolysed of the gluten sample preparations, sample 11 was also used at the same rate of 0.1 gm in a total volume of 1.0 ml. Phosphate buffer, 0.2125M pH 7.6, was diluted to make 0.05M. Enzyme solutions were prepared by dissolving 1 mg of enzyme in 5 ml of water.

For each reaction a total volume of 1.0 ml was used. The reaction was carried out in 15 ml graduated centrifuge tubes immersed in a waterbath controlled at 30 $\pm$ 1°C.

The substrate, 50 ul of N-acetyl-L-gln or CBZ-L-gln solution, or 0.1 gm of gluten or sample 11 plus the required quantity of 0.05M NaOH, was added to 50 ul of 0.05M phosphate buffer and the required quantity of water. After dispersion of the gluten and sample 11, 5 ul of the enzyme solution was added. One reaction tube was prepared for each reaction time. The reaction was stopped by addition of 0.2 ml of 25% trichloroacetic acid (TCA). For the zero reaction time sample the TCA was added prior to the addition of the enzyme. Each reaction was allowed to proceed for 10 minutes, 30 minutes and one hour. After this the reaction mixtures stood overnight. The next day the volume of each tube was made up to 10 ml with water (MQ), the contents mixed and then centrifuged (MSE, 3000 rpm, 20 minutes). From the supernatant, 100 ul samples were taken and the ammonia content determined as previously described.

7.3 Results

The substrates and enzyme additions are shown in Table 7.1 with the measured ammonia levels. The analysis details are shown in Appendices 7.1 and 7.2 in the attached fiche. The data of Table 7.1 are also shown in Figures 7.1 and 7.2.

Table 7.1: The evolution of ammonia when N-acetyl-L-glutamine carbobenzoxy-L-glutamine, gluten and sample 11 were reacted with peptidoglutaminases I and II

REACTION NUMBER	REACTION MIXTURE	AMMONIA CONCENTRATION IN REACTION MIXTURE AFTER TIMES (MINS)			
		0 (mM)	10 (mM)	30 (mM)	60 (mM)
1	Peptidoglutaminase I + CBZ-L-gln	.05	1.2	1.9	2.7
2	Peptidoglutaminase I + N-acetyl-L-glu	0.7	1.8	1.8	5.6
3	Water + CBZ-L-glu	0.6	0.6	0.6	0.7
4	Water + N-acetyl-L-glu	0.6	0.7	0.8	1.1
5	Peptidoglutaminase II+ CBZ-L-glu	0.6	0.6	0.6	0.8
6	Peptidoglutaminase I + gluten	3.1	3.6	3.4	2.9
7	Peptidoglutaminase I + sample 11	1.5	1.8	1.7	2.2
8	Peptidoglutaminase II+ gluten	3.1	2.9	2.2	3.1
9	Peptidoglutaminase II+ sample 11	1.7	1.8	1.5	2.0
10	Peptidoglutaminase I and II + gluten	2.8	3.1	2.1	3.1
11	Peptidoglutaminase I and II + sample 11	1.6	1.8	1.5	2.0
12	Water + gluten	2.4	2.9	3.4	3.6
13	Water + sample 11	1.6	2.0	1.8	2.2
14	Peptidoglutaminase I and II + water	0.7	0.6	0.7	0.6

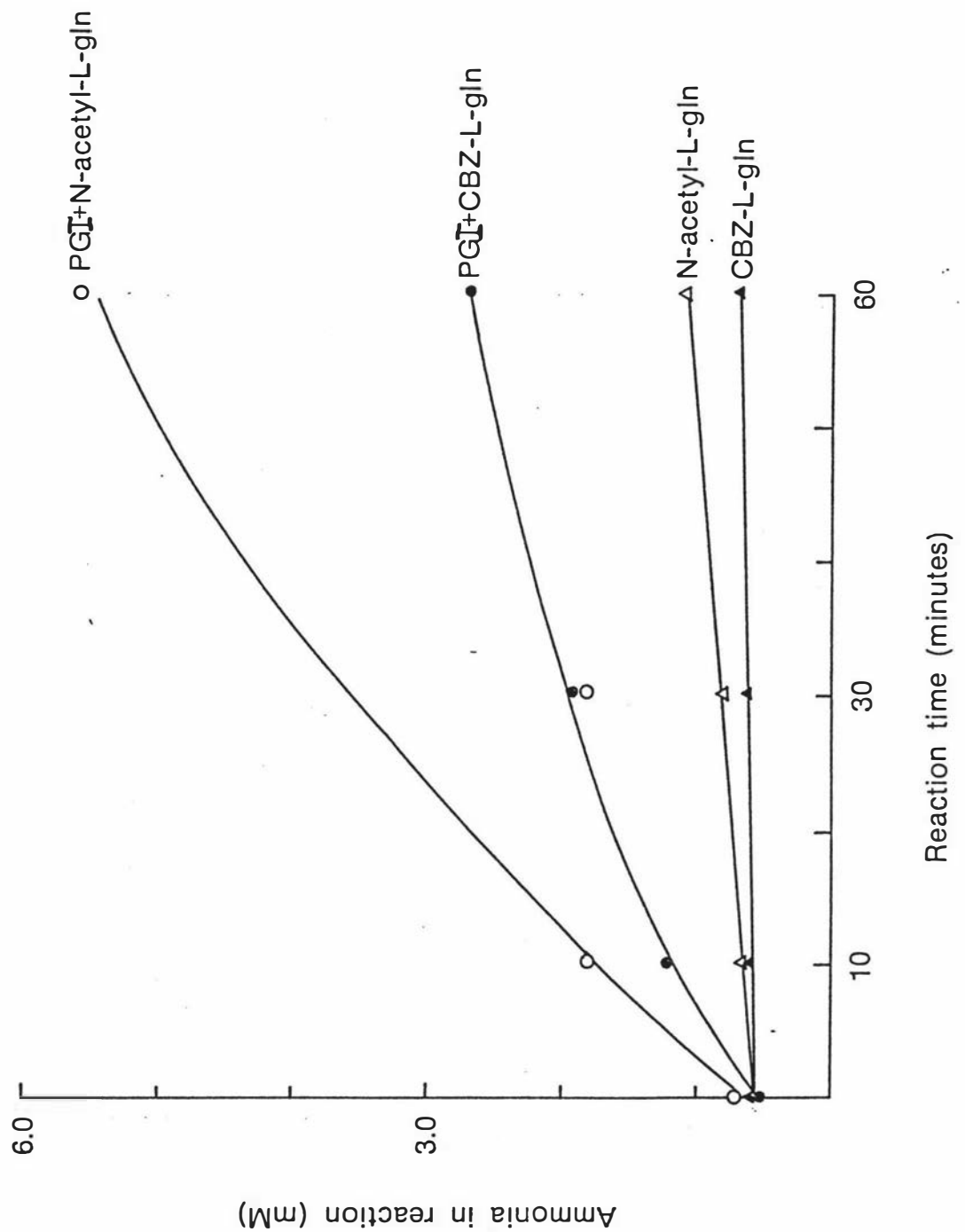


Figure 7.1 The evolution of ammonia when N-acetyl-L-glutamine and carbobenzoxy-L-glutamine were reacted with pepitidoglutaminase I.

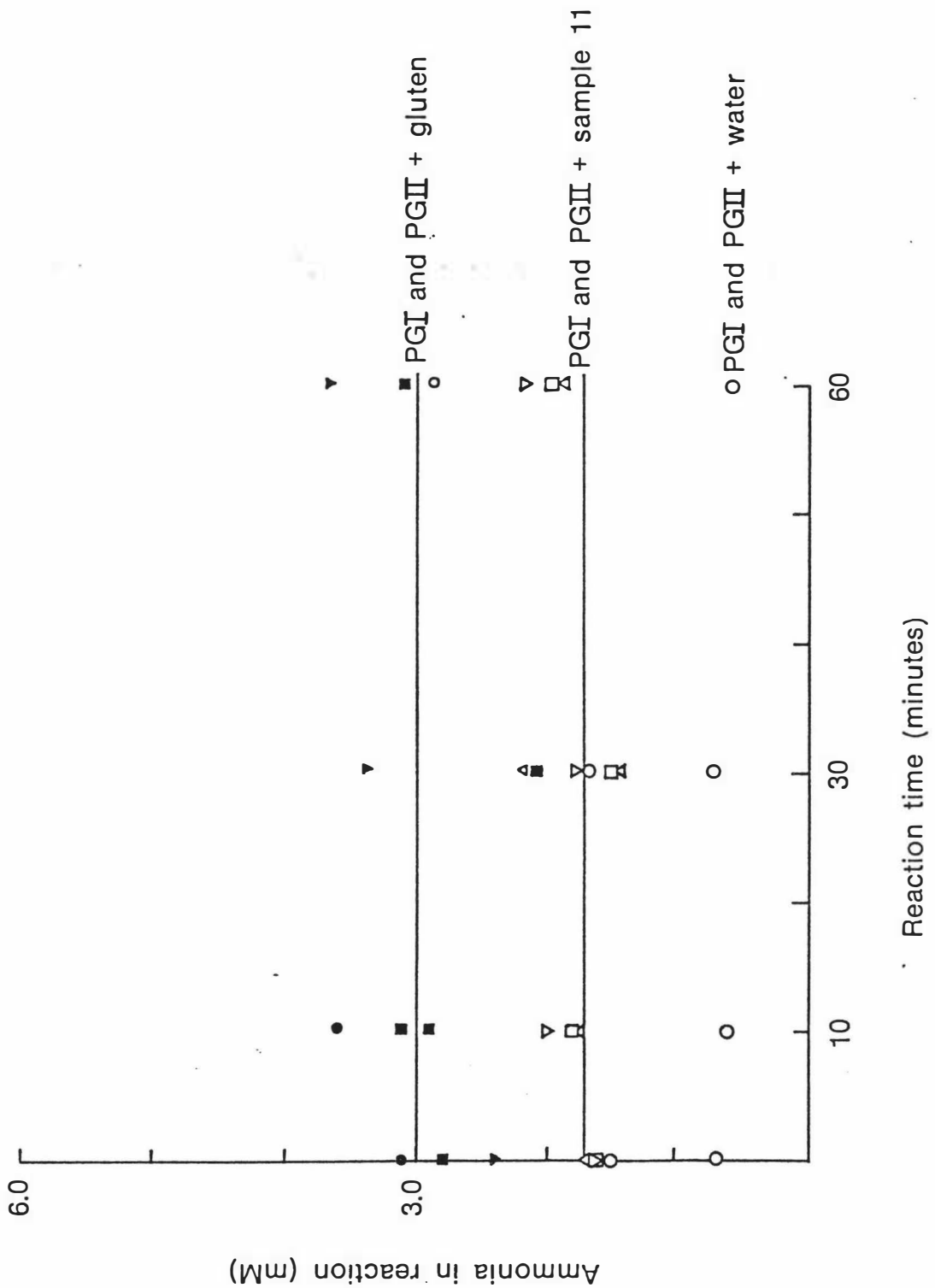


Figure 7.2 The evolution of ammonia when gluten and sample II were reacted with peptidoglutaminase I and peptidoglutaminase II.

The reaction of peptidoglutaminase I with CBZ-L-gln and N-acetyl-L-gln can be seen in Figure 7.1 Specific activities, calculated from the initial reaction rates were, respectively, 70 and 116 μmoles of ammonia formed per minute per mg of protein under the conditions of the assay.

There was no deamidation reaction catalysed by the enzymes when gluten or hydrolysed gluten was the substrate.

7.4 Discussion

The data in Table 7.1 and in Figure 7.1 show that the peptidoglutaminase I had some activity although not as much as claimed by the supplier. There may have been some loss of activity during the twelve months that the enzyme preparation was stored after receipt, and the preparation date is unknown. Because peptidoglutaminase I showed activity it was reasonable to assume that peptidoglutaminase II was also active. Neither of the enzymes was active against gluten or partially hydrolysed gluten. This finding agrees with the more recent work of Gill et al (1985) which showed that peptidoglutaminase II catalysed the release of ammonia from extensively hydrolysed whey proteins and caseins but not from the larger unhydrolysed molecules.

The level of ammonia associated with the gluten and sample 11 seen in Figure 7.2 was probably the result of acid catalysed deamidation due to the addition of TCA and holding overnight. This would explain why the level for sample 11 was approximately half that for the gluten. The amide groups in gluten were present at a level approximately 20 times that of the N-acetyl-L-glu and the CBZ-L-glu. Unless the enzyme catalysed reaction was very slight it would not have been masked by the acid catalysed deamidation. The level of ammonia released in the samples of gluten, reactions 6, 8, 10 and 12 in Table 7.1, represents about 1.5% deamidation. The low level of ammonia associated with reaction number 14 (Table 7.1) may have been due to ammonia associated with the enzyme preparations. A similar level is seen in the data of Figure 7.1.

Peptidoglutaminase enzymes appear to have little potential as an alternative to acid for deamidation of proteins. The potential of transglutaminase, which is active on protein substrates, has yet to be investigated.

8. BIBLIOGRAPHY

Adams, C.A., Robberts, T.C., Butler, K.C. (1976).
Automated determination of proteolytic enzymes and of amino nitrogen by use of trinitrobenzenesulfonic acid.
Analytical Biochemistry, 70, 181-186.

Adler-Nissen, J. (1979).
Determination of the degree of hydrolysis of food protein hydrolysates by trinitrobenzenesulfonic acid.
Journal of Agricultural and Food Chemistry, 27, 1256 - 1262.

Adler-Nissen, J., Sejr-Olsen, H. (1979).
The influence of peptide chain length on taste and functional properties of enzymatically modified soy protein.
In
Functionality and Protein Structure. ACS Symposium Series 92 (Pour-El, A., Ed.). pp 125 - 146.
American Chemical Society, Washington, DC.

Adler-Nissen, J., Sejr-Olsen, H. (1982).
Taste and taste evaluation of soy protein hydrolysates.
In
Chemistry of Foods and Beverages: Recent Developments (Charalambous, G., Inglett, G., Eds). pp. 149 - 169.
Academic Press, New York.

Ali, M., Capindale, J.B. (1975).
The hydrolysis of some N-acylaspartic and N-acylglutamic monoamides in dilute mineral acid.
Canadian Journal of Biochemistry, 53, 1137-1144.

Aoki, H., Taneyama, O., Orimo, N., Kitagawa, I. (1981).
Effect of lipophilization of soy protein on its emulsion stabilizing properties.
Journal of Food Science, 46, 1191 - 1195.

Aoki, H., Shirase, Y., Kato, J., Watanabe, Y. (1984).
Emulsion stabilizing properties of soy protein isolates mixed with sodium caseinates.
Journal of Food Science, 49, 212-216.

Aranyi, C., Hawrylewicz, E.J. (1972).
Preparation and isolation of acid catalyzed hydrolysates from wheat gluten.
Journal of Agricultural and Food Chemistry, 20, 670 - 675.

Bailey, J.E., Ollis, D.F. (1977).
Biochemical Engineering Fundamentals. pp. 96 - 98.
McGraw-Hill, Kogukusha Ltd, Tokyo.

Bates, R.G. (1973)
Determination of pH - Theory and Practice, 2nd edn.
Wiley Interscience, New York.

Bates, R.G., Paabo, M. (1970).
Measurement of pH In
Handbook of Biochemistry, Selected Data for Molecular Biology, 2nd edn. (Sober, H.A., Ed.). pp. J-227 - J-233.
The Chemical Rubber Co, Cleveland, Ohio.

Becher, P. (1966).
Emulsions: Theory and Practice, 2nd edn.
American Chemical Society Monograph Lines.
Reinhold Publishing Corporation, New York.

Beck, W.H., Wynne-Jones, W.F.K. (1952).
The hydrogen electrode function of glass membranes.
Journal de Chimie Physique et de Physicochimie Biologique, 49, C97 - C105.

Beck, W.H., Cavole, J., Covington, A.K., Wynne-Jones, W.F.K. (1963).

Precise measurements with the glass electrode. The time variation of EMF.

Proceedings of the Chemical Society, pp 110 - 111.

Beckwith, A.C., Wall, J.S., Dimler, K.J. (1963).

Amide groups as interaction sites in wheat gluten protein: Effects of amide-ester conversion.

Archives of Biochemistry and Biophysics, 103, 319-330.

Beers, Y. (1953).

Introduction to the Theory of Error.

Addison-Wesley Publishing Company, Inc., Cambridge, Massachusetts.

Bell, D.J., Heywood-Waddington, D., Hoare, M., Dunnill, P. (1982).

'The density of protein precipitates and its effect on centrifugal sedimentation.

Biotechnology and Bioengineering, 24, 127 - 141.

Bigelow, C.C. (1967).

On the average hydrophobicity of proteins and the relation between it and protein structure.

Journal of Theoretical Biology, 16, 187-211.

Blackburn, S., Lee, G.R. (1954).

The liberation of aspartic acid during the acid hydrolysis of proteins.

Biochemical Journal, 58, 227-231.

Blomback, B. (1967).

Derivatives of glutamine in peptides. In Methods in Enzymology,Volume 11, Enzyme Structure (Hirs, C.H.W., Ed.). pp. 398 - 411.

Academic Press, New York.

Bohlen, P., Stein, S., Dairman, W., Volenfriend, S. (1973).
Fluorometric assay of proteins in the nanogram range.
Archives of Biochemistry and Biophysics, 155, 213-220.

Bottomley, R.C., Kearns, H.F., Schofield, J.D. (1982).
Characterization of wheat flour and gluten proteins using buffers containing sodium dodecyl sulphate.
Journal of the Science of Food and Agriculture, 33, 481 - 491.

Boyd, J., Parkinson, C., Sherman, P. (1972).
Factors affecting emulsion stability and the HLB concept.
Journal of Colloid and Interface Science, 41, 359-369.

Breyer, L.M., Walker, C.E. (1983).
Comparative effects of various sucrose-fatty acid esters upon bread and cookies.
Journal of Food Science, 48, 955-958, 987.

Bushuk, W. (1981).
Utilization of cereal proteins. In
Utilization of Protein Resources' (Stanley, D.W., Murray, E.D., Lees, D.H., Eds) pp. 208 - 226.
Food and Nutrition Press, Inc., Westport, Connecticut.

Cappellos, C., Bielski, B.H.J. (1972).
Kinetic Systems.
Wiley Interscience, New York.

Challis, B.C., Challis, J.A. (1970).
Reactions of the carboxamide group.
In
The Chemistry of Amides. (Zabicky, J., Ed.). pp. 731 - 857.
Interscience Publishers, London.

Chen, L.-F., Richardson, T., Amundson, C.H. (1975).
Some functional properties of succinylated proteins from fish protein concentrate.
Journal of Milk and Food Technology, 38, 89 - 93.

Cherry, J.P., McWatters, K.H., Beuchat, L.R. (1979).
Oilseed protein properties related to functionality in emulsions and foams.
In
Functionality and Protein Structure. ACS Symposium Series 92 (Pour-El, A., Ed.). pp. 1 - 26.
American Chemical Society, Washington, D.C.

Christian, J.A., Saffle, R.L. (1967).
Plant and animal fats and oils emulsified in a model system with muscle salt-soluble protein.
Food Technology, 21, 1024-1027.

Clarke, P.H. (1980).
The utilization of amides by microorganisms.
In Microorganisms and Nitrogen Sources: Transport and Utilization of Amino Acids, Peptides, Proteins and Related Substances (Payne, J.W., Ed.). pp. 537 - 562.
John Wiley and Sons, Chichester.

Clegg, K.M., Lee, Y.K., McGilligan, J.F. (1982).
Technical note: Trinitrobenzenesulfonic Acid and ninhydrin reagents for the assessment of protein degradation in cheese samples.
Journal of Food Technology, 17, 517 - 520.

Cohn, E.J., Ferry, J.D. (1965).
Interactions of proteins with ions and dipolar ions.
In
Proteins, Amino Acids and Peptides as Ions and Dipolar Ions (Cohn, E.J., Edsall, J.T., Eds). pp. 586 - 622. ACS Monograph Series
Hafner Publishing Company, New York.

Cooper, H.R. (1985). New Zealand Dairy Research Institute.
Personal Communication.

Cornish-Bowden, A. (1975).
The use of the direct linear plot for determining initial velocities.
Biochemical Journal, 149, 305-312.

Creamer, L.K., Richardson, T. (1984).
Anomalous behaviour of bovine α_{s1}- and β-caseins on gel electrophoresis in sodium dodecyl sulfate buffers.
Archives of Biochemistry and Biophysics, 234, 476-486.

Creamer, L.K., Zoerb, H.F., Olson, N.F., Richardson, T. (1982).
Surface hydrophobicity of α_{s1}-I, $_{s1}$ - Casein A and B and its implications in cheese structure.
Journal of Dairy Science, 65, 902 - 906.

Crocker, J.C. (1907).
The velocity of hydrolysis of aliphatic amides.
Journal of the Chemical Society, 91, 593 - 612.

Danno, G. (1981).
Extraction of unreduced glutenin from wheat flour with sodium dodecyl sulfate.
Cereal Chemistry, 58, 311 - 313.

Danno, G., Kanazawa, K., Natake, M. (1974).
Extraction of wheat flour proteins with sodium dodecyl sulfate and their molecular weight distribution.
Agricultural and Biological Chemistry, 38, 1947 - 1953.

Davies, O.L. (1967).
Statistical methods in Research and Production: With Special Reference to the Chemical Industry, 3rd edn revised.
Imperial Chemical Industries Ltd, London.

Day, J.N.E., Ingold, C.K. (1941).
Mechanism and kinetics of carboxylic ester hydrolysis and carboxyl esterification.
Transactions of the Faraday Society, 37, 686 - 707.

Denoyer, C., Girard, J.P. (1982).
Effect of pH and salt concentration on stability of emulsions made with different protein fractions.
Proceedings of the European Meeting of Meat Research Workers, No. 28, Volume II, 9.20, pp. 449-455.
(Food Science and Technology Abstracts (1984). 16, 5S923).

Dickinson, E., Stainsby, G. (1982).
Colloids in Food.
Applied Science Publishers, London.

Dole, M. (1932).
The theory of the glass electrode. II.
The glass as a water electrode.
Journal of American Chemical Society, 54, 3095 - 3105.

Draper, N.R., Smith, H. (1980).
Applied Regression Analysis, 2nd edn.
John Wiley and Sons, Inc., New York.

Edsall, J.T. (1965).
'Proteins as acids and bases. In
Proteins, Amino Acids and Peptides as Ions and Dipolar Ions (Cohn, E.J. Edsall, J.T., Eds). pp. 444 - 505.
ACS Monograph Series. Hafner Publishing Company, New York.

Edwards, J., Kosikowski, F.V. (1983).
Bitter compounds from Cheddar cheese.
Journal of Dairy Science, 66, 727-734.

Feeney, R.E., Yamasaki, R.B., Geoghegan, K.R. (1982).
Chemical modification of proteins: An overview.
In
Modifications of proteins: Food, Nutritional and
pharmacological Aspects (Feeny, R.E., Whitaker, J.R., Eds).
pp. 3 - 55.
Advances in Chemistry Series 198.
American Chemical Society, Washington, DC.

Fields, R. (1971).
The measurement of amino groups in proteins and peptides.
Biochemical Journal, 124, 581-590.

Fields, R. (1972).
The rapid determination of amino groups with TNBS. In
Methods in Enzymology, Volume 25, Enzyme Structure, Part B
Hirs, C.H.W., Timasheff, S.N., Eds). pp. 464 - 468.
Academic Press, New York.

Finley, J.W. (1975).
Deamidated gluten: a potential fortifier for fruit juices.
Journal of Food Science, 40, 1283-1285.

Finston, H.L., Rychtman, A.C. (1982).
A New View of Current Acid-Base Theories.
John Wiley and Sons, New York.

Fisher, L.R., Parker, N.S. (1985).
How do food emulsion stabilizers work?
CSIRO Food Research Quarterly, 45, 33-39.

Folk, J.E., Chung, S.I. (1973).
Molecular and catalytic properties of transglutaminases.
Advances in Enzymology, 38, 109-191.

Folk, J.E., Finlayson, J.S. (1977).
The ϵ-(γ-glutamyl) lysine crosslink and the catalytic role of transglutaminases.
Advances in Protein Chemistry, 31, 1-133.

Fung, C.P., Fong, A.S., Lam, A.S.M., Lee, G., Nakai, S. (1977).
Preparation and properties of acid-solubilized wheat flour.
Journal of Food Science, 42, 1594-1599.

Gebhardt, E., Newmann, Ch., Eckert, I. (1982).
Modification and derivatization of gluten and its influence on the manufacture of baked goods (in German).
Bacher und Konditor, 30, (2), 37-39.

Gill, B.P., O'Shaughnessey, A.J., Henderson, P., Headon, D.R. (1985).
An assessment of the potential of peptidoglutaminases I and II in modifying the charge characteristics of casein and whey proteins.
Irish Journal of Food Science and Technology, 9, 33 - 41.

Glasstone, S., Lewis, D. (1960).
Elements of Physical Chemistry. 2nd edn., Macmillan and Company Ltd, London.

Goldfarb, A.R. (1966).
A kinetic study of the reactions of amino acids and peptides with trinitrobenzenesulfonic acid.
Biochemistry, 5, 2570-2574.

Govin, R., Leeder, J.G. (1979).
Action of emulsifiers in ice cream using the HLB concept.
Journal of Food Science 36, 718-722.

Graham, D.E., Phillips, M.C. (1976).
The conformation of proteins at interfaces and their roles in stabilizing emulsions.
In
Theory and Practice of Emulsion Technology (Smith, A.L., Ed.) pp. 75 - 98.
Academic Press, London.

Graham, D.E., Phillips, M.C. (1979).
Proteins at liquid interfaces. III. Molecular structures of adsorbed films.
Journal of Colloid and Interface Science,70, 427 - 439.

Graveland, A., Bongers, P., Bosveld, P. (1979).
Extraction and fractionation of wheat flour proteins.
Journal of the Science of Food and Agriculture, 30, 71 - 84.

Graveland, A., Bosveld, P., Lichtenoonk, W.J., Moonen, J.H.E., Scheepstra, A. (1982).
Extraction and fractionation of wheat flour proteins.
Journal of the Science of Food and Agriculture, 33, 1117 - 1128.

Graveland, A., Bosveld, P., Lichtenoonk, W.J., Marseille, J.P., Moonen, J.H.E., Scheepstra, A. (1985).
A model for the molecular structure of the glutenins from wheat flour.
Journal of Cereal Science, 3, 1-16.

Griffin, W.C. (1949).
Classification of surface active agents by HLB.
Journal of the Society of Cosmetic Chemists, 1, 311 - 326.

Halling, P.J. (1981).
Protein-stabilized foams and emulsions.
Critical Reviews in Food Science and Nutrition, 15, 155-203.

Hamauzu, Z., Miyashita, C., Matsutaka, K. (1982).
Properties of gluten deamidated with dilute hydrochloric acid.
Bulletin of Mukogawa Women's University, No. 30, 57 - 61.

Han, K.K., Richard, C., Biserte, G. (1983).
Current developments in chemical cleavage of proteins.
International Journal of Biochemistry, 15, 875 - 885.

Harned, H.S., Ehlers, R.W. (1935).
Journal of the American Chemical Society, 55, 2179 - 2193.

Harris, J.I., Cole, R.D., Pon, N.G. (1956).
The kinetics of acid hydrolysis of dipeptides.
Biochemical Journal, 62, 154-159.

Hartman, S.C. (1968).
Glutaminase of Escherichia coli.
I. Purification and general catalytic properties.
Journal of Biological Chemistry, 243, 853-863.

Hartman, S.C. (1973).
Relationships between glutamine amidotransferases and glutaminases.
In The Enzymes of Glutamine Metabolism
(Prusiner, S., Stadtman, E.R., Eds). pp. 319 - 330.
Academic Press, New York.

Hashimoto, A.G. (1983).
Conversion of straw-manure mixtures to methane at mesophilic and thermophilic temperatures.
Biotechnology Bioengineering, 25, 185-200.

Hayakawa, S., Nakai, S. (1985).
Relationships of hydrophobicity and net charge to the solubility of milk and soy proteins.
Journal of Food Science, 50, 486-491.

Hill, C.G. (1977).
An introduction to Chemical Engineering Kinetics and Reaction Design.
Wiley, New York.

Hill, R.L. (1965).
Hydrolysis of proteins.
Advances in Protein Chemistry, 20, 37-107.

Holme, J., Briggs, D.R. (1959).
Studies on the physical nature of gliadin.
Cereal Chemistry, 36, 321-340.

Hubbard, D., Hamilton, E.H., Finn, A.N. (1939).
Effect of the solubility of glass on the behaviour of the glass electrode.
Journal of Research of the National Bureau of Standards, 22, 339 - 349.
(Data quoted in Beck, W.H., Wynne-Jones, W.F.K. (1952).
The hydrogen electrode function of glass membranes.
Journal de Chimie Physique et de Physiochimie Biologique, 49, C97 - C105.

ICI Americas Inc. (1980).
The HLB System - A Time Saving Guide to Emulsifier Selection.
ICI Americas Inc.,

Ifft, J.B. (1976).
Sedimentation equilibrium of proteins in density gradients.
Biophysical Chemistry, 5, 137-157.

Ikura, K., Kometani, T., Yoshikawa, M., Sasaki, R., Chiba, H. (1980).
Crosslinking of casein components by transglutaminase.
Agricultural and Biological Chemistry, 44, 1567 - 1573.

Ikura, K., Yoshikawa, M., Sasaki, R., Chiba, H. (1981).
Incorporation of amino acids into food proteins by transglutaminase.
Agricultural and Biological Chemistry, 45, 2587 - 2592.

John, P.W.M. (1971).
Statistical Design and Analysis of Experiments. pp. 198 - 201.
McMillan, New York.
Based on Schneider, A.M., Stockett, A.C. (1963).
An experiment to select optimum operating conditions on the basis of arbitrary preference ratings.
Chemical Engineering Progress, Symposium Series No. 42, Volume 59.

Kamata, Y., Ochiai, K., Yamauchi, F. (1984).
'Relationship between the properties of emulsion systems stabilized by soy protein digests and the protein conformation.
Agricultural Biological Chemistry, 48, 1147-1152.

Kasaroa, D.D., Nimmo, C.C., Kohler, G.O. (1971).
Proteins and the amino acid composition of wheat fractions.
In
Wheat Chemistry and Technology (Pomeranz, Y., Ed.). pp. 227 - 299.
American Association of Cereal Chemists Inc., St Paul, Minnesota.

Kasper, C.B. (1970).
Partial acid hydrolysis. In
Protein Sequence Determination (Needleman, S.B., Ed.). pp. 141 - 150.
Chapman and Hall, London.

Kato, A., Nakai, S. (1980).
Hydrophobicity determined by a fluorescence probe method and its correlation with surface properties of proteins.
Biochimica et Biophysica Acta, 624, 13 - 20.

Kato, A., Tsutsui, N., Matsudomi, N., Kobayashi, K., Nakai, S. (1981).
Effects of partial denaturation on surface properties of ovalbumin and lysozyme.
Agricultural and Biological Chemistry, 45, 2755 - 2760.

Kato, A., Osako, Y., Matsudomi, N., Kobayashi, K. (1983).
Changes in the emulsifying and foaming properties of proteins during heat denaturation.
Agricultural and Biological Chemistry, 47, 33 - 37.

Kato, A., Matsuda, T., Matsudomi, N., Kobayashi, K. (1984).
Determination of protein hydrophobicity using a sodium dodecyl sulfate binding method.
Journal of Agricultural and Food Chemistry, 32, 284 - 288.

Kato, A., Komatsu., Fujimoto, K., Kobayashi, K. (1985).
Relationship between surface functional properties and flexibility of proteins detected by the protease susceptibility.
Journal of Agricultural and Food Chemistry, 33, 931 - 934.

Keshavarz, E., Nakai, S. (1979).
The relationship between hydrophobicity and interfacial tensions of proteins.
Biochimical et Biophysica Acta, 576, 269 - 279.

Kikuchi, M., Sakaguchi, K. (1973).
Identification of the organism and some conditions of peptidoglutaminase formation.
Agricultural and Biological Chemistry, 37, 719 - 724.

Kikuchi, M., Sakaguchi, K. (1973).
Peptidoglutaminase-I and II: Isolation in homogeneous form.
Agricultural and Biological Chemistry, 37, 827 - 835.

Kikuchi, M., Sakaguchi, K. (1973).
Some enzymatic properties and substrate specificities of peptidoglutaminase I and II.
Agricultural and Biological Chemistry, 37, 1813 - 1821.

Kikuchi, M., Sakaguchi, K. (1976).
Peptidoglutaminase (Bacillus circulans). In
Methods in Enzymology Volume 45, Proteolytic Enzymes, Part B (Lorand, L., Ed.). pp. 485 - 482. Academic Press, New York.

Kikuchi M., Hayashida, H., Nakano, E., Sakaguchi, K. (1971).
Peptidoglutaminase. Enzymes for selective deamidation of -amide of peptide-bound glutamine.
Biochemistry, 10, 1222 - 1229.

Kinsella, J.E. (1976).
Functional properties of proteins in food: A survey.
Critical Reviews in Food Science and Nutrition, 7, 219 - 280.

Kinsella, J.E. (1982).
Relationships between structure and functional properties of food proteins.
In
Food proteins (Proceedings of the Kellog Foundation International Symposium on Food Proteins) (Fox, P.F., Condon, J.J., Eds). pp. 51 - 103.
Applied Science Publishers, London.

Kitchener, J.A., Mussellwhite, P.R. (1968).
The theory of stability of emulsions.
In Emulsion Science (Sherman, P., Ed). Chapter 2, pp. 77 - 130
Academic Press, London.

Kittrell, J.R. (1970).
Mathematical modeling of chemical reactions.
Advances in Chemical Engineering, 8, 97 - 183.

Knightbridge, J.P. and Goldman, A. (1975).
Water absorptive capacity of dried milk products.
New Zealand Journal of Dairy Science and Technology, 10,
152 - 157.

Kotaki, A., Haraoa, M., Yagi, K. (1964).
Reaction between sulfhydryl compounds and 2, 4, 6-
trinitrobenzene-1-sulfonic acid.
Journal of Biochemistry, 55, 553-561.

Krull, L.H., Wall, J.S. (1966).
Synthetic polypeptides containing side-chain amide groups.
Water-soluble polymers.
Biochemistry, 5, 1521-1527.

Krull, L.H., Wall, J.S., Zobel, H., Dimler, R.J. (1965).
Synthetic polypeptides containing side-chain amide groups:
Water-insoluble polymers.
Biochemistry, 4, 626 - 633.

Kuntz, I.D.Jr, Kauzmann, W. (1974).
Hydration of proteins and polypeptides.
Advances in protein chemistry, 28, 239 - 345.

Laidler, K.J. (1965).
Chemical kinetics, 2nd edn.
McGraw-Hill, New York.

Lawrence, L., Moore, W.J. (1951).
Kinetics of the hydrolysis of simple glycine peptides.
Journal of the American Chemical Society, 73, 3973-3977.

Leach, S.J. (1953).
The rate of hydrolysis of proteins and peptides.
Reviews of Pure and Applied Chemistry (Aust.), 3, 25-60.

Leach, S.J., Lindley, H. (1953).
The kinetics of hydrolysis of the amide group in proteins and peptides. Part 2. Acid hydrolysis of glycyl- and l-leucyl-l-asparagine.
Transactions of the Faraday Society, 49, 921-925.

Leach, S.J., Parkhill, E.M.J. (1955).
Proceedings of the International Wool and Textiles Research Conference Australia C-92.
(Quoted in Hamilton, L.D.G. (1960).
The estimation of side chain groups in the intact protein.
In
A Laboratory Manual of Analytical Methods of Protein Chemistry. Alexander, P., Block, R.J., Eds).
Volume 2, pp. 71 - 72 Pergamon Press, Oxford).

Levenspiel, O. (1972).
'Chemical reaction engineering' 2nd edn.
John Wiley and Sons Inc, New York.

Levitzki, A. (1970).
Determination of submicro quantities of ammonia.
Analytical Biochemistry, 33, 335-340.

Li-Chan, E., Nakai, S., Wood, D.F. (1985).
Relationship between functional (fat binding, emulsifying) and physicochemical properties of muscle proteins. Effect of heating, freezing, pH and species.
Journal of Food Science, 50, 1034-1040.

Light, A. (1967).
Partial acid hydrolysis.
In Methods in Enzymology, Volume 11, Enzyme Structure (Hirs, C.H.W., Ed.). pp. 417 - 420.
Academic Press, New York.

Lin, P.M., Leeder, J.G. (1974).
Mechanism of emulsifier action in an ice cream system.
Journal of Food Science, 39, 108-111.

Lowry, O.H., Passonneau, J.V., Rock, M.K. (1961).
The stability of pryidine nucleotides.
Journal of Biological Chemistry, 236, 2756 - 2759.

Ma, C.Y. (1984).
Functional properties of acylated oat protein.
Journal of Food Science, 49, 1128 - 1131.

McDermott, R.L., Harper, W.J., Whitley, R. (1981).
A centrifugal method for characterization of salad dressing emulsions.
Food Technology, 35, (5), 81, 82, 84, 85, 87.

McDonald, C.E., Pence, J.W. (1961).
Wheat gliadin in foams for food products.
Food Technology, 15, 141-144.

MacInnes, D.A., Belcher, D. (1931).
Further studies on the glass electrode.
Journal of the American Chemical Society, 53, 3315 - 3331.

MacRitchie, F. (1979).
A relation between gluten protein amide content and baking performance of wheat flours.
Journal of Food Technology, 14, 595-601.

MacRitchie, F. (1984).
Baking quality of wheat flours.
Advances in Food Research, 29, 201-277.

McWalters, K.H., Cherry, J.P. (1981).
Emulsification: vegetable proteins.
In
Protein Functionality in Foods. ACS Symposium Series 147
(Cherry, J.P. Ed.). pp. 217 - 242.
American Chemical Society, Washington, DC.

Mangino, M.E., Fritsch, D.A., Liao, S.Y., Fayerman, A.M., Harper, W.J. (1985).
The binding of n-alkanes to whey protein concentrates as a predictor of their functionality.
New Zealand Journal of Dairy Science and Technology, 20, 103-107.

Matsudomi, N., Kaneko, S., Kato, A., Kobayashi, K. (1981).
Functional properties of deamidated gluten. Nippon Nogeikagaku Kaishi, 55, 983-989.

Matsudomi, N., Kato, A., Kobayashi, K. (1982).
Conformation and surface properties of deamidated gluten.
Agricultural and Biological Chemistry, 46, 1583 - 1586.

Matsudomi, N., Mori, H., Kato, A., Kobayashi, K. (1985).
Emulsifying and foaming properties of heat-denatured soybean 11S globulins in relation to their surface hydrophobicity.
Agricultural and Biological Chemistry, 49, 915 - 919.

Meester, C. de (1974).
Extraction et utilization de la gliadine et de la glutenine du froment.
Industries Alimentaires et Agricoles 91, (1), 27-32.

Meister, A., Levintow, L., Greenfield, R.E., Abendschein, P.A. (1955).
Hydrolysis and transfer reactions catalysed by -amidase preparations.
Journal of Biological Chemistry, 215, 441-460.

Melville, J. (1935).
Labile glutamine peptides and their bearing on the origin of the ammonia set free during the enzymic digestion of proteins
Biochemical Journal 29, 179-186.

Ministry of Agriculture and Fisheries (1979).
Chemistry : Standard Laboratory Methods, Section 5, Protein Products.
Dairy Division, Ministry of Agriculture and Fisheries, Wellington, New Zealand.

Mittal, S., Gupta, K.S., Gupta, Y.K. (1981).
Kinetics of carboxylic acid catalysed hydrolysis of formamide: evidence for specific hydronium ion catalysis.
Indian Journal of Chemistry, Section A, 20A, 1220 - 1221.

Mittal, S., Gupta, K.S., Gupta, Y.K. (1982).
Kinetics and mechanism of acid hydrolysis of formamide, acetamide, propanamide and butanamide over an extended concentration range : Kinetic evidence for fast protonation pre-equilibrium.
Indian Journal of Chemistry, Section A, 21A, 357-360.

Mohammedzadeh-K, A., Feeney, R.E., Smith, L.M. (1969).
Hydrophobic binding of hydrocarbons by proteins.
I. Relationship of hydrocarbon structure.
Biochimica et Biophysica Acta, 194, 246-255.

Moore, J.W., Pearson, R.G. (1981).
Kinetics and Mechanism, 3rd edn.
Wiley Interscience, New York.

Morr, C.V. (1981).
Emulsifiers: Milk proteins.
In
Protein Functionality in Foods ACS Symposium Series 147
(Cherry, J.P. Ed.) pp. 201 - 215.
American Chemical Society, Washington, DC.

Mosse, J., Huet, J.C., Bavoet, J. (1985).
The amino acid composition of wheat grain as a function of nitrogen content.
Journal of Cereal Science, 3, 115-130.

Motoki, M., Nio, N. (1983).
Crosslinking between different food proteins by transglutaminase.
Journal of Food Science, 48, 561-566.

Munro, P.A. (1980).
The densities of casein curd particles and caseinate solutions.
New Zealand Journal of Dairy Science and Technology, 15, 225 - 238.

Mycek, M.J., Clarke, D.D., Neidle, A., Waelsch, H. (1959).
Amide incorporation into insulin as catalysed by transglutaminase.
Archives of Biochemistry and Biophysics, 84, 528-540.

Nakai, S. (1983).
Structure-function relationships of food proteins with an emphasis on the importance of protein hydrophobicity.
Journal of Agricultural and Food Chemistry, 31, 676 - 683.

Nakai, S., Ho, L., Helbig, N., Kato, A., Tung, M.A. (1980).
Relationship between hydrophobicity and emulsifying properties of some plant proteins.
Canadian Institute of Food Science and Technology Journal, 13, 23 - 27.

Ohba, N. (1962).
Hydrophile-lipophile balance values for o/w emulsions stabilized by nonionic surfactants.
I. Hydrophile-lipophile balance values of nonionic surfactants determined by emulsification.
Bulletin of the Chemical Society of Japan, 35, 1016-1020.

Ohba, N. (1962).
Hydrophile-lipophile balance values for o/w emulsions stabilized by nonionic surfactants II. "Required hydrophile-lipophile balance values" of the oil mixture.
Bulletin of the Chemical Society of Japan, 35, 1021-1025.

Ollis, D.F. (1983).
Kinetics of enzyme systems.
In
Foundations of Biochemical Engineering:
Kinetics and Thermodynamics in Biological Systems.
ACS Symposium Series 207 Blanch, H.W., Papovtsakis, E.T., Stephanopoulos, G., Eds). pp. 27 - 52.
American Chemical Society, Washington, DC.

Orlowski, M., Richman, P.G., Meister, A. (1969).
Isolation and properties of -L-glutamyl-cyclotransferase from human brain.
Biochemistry, 8, 1048 - 1055.

Osborne, T.B. (1907).
The Proteins of the Wheat Kernel. Publication No. 84.
Carnegie Institute of Washington, Washington, DC.

Payne, J.W. (Ed) (1980).
Microorganisms and Nitrogen Sources: Transport and Utilization of Amino Acids, Peptides, Proteins, and Related Substances.
John Wiley and Sons, Chichester.

Pearce, K.N., Kinsella, J.E. (1978).
Emulsifying properties of proteins: evaluation of a turbidimetric technique.
Journal of Agricultural and Food Chemistry, 26, 716 - 723.

Peltonen, R.I. (1982).
Characterization of whey protein concentrates with regard to factors that affect their function at interfaces.
Ph.D Thesis. The Ohio State University, Columbus, Ohio.

Pfeifer, V.F., Vojnovich, C., Anderson, R.A. (1958).
Vital wheat gluten by drum drying
I. Effect of processing variables
Cereal Chemistry, 35, 458-468.

Pomeranz, Y., Chung, O.K. (1981).
Starch-lipid-protein interactions in cereal systems.
In
Utilization of Protein Resources, (Stanley, D.W., Murray, E.D., Lees, D.H., Eds). pp. 129 - 157.
Food and Nutrition Press, Inc, Westport, Connecticut.

Popineau, Y., Godon, B. (1982).
Surface hydrophobicity of gliadin components.
Cereal Chemistry, 59, 55-62.

Pour-El, A. (1976)
Measurement of functional properties of soy protein products.
In World Soybean Research Conference (Hill, L.D., Ed). pp. 918 - 948.
Interstate, Danville, Illinois.

Pour-El, A. (1981).
Protein functionality : classification, definition, and methodology.
In
Protein Functionality in Foods. ACS Symposium Series 147 (Cherry, J.P., Ed.). pp. 1 - 19.
American Chemical Society, Washington, DC.

Prusiner, S. (1973).
Glutaminases of E. coli: Properties Regulation and Evolution.
In
The Enzymes of Glutamine Metabolism. (Prusiner, S., Stadtman, E.R., Eds). pp. 293 - 315. Academic Press, New York.

Prusiner, S., Stadtman, E.R., (1973).
The Enzymes of Glutamine Metabolism. Academic Press, New York.

Puski, G. (1986).
A Review of methodology for emulsification properties of plant proteins.
Cereal Chemistry, 53, 650 - 655.

Pyler, E.J. (1983).
Flour proteins role in baking performance I.
Bakers Digest, 57, (3), 24, 25, 27, 28 33.
Flour proteins role in baking performance II.
Bakers Digest, 57, (5), 44, 46, 48 - 50.

Ramakrishna, M., Krishnaswamy, P.R. (1967).
A paper chromatographic method for identification and estimation of pyrrolidonecarboxylic acid.
Analytical Biochemistry, 19, 338-343.

Randall, M., Young, L.E. (1928).
The calomel and silver chloride electrodes in acid and neutral solutions. The activity coefficient of aqueous hydrochloric acid and the single potential of the decimolal calomel electrode.
Journal of the Americal Chemical Society, 50, 989 - 1004.

Reynolds, J.A., Tanford, C. (1970).
Binding of dodecyl sulfate to proteins at high binding ratios.
Possible implications for the state of proteins in biological membrane.
Proceedings of the National Academy of Sciences, 66, 1002-1007.

Reynolds, J.A., Tanford, C. (1970).
The gross conformation of protein-sodium dodecyl sulfate complexes.
Journal of Biological Chemistry, 245, 5161-5165.

Riegelman, S., Pichon, G. (1962).
A critical re-evaluation of factors affecting emulsion stability.
American Perfumer, 77, 31-33.

Robbers, J.E., Bhatia, V.N. (1961).
Technique for rapid determination of HLB and required HLB values.
Journal of Pharmaceutical Sciences, 50, 708 709.

Roeper, J. (1974).
The alkali requirement of acid casein.
New Zealand Journal of Dairy Science and Technology, 9, 128-131.

Rose, L.M. (1974).
The Application of Mathematical Modelling to Process Development and Design.
Applied Science Publishers Ltd, London.

Sabharwal, K., Vakaleris, D.G. (1972).
Stability of fluid food emulsions.
I. Effects of emulsifiers, electrolytes and sodium caseinate.
Journal of Dairy Science, 55, 277-282.

Schofield, J.D., Booth, M.R. (1983).
Wheat proteins and their technological significance.
In Developments in Food Proteins - 2 (Hudson, B.J.F., Ed.).
pp. 1 - 65.
Applied Science Publishers, London.

Shelton, J.T., Khuri, A.I., Cornell, J.A. (1983).
Selecting check points for testing lack of fit in response surface models.
Technometrics, 25, 357-366.

Shen, J.L. (1976).
Soy protein solubility: The effect of experimental conditions on the solubility of soy protein isolates.
Cereal Chemistry, 53, 902-909.

Shimizu, M., Kamiya, T., Yamauchi, K. (1981).
The adsorption of whey proteins on the surface of emulsified fat.
Agricultural and Biological Chemistry, 45, 2491-2496.

Shimizu, M., Takahashi, T., Kaminogawa, S., Yamauchi, K. (1983).
Adsorption onto an oil surface and emulsifying properties of bovine α_{s1}-casein in relation to its molecular structure.
Journal of Agrilcutral and Food Chemistry, 31, 1214 - 1218.

Shukla, T.P. (1975).
Cereal proteins: Chemistry and food applications.
Critical Reviews in Food Science and Nutrition, 6, 1-176.

Sinclair, E.F., Martell, A.E. (1950).
The behaviour of glass electrodes in acid solutions.
Journal of Chemical Physics, 18, 224-227.

Southward, C.R. (1975).
New Zealand Dairy Research Institute, Personal Communication.

Stein, S., Bohler, P., Stone, J., Davma, W., Volvenfriend, S. (1973).
'Amino acid analysis with fluorescamine at the picomole level.
Archives of Biochemistry and Biophysics, 55, 202 - 212.

Steinhardt, J., Fugitt, C.H. (1942).
Catalyzed hydrolysis of amide and peptide bonds in proteins.
Journal of Research of the National Bureau of Standards, 29, 315-327.

Stewart, K.K. (1977).
Thin film dialysis.
Advances in Protein Chemistry, 31, 135 - 186.

Sullivan, J.J., Jago, G.R. (1970).
A model for bitter peptide formation and degradation in cultured dairy products.
The Australian Journal of Dairy Technology, 25, 111.

Titus, T.C., Wiancko, N.N., Barbour, H.F., Mickle, J.B. (1968).
Emulsifier efficiency in model systems of milk fat or soybean oil and water.
Food Technology, 22, 1449-1451.

Tornberg, E. (1979).
The adsorption behaviour of proteins at an interface as related to their emulsifying properties.
In
'Functionality and protein structure' ACS Symposium Series 92, (Pour-El, A., Ed.). pp. 105 - 123. American Chemical Society, Washington, DC.

Tornberg, E., Hermansson, A.-M. (1977).
Functional characterization of protein stabilized emulsions: Effect of processing.
Journal of Food Science, 42, 468-472.

Tower, D.B. (1967).
Enzymatic determination of glutamine and asparagine.
In
Volume 11, Enzyme Structure (Hirs, C.H.W., Ed.). pp. 76 - 93.
Academic Press, New York.

Udenfriend, S., Stein, S., Bohlen, P., Dairman, W., Leimgruber, W., Weigele, M. (1972).
Fluorescamine: A reagent for assay of amino acids, peptides, proteins and primary amines in the picomole range.
Science, 178, 871 - 872.

van Eerd, J.-P. (1971).
Meat emulsion stability. Influence of hydrophilic lipophilic balance salt concentration and blending with surfactants.
Journal of Food Science, 36, 1121 - 1124.

Vickery, H.B. (1922).
The rate of hydrolysis of wheat gliadin.
Journal of Biological Chemistry, 53, 495 - 511.

Vidal, F.D. (1972).
Treatment of flour and dough.
US Patent 3,655,403.

Vogel, A.I. (1961).
A Text-Book of Quantitative Inorganic Analysis Including Elementary Instrumental Analysis, 3rd edn. Longman, London.

Walker, N.J., Connolly, P.B. (1984).
Protein isolates and method of producing them.
US patent 4,486,343.

Weast, R.C. (1977).
CRC Handbook of Chemistry and Physics' 58th edn.
CRC Press, Inc, Boca Raton, Florida.

Weigele, M., De Bernando, S., Leimgruber, W. (1973).
Fluorometric assay of secondary amino acids.
Biochemical and Biophysical Research Communications, 50, 352-356.

Whitaker, J.R., Puigserver, A.J. (1982).
Fundamentals and applications of enzymatic modifications of proteins: An overview. In
Modification of Proteins: Food, Nutritional, and Pharmacological Aspects. (Feeney, R.E., Whitaker, J.R., Eds). Advances in Chemistry Series 198. pp. 57 - 87.
American Chemical Society, Washington, DC.

Whitaker, J.R., Granvin, P.E., Aasen, G. (1980).
Reaction of ammonia with trinitrobenzene sulfonic acid.
Analytical Biochemistry, 108, 72 - 75.

Wu, C.H. (1975).
Preparation, characterization and functionality of acid-modified gluten.
Ph.D. Thesis. University of British Columbia, Vancouver, Canada.

Wu, Y.V., Dimler, R.J. (1963).
Hydrogen ion equilibria of wheat gluten.
Archives of Biochemistry and Biophysics, 102, 230-237.

Wu, Y.V., Dimler, R.J. (1963).
Hydrogen ion equilibria of wheat glutenin and gliadin.
Archives of Biochemistry and Biophysics, 103, 310-318.

Wu, C.H., Nakai, S., Powrie, W.D. (1976).
Preparation and properties of acid-solubilized gluten.
Journal of Agricultural and Food Chemistry, 24, 504 - 510.

Yates, K., Riordan, J.C. (1965).
The H_A acidity function and the mechanism of amide hydrolysis in hydrochloric acid.
Canadian Journal of Chemistry, 43, 2328-2335.

Yates, K., Stevens, J.B. (1965).
The ionization behaviour of amides in concentrated sulfuric acids.
II. Applications of the H_A function to rates and equilibria.
Canadian Journal of Chemistry, 43, 529 - 537.

Yokotsuka, T., Iwaasa, T., Fujii, M. (1974).
Process for producing a protein hydrolysate.
US Patent 3,852,479.

Yokotsuka, T., Iwaasa, T., Fujii, M. (1975).
Process for producing a protein hydrolysate.
US Patent 3,912,822.

Appendix 1.1 The solution properties of ammonia

The solution properties of ammonia can be considered in terms of the theory of conjugate acids and bases (Glasstone and Lewis, 1960)

Now

$$\underset{\text{Base 2}}{NH_3} + \underset{\text{Acid 1}}{H_2O} \rightleftharpoons \underset{\text{Acid 2}}{NH_4^+} + \underset{\text{Base 1}}{OH^-}$$

and

$$\underset{\text{Acid 2}}{NH_4^+} + \underset{\text{Base 1}}{H_2O} \rightleftharpoons \underset{\text{Base 2}}{NH_3} + \underset{\text{Acid 1}}{H_3O^+}$$

And since

$$K_b = \frac{[NH_4^+][OH^-]}{[NH_3]} = 1.710 \times 10^{-5} \quad (20°C)$$

and

$$K_w = \frac{[H_3O^+][OH^-]}{1} = 6.80926 \times 10^{-15} \quad (20°C)$$

(Weast, 1977)

It can be shown, by substitution in the above equations, that:

$$K_a = \frac{[NH_3][H_3O^+]}{[NH_4^+]} = 11.297 \times 10^{-10}$$

Similar values were calculated by Hashimoto (1983). The practical effect of this calculation is to show that at moderate concentrations of acid $[H_3O^+]$ is large compared to $[NH_3]$ plus $[NH_4^+]$ and practically all of the ammonia must be present as NH_4^+.

Appendix 1.2: Derivation of rate equations for amide hydrolysis using the steady state treatment

$$H_3O^+ + S \underset{}{\overset{K_1}{\rightleftharpoons}} SH^+ + H_2O$$

$$SH^+ + H_2O \overset{K_2}{\rightleftharpoons} X^* \xrightarrow{k} \text{products}$$

$$K_2 = \frac{[X^*]}{[SH^+]\,[H_2O]} \quad \frac{f_{X^*}}{f_{SH^+} f_{H_2O}}$$

$$v = k[X^*] = kK_2\,[SH^+][H_2O] \quad \frac{f_{SH^+} f_{H_2O}}{f_{X^*}} \qquad \text{- - - -} \quad (1)$$

but

$$K_1 = \frac{[SH^+]\,[H_2O]}{[H_3O^+]\,[S]} \quad \frac{f_{SH^+}\, f_{H_2O}}{f_S\, f_{H_3O^+}}$$

$$f_{SH^+}\,[SH^+] = \frac{K_1\,[H_3O^+]\,[S]}{[H_2O]} \quad \frac{f_{H_3O^+}\, f_S}{f_{H_2O}}$$

$$v = k\,K_2\,K_1\,\frac{[H_3O^+][S][H_2O]}{[H_2O]} \quad \frac{f_{H_2O}}{f_{X^*}} \frac{f_{H_3O^+}}{f_{H_2O}} f_S$$

$$= kK_2\,K_1\,[H_3O^+]\,[S] \quad \frac{f_{H_3O^+}\, f_S}{f_{X^*}} \qquad \text{- - - -} \quad (2)$$

Appendix 1.3: Further derivation of amide hydrolysis rate equations

Mittal et al (1981) showed that

$$\text{Amide} + H^+ \underset{}{\overset{K}{\rightleftharpoons}} [\text{Amide}.H^+] \qquad \text{rapid} \quad ---(1)$$

$$[\text{Amide}.H^+] + H_2O \xrightarrow{k} \text{products} \qquad \text{slow} \quad ---(2)$$

- analgous to enzyme reaction where the concentration of the enzyme is limiting so that;

$$\frac{d[\text{Amide}.H^+]}{dt} = k_2 \; [\text{Amide}.H^+] \qquad (3)$$

and

$$\frac{[\text{Amide}.H^+]}{[\text{Amide}]\;[H^+]} = K \qquad (4)$$

$$\therefore \quad \frac{d[\text{Amide}.H^+]}{dt} = k_2 \; K \; [\text{Amide}] \; [H^+] \qquad (5)$$

but $[H_o^+] = [H^+] + [\text{Amide}.H^+]$

$[H_o^+] = [H^+] + K \; [\text{Amide}].[H^+]$

$$\therefore \quad [H^+] = \frac{[H_o^+]}{1 + K\,[\text{Amide}]}$$

$\therefore$ subst. in (5)

$$\frac{d\,[Amide.H^+]}{dt} = \frac{k_2\,K\,[Amide]\,[H_o^+]}{1 + K\,[Amide]}$$

This is of the Michaelis-Menten type but note that 'usual' derivation is from Briggs-Haldane where the steady state assumption is made.

i.e. $Amide + H^+ \underset{K_{-1}}{\overset{K_1}{\rightleftharpoons}} [Amide.H^+]$

and $[Amide.H^+] \xrightarrow{k}$ products

then the steady state assumption

$$K_1\,[Amide]\,[H^+] - K_{-1}\,[Amide.H^+] - k\,[Amide.H^+] = 0$$

similarly $[H_o^+] = [H^+] + [Amide.H^+]$

and substituting

$$v = k\,[Amide.H^+] = \frac{k\,[Amide]\,[H_o^+]}{\frac{k + K_{-1}}{K_1} + [Amide]}$$

where $\frac{k + K_{-1}}{K_1} = Km$

whereas in earlier derivation $Km = \frac{1}{K}$

Difference between the two is steady-state assumption (Briggs-Haldane) or equilibrium assumption.

These assumptions are discussed by Bailey and Ollis (1977) and Ollis (1983).

Appendix 3.1 Nitrogen analysis results to show solubilization of gluten by SDS in various buffers

TREATMENT(1)	TN (%)	NPN (%)	TN-NPN	pH
A1a	0.118	0.010	0.108	2.856
A1ac	0.116	0.010	0.106	
A1b	0.122	0.010	0.112	
A1bc	0.118	0.010	0.108	
A2a	0.122	0.010	0.112	6.752
A2ac	0.126	0.010	0.116	
A2b	0.124	0.010	0.114	
A2bc	0.124	0.010	0.114	
A3a	0.124	0.012	0.112	
A3ac	0.122	0.015	0.107	
A3b	0.118	0.012	0.106	2.808(2)
A3bc	0.116	0.012	0.104	
D2a	0.135	0.002	0.133	7.907
D2ac	0.118	0.000	0.118	
D2b	0.133	0.002	0.131	
D2bc	0.118	0.005	0.113	
C2a	0.129	0.005	0.124	7.457
C2ac	0.129	0.005	0.124	
C2b	0.126	0.005	0.121	
C2bc	0.124	0.005	0.119	

Notes (1)

The letter codes refer to gluten source and numbers to diluent buffers as described in Section 3.1.1, a, b are duplicate samples and c is the centrifuged or supernatant sample.

Note (2)

The low pH indicates that diluent (1) and not (3) was used in error.

Appendix 3.2 Peak height data of fluorescence response for L-glu standards and gluten with fluorescamine

SAMPLE COMPOSITION				
1% SDS (µl)	GLUTEN (A 10^{-2}) (µl)	L-GLU (0.5 mM) (µl)	PEAK HEIGHTS (mm)	MEAN (mm)
200	–	–	1, 1	1
175	25	–	17, 18	18
150	50	–	29, 28	29
125	75	–	45, 46	46
100	100	–	60, 60	60
50	150	–	88, 87	87
150	–	50	30, 33	32
100	–	100	54, 53	54
50	–	150	87, 84	86
–	–	200	103, 105	104
200	–	–	4, 2	3
130	50	20	46, 44	45
100	50	50	58, 60	59
50	50	100	81, 84	83
–	50	150	105, 105	105

Appendix 3.3 Peak height of fluorescence response for ammonia with fluorescamine

SAMPLE COMPOSITION					
1% SDS (µl)	GLUTEN (A 10^{-2}) (µl)	NH_4Cl (0.004M) (µl)	L-GLU (0.5 mM) (µl)	PEAK HEIGHTS (mm)	MEAN (mm)
200	-	-	-	1, 1	1
190		10	-	1, 1	1
180		20	-	1.5, 1.5	1.5
160		40	-	2.0, 2.0	2.0
140		60	-	2.5, 2.5	2.5
100		100	-	3.0, 3.0	3.0
150	50	-	-	29, 28	21
140	50	10	-	30, 31	31
130	50	20	-	31, 32	33
110	50	40	-	33, 32	32
90	50	100	-	32, 32	32
50	50	100	-	33, 33	33
100	-	-	100	54, 53	54
90	-	10	100	53, 53	53
80	-	20	100	55, 52	53
60	-	40	100	55, 52	54
40	-	60	100	53, 55	54
-	-	100	100	56, 57	57

Appendix 3.4 Further peak height data of fluorescence response for ammonia with fluorescamine

NH_4^+ concentration in sample containing 0.75 mM L-glu (M)	PEAK HEIGHTS (mm)	MEAN (mm)	LESS NIL VALUE
nil	96, 90	93	-
0.01	84, 100	92	-1
0.05	111, 116	114	21
0.1	114, 123	119	26
0.5	124, 124	124(132)(2)	(39)(2)

(1) $NH4^+$ added to buffers as explained in Section 3.6.1

(2) Take pH corrected value using the data of Table 3.2.

Now the peak height for 0.75 mM L-glu in this case is 93 mm. For the data shown in Appendix 3.2 the equivalent set of standards give a peak height of 155 mm (by extrapolution of the data in Appendix 3.2). The ratio of 93/155 was used to reduce the data from Appendix 3.3 as shown below.

$NH4^+$ CONCENTRATION IN SAMPLE (M)	MEAN - BLANK (mm)	REDUCED VALUE (mm)
nil	-	-
0.005	0.5	0.3
0.001	1	0.6
0.002	2	1.2

Appendix 3.5 The reaction of L-glu and L-glu plus gluten with TNBS

SAMPLE COMPOSITION				ABSORBANCE[1]
SDS (1% w/v) (µl)	L-GLU (2.5 mM)[2] (µl)	GLUTEN A (DILUTED 10^{-1}) (µl)	GLUTEN A (DILUTED 10^{-2}) (µl)	(A_{340})
250	-	-	-	0.550[3]
225	25	-	-	0.655
125	125	-	-	0.994
75	175	-	-	1.222
-	250	-	-	1.517
112.5	12.5	125	-	0.917
62.5	62.5	125	-	1.129
37.5	87.5	125	-	1.210
112.5	12.5	-	125	0.639
62.5	62.5	-	125	0.824
37.5	87.5	-	125	0.926
-	125	-	125	1.065
250	-	-	-	0.537[3]

Note (1)
Mean of duplicate determinations

Note (2)
Samples were diluted from 2.5 mM L-glu in 5 and 10 ml lots and 250 µl or 125 µl of ready diluted sample was added. Figures presented in above form however for consistency with subsequent practice.

Note (3)
Some drift in calibration of the spectrophotometer is evident, no corrections were made.

Appendix 3.6 The reaction of L-glu and ammonia with TNBS

SAMPLE COMPOSITION			ABSORBANCE[1]	ABSORBANCE[3] (CORRECTED)	Δ ABSORBANCE[4]
SDS (1% w/v) (μl)	L-GLU (2.5 mM) (μl)	NH_4Cl (0.05M) (μl)	(A_{340})	(A_{340})	(A_{340})
250	-	-	0.513[2]	0.513	-
100	150	-	1.075	1.082	-
95	150	5	1.339	1.354	0.279
90	150	10	1.391	1.413	0.316
80	150	20	1.473	1.502	0.427
70	150	30	1.700	1.737	0.662
60	150	40	1.832	1.876	0.801
50	150	50	1.910	1.961	0.886
40	150	60	2.074	2.132	1.056
-	150	100	2.546	2.612	1.537
250	-	-	0.440[2]	0.513	-

NOTES:

(1) Mean of duplicate determinations

(2) Difference indicates a drift in the calibration of the spectrophotometer. This was later found to be approximately linear. Because of this phenomenon a change from the use of the Gilson spectrophotometer to Perkin-Elmer spectrophotometer was made.

(3) To correct the absorbance the difference between the readings using the reaction blank readings, 0.513 and 0.440 was divided into ten steps and corrected as shown.

(4) The Δ absorbance is the correction for the increase in absorbance due to ammonia.

Appendix 3.7 The reaction of L-glu, gluten and ammonia with TNBS

SAMPLE COMPOSITION				ABSORBANCE[1]	ABSORBANCE[3]	ABSORBANCE[4]
SDS (1% w/v) (µl)	L-GLU (2.5mM) (µl)	GLUTEN (diluted 10^{-1}) (µl)	NH_4CL (0.05M) (µl)	(A_{340})	(CORRECTED) (A_{340})	(A_{340})
250	-	-	-	0.564[2]	0.561	-
240	-	-	10	-.838	0.833	0.272
225	-	-	25	1.058	1.050	0.489
200	-	-	50	1.496	1.485	0.924
175	-	-	75	1.838	1.825	1.264
150	-	-	100	2.154	2.138	1.577
90	150	-	10	1.474	1.455	0.280
75	150	-	25	1.660	1.639	0.464
50	150	-	50	2.061	2.037	0.862
25	150	-	75	2.379	2.352	1.177
-	150	-	100	2.608	2.579	1.404
100	-	150	-	0.944	0.912	-
50	-	150	50	1.770	1.735	0.823
-	-	150	100	2.404	2.367	1.455
200	50	-	-	0.819	0.779	-
100	150	-	-	1.218	1.175	-
-	250	-	-	1.694	1.649	-
250	-	-	-	0.611[2]	0.563	-

NOTES:

(1) Mean of duplicate determinations

(2) Difference indicates drift as noted in Appendix 3.5

(3) To correct the absorbance the difference between the readings for water, 0.000 initially, and 0.048 finally was divided into 18 steps and the absorbance corrected as shown.

(4) Increase in absorbance due to ammonia - data for Figure 3.7.

Appendix 4.1: Calculation of pH values from other authors

The data in Table 4.3 show the pH measured and the pH which would be expected if the electrode was behaving as a hydrogen electrode i.e. measuring the solution activity.

Various authors, listed below, present data showing the acid error found with various glass electrodes as changes in measured voltage. These data have been combined with the pH/voltage response of the electrode used in this present study to show the difference between measured and true (i.e. hydrogen electrode) pH. Although not strictly correct, because different electrodes will have slightly different voltage response the calculation is useful in illustrating that electrodes respond similarly.

Now the behaviour of electrodes is described by the Nernst equation

$$E = E^{o'} + 2.3 \frac{RT}{nF} \log a_{H^+} \qquad (1)$$

where E is the observed electrode potential,

$E^{o'}$ is the stable fixed potential including reference potential

pH is defined as $pH = -\log a_{H^+}$

and at 25°C

$2.3 \frac{RT}{nF} = 0.059157$ also termed Nernst's voltage

R is the gas constant, T the absolute temperature, F is the Faraday and n an integer

(Bates, 1973)

For this pH meter S_{rel} is shown during calibration where:

$$S_{rel} = \frac{\Delta v}{\Delta pH.V_n\ (t)}$$

(where $V_n(t)$ is Nernst's voltage) at temperature (t)

Now if S_{rel} = 1.049 @ 25°C (Table 4.3)

and pH value at zero voltage is 6.653

$$\text{then} \quad 1.049 = \frac{\Delta v}{\Delta pH\ 0.059157} \qquad (2)$$

Equation (2) can therefore be used to calculate, pH from electrode voltage response data

The data of Sinclair and Martell (1950) show from Table II for HCl (average error)

CONCN. (M)	ERROR (volts)	ACTIVITY COEFFICIENT	'CORRECT' pH	CORRECT VOLTAGE	CORRECT + ERROR VOLTAGE	- EXPECTED pH
0.001	0.0001	0.96	3.02	0.2254	0.2255	3.019
0.01	0.0000	0.91	2.04	0.2863	0.2863	2.039
0.1	-0.0003	0.8	1.10	0.3446	0.3443	1.105
1	-0.0010	0.81	0.092	0.4071	0.4061	0.109
5	-0.0082	1.045	-0.718	0.4574	0.3392	-0.536

and from Table III for HCl (greatest error)

0.001	0.0000	0.96	3.02	0.2254	0.2254	3.02
0.01	-0.0000	0.91	2.04	0.2863	0.2863	2.04
0.1	-0.0001	0.8	1.10	0.3446	0.3445	1.10
1	-0.0022	0.81	0.092	0.4071	0.4049	0.128
5	-0.0600	1.045	-0.718	0.4574	0.3974	0.249

The data of McInnes and Belcher (1931) for HCl

CORRECT(1) pH	ERROR (volts)	CORRECT VOLTAGE	CORRECT + ERROR VOLTAGE	EXPECTED pH
3	0.3522	0.2268	0.2268	3
2	0.3522	0.2889	0.2268	2
1	0.3521	0.3509	0.3508	1.002
0	0.3512	0.4130	0.4120	0.0158
-0.5	0.3500	0.4440	0.4418	-0.464
-0.75	0.3494	0.4595	0.4567	-0.7045

The data of Dole (1932) for HCl

CORRECT pH	ERROR (mV)	CORRECT VOLTAGE	EXPECTED VOLTAGE	EXPECTED pH
-0.272	-1.1	0.4299	0.4288	-0.252
-0.458	-1.1	0.4414	0.4403	-0.440
-0.543	-2.8	0.4467	0.4439	-0.498
-0.680	-6.6	0.4522	0.4486	-0.574
-0.730	-15.1	0.4583	0.4432	-0.487
-1.67	-18.4	0.5166	0.4982	-1.373

The data of Hubbard, Hamilton and Finn (1939) quoted by Beck and Wynne-Jones (1952) for HCl

CONCN (M)	DIFFERENCE(1) (volts)	ACTIVITY COEFFICIENT	CORRECT' pH	CORRECT VOLTAGE	CORRECT + ERROR VOLTAGE	EXPECTED pH
0.001	0.218	0.96	3.02	0.226	0.226	3.02
0.01	0.218	0.91	2.04	0.286	0.286	2.04
0.1	0.216	0.8	1.10	0.345	0.343	1.127
1.0	0.213	0.81	0.092	0.407	0.402	0.177
5.0	0.194	1.045	-0.718	0.458	0.434	-0.338
10.0	0.175	-	-	-	-	-

NOTE (1) The value is the difference between the hydrogen and glass electrode voltage readings. The first two values, for 0.001 and 0.01 do not represent a deviation in behaviour.
Therefore for 5.0 M the error is
Error = 0.194 - 0.218 volts

Appendix 4.3: Calculation of the amount of reaction due to acidified gluten standing at room temperature

Simulation of the reaction used initial conditions

Amide = 0.150 M
Peptide = 0.595 M
H^+ = 1.5 M
Temperature = 20°C
1/T = 3.4×10^{-3} $^oK^{-1}$
k_1 = 6.3×10^{-3} (by extrapolation of Line B Fig. 4.12)
k_2 = 6.8×10^{-8} (by extrapolation of line D Fig. 4.13)

Activity correction factor = 0.60

REACTION TIME (Secs)	AMIDE (M)	PEPTIDE (M)	HYDROGEN ION (M)	AMIDE BOND HYDROLYSIS (%)	PEPTIDE BOND HYDROLYSIS (%)	pH
1×10^1	0.1500	0.5950	1.500	0.00	0.00	0.176
1×10^2	0.1499	0.5950	1.500	0.07	0.00	0.176
3×10^2	0.1496	0.5950	1.500	0.27	0.00	0.176
1×10^3	0.1486	0.5950	1.499	0.93	0.00	0.176
3×10^3	0.1458	0.5948	1.497	2.80	0.03	0.175
1×10^4	0.1365	0.5944	1.492	9.00	0.10	0.174
1.8×10^4	0.1266	0.5939	1.485	15.6	0.18	0.172

Appendix 4.4: Method and example of calculation of confidence limits for components of variance

Method

the method followed is that given by Davies (1967)

$\hat{\sigma}_d$ is first estimated as shown in Table 4.22 using the data in Tables 4.23 or 4.24. Upper and lower limits can then be calculated by using the tables given.

$\hat{\sigma}_s$ is then estimated using the equation as shown in Table 4.22 and data in Tables 4.23 or 4.24.

Confidence limits are:

$$\sqrt{\left\{\frac{1}{\bar{n}_1}\right.} (M_1 L_1^2 - M_o) \quad \text{and} \quad \sqrt{\left\{\frac{1}{\bar{n}_1}\right.} (M_1 L_2^2 - M_o)$$

where: L_1 L_2 are multipliers from given tables and:

$\bar{n}_1 = 2$ $\bar{n}_2 = 2$ $\bar{n}_3 = 4$ i.e. the multiplier in the equations in Table 4.22 and: M_2, M_1, M_o are the mean squares shown in Tables 4.22, 4.23, 4.24.

$\hat{\sigma}_b$ is similarly estimated and the confidence limits calculated from

$$\sqrt{[(M_2 L_1^2 - M_1)/\bar{n}_3]} \quad \text{and} \quad \sqrt{[(M_2 L_2^2 - M)/\bar{n}_3]}$$

Example:

Ammonia data

$m_2 = 165.99$
$m_1 = 28.63$
$m_o = 12.31$

1) CI for σ_d

now $\hat{\sigma}_d = \sqrt{12.31} = 3.509$

Therefore LL = .93 x 3.509 = 3.26 (3.26, 3.82)
and UL = 1.09 x 3.509 = 3.82

2) CI for σ_s

Now $M_1 = 28.63 = \sigma_d^2 + 2\sigma_s^2$ therefore $\sigma_s = 2.857$

$\sqrt{\frac{1}{2}(28.63 \times 0.89^2 - 12.31)}$, $\sqrt{\frac{1}{2}(28.63 \times 1.12^2 - 12.31)}$

= (2.277, 3.435)

3) CI for σ_b

$$\sqrt{\frac{165.99 \times 0.87^2 - 28.63}{4}} \quad , \quad \sqrt{\frac{165.99 \times 1.16^2 - 28.63}{4}}$$

= (4.925, 6.997)

$\hat{\sigma}_b = 5.862$

Fluorescamine data

1) $_d$ = 1.775 CI (1.65, 1.93)
2) $_s$ = 7.13 CI (6.32, 8.01)
3) $_b$ = 5.85 CI (4.42, 7.42)

Appendix 4.7: Example of information generated by the Minitab programme and calculation of lack of fit (LOF)

Numbers in the margin refer to descriptive notes which follow. The example is for the calculation of the type (iii) analysis in Table 4.33.

```
ELEASE 82.1 *** COPYRIGHT - PENN STATE UNIV. 1982
 1984 *** DEPARTMENT OF SCIENTIFIC AND INDUSTRIAL RESEARCH
VAILABLE  385704
RELEASE OF MINITAB IS OBSOLETE ***

HELP on new features of Rel. 82.1.  Type NEWS for details.

.EVMODELX.MTB'
PUT 132 0
.D 'modeLX.kin' c1 c2 c18 c19
WS READ
```

C1	C2	C18	C19
.229	2.755	0.02	1
.905	3.096	0.28	1
.900	2.755	0.28	1
.295	2.755	0.28	1

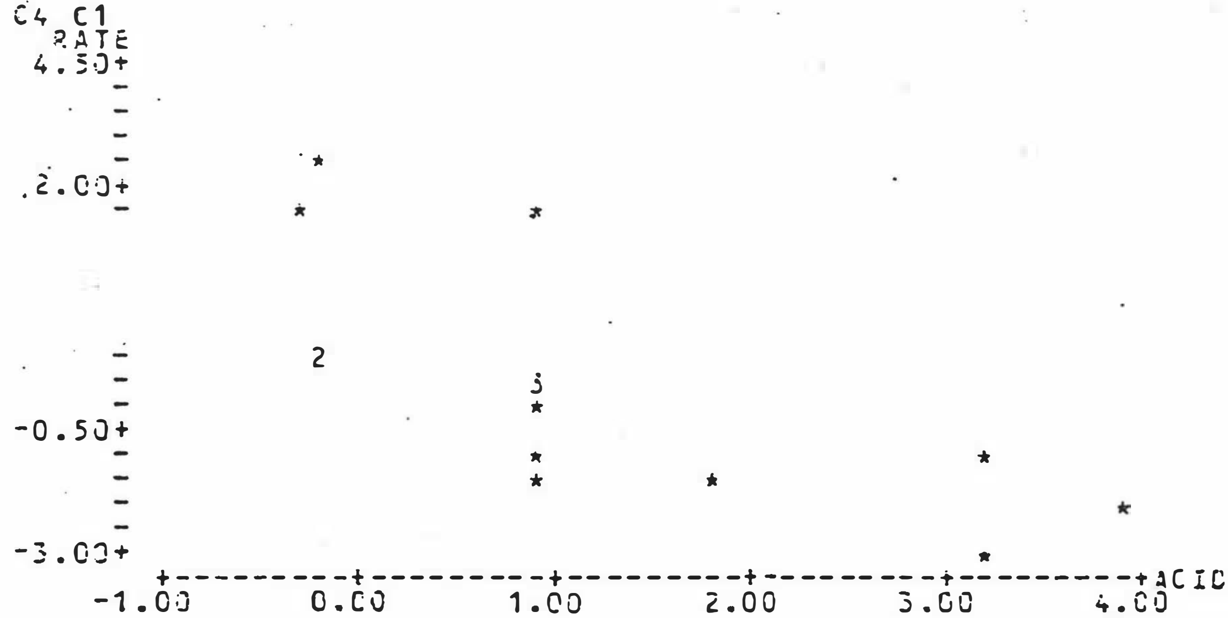
```
'REVRATEX.DAT' C20

    0.077      0.007     50.500    .  .  .

: c3=logTEN (c18)
: c4=logTEN (c20)
E C1 'ACID' C2 'TEMP' C3 'GLUTEN' C4 'RATE'
INT C1-C4 c18 c19 c20
```

ACID	TEMP	GLUTEN	RATE	C18	C19	C20	
.229	2.755	-1.69897	0.82930	0.02	1	6.750	*
.905	3.096	-0.55284	-1.11351	0.28	1	0.077	
.900	2.755	-0.55284	-2.15490	0.28	1	0.007	*
.295	2.755	-0.55284	1.70329	0.28	1	50.500	*
.340	2.755	-1.69897	-1.46852	0.02	1	0.034	
.897	2.755	-0.82391	0.18184	0.15	0	1.520	
.179	3.096	-0.82391	0.80277	0.15	1	6.350	
.900	2.755	-0.82391	0.46982	0.15	C	2.950	
.8[illegible]	3.096	-1.69897	-1.72125	0.02	1	0.019	
.871	2.481	-1.69897	0.44404	0.02	1	2.780	
.880	2.481	-0.55284	1.35218	0.28	1	22.500	
.200	2.481	-0.82391	-0.87615	0.15	1	0.133	
.130	3.096	-0.82391	-3.00000	0.15	1	0.001	
.174	2.481	-0.82391	2.49136	0.15	1	310.000	
.870	2.755	-0.82391	0.34830	0.15	C	2.230	

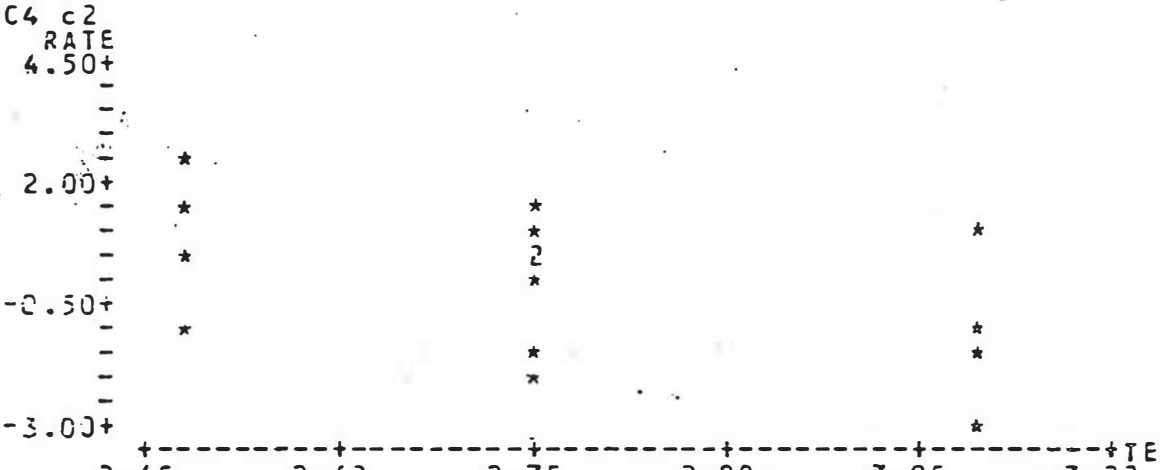
```
T C9
ME C9'ORDER'
RR C1-C4
```

	ACID	TEMP	GLUTEN
	-0.001		
	0.157	0.002	
	-0.797	-0.520	0.135

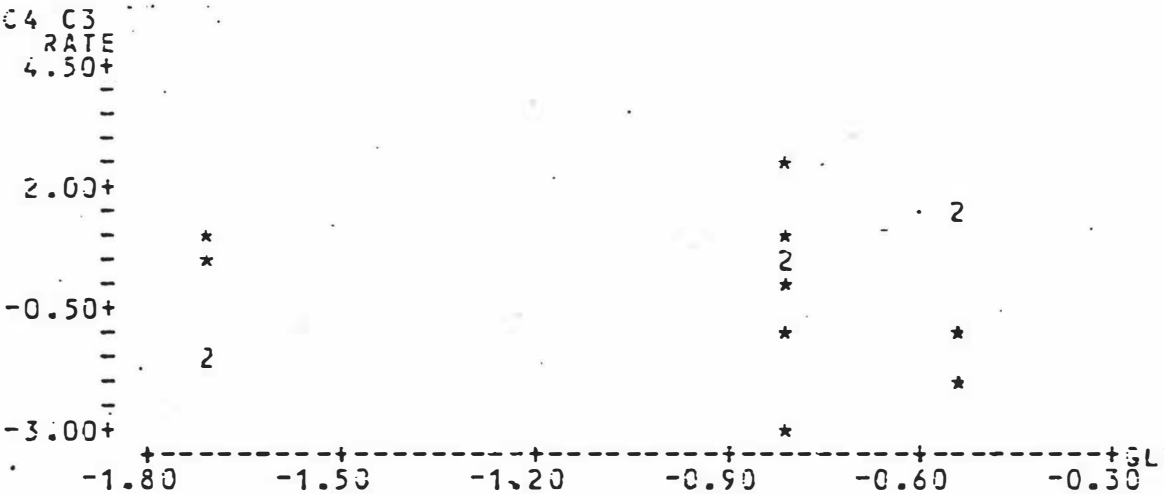
```
OT C4 C1
```



```
MTB > CORR C4 C1

   CORRELATION OF      RATE AND ACID     =-0.797

MTB > PLOT C4 C2
```




```
MTB > CORR C4 C2

   CORRELATION OF      RATE AND TEMP     =-0.520

MTB > PLOT C4 C3
```

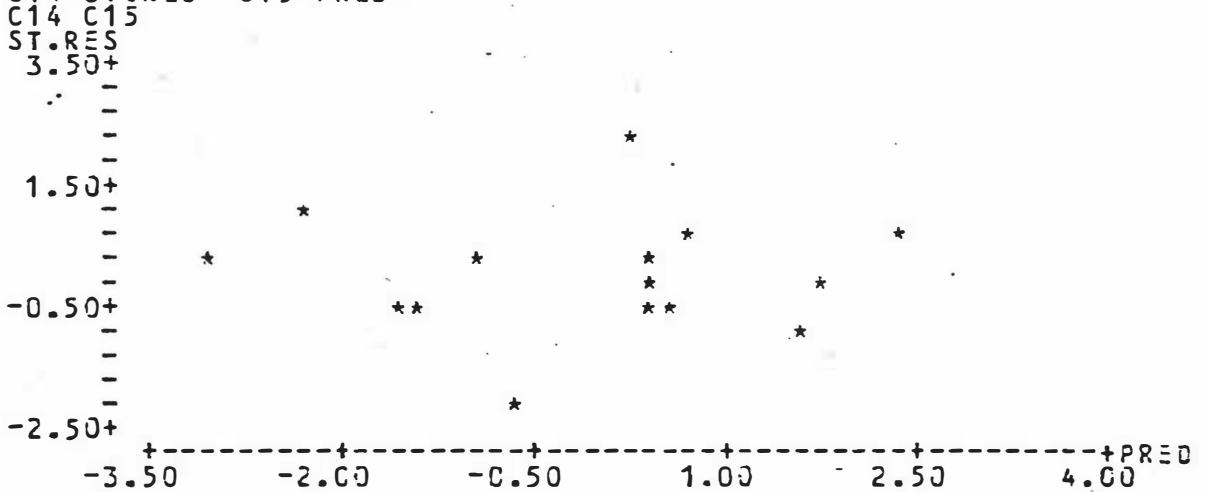


```
> CORR C4 C3
ORRELATION OF      RATE AND GLUTEN   = 0.135
> PLOT C4 C9
          RATE
        4.50+
            -
            -
            -
            -
        2.00+
            -
            -
            -
            -
       -0.50+
            -
            -
            -
            -
       -3.00+
            +---------+---------+---------+---------+---------+---------+ORDE
           0.00      4.00      8.00     12.00     16.00     20.00
```

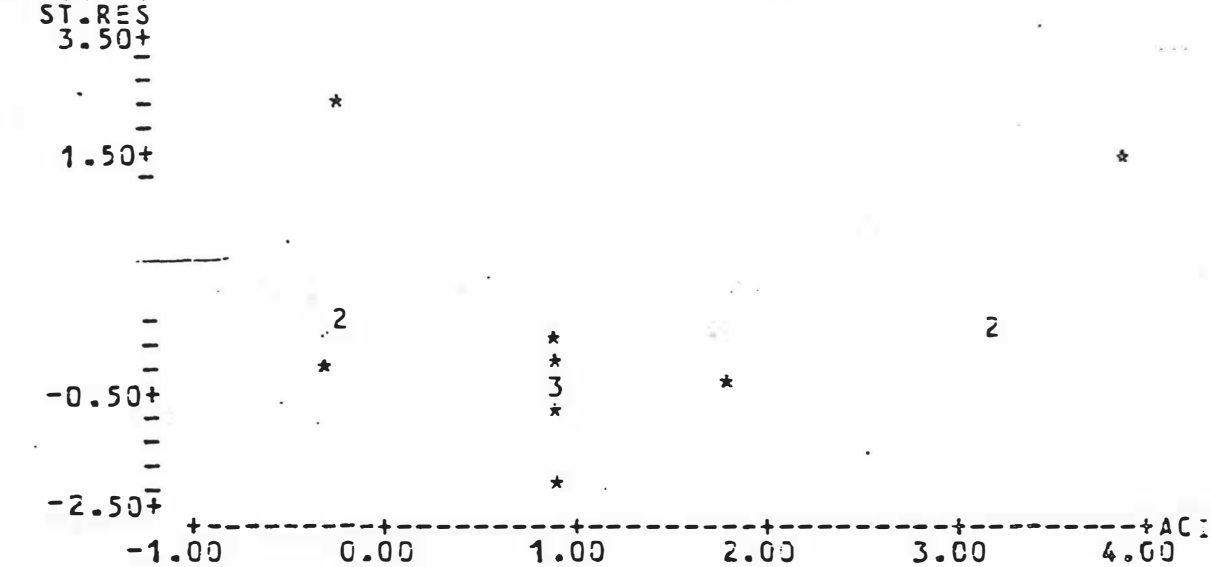
```
> CORR C4 c9
ORRELATION OF      RATE AND ORDER    = 0.105
> STEPWISE REGRESSION OF C4 ON C1-C3;
> FENTER=1;
> FREMOVE=1.
WISE REGRESSION OF  RATE   ON  3 PREDICTORS, WITH N =    15

STEP          1         2         3
TANT      0.9710   10.5323   11.4843

         -0.935    -0.936    -0.935
TIO       -4.75     -8.97     -17.49

                    -3.45     -3.45
TIO                 -5.86     -10.99

EN                              0.90
TIO                             5.58

          0.969     0.513     0.274
          63.47     90.53     97.53
> REGRESS C4 3 PREDS C1-C3, ST. RES C14,pred c15;
> PURE ERROR LOF TEST.

REGRESSION EQUATION IS
 = 11.5 - 0.985 ACID - 3.45 TEMP + 0.899 GLUTEN

                                ST. DEV.     T-RATIO =
AN      COEFFICIENT             OF COEF.     COEF/S.D.
          11.4843                0.8925         12.87
         -0.98518                0.05632       -17.49
          -3.4514                 0.3140        -10.99
EN         0.8988                 0.1610          5.58
```

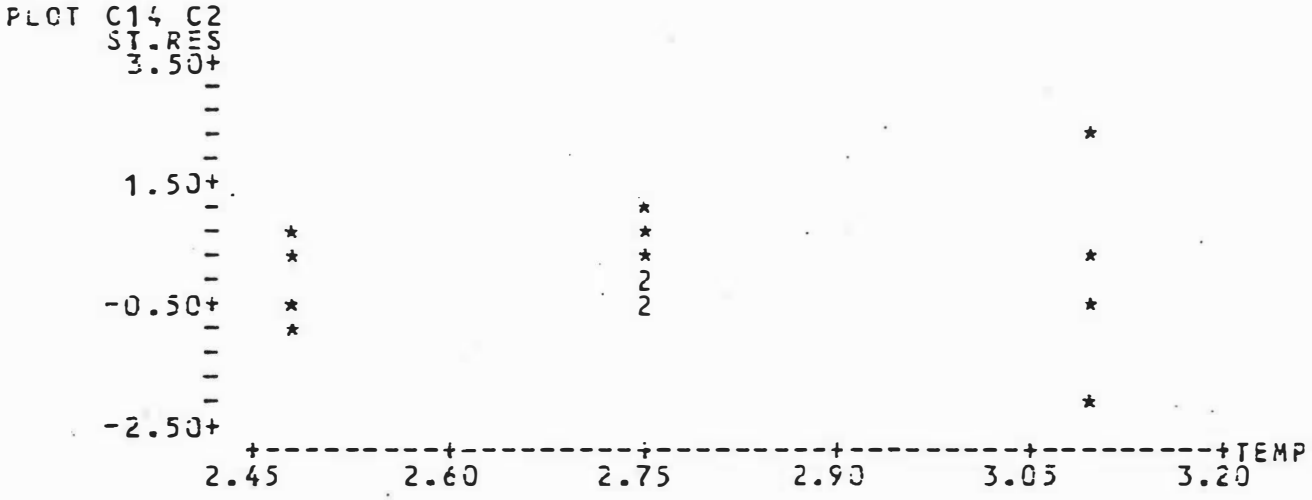
```
3.2739

JARED = 97.5 PERCENT
JARED = 96.9 PERCENT, ADJUSTED FOR D.F.

SIS OF VARIANCE

TO       DF            SS          MS=SS/DF
SSION     3         32.591            10.864
IDUAL    11          0.825             0.075
         14         33.416
```

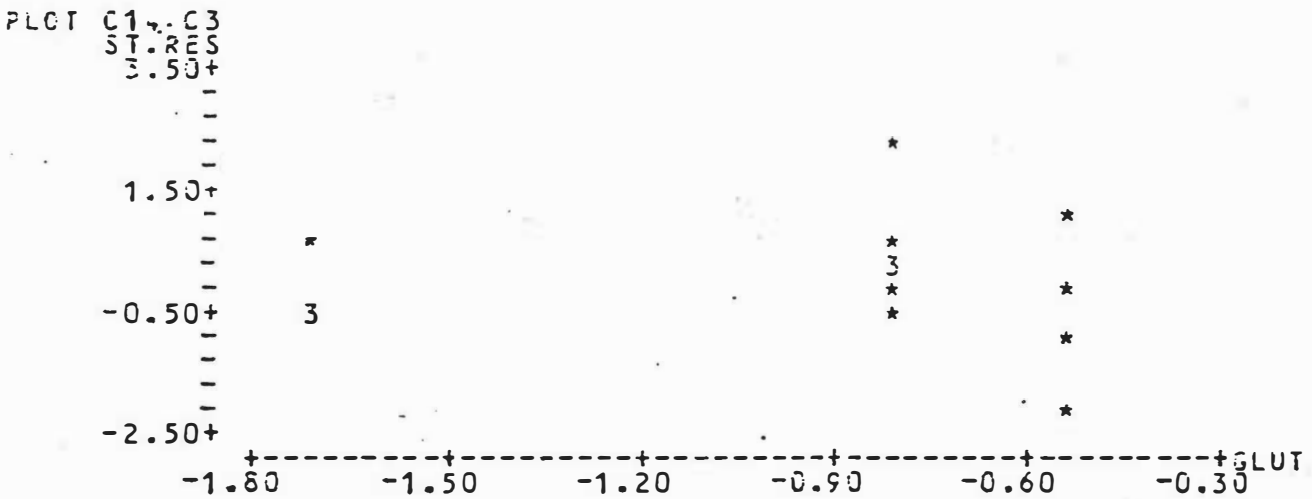
```
FURTHER ANALYSIS OF VARIANCE
SS EXPLAINED BY EACH VARIABLE WHEN ENTERED IN THE ORDER GIVEN
  DUE TO      DF          SS
REGRESSION     3      32.591
ACID           1      21.210   63.5
TEMP           1       9.043   90.5
GLUTEN         1       2.338   97.5

                          Y     PRED. Y    ST.DEV.
 ROW      ACID          RATE      VALUE    PRED. Y   RESIDUAL     ST.RES.
   2      0.90       -1.1135    -0.5896     0.1436    -0.5239      -2.25R
   7     -0.18        0.8028     0.2347     0.1492     0.5681       2.47R

R DENOTES AN OBS. WITH A LARGE ST. RES.

DURBIN-WATSON STATISTIC = 2.47
CANNOT DO PURE ERROR TEST

MTB > NAME C14'ST.RES' C15'PRED'
MTB > PLOT C14 C15
```

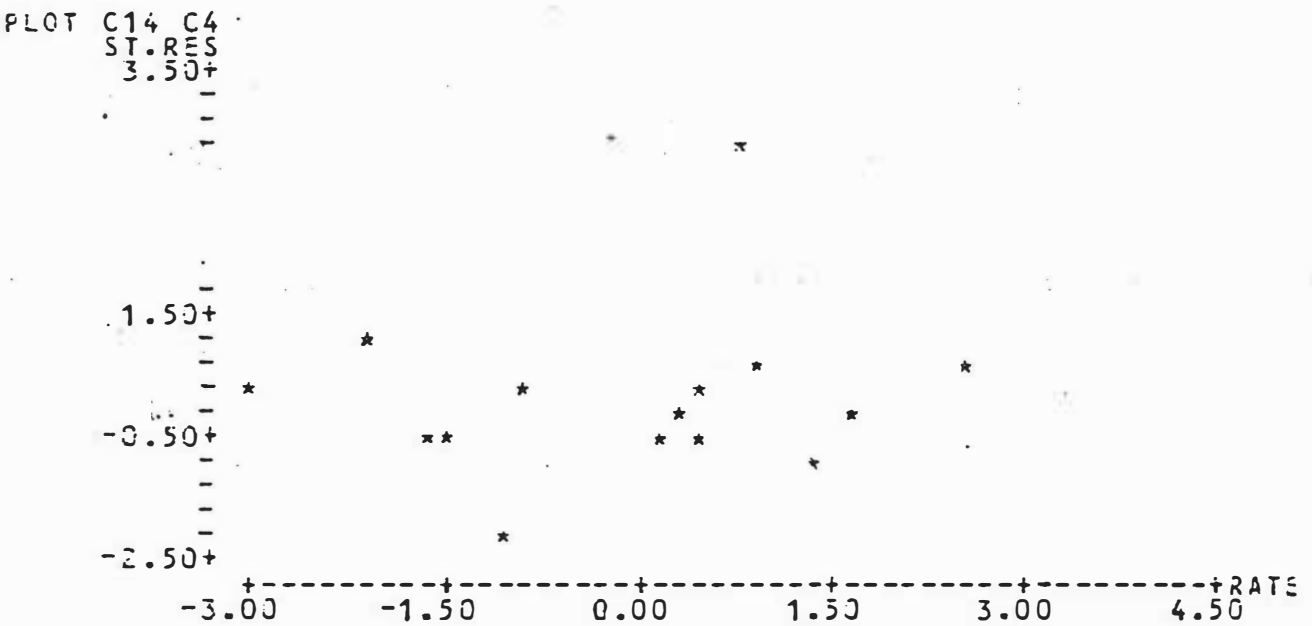



```
MTB > PLOT C14 C1
```




PLOT C14 C2
ST.RES
3.50+
1.50+
-0.50+
-2.50+
2.45
2.60
2.75
2.90
3.05
3.20
TEMP


PLOT C14 C3
ST.RES
3.50+
1.50+
-0.50+
-2.50+
-1.80
-1.50
-1.20
-0.90
-0.60
-0.30
GLUT


PLOT C14 C4
ST.RES
3.50+
1.50+
-0.50+
-2.50+
-3.00
-1.50
0.00
1.50
3.00
4.50
RATE

```
MTB > PLOT C14 C9
          ST.RES
          3.50+
              -
              -
              -                    *
              -
          1.50+
              -          *
              -     *                        *
              -                    *     *     *
              -          *                   *
          -0.50+               *  *     * *
              -                             *
              -
              -
              -       *
          -2.50+
              +---------+---------+---------+---------+---------+---------+ORD
             0.00      4.00      8.00      12.00     16.00     20.00

MTB > CHOOSE 0 IN C19  CORRESP ROWS c1 C2 C3 C4 PUT IN c21  C5-C8
MTB > PRINT C5-C8
 ROW       C5       C6         C7          C8

   1    0.397    2.755   -0.82391    0.181844
   2    0.900    2.755   -0.82391    0.469822
   3    0.370    2.755   -0.82391    0.343305

MTB > STANDARD DEV C5 PUT IN K1
   ST.DEV. =      0.14457
MTB > LET K2=K1*K1
MTB > PRINT K1,K2
K1          0.144573
K2          0.0209012
MTB > TABLE C1 C2;
MTB > MEAN C4.
* ERROR * VALUES OF VAR.  1, DIMENSION  1 NOT ALL INTEGER

* ERROR * VALUES OF VAR.  2, DIMENSION  2 NOT ALL INTEGER

MTB > TABLE C1 C3;
MTB > MEAN C4.
* ERROR * VALUES OF VAR.  1, DIMENSION  1 NOT ALL INTEGER

* ERROR * VALUES OF VAR.  3, DIMENSION  2 NOT ALL INTEGER
```

```
> TABLE C2 C3;
> MEAN C4.
ROR * VALUES OF VAR.  2, DIMENSION  1 NOT ALL INTEGER

ROR * VALUES OF VAR.  3, DIMENSION  2 NOT ALL INTEGER

> TABLE C1 C2 C3;
> MEAN C4.
ROR * VALUES OF VAR.  1, DIMENSION  1 NOT ALL INTEGER

ROR * VALUES OF VAR.  2, DIMENSION  2 NOT ALL INTEGER

ROR * VALUES OF VAR.  3, DIMENSION  3 NOT ALL INTEGER

> PRINT C1-C9
    ACID     TEMP     GLUTEN       RATE        C5        C6          C7

  -0.229    2.755   -1.69397    0.32930    0.897    2.755   -0.82391    0.1
   0.905    3.096   -0.55284   -1.11351    0.900    2.755   -0.32391    0.4
   3.900    2.755   -0.55284   -2.15490    0.870    2.755   -0.82391    0.3
  -0.295    2.755   -0.55284    1.70329
   1.340    2.755   -1.69397   -1.46852
   0.897    2.755   -0.82391    0.18184
  -0.179    3.096   -0.82391    0.30277
   0.900    2.755   -0.82391    0.46982
   0.866    3.096   -1.69397   -1.72125
   0.871    2.481   -1.69397    0.44404
   0.880    2.481   -0.55284    1.35213
   3.200    2.481   -0.82391   -0.87615
   3.150    3.096   -0.82391   -3.00000
  -0.174    2.481   -0.82391    2.49136
   0.870    2.755   -0.82391    0.34830


INITAB *** STATISTICS DEPT * PENN STATE UNIV. * RELEASE 82.1 *
SE AVAILABLE  885704
```

Notes:

(1) Experimental data of pH, temperature (°K), initial amide concentration and reaction rate are read from a data file.

(2) Some of the data are expressed as logarithms.

(3) A plotting facility is used to show obvious trends.

(4) A regression equation is calculated using nominated predictors.

(5) The coefficients and standard deviations of the coefficient are given. These data were used as explained in Appendix 4.8.

(6) The R-squared values are given and below these the analysis of variance. The residual sum of squares is carried forward to calculate LOF.

(7) Data rows with a large standard residual are identified to allow checking e.g. data entry errors.

(8) From the repetition runs a pure error sum of squares can be calculated and this too is carried forward to calculate LOF.

(9) The Minitab package will calculate the LOF (see printout just above Note 4). A requirement however is that the data variables are all integers e.g. 0, 1 01 etc as in type (i) analysis. If this is not so the error statements are generated. A manual LOF calculation is possible and is shown below.

To apply pure error lack of fit test using the method given by Draper and Smith (1981).

From the Minitab calculation:

Residual sum of squares = 0.825 with 11 degrees of freedom

Pure error sum of squares = 0.0209012 with 2 degrees of freedom

Now:

Residual SS = Pure error SS + LOF SS

$\therefore$ LOF SS = 0.825 - 0.0209012

= 0.804 with 9 d.f.

MS_L = 0.804/9

and MS_e = S_e^2 = 0.0209012/2

$\therefore$ $F = \frac{MS_L}{Se^2} = \frac{0.08934}{0.01045} = 8.549$

Tables of the F distribution are consulted at 9 and 2 degrees of freedom. At the 1% level F = 99. It is concluded that since the calculated value is much lower it is not significant and the regression equation does give a satisfactory explanation of the data.

Appendix 4.8: Calculation of t-tests for kinetic equation coefficients

From Table 4.33

The equation is:

$$\log_{10} (\text{Rate} \times 10^{5}) = 11.5 - 0.985\,(\text{pH}) - 3.45\,\frac{1000}{T(^{\circ}K)} + 0.899 \log_{10} [\text{Amide}]$$

To test if the coefficients for pH and $\log_{10}$ [Amide] are significantly different from one, a t-test is used.

The regression analysis gives the standard deviation of each coefficient (an example is shown in Appendix 4.7).

i.e.

VARIABLE	COEFFICIENT	STANDARD DEV. OF COEFFICIENT	d.f. (DEGREE OF FREEDOM OF RESIDUAL)
pH	-0.98518	0.05632	11
Amide	0.8988	0.1610	11

To test if the coefficient is significantly different from 1.00 for pH:

$$t = \frac{-0.985 - (-1.0)}{0.05632} = 0.266 \quad \text{n.s.*}$$

for Amide:

$$t = \frac{0.899 - 1}{0.1610} = -0.627 \quad \text{n.s.*}$$

* - from statistical tables at 11 degrees of freedom

From Table 4.34

The equation is:

$$\log_{10} (\text{Rate} \times 10^5) = 10.3 - 0.329\,(\text{pH}) - 3.58\,\frac{1000}{T(^\circ K)} + 0.605 \log_{10} [\text{Peptide}]$$

and, as described above:

VARIABLE	COEFFICIENT	STANDARD DEV. OF COEFFICIENT	d.f.
pH	-0.3293	0.1217	11
Peptide	0.6054	0.3478	11

and

for pH:

$$t = \frac{0.3293 - (-1.00)}{0.1217} = 5.51 \quad \text{significant}$$

for peptide:

$$t = \frac{0.6054 - 1.00}{0.3478} = 1.13 \quad \text{n.s.}$$

From Table 4.35

Hydrogen ion consumption rate $\times 10^5$

$$= 0.109^{*} (\text{Amide} + \text{Peptide hydrolysis rate}) \times 10^5)^{0.998}$$

but, as discussed in Section 4.2.3

$$\frac{[H^+] \text{ measured}}{[H^+] \text{ actual}} \simeq 0.65$$

The expected stoichiometry is:

H^+_{actual} consumption rate = 1.0 x (Amide + Peptide hydrolysis rate)$^{1.0}$

or

$H^+_{measured}$ consumption rate = 0.65 x (Amide + Peptide hydrolysis rate)$^{1.0}$

In logarithmic form, therefore, as in Table 4.35 the expected equation is:

$\log_{10}$ (rate x 10^5) = -0.187 + $\log_{10}$ (Amide + Peptide hydrolysis rate x 10^5)

The equation obtained by regression analysis was

$\log_{10}$ (rate x 10^5) = -0.963 + 0.998 $\log_{10}$ (Amide + Peptide hydrolysis rate x 10^5)

The coefficients are tested for significant difference as previously described with, in this case, 13 degress of freedom.

i.e. $t = \frac{-0.187 - (-0.963)}{0.2899} = 2.68$ n.s.

Appendix 4.9: Calculation of activation energy for hydrolysis reactions

Figure 4.12:

For line B at $\frac{1}{T}$ $(°K^{-1})$ = 2.5×10^{-3} and 3.0×10^{-3}

$$-\frac{E}{R} = \frac{\ln k_1 - \ln k_2}{T_1 - T_2} = \frac{-4.8665 - (-8.8388)}{3 \times 10^{-3} - 2.5 \times 10^{-3}}$$

$$= -7944.6$$

Now R = 1.98 cal/°K

$\therefore$ E = 15730 calories.mol^{-1}

Figure 4.13:

For Line A

$$\frac{E}{R} = \frac{12.5345 - (-8.3774)}{3.0 \times 10^{-3} - 2.5 \times 10^{-3}} = -8314.2$$

$\therefore$ E = 16462 calories.mol^{-1}

For Line D

$$-\frac{E}{R} = \frac{-12.4045 - (-7.2502)}{3.0 \times 10^{-3} - 2.5 \times 10^{-3}} = -10309$$

$\therefore$ E = 20411 calories.mol^{-1}

Appendix 4.10: Calculation of standard deviation for hydrogen ion stoichiometry data

RUN	H^+ RATE	AMIDE RATE	PEPTIDE RATE	SUM
13	1.990×10^{-5}	2.95×10^{-5}	1.05×10^{-5}	4×10^{-5}
14	1.684×10^{-5}	2.23×10^{-5}	4.75×10^{-6}	2.705×10^{-5}
15	2.412×10^{-5}	1.52×10^{-5}	1.92×10^{-5}	3.44×10^{-5}
Mean	2.029		3.382	
	($\sigma_{n-1} = 0.366$)		($\sigma_{n-1} = 0.649$)	

Now ratio 2.029/3.382 = 0.6

Calculation of σ for the ratio above using the formula given by Beers (1953)

Now If $x = 2.029$ and $y = 3.382$

$\therefore \quad S_x = \frac{0.366}{2.029} \qquad S_y = \frac{0.649}{3.382}$

And $V = \frac{x}{y} = 0.6$

$S_v = \sqrt{S_x^2 + S_y^2} = 0.263$
(Fractional)

.5.4. $S_v = 0.263 = \frac{\sigma_v}{V} = \frac{\sigma_v}{0.6}$

$\therefore \quad \sigma_v = 0.158$

$\therefore$ Ratio = 0.6 ± 0.3 (i.e. $2 \times \sigma_v$)

Appendix 4.11: Further calculation of stoichiometry of hydrogen ion consumption

RUN NO.	ΔNH_4 (M)	ΔL-glu (M)	$\Delta NH_4 + \Delta$L-glu (M)	ΔH^+ (M)	$\Delta H+/\Delta sum$
1	-	-	-	-	-
2	-	-	-	-	-
3	1.6×10^{-2}	1.6×10^{-2}	3.2×10^{-2}	5.52×10^{-4}	0.02
4	-	-	-	-	-
5	3.55×10^{-3}	1.2×10^{-3}	4.75×10^{-3}	4.73×10^{-3}	1.0
6	1.65×10^{-2}	7.2×10^{-2}	8.85×10^{-2}	3.0×10^{-2}	0.34
7	-	-	-	-	-
8	3.2×10^{-1}	4.7×10^{-1}	7.9×10^{-1}	2.28×10^{-1}	0.29
9	2.6×10^{-3}	1.0×10^{-3}	3.6×10^{-3}	1.55×10^{-3}	0.43
10	1.8×10^{-2}	3.6×10^{-2}	5.4×10^{-2}	1.65×10^{-2}	0.31
11	-	-	-	-	-
12	1.35×10^{-1}	6.7×10^{-2}	2.02×10^{-1}	6.4×10^{-2}	0.32
13	7.85×10^{-2}	2.7×10^{-2}	1.05×10^{-1}	6.2×10^{-2}	0.59
14	8.0×10^{-2}	2.4×10^{-2}	1.04×10^{-1}	6.72×10^{-2}	0.65
15	6.8×10^{-2}	3.2×10^{-2}	1.0×1^{-2}	6.8×10^{-2}	0.68

Mean = 0.46

Standard Deviation = 0.27

(σ_{n-1})

Appendix 4.12: Manipulation of data from runs 2, 6 and 9 to test for fit to reaction model

Run 6

REACTION TIME (mins)	AMMONIA (mM)	FRACTION[1] REMAINING (%)	L-GLU (FLUORESCAMINE) (mM)	FRACTION[3] REMAINING
0	5.6	100	1.5[2]	100
2	10.8	68	3.4	98
5.33	14.6	45	4.5	96
13	17.0	30	9.8	90
26	18.8	19.5	17.5	80
60	21.8	1	33.4	60
120	21.9	-	53.5	35
240	22.2	-	69.2	15
360	21.7	-	75.8	6.5

(1) Calculated by taking total potential amide = 22 mM (cf lower figure used in Section 4.7.3) and initial value = 5.6

(2) Value obtained by extrapolation on Figure 4.3

(3) Calculated by taking total potential peptide = 81 mM from value of 8.12×10^{-3} moles per gram calculated in Table 4.26

Run 9

REACTION TIME (mins)	AMMONIA mM	FRACTION[1] REMAINING (%)
0	0	100
10.5	0.12	99.4
25.0	0.30	98.5
60	0.56	97.2

Run 2

REACTION TIME (mins)	AMMONIA (mM)	FRACTION[2] REMAINING (%)
0	14	100
10	27	90
25	35	85
60	60	66

(1) Based on 20 mM

(2) Based on 150 mM

total $\simeq$ 150-14

Appendix 4.13: A programme to simulate mild acid hydrolysis of gluten

```
10 REM A PROGRAMME TO SIMULATE MILD ACID
HYDROLYSIS OF GLUTEN USING INTEGRATION SUBROUTINE D02BAF
20 COMMON AMIDE, HPLUS, PEPTIDE, K(10)
25 REM THE REACTION CONDITIONS ARE DEFINED
100 INPUT 'ENTER AM(0)'; AM(0)
101 INPUT 'ENTER HION(0)'; HION(0)
103 INPUT 'ENTER TEMP'; TEMP
104 INPUT 'ENTER K(1)'; K(1)
105 INPUT 'ENTER K(2)'; K(2)
106 INPUT 'ENTER TOTAL TIME': TOTALTIME
107 REM THE DATA OUTPUT IS ORGANIZED
111 OPEN 'SIM. DAT' FOR OUTPUT AS FILE 2
112 NOMARGIN #2
113 PRINT #2, 'HION(0), 'PEP(0)', 'AM(0)', 'TEMP', 'K(1)',
'K(2)'
114 PRINT #2
115 PRINT #2, HION(0)', 'PEP(0)', 'AM(0), TEMP, K(1), K(2)
116 PRINT #2
117 PRINT #2
118 PRINT #2, 'Y(0)', 'Y(1)', 'IFAIL%', 'RTIME', 'AMIDE',
'PEPTIDE', 'HPLUS'
119 PRINT #2
200 AMIDE=AM(0)
205 PEPTIDE=PEP(0)
210 HPLUS=HION(0)
215 REM THE INTEGRATION STEP SIZE IS SET
220 FOR RTIME=10 TO TOTAL TIME STEP 10
230 EXTERNAL INTEGER FCN
240 DIM W(20), Y(1)
250 X=RTIME
260 XEND=RTIME+10
270 N%=2
280 TOL=0.0001
290 IFAIL%=0
300 Y(0)=0
```

```
310  Y(1)=0
315 REM THE INTEGRATION ROUTINE IS CALLED
320 CALL D)2BAF(X,XEND,N%,Y(0),TOL,FCN,W(0),IFAIL%)
330 REM NEW VALUES OF THE CONCENTRATIONS ARE CALCULATED
340 AMIDE=AMIDE-Y(0)
350  PEPTIDE=PEPTIDE-Y(1)
360 HPLUS = HPLUS- ACTIVITY CORRECTION FACTOR* (Y(0) + Y(1))
365 PRINT Y(0);Y(1);I FAIL%;RTIME;AMIDE;PEPTIDE;HPLUS
375 NEXT RTIME
820  PRINT 'EXECUTION COMPLETE'
999 END
1000 REM A SUBROUTINE DEFINES THE REACTION RATE EQUATIONS FOR
INTEGRATION
1003 SUB FCN(X,Y() BY REF,F() BY REF)
1005  COMMON AMIDE,HPLUS,PEPTIDE,K(10)
1010 F(0)=K(1)*AMIDE*HPLUS
1020 F(1)=K(2)*PEPTIDE*HPLUS
1030 END SUB
```

Appendix 4.14: Calculation of reaction rate coefficients from the data of Figures 4.15 and 4.17

To find k_1, k_2 using the equation $- \ln (1-X_A) = kt$

From Figure 4.15:

For k_2 $t = 120$ minutes $t_2 = 0$ minutes

$(1 - X_A)_{120} = 0.35$ $\qquad (1-X_A)_0 = 1.0$

$$\text{slope} = \frac{\ln 0.35 - \ln 1.0}{120 - 0}$$

i.e. $k_2[H^+] = 1.458 \times 10^{-4} \text{ sec}^{-1}$

From Figure 4.17:

For k_1 $t_1 = 60$ minutes $t_2 = 0$ minutes

$(1-X_A)_{60} = 0.01$ $\quad (1-X_A)_0 = 1.0$

$$\text{slope} = \frac{\ln 0.01 - \ln 1.0}{60 - 0}$$

i.e. $k_1[H^+] = 1.279 \times 10^{-3} \text{ sec}^{-1}$

Appendix 4.15: Data from reaction simulation for test of fit to experimental data for Run 6 of the experimental design

REACTION TIME (mins)	AMIDE (M)	FRACTION REMAINING (%)	PEPTIDE (M)	FRACTION REMAINING (%)	HYDROGEN ION (M)
0	0.02	100	0.079	100	1.702
20	0.0043	21.5	0.066	83.5	1.685
40	0.00096	4.8	0.056	70.9	1.677
60	0.0002	1	0.047	59.5	1.671
120	-		0.028	35.4	1.659

Appendix 4.16: Smoothed data from repetition runs of the experimental design

Experiment No.	15	13	14	15	13	14					15	13	14				
REACTION TIME (mins)		pH			$[H^+]$		MEAN	s.d.	+2s.d.	-2s.d.	AMMONIA (mM)			MEAN	s.d.	+2s.d.	-2s.
0	0.896	0.910	0.870	0.127	0.123	0.135	0.128	0.0061	0.140	0.116	0	0	0	0	0	0	0
10	0.938	0.932	0.900	0.115	0.115	0.126	0.119	0.0064	0.132	0.106	7.4	15	17.5	13.3	5.3	23.9	2.7
60	1.07	1.052	1.012	0.085	0.085	0.097	0.089	0.0069	0.103	0.075	35.6	51	45	43.9	7.8	59.5	28.3
120	1.16	1.135	1.103	0.069	0.073	0.079	0.073	0.0050	0.0830	0.063	57.4	68.5	62.5	62.8	5.6	74.0	51.(
180	1.23	1.195	1.168	0.059	0.064	0.068	0.064	0.0045	0.073	0.055	68.0	78.2	79	75.2	6.2	87.6	62.8

Experiment No.	15	13	14				
REACTION TIME (mins)	L-glu (mM)			MEAN	s.d.	+ 2 s.d.	- 2 s.d.
0	19	16.5	12.5	16.0	3.3	22.6	9.4
10	26	21	17	21.3	4.5	30.3	12.3
60	41	33	27.5	33.8	6.8	47.4	20.2
120	47	40	33.5	40.2	6.8	53.8	26.6
180	52	43.5	36.5	44.0	7.8	59.6	28.4

Appendix 4.17: Data from reaction simulation for test to fit to reaction data from repetition runs of the experimental design

	CELL (2)			CELL (3)			CELL (4)			CELL (5)			CELL (8)		
TIME (s)	[AMIDE] (M)	[PEPTIDE] (M)	$[H^+]$ (M)	[AMIDE] (M)	[PEPTIDE] (M)	$[H^+]$ (M)	[AMIDE] (M)	[PEPTIDE] (M)	$[H^+]$ (M)	[AMIDE] (M)	[PEPTIDE] (M)	$[H^+]$ (M)	[AMIDE] (M)	[PEPTIDE] (M)	$[H^+]$ (M)
600	0.1390	0.5927	0.1200	0.1390	0.5927	0.1194	0.1388	0.5891	0.1261	0.1301	0.5873	0.1181	0.1392	0.5855	0.11
3600	0.1010	0.5831	0.0915	0.1016	0.5832	0.0889	0.0955	0.5618	0.1184	0.1028	0.5670	0.0829	0.1048	0.5507	0.07
7200	0.0752	0.5744	0.0708	0.0765	0.5749	0.0672	0.0627	0.5324	0.1115	0.0797	0.5489	0.0581	0.0843	0.5254	0.04
10800	0.0595	0.5676	0.0573	0.0614	0.5685	0.0532	0.0420	0.5059	0.1063	0.0663	0.5362	0.0426	0.0733	0.5098	0.03

TIME (min)	NH_4 (mM)	L-glu[1] (mM)	H^+ (M)	NH_4 (mM)	L-glu[1] (mM)	H^+ (M)	NH_4 (mM)	L-glu[1] (mM)	H^+ (M)	NH_4 (mM)	L-glu[1] (mM)	H^+ (M)	NH_4 (mM)	L-glu[1] (mM)	H^+ (M)
10	11	19	0.120	11	22	0.118	11	25	0.116	11	18	0.119	11	22	0.12
60	49	28	0.092	47	44	0.083	45	60	0.074	48	28	0.089	54	49	0.11
120	75	37	0.071	70	62	0.058	66	86	0.047	73	36	0.067	87	79	0.1
180	90	43	0.057	84	75	0.043	77	101	0.031	89	44	0.053	108	105	0.1

	CELL (6)			CELL(9)			CELL (10)			CELL (11)		
TIME (s)	[AMIDE] (M)	[PEPTIDE] (M)	$[H^+]$ (M)	[AMIDE] (M)	[PEPTIDE] (M)	$[H^+]$ (M)	[AMIDE] (M)	[PEPTIDE] (M)	$[H^+]$ (M)	[AMIDE] (M)	[PEPTIDE] (M)	$[H^+]$ (M)
600	0.1391	0.5893	0.1173	0.1393	0.5856	0.1149	0.1390	0.5919	0.1200	0.1390	0.5915	0.1200
3600	0.1035	0.5675	0.799	0.1056	0.5516	0.0709	0.1011	0.5791	0.910	0.1010	0.5768	0.0909
7200	0.0812	0.5503	0.0542	0.0861	0.5279	0.0429	0.0754	0.568	0.0698	0.0754	0.5638	0.0699
10800	0.0687	0.5386	0.0385	0.0759	0.5138	0.0271	0.0600	0.5587	0.0560	0.0599	0.5537	0.0558

TIME (min)	NH_4 (mM)	L-glu[1] (mM)	H^+ (M)	NH_4 (mM)	L-glu[1] (mM)	H^+ (M)	NH_4 (mM)	L-glu[1] (mM)	H^+ (M)	NH_4 (mM)	L-glu[1] (mM)	H^+ (M)
10	11	22	0.117	11	25	0.115	11	20	0.120	11	19	0.120
60	46	43	0.080	44	59	0.071	49	32	0.091	49	34	0.091
120	69	61	0.054	64	83	0.043	75	43	0.070	75	47	0.070
180	81	72	0.039	74	97	0.027	90	52	0.056	90	57	0.056

NOTE (1) All L-glu data have 16.0 added to take account of initial value as discussed in Section 4.7.2

Appendix 4.18: Calculation of slopes from Figures 4.21 and 4.22

Ammonia data

Figure 4.21

For 0.027 M HCl slope = $\frac{-0.77}{18}$ = -0.043

For 0.1 M HCl slope = $\frac{-1.0}{2.5}$ = 0.4

For 0.2 M HCl slope = $\frac{-1.0}{1.2}$ = 0.83

For 0.5 M HCl slope = $\frac{-1.7}{0.8}$ = 2.13

Amino N data:

Figure 4.23

For 0.1 M HCl slope = $\frac{-0.19 - (-0.01)}{40-0}$ = -4.5×10^{-3}

For 0.2 M HCl slope = $\frac{0.26 - (-0.01)}{24}$ = -0.010

For 0.5 M HCl slope = $\frac{0.29 - (-0.01)}{10}$ = -0.028

For 1.0 M HCl slope = $\frac{0.33 - (-0.01)}{6}$ = -0.053

For 2.0 M HCl slope = $\frac{-0.245 - (-0.01)}{1}$ = -0.235

Appendix 5.10: Reaction progress calculated from simulation for Figures 5.1, 5.2 and 5.3

For Line A:

REACTION TIME (secs)	AMIDE (M)	PEPTIDE (M)	HYDROGEN ION (M)	AMIDE BOND HYDROLYSIS (%)	PEPTIDE BOND HYDROLYSIS (%)	pH
1×10^2	0.1997	0.7930	0.4758	0.15	0	0.3226
1×10^2	0.1977	0.7927	0.4742	1.15	0.04	0.3240
3×10^2	0.1923	0.7920	0.4708	3.85	0.13	0.3272
3×10^3	0.1377	0.7830	0.4326	31.15	1.26	0.3639
1×10^4	0.0636	0.7629	0.3761	68.20	3.80	0.4247
1.8×10^4	0.0289	0.7428	0.3433	85.55	6.33	0.4643

For Line B:

REACTION TIME (secs)	AMIDE (M)	PEPTIDE (M)	HYDROGEN ION (M)	AMIDE BOND HYDROLYSIS (%)	PEPTIDE BOND HYDROLYSIS (%)	pH
1×10^1	0.1997	0.7929	0.4758	0.15	0.01	0.3226
1×10^2	0.1974	0.7919	0.4738	1.30	0.14	0.3244
3×10^2	0.1924	0.7896	0.4694	3.80	0.43	0.3285
1×10^3	0.1760	0.7820	0.4550	12.00	1.39	0.3420
3×10^3	0.1384	0.7618	0.4203	30.80	3.93	0.3764
1×10^4	0.0669	0.7037	0.3426	66.55	11.26	0.4652
1.8×10^4	0.0334	0.6525	0.2918	83.30	17.71	0.5349

NEW ZEALAND DAIRY RESEARCH INSTITUTE
PRIVATE BAG ■ PALMERSTON NORTH ■ NEW ZEALAND
Telegrams: DAISEARCH
Telex: NZ31312 NZDRI
Telephone: (63) 74 129

APPENDIX 6.1 PRODUCT USE AND EVALUATION

Please Quote:

GLUTEN EVALUATION: Effect of acid hydrolysis on the flavour of gluten.

SE85 Gluten
Evaluation dates: 16, 17, 18, 19, 22, 23, 24, 26 April 1985.

AIM:

The aim of the project was to determine the effect of acid hydrolysis on specific flavour attributes of gluten.

SAMPLES:

The following samples, in increasing order of acid hydrolysis, were submitted for evaluation:

		Sample Numbers as shown in Table 5.1
Sample 1:	JH 11:265	(1)
Sample 2:	JH 12:8A	(4)
Sample 3:	JH 11:282	(2)
Sample 4:	JH 12:2	(5)
Sample 5:	JH 11:284	(3)
Sample 6:	JH 11:277	(6)
Sample 7:	JH 11:280	(7)
Sample 8:	JH 11:270	(8)
Sample 9:	JH 11:266	(10)
Sample 10:	JH 11:286	(9)
Sample 11:	JH 11:268	(11)
Sample 12:	JH 11:272	

TEST METHOD:

For the preparation of each sample at 5% total solids, 22g of gluten was mixed into 400 mls of R.O. water. Sodium hydroxide (0.5 M) was added according to the degree of acid hydrolysis (Appendix A). The solution was heated to 60°C for 20 - 25 minutes while being mixed with stirrers (Type RZRI-64, Caframo, Wairton, Ontario, Canada). The solution was poured through muslin and the supernatent was cooled to 25°C. The pH of the supernatent was measured and adjusted to 7.5 ± 0.2. Thirty millilitres of each sample was poured into 50 ml Kimax beakers and served to panelists in the Institute sensory panel room under standard red lighting.

A panel consisting of 10 members was drawn from the Institute staff. This panel had been previously trained in the profiling of a similar product. Prior

to testing the gluten product four training sessions were held during which the panel identified and quantified various flavour attributes. The samples were evaluated on a 0 - 10 scale where 0= absent and 10= intense (Appendix B). The twelve samples were randomised with three being tested at each session. Testing was repeated the following week.

RESULTS:

The means for the three attributes tested over two weeks were calculated and the difference between the means were analysed using Analysis of Variance. Table 1 shows the means scores for each attribute.

Table 1: Mean sensory scores for evaluation of gluten.

	Sample Number												
	1	2	3	4	5	6	7	8	9	10	11	12	F value
Wheaty/ cereal	5.9	5.6	4.2	5.7	4.3	4.7	4.5	3.1	3.5	3.1	3.1	2.5	11.17***
Rancid/ nut	1.8	1.8	3.2	2.1	3.8	3.1	3.9	5.9	5.5	5.7	6.2	6.1	19.44***
Burning/ bitter	1.2	1.3	2.9	1.3	3.2	2.2	3.2	4.1	3.6	5.3	6.9	8.1	28.79***

Significant differences (p=0.001) were found between the samples for all the tested attributes. Each of these will be considered in turn.

Wheaty/cereal:

This attribute was defined as being characteristic of the presence of wheat flour. Sample 1 (JH 11:265) received the highest mean score for this attribute with the lowest score given to Sample 12 (JH 11:272). Other differences between samples are shown below (lines join those samples not significantly different).

	Sample Number											
	1	4	2	6	7	5	3	9	10	11	8	12
Sample mean	5.9	5.7	5.6	4.7	4.5	4.3	4.2	3.5	3.1	3.1	3.1	2.5

Rancid/nut:

This attribute described the rancid flavour present in the sample. Panelists defined it as that flavour present in nuts which had become rancid. This attribute was found in moderate levels in Sample 11 (JH 11:268) with a threshold level being reported in Sample 1 (JH 11:265). Significant differences between samples are shown below (lines join those samples not significantly different).

	Sample Number											
	11	12	8	10	9	7	5	3	6	4	2	1
Sample mean	6.2	6.1	5.9	5.7	5.5	3.9	3.8	3.2	3.1	2.1	1.8	1.8

Burning/bitter:

This particular attribute was defined as a burning sensation perceived at the back of the mouth. With the gluten samples it was found that the sensation developed over time and took up to a minute before it appeared. Sample 12 (JH 11:272) was found to be strongly burning/bitter with only a threshold level found in Sample 1 (JH 11:265). Other differences between samples are shown below (lines join those samples not significantly different).

	Sample Number											
	12	11	10	8	9	7	5	3	6	4	2	1
Sample mean	8.1	6.9	5.3	4.1	3.6	3.2	3.2	2.9	2.2	1.3	1.3	1.2

A further way of representing these results is by graphing the mean score against the level of hydrolysis. Figures 1, 2 and 3 illustrate the effect of acid hydrolysis on the attributes of wheaty/cereal, rancid/nut and burning/bitter respectively. The samples are shown in increasing order of acid hydrolysis.

The wheaty/cereal attribute decreased with increasing acid hydrolysis but was present at only a moderate level in the least hydrolysed sample. The rancid/nut characteristic was present at threshold levels in Samples 1 and 2 but increased to a moderate level with increasing acid hydrolysis.

The burning/bitter attribute became quite distinct with increasing acid hydrolysis. This characteristic was perceived at a strong level in the most hydrolysed sample.

CONCLUSIONS:

The sensory attributes of acid hydrolysed gluten changed with increasing levels of acid hydrolysis. The rancid/nut and burning/bitter characteristics increased with increasing acid hydrolysis. In comparison the wheaty/cereal attribute decreased.

Sharon Wards

Tony McKenna

Product Use and Evaluation Section

June 1985

FIGURE 1: Effect of incre sing levels of cid hydrolysis on the wheaty/cereal attribute.

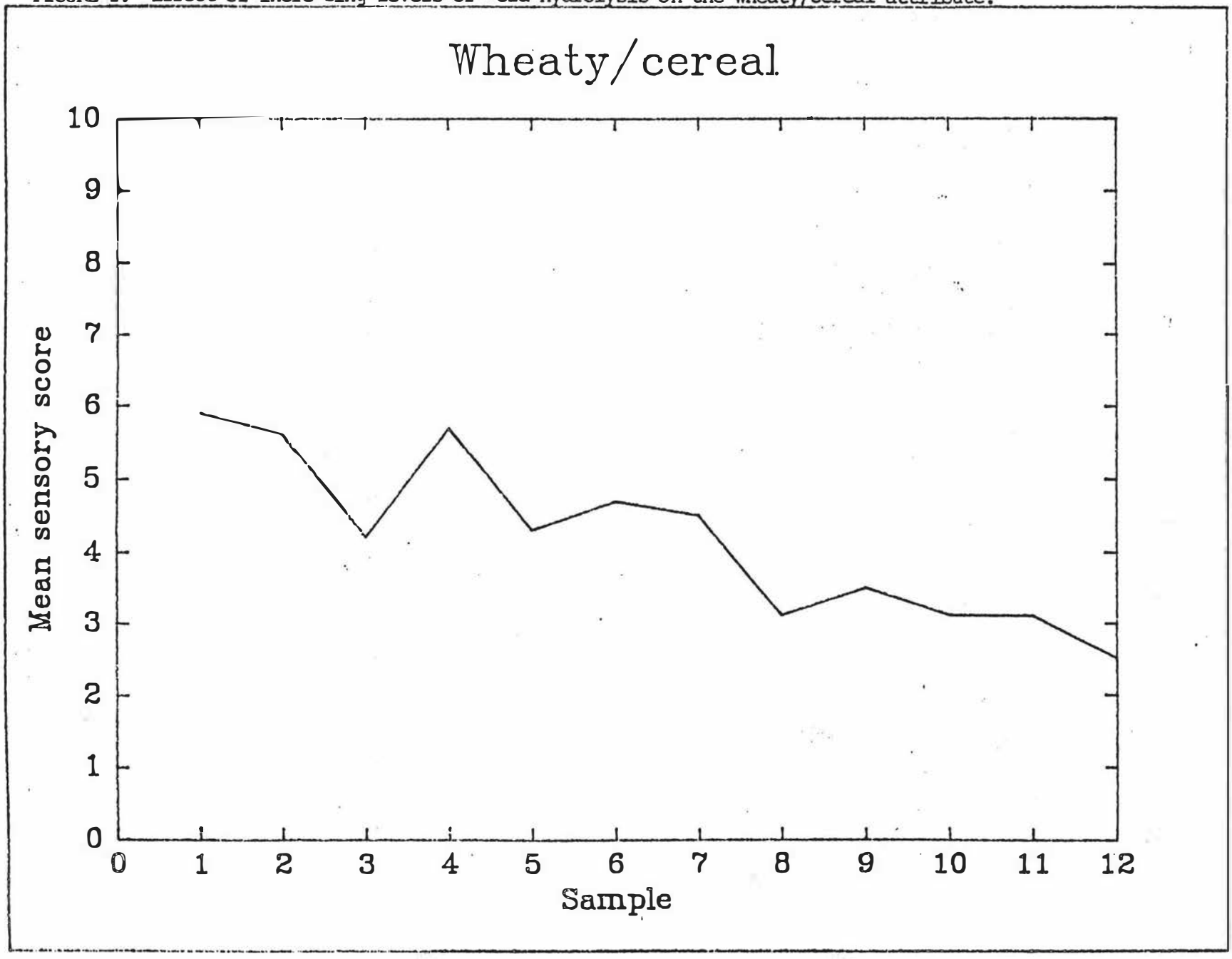


FIGURE 2: Effect of increasing levels of acid hydrolysis on the rancid/nut attribute of gluten.

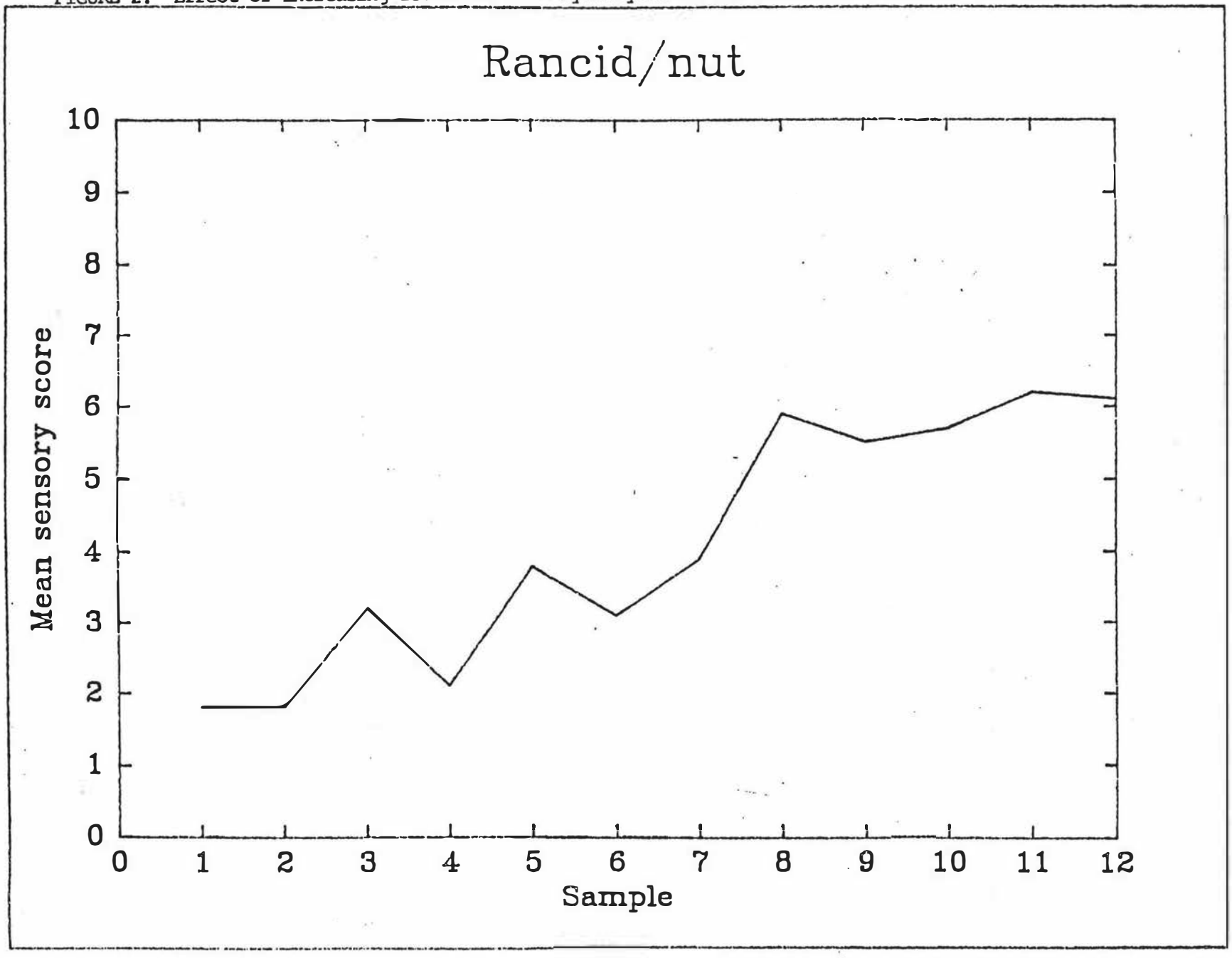
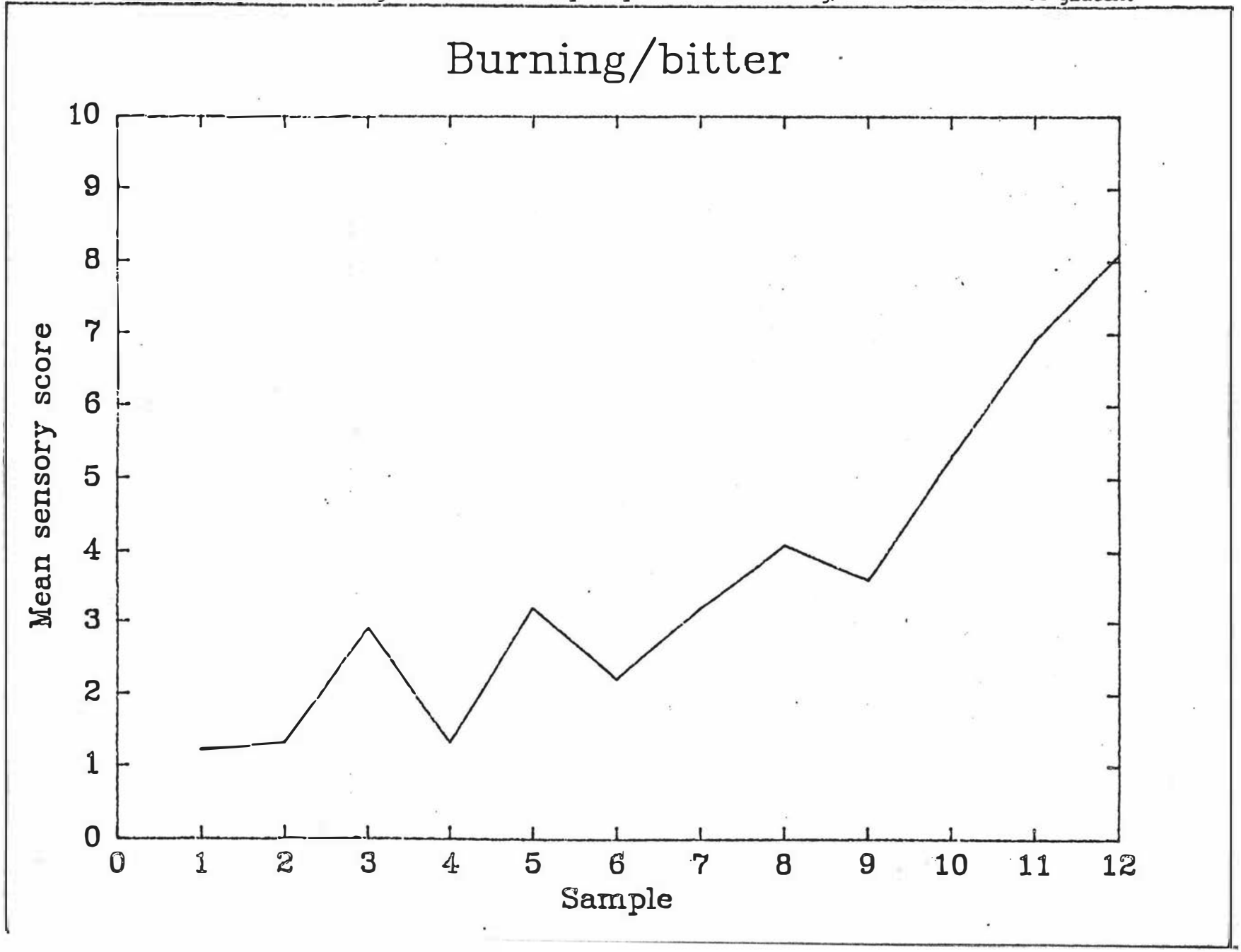


FIGURE 3: Effect of increasing levels of acid hydrolysis on the burning/bitter attribute of gluten.



APPENDIX A

Sample Number	Description	Amount of NaOH (mls)
1	JH 11:265	4.8
2	JH 12:8A	5.2
3	JH 11:282	9.7
4	JH 12:2	10.6
5	JH 11:284	11.4
6	JH 11:277	11.7
7	JH 11:280	14.0
8	JH 11:270	21.6
9	JH 11:266	25.3
10	JH 11:286	33.0
11	JH 11:268	45.3
12	JH 11:272	61.9

APPENDIX B

GLUTEN PANEL

In front of you are several samples of gluten supernatent. Please evaluate them for the following attributes using a 0-10 scale where:

0 = Absent
2 = Threshold
4 = Weak
6 = Moderate
8 = Strong
10 = Intense

FLAVOUR	Sample numbers		
	_____	_____	_____
Wheaty/cereal	_____	_____	_____
Rancid/nut	_____	_____	_____
Burning/bitter	_____	_____	_____
Other			

COMMENTS

Appendix 4.2: pH determination for hydrolysis experiment, run 1

Sample	A	B	Mean
B1	3.118	3.145	3.132
1	3.216	3.194	3.205
2	3.221	3.164	3.193
3	3.246	3.196	3.221
4	3.267	3.283	3.275
5	3.219	3.246	3.233

Appendix 4.2: pH determination for hydrolysis experiment, run 2

Sample	A	B	Mean
B1	-0.178	-0.179	-0.179
1	-0.176	-0.177	-0.177
2	-0.188	-0.178	-0.183
3	-0.177	-0.176	-0.177
4	-0.181	-0.172	-0.177
5	-0.160	-0.152	-156*

* Value low due to electrode draft.
- 'correct' value probably ca -0.164

Appendix 4.2: pH determination for hydrolaysis experiment, run 3

Sample	A	B	Mean
B1	3.197	3.219	3.208
1	3.324	3.311	3.318
2	3.550	3.453	3.502
3	3.599	3.660	3.630
4	3.807	3.815	3.811
5	-	4.112	4.112

Appendix 4.2: pH determination for hydrolysis experiment, run 4*

Sample	A	B	Mean
B1	-0.227	-0.233	-0.23
1	-0.213	-0.215	-0.21
2	-0.200	-0.200	-0.20
3	-0.184	-0.197	-0.191
4	-0.182	-0.176	-0.179
5	-0.165	-0.166	-0.166

* First experiment

Appendix 4.2: pH determination for hydrolysis experiment, run 4*

Sample	A	B	Mean
B1	-0.172	-0.176	-0.174
1	-0.164	-0.166	-0.165
2	-0.159	-0.156	-0.158
3	-0.147	-0.143	-0.145
4	-0.139	-0.140	-0.140
5	-0.336	-0.118	-0.118

* Repeat experiment, A repeat of the hydrolysis was carried out wit analysis of pH and ammonia only.

Appendix 4.2: pH determination for hydrolysis experiment, run 5

Sample	A	B	Mean
B1	1.845	1.840	1.842
1	1.851	1.853	1.852
2	1.867	1.863	1.865
3	1.904	1.857	1.880
4	1.928	1.922	1.925
5	1.992	2.034	2.013

Appendix 4.2: pH determination for hydrolaysis experiment, run 6

Sample	A	B	Mean
B1	-0.228	-0.233	-0.231
1	-0.227	-0.229	-0.228
2	-0.229	-0.230	-0.230
3	-0.230	-0.230	-0.230
4	-0.215	-0.219	-0.217
5	-0.229	-0.219	-0.224
6	-0.227	-0.229	-0.228
7	-0.215	-0.225	-0.220
8	-0.223	-0.225	-0.222

Appendix 4.2: pH determination for hydrolysis experiment, run 7

Sample	A	B	Mean
B1	3.949	3.924	3.937
1	3.942	3.854	3.898
2	3.994	3.859	3.927
3	3.956	4.019	3.988
4	4.010	3.932	3.971
5	4.065	3.994	4.030

Appendix 4.2: pH determination for hydrolysis experiment, run 8

Sample	A	B	Mean
R1	-0.29		-0.29
1	-0.302	-0.304	-0.304
2	-0.290	-0.287	-0.289
3	-0.280	-0.275	-0.278
4	-0.258	-0.262	-0.260
5	-0.250	-0.257	-0.254

Appendix 4.2: pH determination for hydrolysis experiment, run 9

Sample	A	B	Mean
B1	0.865	0.870	0.868
1	0.868	0.867	0.868
2	0.868	0.871	0.870
3	0.869	0.868	0.869
4	0.874	0.869	0.871
5	0.872	0.874	0.873

Appendix 42: pH determination for hydrolysis experiment, run 10

Sample	A	B	Mean
B1	0.871	0.873	0.872
1	0.873	0.875	0.875
2	0.886	0.886	0.886
3	0.906	0.908	0.907
4	0.921	0.934	0.928
5	0.935	0.935	0.935

Appendix 4.2: pH determination for hydrolysis experiment, run 11

Sample	A	B	Mean
B1	0.905	0.905	0.905
1	0.913	0.912	0.913
2	0.917	0.913	0.915
3	0.918	0.919	0.919
4	0.924	0.934	0.929
5	0.955	0.959	0.957

Appendix 4.2: pH determination for hydrolysis experiment, run 12

Sample	A	B	Mean
B1	0.905	0.871	0.888
1	0.974	0.990	0.982
2	1.204	1.200	1.202
3	1.775	1.763	1.769
4	2.318	2.249	2.281
5	2.689	2.808	2.749

Appendix 4.2: pH determination for hydrolysis experiment, run 13

Sample	A	B	Mean
B1	0.912	0.907	0.910
1	0.918 (0.939)	0.906 (0.930)	0.912
2	0.943	0.943	0.943
3	0.993	0.994	0.994
4	1.071	1.074	1.073
5	1.175	1.216	1.196

Appendix 4.2: pH determination for hydrolysis experiment, run 14

Sample	A	B	Mean
B1	0.868	0.871	0.870
1	0.885	0.888	0.887
2	0.910	0.909	0.910
3	0.949	0.960	0.955
4	1.036	1.030	1.033
5	1.162	1.182	1.172

Appendix 4.2: pH determination for hydrolysis experiment, run 15

Sample	A	B	Mean
B1	0.897	0.896	0.897
1	0.903	0.902	0.907
2	0.933	0.937	0.935
3	0.986	1.024	1.005
4	1.074	1.075	1.075
5	1.236	1.233	1.235

Appendix 4.2: Ammonia determination standards for hydrolysis experiment, run 1

(A340)

Label	a	b	Mean	Concentration (mM)
0	2.367	2.302	-	0
5	2.254	2.236	-	0.2
10	2.133	2.100	-	0.4
20	1.850	1.865	-	0.8
30	1.619	1.605	-	1.2
40	1.469	1.360	-	1.6
50	1.157	1.155	-	2.0
60	1.140	1.141	-	2.4

Appendix 4.2: Ammonia determination standards for hydrolysis experiment, run 2

(A340)

Label	a	b	Mean	Concentration (mM)
0	2.430	2.350	2.390	-
5	2.346	2.279	2.313	0.2
10	2.210	2.156	2.183	0.4
20	1.924	1.888	1.906	0.8
30	1.615	1.632	1.624	1.2
40	1.406	1.406	1.406	1.6
50	1.129	1.145	1.137	2.0
60	1.094	1.090	1.092	2.4

Appendix>402: Ammonia determinaticn standards for hydrolysis experiment, run 2 (repeat determination)

(A340)

Label	a	b	Mean	Concentration (mM)
0	2.240	2.265	2.253	-
5	2.150	2.129	2.140	0.2
10	2.028	2.022	2.025	0.4
20	1.791	1.771	1.784	0.8
30	1.544	1.513	1.529	1.2
40	1.295	1.278	1.287	1.6
50	1.090	1.083	1.087	2.0
60	1.074	1.070	1.072	2.4

Appendix 4.2: Ammonia determination standards for hydrolysis experiment, run 3

(A340)

Label	a	b	Mean	Concentration (mM)
0	2.400	2.405		-
5	2.285	2.290		0.2
10	2.162	2.160		0.4
20	1.912	1.911		0.8
30	1.653	1.640		1.2
40	1.491	1.431		1.6
50	1.186	1.199		2.0
60	1.165	1.162		2.4

Appendix 4.2: Ammonia determination standards for hydrolysis experiment, run *4

(A340)

Label	Absorbance	Mean	Concentration (mM)
0	2.085 2.127	2.106	-
10	1.948 2.075	2.012	0.4 -
20	1.890 1.706	1.798	0.8
30	1.482 1.515	1.499	1.2
40	1.247 1.249	1.248	1.6
50	1.022 0.993	1.008	2.0

* First determination

Appendix 4.2: Ammonia determination standards for hydrolysis experiment, run *4

(A340)

Label	Absorbance	Mean	Concentration (mM)
0	2.284 2.254	2.269	0
10	2.117 2.125	2.121	0.4
20	1.838 1.945	1.892	0.8
30	1.714 1.602	1.658	1.2
40	1.409 1.382	1.396	1.6
50	1.192 1.106	1.149	2
60	1.078 1.081	1.080	2.4

* Repeat analysis of original samples

Appendix 4.2: Ammonia determination standards for hydrolysis experiment, run *4

Label	(A340)	Mean	Concentration (mM)
0	2.496	-	-
	2.509		
5	2.329	-	-
	2.344		
10	2.187	-	-
	2.194		
20	2.029	-	-
	1.919		
30	1.639	-	-
	1.675		
40	1.404	-	-
	1.399		
50	1.182	-	-
	1.187		
60	1.156	-	-
	1.156		

* Repeat determination

Appendix 4.2: Ammonia determination standards for hydrolysis experiment, run 5

(A340)

Label	a	b	Mean	Concentration (mM)
0	2.158	2.165	2.162	0
10	1.995	1.975	1.985	0.4
20	1.754	1.745	1.750	0.8
30	1.524	1.515	1.520	1.2
40	1.277	1.275	1.276	1.6
50	1.050	1.050	1.050	2

Appendix 4.2: Ammonia determination standards for hydrolysis experiment, run 6

(A340)

Label	Absorbance	Mean	Concentration (mM)
0	1848 1831	1.840	-
10	1731 1725	1.728	0.04
20	1503 1453	1.478	0.08
30	1229 1163	1.196	1.2
40	0951 0907	0.929	1.6
50	0735 0678	0.707	2.0

Appendix 4.2: Ammonia determination standards for hydrolysis experiment, run 7

(A340)

Label	Absorbance	Mean	Concentration (mM)
0	1.941 1.913	1.927	
10	1.841 1.835	1.838	0.4
20	1.700 1.684	1.692	0.8
30	1.514 1.477	1.496	1.2
40	1.529 1.307	1.418	1.6
50	1.402	1.402	2.0
60	1.089 1.072	1.081	2.4
80	1.080 1.072	1.076	3.2

Appendix 4.2: Ammonia determination standards for hydrolysis experiment, run 8

(A340)

Label	a	b	Mean	Concentration (mM)
0	2.426	2.329	2.378	nil
10	2.185	2.158	2.172	0.4
20	1.934	1.930	1.932	0.8
30	1.688	1.674	1.681	1.2
40	1.433	1.420	1.427	1.6
50	1.191	1.188	1.190	2.0
60	0.943	0.930	0.937	2.4

Appendix 4.2: Ammonia determination standards for hydrolysis experiment, run 9

(A340)

Label	a	b	Mean	Concentration (mM)
0	2.236	2.237	2.237	0
5	2.107	2.114	2.111	0.2
10	2.018	2.013	2.016	0.4
20	1.788	1.747	1.768	0.8
30	1.585	1.427	1.506	1.2
40	1.277	1.313	1.295	1.6
50	1.070	1.070	1.070	2.0
60	1.061	1.058	1.060	2.4

Appendix 4.2: Ammonia determination standards for hydrolysis experiment, run 10

(A340)

Label	a	b	Mean	Concentration (mM)
0	2.275	2.301	2.285	-
5	2.180	2.160	2.170	0.2
10	2.053	2.053	2.053	0.4
20	1.796	1.810	1.803	0.8
30	1.567	1.562	1.565	1.2
40	1.336	1.308	1.322	1.6
50	1.102	1.082	1.092	2.0
60	1.060	1.057	1.059	2.4

Appendix 4.2: Ammonia determination standards for hydrolysis experiment, run 11

(A340)

Label	Absorbance	Mean	Concentration (mM)
0	2.287 2.290	2.289	nil
10	2.243 2.193	2.218	0.4
20	1.972 1.962	1.967	0.8
30	1.655 1.663	1.657	1.2
40	1.392 1.526	1.459	1.6
50	1.143 1.206	1.173	2.0

Appendix 4.2: Ammonia determination standards for hydrolysis experiment, run 12

(A340)

Samples	a	b	Mean	Concentration (mM)
0	2.233	2.237	2.235	0
5	2.185	2.145	2.165	0.2
10	2.102	2.039	2.071	0.4
20	1.868	1.781	1.825	0.8
30	1.576	1.511	1.544	1.2
40	1.305	1.289	1.297	1.6
50	1.152	1.136	1.144	2.0
60	1.142	1.122	1.132	2.4

Appendix 4.2: Ammonia determination standards for hydrolysis experiment, run 13

(A340)

Label	a	b	Mean	Concentration (mM)
0	2.220	2.230	2.225	0
5	-	2.154	2.154	0.2
10	2.006	2.022	2.014	0.4
20	1.796	1.766	1.781	0.8
30	1.550	1.520	1.535	1.2
40	1.278	1.302	1.290	1.6
50	1.084	1.080	1.082	2.0
60	1.023	1.026	1.025	2.4

Appendix 4.2: Ammonia determination standards for hydrolysis experiment, run 14

(A340)

Sample Label	a	b	Concentration (mM)
0	2.460	2.455	-
5	2.300	2.330	0.2
10	2.232	2.202	0.4
20	1.983	1.999	0.8
30	1.783	1.738	1.2
40	1.486	-	1.6
50	1.264	1.282	2.0
60	1.171	1.160	2.4

Appendix 4.2: Ammonia determination standards for hydrolysis experiment, run 15

(A340)

Label	a	b	Mean	Concentration (mM)
0	2.365	2.240	2.303	-
5	2.262	2.188	2.132	0.2
10	2.155	2.114	2.134	0.4
20	1.900	1.894	1.897	0.8
30	1.665	1.664	1.665	1.2
40	1.422	1.420	1.421	1.6
50	1.195	1.182	1.187	2.0
60	1.081	1.070	1.086	2.4

Appendix 4.2: Ammonia determination for hydrolysis experiment, run 1

(A340)

Sample	Aa	Ab	Ba	Bb	Mean (σ_{n-1})	Concentration* in reactor (mM)
B1	2.286	2.325	2.335	2.338	2.327	0.16
	2.327	2.330	2.339	2.338	(0.017)	
1	2.340	2.351	2.347	2.361	2.351	0.07
	2.347	2.351	2.352	2.361	(0.007)	
2	2.340	2.347	2.351	2.344	2.347	0.08
	2.342	2.349	2.348	2.352	(0.004)	
3	2.341	2.343	2.342	2.349	2.346	0.08
	2.345	2.350	2.347	2.351	(0.004)	
4	2.340	2.339	2.337	2.341	2.338	0.11
	2.335	2.334	2.330	2.347	(0.005)	
5	2.291	2.302	2.307	2.317	2.303	0.22
	2.291	2.296	2.301	2.317	(0.010)	

* Calculation includes correction for sample dilution.

Appendix 4.2: Ammonia determination for hydrolysis experiment, run 2

(A340)

Sample	Aa	Ab	Ba	Bb	Mean (σ_{n-1})	Concentration * in reactor (Mm)
B1	1.006	1.100	1.132	1.101	1.092	21
	1.103	1.108	1.114	1.074	(0.038)	
3	2.053	2.019	2.061	2.049	2.041	60
	2.010	2.050	2.057	2.025	(0.019)	
4	1.930	1.924	1.865	1.884	1.906	82
	1.912	1.909	1.932	1.889	(0.024)	
5	1.765	1.641	1.786	1.708	1.721	110
	1.725	1.644	1.784	1.715	(0.057)	

* Calculation includes correction for dilution.

Appendix 4.2: Ammonia determination for hydrolysis experiment, run 2 (repeat determination)

(A340)

Sample	Aa	Ab	Ba	Bb	Mean (σ_{n-1})	Concentration* in reactor (mM)
B1	1.946	2.018	2.123	2.101	2.056	14.0
	2.030	2.043	2.118	2.066	(0.060)	
1	1.937	1.855	1.846	1.864	1.854	27.2
	1.884	1.828	1.765	1.851	(0.049)	
2	1.702	1.658	1.718	1.810	1.731	35.2
	1.744	1.715	1.777	1.726	(0.047)	
3	1.566	1.548	1.318	1.421	1.471	52
	1.445	1.517	1.450	1.504	(0.080)	

* Calculation includes correction for dilution

Appendix 4.2: Ammonia determination for hydrolysis experiment, run 3

(A340)

Sample	Aa	Ab	Ba	Bb	Mean (σ_{n-1})	Concentration* in reactor (mM)
B1	2.270	2.326	2.320	2.315	2.312	0.3
	2.320	2.315	2.319	2.313	(0.018)	
1	2.067	2.049	2.030	2.033	2.047	1.2
	2.055	2.079	2.035	2.025	(0.019)	
2	1.779	1.755	1.683	1.631	1.701	2.3
	1.769	1.710	1.660	1.624	(0.062)	
3	1.214	1.209	1.214	1.223	1.215	3.9
	1.218	1.210	1.223	1.205	(0.006)	
4	1.213	1.218	1.225	1.217		-
	1.213	1.221	1.220	1.212		
5	-	-	1.269	1.274		-
	-	-	1.262	1.271		
3+	2.103	2.139	2.149	2.125	2.132	4.4
	2.150	2.133	2.147	2.108	(0.018)	
4+	1.882	1.791	1.850	1.890	1.853	8.9
	1.860	1.809	1.869	1.874	(0.035)	
5+	-	-	1.463	1.398	1.409	16.1
	-	-	1.429	1.345	(0.050)	

* Calculation include correction for dilution

+ Repeat determinations at different dilution

Appendix 4.2: Ammonia determination for hydrolysis experiment, run 4 first determination

(A340)

Sample	Aa	Ab	Ba	Bb	Mean (σ_{n-1})	Concentration* in reactor (mM)
B1	1.860	1.894	1.946	1.911	1.913	22.0
	1.762	1.885	1.906	1.940	(0.034)	
1	1.535	1.397	1.451	1.407	1.409	54.4
	1.322	1.332	1.478	1.352	(0.075)	
2	1.797	1.734	1.647	1.680	1.706	89
	1.738	1.687	1.656	1.712	(0.049)	
3	1.728	1.687	1.786	1.675	1.737	84
	1.791	1.755	1.800	1.671	(0.054)	
4	1.655	1.782	1.683	1.735	1.720	87
	1.681	1.797	1.702	1.754	(0.056)	
5	1.583	1.585	1.732	1.475	1.572	107
	1.683	1.590	1.546	1.544	(0.081)	

* Calculation includes correction for dilution.

Appendix 4.2: Ammonia determination for hydrolysis experiment, run 4 (repeat analysis of original samples)

(A340)

Samples	Aa	Ab	Ba	Bb	Mean (σ_{n-1})	Concentration* in reactor (mM)
B1	1.856	1.949	1.938	2.004	1.936	14.4
	1.918	1.956	1.917	1.951	(0.042)	
1	1.729	1.591	1.556	1.582	1.611	50.0
	1.739	1.444	1.623	1.723	(0.114)	
2	1.327	1.089	1.107	1.411	1.312	69.6
	1.464	1.283	1.272	1.539	(0.160)	
3	1.076	1.137	1.088	1.101	1.151	80.0
	1.281	1.086	1.318	1.120	(0.094)	
4	1.772	1.990	1.684	1.876	1.865	66.4
	1.928	2.028	1.801	1.837	(0.115)	
5	1.695	1.564	1.684	1.783	1.732	84
	1.743	1.791	1.747	1.847	(0.086)	

* Calculation includes correction for dilution.

Appendix 4.2: Ammonia determination for hydrolysis experiment, run 4 (further analysis of original samples)+

(A340)

Sample	Aa	Ab	Ba	Bb	Mean (σ_{n-1})	Concentration* in reactor (mM)
B1	1.600	1.596	1.526	1.540	1.563	27
	1.552	1.643	1.509	1.536	(0.046)	
1	1.150	1.147	-	-	-	100
	1.145	-	-	-	(0.003)	
2	1.514	1.616	1.478	1.491	1.537	140
	1.576	1.556	1.550	1.513	(0.046)	
3	1.530	1.399	1.445	1.405	1.443	154
	1.456	1.447	1.524	1.334	(0.065)	
4	1.439	1.605	-	-	1.583	-
	1.559	1.729	-	-	(6.120)	
				colour cor	1.579	
5	1.453	-	1.610	-	1.515	-
	1.564	1.335	-	1.612	(0.119	
				colour cor	1.503	

* Calculation includes correction for dilution

\+ Standards were those of run 3

Appendix 4.2: Ammonia determination for hydrolysis experiment, run 4

Checking the effect of solution colour on ammonia determination

Sample	(A340)*
B1Aa	-0.003
1Aa	-0.002
2Aa	-0.002
3Aa	0.008
4Aa	0.006
5Aa	0.011

These data were applied to the correction of the fourth analysis of samples from run 4.

* Absorbance of determination dilution with water, no enzyme added to reaction mixture.

Appendix 4.2: Ammonia determination for hydrolysis experiment, run 4 (4th analysis of original samples)+

Sample	Aa	Ab	Ba	Bb	Mean (σ_{n-1}) ( )°	Concentration* in reactor (mM)
B1	1.810	1.875	1.764	1.623	1.784	22.8
	1.854	1.910	1.810	1.627	(0.107)	
					(1.787)	
1	1.960	1.779	2.054	2.034	2.006	77
	2.033	1.770	1.780	2.038	(0.037)	
					(2.008)	
2	2.134	2.277	2.223	2.124	2.185	93
	2.128	2.196	2.233	2.168	(0.056)	
3	2.132	2.122	2.226	2.270	2.192	92
	2.142	2.098	2.202	2.346	(0.085)	
					(2.189)	
4	2.220	2.275	2.215	2.295	2.237	78
	2.125	2.307	2.286	2.174	(0.065)	
					(2.231)	
5	1.956	2.141	2.287	2.328	2.203	90
	2.128	2.256	2.263	2.268	(0.122)	
					(2.192)	

+ Standards were those of run 14.

* Calculation includes correction for dilution.

0 Smoothed correction for solution colour applied

Appendix 4.2: Ammonia determination for hydrolysis experiment run 4 (repeat experiment)+

Sample	Aa	Ab	Ba	Bb	Mean (σ_{n-1})	Concentration* in reactor (mM)
B1	2.169	2.120	2.156	2.138	2.148	10
	2.188	2.113	2.139	2.159	(0.025)	
1	1.486	1.537	1.525	1.438	1.492	76
	1.457	1.557	1.491	1.448	(0.044)	
2	1.353	1.245	1.249	1.323	1.296	91
	1.310	1.252	1.230	1.406	(0.063)	
2	2.016	1.825	2.002	1.810	1.925	85
	1.980	1.836	1.951	1.979	(0.086)	
3	1.742	1.512	1.657	1.838	1.762	110
	1.717	1.864	1.875	1.792	(0.097)	
4	1.695	1.923	1.836	1.590	1.731	115
	1.837	1.839	1.563	1.569	(0.145)	
5	1.765	1.441	1.914	1.696	1.603	130
	1.296	1.212	1.881	1.620	(0.262)	

* Calculation includes correction for dilution.

+ A repeat of the hydrolysis was carried out with analysis of pH and ammonia only.

Appendix 4.2: Ammonia determination for hydrolysis experiment, run 5

(A340)

Sample	Aa	Ab	Ba	Bb	Mean (σ_{n-1})	Concentration* in reactor (mM)
B1	2.219	2.184	2.135	2.148	2.165	0
	2.182	2.145	2.163	2.145	(0.028)	
1	2.195	2.186	2.162	2.169	2.174	0
	2.185	2.175	2.163	2.157	(0.014)	
2	2.120	2.127	2.098	2.093	2.108	0.36
	2.119	2.120	2.080	2.103	(0.017)	
3	2.014	1.975	1.926	1.929	1.943	0.92
	1.975	1.972	1.805	1.950	(0.063)	
4	1.655	1.599	1.572	1.597	1.606	2.08
	1.622	1.605	-	1.591	0.026	
5	1.171	1.176	1.170	1.177	1.175	3.56
	1.178	1.179	1.165	1.183	(0.006)	

* Calculation includes correction for sample dilution.

Appendix 4.2: Ammonia determination for hydrolysis experiment, run 6

(A340)

Sample	Aa	Ab	Ba	Bb	Mean (σ_{n-1})	Concentration* in reactor (mM)
B1	1.735	1.774	1.744	1.751	1.749	5.6
	1.757	1.749	1.739	1.741	(0.014)	
1	1.642	1.655	1.662	1.650	1.650	10.8
	1.651	1.660	1.647	1.632	(0.010)	
2	1.559	1.545	1.483	1.493	1.525	14.6
	1.552	1.545	1.513	1.512	(0.029)	
3	1.423	1.407	1.358	1.383	1.392	17.0
	1.417	1.419	1.338	1.388	(0.032)	
4	1.356	1.344	1.293	1.323	1.341	18.8
	1.359	1.341	1.356	1.354	(0.023)	
5	1.824	1.828	1.208	1.262	1.257	21.8
	1.838	1.824	1.273	1.285	(0.034)	
6	1.306	1.270	1.148	1.242	1.251	21.9
	1.302	1.281	1.200	1.257	(0.054)	
7	1.273	1.247	1.176	1.205	1.232	22.2
	1.257	1.287	1.224	1.179	(0.043)	
8	1.260	1.221	1.289	1.210	1.257	21.7
	1.259	1.265	1.245	1.304	(0.032)	

* Calculation includes correction for sample dilution.

Appendix 4.2: Ammonia determination for hydrolysis experiment, run 7

(A340)

Sample	Aa	Ab	Ba	Bb	Mean (σ_{n-1})	Concentration* in reactor (mM)
B1	1.895	1.919	1.898	1.902	1.907	0.54
	1.919	1.924	1.908	1.893	(0.012)	
1	1.921	1.929	1.909	1.912	1.917	0.36
	1.927	1.925	1.905	1.904	(0.010)	
2	1.919	1.923	1.902	1.898	1.909	0.51
	1.921	1.924	1.893	1.886	(0.015)	
3	1.898	1.898	1.892	1.880	1.892	0.72
	1.902	1.906	1.887	1.876	(0.011)	
4	1.859	1.863	1.824	1.818	1.834	1.23
	1.864	1.868	1.816	1.762	(0.037)	
5	1.751	1.756	1.730	1.717	1.735	1.92
	1.742	1.744	1.728	1.710	(0.016)	

* Calculation includes correction for sample dilution.

Appendix 42: Ammonia determination for hydrolysis experiment, run 8

(A340)

Sample	Aa	Ab	Ba	Bb	Mean (σ_{n-1})	Concentration in reactor (mM)
B1	2.130	2.230	2.190	2.218	2.202	35
	2.247	2.190	2.205	2.202	(0.035)	
1	1.929	1.943	1.899	1.850	1.910	82
	1.969	1.941	1.899	1.848	(0.044)	
2	1.595	1.575	1.534	1.466	1.543	141
	1.606	1.592	1.511	1.468	(0.057)	
3	1.495	1.372	1.355	1.459	1.420	323
					(0.068)	
4	1.434	1.372	1.488	1.368	1.416	313
					(0.057)	
5	1.434	1.378	1.239	1.318	1.342	348
					(0.084)	

*Calculation includes correction for sample dilution.

Appendix 4.2: Ammonia determination for hydrolysis experiment, run 9

(A340)

Sample	Aa	Ab	Ba	Bb	Mean (σ_{n-1})	Concentration* in reactor (mM)
B1	2.207	2.240	2.243	2.237	2.237	0
	2.271	2.237	2.234	2.226	(0.018)	
1	2.221	2.212	2.201	2.192	2.206	0.12
	2.221	2.207	2.191	2.197	(0.012)	
2	2.152	2.150	2.166	2.160	2.153	0.30
	2.151	2.146	2.152	2.150	(0.006)	
3	2.076	2.085	2.084	2.066	2.076	0.56
	2.082	2.080	2.079	2.062	(0.008)	
4	1.881	1.880	1.859	1.851	1.864	1.28
	1.873	1.872	1.836	1.860	(0.016)	
5	1.500	1.455	1.469	1.476	1.472	2.59
	1.461	1.462	1.477	1.475	(0.014)	

* Calculation includes correction for sample dilution.

Appendix 4.2: Ammonia determination for hydrolysis experiment, run 10

(A349)

Sample	Aa	Ab	Ba	Bb	Mean (σ_{n-1})	Concentration* in reactor (mM)
B1	2.337	2.344	2.359	2.354	2.350	> 0
	2.355	2.350	2.352	2.345	(0.007)	
1	2.234	2.216	2.234	2.236	2.227	1.0
	2.218	2.206	2.230	2.243	(0.012)	
2	1.944	1.930	1.916	1.914	1.925	6.1
	1.937	1.916	1.931	1.911	(0.012)	
3	1.425	1.447	1.469	1.480	1.455	13.9
	1.392	1.442	1.491	1.492	(0.035)	
4	1.214	1.302	1.263	1.233	1.261	17.1
	1.220	1.345	1.280	1.230	(0.045)	
5	1.116	1.128	1.167	1.170	1.150	19.0
	1.141	1.127	1.164	1.186	(0.025)	
5+	1.708	1.699	1.734	1.737	1.714	19.1
	1.690	1.672	1.727	1.742	(0.025)	

+ Repeat determination at different dilution.

* Calculation includes correction for sample dilution.

Appendix 4.2: Ammonia determination for hydrolysis experiment, run 11

(A340)

Samples	Aa	Ab	Ba	Bb	Mean (σ_{n-1})	Concentration* in reactor (mM)
B1	2.001	2.062	2.058	2.048	2.055	7.2
	2.063	2.064	2.076	2.066	(0.023)	
1	2.039	2.054	2.079	2.052	2.062	7.2
	2.059	2.074	2.081	2.063	(0.015)	
2	1.983	2.078	2.022	2.003	2.023	7.3
	2.021	2.028	2.037	2.014	(0.028)	
3	1.901	-	1.902	1.927	1.921	8.5
	1.941	-	1.908	1.944	(0.019)	
4	1.663	1.649	1.678	1.664	1.663	12.4
	1.597	1.662	1.709	1.682	(0.032)	
5	1.084	1.158	1.121	1.172	1.143	23
	1.107	1.164	1.142	1.198	(0.037)	

* Calculation includes correction for sample dilution.

Appendix 2.2: Ammonia determination for hydrolysis experiment, run 12+

(A340)

Sample	Aa	Ab	Ba	Bb	Mean (σ_{n-1})	Concentration* in reactor (mM)
1	-	-	1.264	1.264	1.341	16.3
	1.406	1.431	-	-	(0.090)	
2	1.669	-	1.576	1.541	1.602	61
	1.631	1.576	-	1.619	(0.046)	
3	1.608	-	1.527	1.506	1.572	126
	1.568	1.581	-	1.644	(0.051)	
4	1.690	-	1.475	1.466	1.602	122
	1.526	1.846	-	1.606	(0.147)	
5	1.496	-	1.385	1.400	1.457	145
	1.634	1.449	-	1.377	(0.098)	

+ Repeat determinations due to unsatisfactory initial dilutions (standards from run 1).

* Calculation includes correction for sample dilution.

Appendix 4.2: Ammonia determination for hydrolysis experiment, run 12

(A340)

Sample	Aa	Ab	Ba	Bb	Mean (σ_{n-1})	Concentration* in reactor (mM)
B1	2.280	2.341	2.309	2.324	2.317	< 0
	2.317	2.342	2.298	2.329	(0.022)	
1	1.512	1.508	1.234	1.259	1.374	14.4
	1.474	1.505	1.240	1.259	(0.135)	
2	1.165	1.173	1.146	1.156	1.160	34.0
	1.158	1.172	1.156	1.155	(0.009)	
3	1.551	1.607	1.470	1.702	1.529	119
	1.536	1.533	1.419	1.412	(0.097)	
4	1.452	1.421	1.388	1.612	1.539	117
	1.434	1.736	1.705	1.563	(0.135)	
5	1.852	1.713	1.443	1.288	1.527	119
	1.717	1.388	1.491	1.321	(0.208)	

* Calculation includes correction for sample dilution..

Appendix 4.1: Ammonia determination for hydrolysis experiment, run 13

(A340)

Sample	Aa	Ab	Ba	Bb	Mean (σ_{n-1})	Concentration* in reactor (mM)
B1	2.232	2.276	2.220	2.392	2.287	0
	2.242	2.285	2.233	2.417	(0.076)	
1	1.812	1.839	1.771	1.756	1.788	8
	1.785	1.833	1.738	1.772	(0.036)	
2	1.156	1.187	1.104	1.142	1.135	18.6
	1.122	1.192	1.041	1.133	(0.048)	
3	1.038	1.037	1.033	1.032	1.034	< 23
	1.033	1.035	1.036	1.033	(0.002)	
4	1.609	1.601	1.515	1.555	1.578	56.5
	1.581	1.594	1.631	1.534	(0.040)	
5	1.336	1.387	1.182	1.228	1.307	78.5
	1.352	1.428	1.188	1.352	(0.094)	

* Calculation includes correction for sample dilution.

Appendix 4.2: Ammonia determination for hydrolysis experiment, run 14

(A340)

Sample	Aa	Ab	Ba	Bb	Mean (σ_{n-1})	Concentration in reactor (mM)
B1	2.350	2.415	2.410	2.560	2.444	0.3
	2.388	2.432	2.415	2.585	(0.083)	
1	1.992	1.946	2.004	2.018	1.983	8.1
	1.968	1.920	2.008	2.008	(0.035)	
2	1.403	1.338	1.363	1.345	1.351	18.8
	1.339	1.332	1.326	1.360	(0.025)	
3	2.083	2.129	2.028	2.012	2.077	32.5
	2.173	2.105	2.050	2.034	(0.056)	
4	1.906	1.946	1.865	1.756	1.873	49.5
	1.880	1.892	1.855	1.883	(0.055)	
5	1.616	1.585	1.395	1.385	1.516	80
	1.567	1.575	1.475	1.533	(0.088)	

* Calculation includes correction for sample dilution.

Appendix 4.2: Ammonia determination for hydrolysis experiment, run 15

(A240)

Sample	Aa	Ab	Ba	Bb	Mean ($\bar{6}_n$ - 1)	Concentration* in reactor (mM)
B1	2.345	2.305	2.215	2.255	2.283	1.0
	2.346	2.275	2.276	2.250	(0.046)	
1	2.067	2.062	2.072	2.085	2.076	5.0
	2.075	2.068	2.085	2.097	(0.012)	
2	1.879	1.808	1.782	1.785	1.812	9.4
	1.868	1.787	1.795	1.789	(0.039)	
3	1.332	1.180	1.248	1.380	1.292	18.2
	1.329	1.146	1.270	1.452	(0.102)	
4	2.023	2.112	2.077	2.088	2.076	50
	2.012	2.107	2.096	2.089	(0.038)	
5	2.000	1.943	1.996	1.962	1.965	68
	1.970	1.932	1.990	1.928	(0.029)	

* Calculation includes correction for sample dilution.

Appendix 4.2: N-terminal amino groups determination standards (TNBS) hydrolysis experiment, run 1

(A340)

Label	a	b	Mean	Concentration L-glu (mM)
0	0.516	0.521	0.519	-
25	0.585	0.583	0.554	0.25
50	0.642	0.640	0.641	0.5
100	0.760	0.762	0.761	1.0
150	0.886	0.888	0.887	1.5
250	1.075	1.133	1.104	2.5

(Absorbance generally low but data appears satisfactory)

Appendix 4.2: N-terminal amino groups determination standards (TNBS) for hydrolysis experiment, run 2

(A340)

Label	a	b	Mean	Concentration L-glu (mM)
0	0.537	0.544	0.541	-
25	0.607	0.614	0.611	0.25
50	0.679	0.675	0.677	0.50
100	0.801	0.828	0.815	1.00
150	0.926	0.990	0.958	1.50
250	1.186	1.320	1.253	2.5

Appendix 4.2: N-terminal amino groups determination standards (TNBS) for hydrolysis experiment, run 3

(A340)

Label	a	b	Mean	Concentration L-glu (mM)
0	0.511	0.530		0
25	0.596	0.599		0.25
50	0.663	0.672		0.5
100	0.816	0.826		1.0
150	0.942	1.018		1.5
250	1.364	1.395		2.5
50	0.654	0.667		0.5
100	0.768	0.814		1.0
150	0.937	0.932		1.5

Appendix 4.2: N-terminal amino groups determination standards (TNBS) for hydrolysis experiment, run 4

(A340)

Label	Absorbance		Mean	Concentration L-glu (mM)
0	0.513	0.514	0.516	-
25	0.608	0.586	0.597	0.25
75	0.811	0.768	0.790	0.75
125	1.012	0.957	0.985	1.25
175	1.244	1.166	1.205	1.75
250	1.513	1.450	1.482	2.5
0	1.513	0.529	0.523	-
25	0.606	0.610	0.608	0.25
75	0.804	0.836	0.820	0.75
125	1.001	1.013	1.007	1.25
175	1.202	1.228	1.215	1.75
250	1.471	1.463	1.467	2.5

Appendix 4.2: N-terminal animo groups determination standards (TNBS) for hydrolysis experiment, run 4

(A340)

Label	Asorbance	Mean	Concentration L-glu (mM)
0	0.519 0.520	0.520	nil
20	0.596 0.586	0.591	0.2
50	0.719 0.700	0.710	0.5
100	0.922 0.893	0.908	1.0
150	1.158 1.086	1.122	1.5
250	1.555 1.485	1.520	2.5

Appendix 4.2: N-terminal amino groups determination standards (TNBS) for hydrolysis experiment, run 5

(A340)

Label	a	b	Mean	Concentration L-glu (mM)
0	0.528	0.530	0.529	-
20	0.597	0.603	0.600	0.2
50	0.700	0.698	0.699	0.5
100	0.885	0.868	0.877	1.0
150	1.071	1.070	1.071	1.5
250	1.403	1.421	1.412	2.5

Appendix 4.2: N-terminal amino groups determination standards (TNBS for hydrolysis experiment, run 6

(A340)

Label	Absorbance	Mean	Concentration L-glu (mM)
0	0.542 0.519	0.531	nil
25	0.638 0.615	0.627	0.25
75	0.841 0.790	0.815	0.75
125	1.059 0.981	1.020	1.25
175	1.270 1.173	1.222	1.75
250	1.584 1.464	1.524	2.5

Appendix 4.2: N-terminal amino groups determination standards (TNBS) for hydrolysis experiment, run 6*

(A340)

Label	Absorbance	Mean	Concentration L-glu (mM)
0	0.546 0.542	0.544	nil
25	0.637 0.621	0.629	0.25
75	0.845 0.811	0.828	0.75
125	1.075 1.004	1.040	1.25
175	1.304 1.207	1.256	1.75
250	1.588 1.529	1.559	2.5

* Repeat determination

Appendix 4.2: N-terminal animo groups determination standards (TNBS for hydrolysis experiment, run 7

(A340)

Sample Lable	Absorbance	Mean	Concentration L-glu (mM)
0	0.546 0.544	0.545	-
20	0.590 0.593	0.592	0.2
50	0.670 0.667	0.669	0.5
100	0.821 0.808	0.815	1.0
150	0.930 0.952	0.941	1.5
250	1.247 1.226	1.237	2.5

Appendix 4.2: N-terminal amino groups determination standards (TNBS) for hydrolysis experiment, run 8

(A340)

Label	a	b	Mean	Concentration L-glu (mM)
0	0.530	0.518	0.524	0
20	0.593	0.580	0.587	0.2
50	0.695	0.677	0.686	0.5
100	0.882	0.859	0.871	1.0
150	1.079	1.037	1.058	1.5
250	1.415	1.382	1.399	2.5

Appendix 4.2: N-terminal amino groups determination standards (TNBS) for hydrolysis experiment, run 9

(A340)

Label	a	b	Mean	Concentration L-glu (mM)
0	0.509	0.529	0.519	-
25	0.636	0.631	0.634	0.25
50	0.724	0.728	0.726	0.5
100	0.946	0.951	0.949	1.0
150	1.152	1.145	1.149	1.5
250	1.586	1.588	1.587	2.5

Appendix 4.2: N-terminal amino groups determination standards (TNBS) for hydrolysis experiment, run 10

(A340)

Label	a	b	Mean	Concentration L-glu (mM)
0	0.540	0.545	0.543	-
25	0.636	0.639	0.638	0.25
50	0.725	0.741	0.733	0.5
100	0.868	0.932	0.900	1.0
150	1.130	1.110	1.120	1.5
250	1.560	1.523	1.547	2.5

Appendix 4.2: N-terminal amino groups determination standards (TNBS) for hydrolysis experiment, run 11

(A340)

Label	Absorbance	Mean	Concentration L-glu (mM)
0	0.514 0.502	0.508	0
20	0.609 0.557	0.583	0.20
50	0.710 0.682	0.696	0.50
100	0.931 0.876	0.904	1.00
150	1.116 1.090	1.103	1.5
250	1.563 1.463	1.513	2.5

Appendix 4.2: N-terminal amino groups determination standards (TNBS) for hydrolysis experiment, run 12

(A340)

Label	a	b	Mean	Concentration L-glu (mM)
0	0.509	0.520	0.515	0
25	0.610	0.611	0.611	0.25
50	0.709	0.700	0.705	0.5
100	0.898	0.884	0.891	1.0
150	1.109	1.120	1.115	1.5
250	1.510	1.499	1.505	2.5

Appendix 4.2: N-terminal amino groups determination standards (TNBS for hydrolysis experiment, run 13

(A340)

Label	a	b	Mean	Concentration L-glu (mM)
0	0.544	0.545	0.545	-
25	0.622	0.614	0.618	0.25
50	0.701	0.683	0.692	0.50
100	0.873	0.865	0.869	1.00
150	1.036	1.047	1.042	1.50
250	1.334	1.340	1.337	2.5

Appendix 4.2: N-terminal amino groups determination standards (TNBS for hydrolysis experiment, run 14

(A340)

Label	a	b	Concentration L-glu (mM)
0	0.527	0.531	0
25	0.620	0.611	0.25
50	0.687	0.695	0.50
100	0.869	0.920*	1.0
150	1.066	0.968	1.5
250	1.387	1.375	2.5

* Preparation error - see notebook, p 205

Appendix 4.2: N-terminal amino groups determination standards (TNBS) for hydrolysis experiment, run 15

(A340)

Label	a	b	Mean	Concentration L-glu (mM)
0	0.524	0.530	0.527	-
25	0.618	0.612	0.615	0.25
50	0.717	0.717	0.717	0.5
100	0.915	0.899	0.907	1.00
150	1.120	1.106	1.113	1.5
250	1.509	1.482	1.496	2.5

Appendix 4.2: N-terminal amino groups determination (TNBS) for hydrolysis experiment, run 1

(A340)

Sample	Aa	Ab	Ba	Bb	Mean (σ_{n-1})
B1	0.594	0.616	0.596	0.631	0.616
	0.603	-	-	0.654	(0.023)
1	0.619	0.604	0.589	0.587	0.599
	0.602	-	-	0.590	(0.012)
2	0.593	0.597	0.598	0.611	0.598
	0.597	-	-	0.589	(0.007)
3	0.601	0.627	0.609	0.600	0.607
	0.606	-	-	0.601	(0.010)
4	0.644	0.631	0.608	0.611	0.623
	0.626	-	-	0.617	(0.014)
5	0.661	0.662	0.626	0.635	0.643
	0.650	-	0.635	0.631	(0.015)

Appendix 4.2: N-terminal amino groups determination (TNBS) for hydrolysis experiment, run 2

(A340)

Sample	Aa	Ab	Ba	Bb	Mean (σ_{n-1})
1	0.648	0.642	0.632	0.650	0.638
	0.646	0.630	0.631	0.628	(0.009)
2	0.688	0.703	0.724	0.707	0.700
	0.692	0.693	0.711	0.686	(0.013)
3	0.757	0.812	0.800	0.844	0.798
	0.795	0.748	0.829	0.804	(0.032)
4	0.870	0.887	0.881	0.930	0.890
	0.584	0.858	0.913	0.879	(0.023)
5	1.003	1.045	0.984	1.050	1.021
	1.017	1.026	1.013	1.030	(0.022)

Appendix 4.2: N-terminal amino groups determination (TNBS) for hydrolysis experiment, run 3

(A340)

Sample	Aa	Ab	Ba	Bb	Mean (σ_{n-1})
B1	0.644	0.660	0.685	0.666	0.664
	0.654	0.653	0.676	0.678	(0.014)
1	0.730	0.728	0.819	0.716	0.753
	0.730	0.741	0.795	0.763	(0.037)
2	0.741	0.742	0.783	0.786	0.766
	0.752	0.744	0.802	0.779	(0.024)
3	0.740	0.767	0.790	0.787	0.782
	0.780	0.770	0.835	0.784	(0.027)
4	0.858	0.860	0.863	0.904	0.874
	0.884	0.870	0.863	0.887	(0.016)
5	-	-	0.940	0.958	0.951
	-	-	0.849	0.959	(0.009)

Appendix 4.2: N-terminal amino groups determination (TNBS) for hydrolysis experiment, run 4

(A340)

Sample	Aa	Ab	Ba	Bb	Mean (σ_{n-1})
1	0.720	0.735	0.719	0.714	0.725
	0.747	0.737	0.714	0.714	(0.013)
2	0.888	0.899	0.923	0.931	0.913
	0.913	0.901	0.920	0.928	(0.015)
3	1.249	1.263	1.326	1.337	1.293
	1.270	1.242	1.324	1.336	(0.041)
4	1.242	1.269	1.226	1.234	1.239
	1.247	1.229	1.241	1.220	(0.015)
5	1.524	1.522	1.531	1.520	1.525
	1.530	1.537	1.525	1.513	(0.007)

Appendix 4.2: N-terminal amino groups determination (TNBS) for hydrolysis experiment, run 4

(A340)

Sample	Aa	Ab	Ba	Bb	Mean
1	0.685	0.690	0.664	0.646	0.676
	0.705	0.699	0.671	0.649	
2	0.851	0.846	0.849	0.851	0.850
	0.866	0.843	0.837	0.856	
3	1.156	1.146	1.180	1.211	1.174
	1.163	1.147	1.192	1.197	
4	1.704	1.740	1.716	1.717	1.720
	1.724	1.729	1.711	1.722	
5	2.197	2.210	2.266	2.163	2.214
	2.240	2.225	2.243	2.168	
4*	0.969	0.919	0.925	0.847	0.912
	0.980	0.937	0.906	0.816	(0.912)
5*	0.981	0.930	0.946	0.927	0.961
	0.985	0.973	0.969	0.976	

* Repeat determinations at different dilution

Appendix 4.2: N-terminal amino groups determination (TNBS) for hydrolysis experiment, run 5

(A340)

Sample	Aa	Ab	Ba	Bb	Mean (σ_{n-1})
B1	0.555	0.554	0.557	0.561	0.558
	0.552	0.558	0.557	0.571	(0.006)
1	0.566	0.576	0.575	0.576	0.573
	0.566	0.566	0.579	0.579	(0.006)
2	0.560	0.576	0.580	0.583	0.577
	0.575	0.582	0.578	0.585	(0.008)
3	0.562	0.585	0.585	0.591	0.584
	0.582	0.590	0.591	0.588	(0.010)
4	0.590	0.606	0.607	0.606	0.605
	0.605	0.606	0.609	0.607	(0.006)
5	0.628	0.632	0.634	0.636	0.634
	0.631	0.632	0.635	0.642	(0.004)

Appendix 4.2: N-terminal amino groups determination (TNBS) for hydrolysis experiment, run *6

(A340)

Sample	Aa	Ab	Ba	Bb	Mean (σ_{n-1})
1	0.524	0.546	0.526	0.525	0.531
	0.528	0.546	0.526	0.529	(0.009)
2	0.539	0.547	0.549	0.547	0.543
	0.544	0.530	0.550	0.536	(0.007)
3	0.556	0.576	0.574	0.561	0.567
	0.569	0.575	0.564	0.561	(0.008)
4	0.574	0.586	0.599	0.584	0.586
	0.589	0.584	0.591	0.580	(0.007)
5	-	-	0.658	0.648	0.649
	-	-	0.653	0.638	(0.009)
6	0.727	0.716	0.720	0.722	0.724
	0.726	0.735	0.728	0.714	(0.007)
7	0.827	0.791	0.825	0.833	0.724
	0.845	0.812	0.818	0.797	(0.018)
8	0.870	0.910	0.878	0.875	0.885
	0.908	0.893	0.898	0.839	(0.023)

* First determination, repeated when ammonia connected data gav results less than blank.

Appendix 4.2: N-terminal amino groups determination (TNBS) for hydrolysis experiment, run *6

(A340)

Sample	Aa	Ab	Ba	Bb	Mean (σ_{n-1})
1	0.524	0.534	0.549	0.557	0.540
	0.533	0.529	0.546	0.551	(0.012)
2	0.557	0.556	0.566	0.569	0.563
	0.568	0.551	0.569	0.570	(0.007)
3	0.570	0.591	0.607	0.604	0.594
	0.578	0.583	0.606	0.612	(0.016)
4	0.620	0.597	0.648	0.634	0.623
	0.614	0.597	0.636	0.635	(0.019)
5	-		0.689	0.742	0.703
	-		0.680	0.701	(0.027)

* Repeat determination carrued out when ammonia corrected data g results less than blank.

Appendix 4.2: N-terminal amino groups determination (TNBS) for hydrolysis experiment, run 7

(A340)

Sample	Aa	Ab	Ba	Bb	Mean (σ_{n-1})
B1	0.738	0.704	0.756	0.740	0.731
	0.749	0.702	0.735	0.726	(0.020)
1	0.699	0.711	0.694	0.690	0.698
	0.703	0.707	0.694	0.682	(0.009)
2	0.667	0.696	0.692	0.724	0.692
	0.671	0.696	0.689	0.703	(0.018)
3	0.679	0.737	0.670	0.715	0.701
	0.686	0.748	0.675	0.701	(0.029)
4	0.763	0.750	0.738	0.730	0.744
	0.759	0.751	0.745	0.716	(0.016)
5	0.680	0.756	0.756	0.745	0.731
	0.686	0.748	0.751	0.733	(0.031)

Appendix 4.2: N-terminal amino groups determination (TNBS) for hydrolysis experiment, run 8

(A340)

Sample	Aa	Ab	Ba	Bb	Mean (σ_{n-1})
B1	0.538	0.542	0.538	0.537	0.540
	0.547	0.545	0.534	0.539	(0.004)
1	0.588	0.582	0.577	0.580	0.582
	0.587	0.593	0.569	0.579	(0.007)
2	0.619	0.627	0.619	0.630	0.639
	0.637	0.652	0.699	0.631	(0.026)
3	0.691	0.671	0.685	0.694	0.686
	0.690	0.673	0.689	0.692	(0.009)
4	0.780	–	0.782	0.809	0.794
	0.791	–	0.792	0.811	(0.013)
5	0.959	0.930	0.930	0.925	0.939
	0.970	0.930	0.937	0.934	(0.016)

Appendix 4.2: N-terminal amino groups determination (TNBS) for hydrolysis experiment, run 9

(A340)

Sample	Aa	Ab	Ba	Bb	Mean (σ_{n-1})
B1	0.526	0.527	0.529	0.536	0.530
	0.530	0.527	0.532	0.536	(0.004)
1	0.536	0.542	0.531	0.532	0.537
	0.537	0.550	0.531	0.536	(0.006)
2	0.541	0.539	0.543	0.543	0.541
	0.537	0.541	0.545	0.538	(0.003)
3	0.543	0.542	0.544	0.540	0.542
	0.541	0.543	0.543	0.537	(0.002)
4	0.550	0.552	0.556	0.553	0.554
	0.555	0.555	0.555	0.552	(0.002)
5	-	0.584	0.591	0.592	0.588
	0.585	0.586	0.591	0.588	(0.003)

Appendix 4.2: N-terminal amino groups determination (TNBS) for hydrolysis experiment, run 10

(A340)

Sample	Aa	Ab	Ba	Bb	Mean (σ_{n-1})
B1	0.531	0.540	0.537	0.537	0.535
	0.532	0.532	0.536	0.532	(0.003)
1	0.555	0.567	0.567	0.560	0.563
	0.562	0.565	0.563	0.563	(0.004)
2	0.607	0.621	0.621	0.620	0.617
	0.612	0.614	0.620	0.622	(0.006)
3	0.694	0.715	0.721	0.732	0.716
	0.708	0.715	0.721	0.721	(0.011)
4	0.785	0.806	0.795	0.790	0.797
	0.793	0.810	0.788	0.808	(0.010)
5	0.930	0.969	0.976	0.945	0.960
	0.936	0.976	0.986	0.959	(0.021)

Appendix 4.2: N-terminal amino groups determination (TNBS) fo hydrolysis experiment, run 11

(A340)

Sample	Aa	Ab	Ba	Bb	Mean ($\sigma_n - 1$)
B1	0.568	0.592	0.565	0.550	0.568
	0.584	0.583	0.553	0.550	(0.017)
1	0.615	0.630	0.585	0.576	0.599
	0.605	0.627	0.580	0.570	(0.024)
2	0.603	0.617	0.574	0.553	0.587
	0.607	0.627	0.564	0.552	(0.030)
3	0.660	0.659	0.622	0.592	0.630
	0.657	0.645	0.616	0.585	(0.030)
4	0.668	0.670	0.620	0.604	0.636
	0.658	0.662	0.606	0.597	(0.032)
5	0.694	0.710	0.651	0.647	0.678
	0.707	0.720	0.660	0.637	(0.033)

Appendix 4.2: N-terminal amino groups determination (TNBS) for hydrolysis experiment, run 12

(A340)

Sample	Aa	Ab	Ba	Bb	Mean (σn - 1)
B1	0.644	0.652	0.647	0.651	0.650
	0.655	0.657	0.651	0.603	(0.005)
1	0.780	0.781	0.818	0.845	0.811
	0.781	0.794	0.835	0.854	(0.031)
2	1.202	1.248	1.270	1.268	1.252
	1.230	1.252	1.294	1.249	(0.028)
3	0.852	0.870	0.862	0.860	0.858
	0.851	0.848	0.857	0.866	(0.008)
4	0.895	0.918	0.934	0.921	0.924
	0.930	0.923	0.953	0.917	(0.017)
5	0.904	0.960	0.948	0.940	0.945
	0.934	0.966	0.961	0.945	(0.020)
3*	1.740	1.784	1.742	1.767	1.762
	1.738	1.797	1.761	1.770	(0.022)

* Repeat determination at different dilution.

Appendix 4.2: N-terminal amino groups determination (TNBS) for hydrolysis experiment, run 13

(A340)

Sample	Aa	Ab	Ba	Bb	Mean ($\sigma n - 1$)
B1	0.626	0.702	0.622	0.650	0.651
	0.647	0.658	0.650	0.655	(0.024)
1	0.702	0.871	0.716	0.713	0.732
	0.706	0.713	0.720	0.711	(0.057)
2	0.799	0.821	0.824	0.813	0.816
	0.810	0.822	0.828	0.807	(0.010)
3	0.965	0.990	0.968	0.958	0.977
	0.979	0.996	0.982	0.981	(0.013)
4	0.857	0.887	0.900	0.877	0.881
	0.865	0.889	0.901	0.874	(0.016)
5	0.981	0.997	0.994	0.981	0.993
	1.002	1.007	0.996	0.984	(0.010)

Appendix 4.2: N-terminal amino groups determination (TNBS) fo hydrolysis experiment, run 14

(A340)

Sample	Aa	Ab	Ba	Bb	Mean (σ_{n-1})
B1	0.602	0.647	0.606	0.621	0.619
	0.613	0.637	0.604	0.622	(0.016)
1	0.671	0.690	0.688	0.679	0.684
	0.678	0.697	0.694	0.672	(0.010)
2	0.753	0.754	0.788	0.766	0.770
	0.771	0.769	0.794	0.761	(0.015)
3	0.843	0.861	0.924	0.880	0.877
	0.858	0.856	0.927	0.866	(0.032)
4	0.795	0.812	0.804	0.808	0.808
	0.810	0.814	0.818	0.803	(0.007)
5	0.900	0.915	0.965	0.943	0.938
	0.936	0.932	0.978	0.935	(0.025)

Appendix 4.2: N-terminal amino groups determination (TNBS) for hydrolysis experiment, run 15

(A340)

Sample	Aa	Ab	Ba	Bb	Mean (σ_{n-1})
B1	0.654	0.613	0.620	0.645	0.630
	0.624	0.609	0.637	0.637	(0.016)
1	0.679	0.694	0.724	0.742	0.712
	0.683	0.697	0.734	0.740	(0.026)
2	0.732	0.779	0.772	0.791	0.771
	0.738	0.791	0.771	0.793	(0.024)
3	0.864	0.922	0.875	0.867	0.881
	0.869	0.922	0.868	0.863	(0.025)
4	0.819	0.819	0.844	0.834	0.831
	0.824	0.843	0.839	0.829	(0.010)
5	0.950	0.967	0.972	0.953	0.966
	0.969	0.976	0.969	0.973	(0.009)

Appendix 4.2: N-terminal amino groups determination (TNBS), correction for the effect of ammonia, for hydrolysis experiment, run 11

Sample	Ammonia in reactor (mM)	Ammonia in sample (mM)	Measured A340	A340 correction	Corrected A340	L-glu (mM)
B1	7.2	0.29	0.568	0.035	0.533	2.0
1	7.2	0.29	0.599	0.035	0.564	4.0
2	7.3	0.29	0.587	0.035	0.552	3.25
3	8.5	0.34	0.630	0.038	0.592	5.75
4	12.4	0.50	0.636	0.052	0.584	5.25
5	23	0.92	0.678	0.090	0.588	5.5

Appendix 4.2: N-terminal amino groups determination (TNBS), correction for the effect of ammonia, for hydrolysis experiment, run 10

Sample	Ammonia in reactor (mM)	Ammonia in sample (mM)	A340 correction	Measured A340	Corrected A340	L-glu (mM)
B1	0	0	0	0.535	0.535	0.063
1	1.0	0.08	0.005	0.563	0.558	0.75
2	6.1	0.49	0.050	0.617	0.567	1.0
3	13.0	1.11	0.105	0.716	0.611	2.44
4	17.1	1.37	0.127	0.797	0.670	4.25
5	19.0	1.52	0.142	0.960	0.818	8.94

Appendix 4.2: N-terminal amino groups determination (TNBS), correction for the effect of ammonia, for hydrolysis experiment, run 9

Sample	Ammonia in reactor (mM)	Ammonia in sample (mM)	A340 correction	Measured A340	Corrected A340	L-glu (mM)
B1	0	0	0	0.530	0.530	0.05
1	0.12	0.024	0.002	0.537	0.535	0.10
2	0.30	0.060	0.005	0.541	0.537	0.115
3	0.56	0.112	0.015	0.542	0.527	0.03
4	1.28	0.256	0.030	0.554	0.524	0.02
5	2.59	0.518	0.055	0.588	0.533	0.09

Appendix 4.2: N-terminal amino groups determination (TNBS), correction for the effect of ammonia, for hydrolysis experiment, run 8

Sample	Ammonia in reactor (mM)	Ammonia in sample (mM)	Measured A340	A340 correction	Corrected A340	L-glu (mM)
B1	35	0.28	0.540	0.033	0.507	0
1	82	0.66	0.582	0.065	0.517	3.8
2	141	1.13	0.639	0.107	0.532	7.5
3	230	1.84	0.686	0.170	0.516	3.8
4	326	2.60	0.794	0.230	0.564	18.8
5	348	2.78	0.939	0.253	0.686	62.5

Appendix 4.2: N-terminal amoni groups determination (TNBS), correction for the effect of ammonia, for hydrolysis experiment, run 7

Sample	Ammonia in reactor (mM)	Ammonia in sample (mM)	A340 correction	Measured A340	Corrected A340	L-glu (mM)
B1	0.54	0.0216	.002	0.731	0.729	17
1	0.36	0.0144	.002	0.698	0.696	14
2	0.51	0.0204	.002	0.692	0.690	14
3	0.72	0.0288	.002	0.701	0.699	15
4	1.23	0.0492	0.004	0.744	0.740	19
5	1.92	0.0768	.005	0.731	0.726	17

Appendix 4.2: N-terminal amino groups determination (TNBS), correction for the effect of ammonia, for hydrolysis experiment, run 6

Sample	Measured A340	Ammonia in reactor (mM)	Ammonia in sample (mM)	A340 correction	Corrected A340	L-glu (mM)
1	0.531	10.8	0.432	0.062	0.469	less than blank
2	0.543	14.6	0.584	0.086	0.455	
3	0.567	17.0	0.68	0.102	0.465	
4	0.586	18.8	0.752	0.106	0.48	
5	0.669	21.8	0.872	0.130	0.569	3
6	0.724	21.9	0.876	0.132	0.592	4.25
7	0.819	22.2	0.888	0.133	0.686	10.25
8	0.885	21.7	0.868	0.130	0.755	14.5

Appendix 4.2: N-terminal amino groups determination (TNBS), correction for the effect of ammonia, for hydrolysis experiment, run 5

Sample	Ammonia in reactor (mM)	Ammonia in sample (mM)	A340 correction	Measured A340	Corrected A340	L-glu (mM)
B1	0	0	0	0.558	0.558	0.8
1	0	0	0	0.573	0.573	1.0
2	0.36	0.036	0.006	0.577	0.571	1.0
3	0.92	0.092	0.015	0.584	0.569	1.0
4	2.08	0.208	0.025	0.605	0.580	1.3
5	3.56	0.356	0.04	0.634	0.630	2.7

Appendix 4.2: N-terminal amino groups determination (TNBS), correction for the effect of ammonia, for hydrolysis experiment, run 4

Sample	Measured A340	Ammonia in reactor (mM)	Ammonia in sample (mM)	A340 connection	Connected A340	L-glu (mM)
1	0.725	54.5	1.088	0.103	0.622	13.5
2	0.913	89	1.78	0.165	0.748	17
3	1.293	84	1.68	0.155	1.138	77.5
4	1.239	87	0.87	0.085	1.154	159
5	1.525	107	1.07	0.102	1.423	226

Appendix 4.2: N-terminal amino groups determination (TNBSO, correction for the effect of ammonia, for hydrolysis experiment, run 3

Sample	Ammonia in reactor (mM)	Ammonia in sample (mM)	A340 correction	Measured A340	Corrected A340	L-glu (mM)
B1	0.3	0.02	-	0.664	0.664	5.8
1	1.2	0.10	0.005	0.753	0.748	8.9
2	2.3	0.18	0.02	0.766	0.746	8.9
3	4.4	0.35	0.035	0.782	0.747	8.9
4	8.9	0.71	0.07	0.874	0.804	10.9
5	16.1	1.29	0.12	0.951	0.831	12.0

Appendix 4.2: N-terminal anomi groups determination (TNBS), correction for the effect of ammonia, for hydrolysis experiment, run 2

Sample	Ammonia in reactor (mM)	Ammonia in sample (mM)	A340 correction	Measured A340	Corrected A340	L-glu (mM)
B1	14	1.12	0.106	-	-	-
1	27.2	2.18	0.200	0.638	0.438	
2	35.2	2.82	0.257	0.700	0.443	
3	56	4.48	0.400	0.798	0.398	
4	82	6.56	0.540	0.890	0.350	
5	110	8.8	0.800	1.021	0.221	

Appendix 4.2: N-terminal amino groups determination (TNBS), correction for the effect of ammonia, for hydrolysis experiment, run 1

Sample	Ammonia in reactor (mM)	Ammonia in sample (mM)	A340 correction	Measured A340	Connected A340	L-glu (mM)
B1	0.16	0.013	-	0.616	-	5
1	0.07	0.006	-	0.599	-	4
2	0.08	0.006	-	0.598	-	4
3	0.08	0.006	-	0.607	-	4.5
4	0.11	0.009	-	0.623	-	5.3
5	0.22	0.020	-	0.643	-	6.25

Sample 4.2: N-terminal amino groups determination (TNBS), correction for the effect of ammonia, for hydrolysis experiment, run 11

Label	Measured A340	Ammonia in sample (mM)	A340 correction
0	0.507	0	nil
25	0.560	0.4 mM	0.04
50	0.592	0.8 mM	0.07
100	0.648	1.6 mM	0.128
250	0.849	4 mM	0.329

Appendix 4.2: N-terminal amino groups determination (TNBS), correction for the effect of ammonia, for hydrolysis experiment, run 12

Sample	Ammonia in reactor (mM)	Ammonia in sample (mM)	A340 correction	Measured A340	Corrected A340	L-glu (mM)
B1	0	-	-	0.650	0.650	4.4
1	15	1.2	0.110	0.811	0.701	6.0
2	61	4.9	0.44	1.252	0.812	9.5
3	120	2.4	0.22	0.858	0.638	16
4	120	2.4	0.22	0.924	0.704	24
5	130	2.6	0.236	0.945	0.725	27

Appendix 4.2: N-terminal amino groups determination (TNBS), correction for the effect of ammonia, for hydrolysis experiment, run 15

Sample	Ammonia in reactor (mM)	Ammonia in sample (mM)	A340 correction	Measured A340	Corrected A340	L-glu (mM)
B1	1.0	0.08	0.003	0.630	0.627	3.4
1	5.0	0.4	0.045	0.712	0.667	4.6
2	9.4	0.752	0.075	0.771	0.696	5.6
3	18.2	1.46	0.135	0.881	0.746	7.2
4	50	2.0	0.184	0.831	0.647	8
5	68	2.72	0.250	0.966	0.716	12.5

Appendix 4.2: N-terminal amino groups determination (TNBS), correction for the effect of ammonia, for hydrolysis experiment, run 14

Sample	Ammonia in reactor (mM)	Ammonia in reactor (mM)	A340 correction	Measured A340	Corrected A340	L-glu (mM)
B1	0.3	0.024	-	0.619	0.619	3.25
1	8.1	0.648	0.065	0.654	0.619	3.25
2	18.8	1.504	0.140	0.770	0.630	3.75
3	32.5	2.6	0.236	0.877	0.641	4.25
4	49.5	1.98	0.184	0.808	0.624	7.13
5	80	3.2	0.280	0.938	0.658	9.5

Appendix 4.2: N-terminal amino groups determination (TNBS), correction for the effect of ammonia, for hydrolysis experiment, run 13

Sample	Ammonia in reactor (mM)	Ammonia in sample (mM)	A340 correction	Measured A340	Corrected A340	L-glu (mM)
B1	0	0	0	0.651	0.651	4.25
1	8	0.64	0.065	0.732	0.667	4.88
2	18.6	1.488	0.138	0.816	0.678	5.25
3	34.5*	2.76	0.250	0.977	0.727	7.13
4	56.5	2.26	0.210	0.881	0.671	10
5	78.5	3.14	0.285	0.993	0.708	12.75

* by interpolation

Appendix 4.2: N-terminal amino groups determination standards (fluorescamine) for hydrolysis experiment, run 1

Label	Peak Height (mm) a	b	Mean	Concentration L-glu (mM)
0	0.5	0	0	0
10	21	21	21	0.125
20	32	40	36	0.25
30	59	58	59	0.375
40	77	75	76	0.50
50	92	94	93	0.625
60	104	105	105	0.75

Appendix 4.2: N-terminal amino groups determination standards (fluorescamine) for hydrolysis experiment, run 2

Label	Peak Height (mm) a	b	Mean	Concentration L-glu (mM)
0	1	4	3	-
10	33	31	32	0.125
20	57	58	58	0.250
30	80	84	82	0.375
40	106	106	106	0.500
50	130	130	130	0.625
60	145	149	147	0.75

Appendix 4.2: N-terminal amino groups determination standards (fluorescamine) for hydrolysis experiment, run 3

Label	Peak Height (mm) a	b	Mean	Concentration L-glu (mM)
0	3	1	2	0
10	24	24	24	0.125
20	47	46	47	0.25
30	64	62	63	0.375
40	85	85	85	0.5
50	104	97	101	0.625
60	116	122	119	0.75

Appendix 4.2: N-terminal amino groups determination standards (fluorescamine) for hydrolysis experiment, run 4

Label	Peak Height (mm) a	b	Mean	Concentration L-glu (mM)
0	3	3	3	-
10	19	21	20	0.125
20	41	37	39	0.25
30	57	56	57	0.375
40	78	69	74	0.50
60	94	96	95	0.75

Appendix 4.2: N-terminal amino groups determination standards (fluorescamine) for hydrolysis experiment, run 5

Label	Peak Height (mm) a	b	Mean	Concentration L-glu (mM)
0	3	3	3	-
10	22	20	21	0.125
20	40	39	40	0.25
30	56	59	58	0.375
40	70	70	70	0.5
50	85	85	85	0.625
60	91	98	95	0.75

Appendix 4.2: N-terminal amino groups determination standards (fluorescamine) for hydrolysis experiment, run 6

Label	Peak Heights (mm) a/b	Mean	Concentration L-glu (mM)
0	3 3	3	nil
10	21 19	20	0.125
20	36 -	36	0.25
30	50 48	49	0.375
40	65 68	67	0.50
50	82 77	80	0.675
60	93 90	92	0.75

Appendix 4.2: N-terminal amino groups determination standards (fluorescamine) for hydrolysis experiment, run 7

Label	Peak Height (mm) a/b	Mean	Concentration L-glu (mm)
0	1 2	2	-
10	19 19	19	0.125
20	34 34	34	0.25
30	51 52	52	0.375
40	64 61	63	0.500
50	76 79	78	0.625
60	95 92	94	0.75

Appendix 4.2: N-terminal amino groups determination standards (fluorescamine) for hydrolysis experiment, run 8

Label	Peak Height (mm) a/b	Mean	Concentration L-glu (mm)
0	2 2	2	nil
10	20 19	20	0.125
20	35 37	36	0.25
30	52 52	52	0.375
40	64 66	65	0.500
50	79 80	80	0.625
60	94 93	94	0.75

Appendix 4.2: N-terminal amino groups determination standards (fluorescamine) for hydrolysis experiment, run 9

Label	Peak Height (mm) a	b	Mean	Concentration L-glu (mM)
0	1	0.5	1	-
10	22	22	22	0.125
20	42	43	43	0.25
30	57	59	58	0.375
40	81	76	79	0.5
50	97	93	95	0.625
60	106	103	105	0.75

Appendix 4.2: N-terminal amino groups determination standards (fluorescamine) for hydrolysis experiment, run 10

Label	Peak Height (mm) a	b	Mean	Concentration L-glu (mM)
0	4	3	3	0
10	18	20	19	0.125
20	39	35	37	0.25
30	53	53	53	0.375
40	71	66	69	0.50
50	87	83	85	0.625
60	101	90	96	0.75

Appendix 4.2: N-terminal amino groups determination standards (fluorescamine) for hydrolysis experiment, run 11

Label	Peak Height (mm) a/b	Mean	Concentration L-glu (mM)
0	4 5	5	-
10	22 22	22	0.125
20	39 39	39	0.25
30	52 55	54	0.375
40	68 70	69	0.50
50	82 80	81	0.625
60	93 90	92	0.75

Appendix 4.2: N-terminal amino groups determination standards (fluorescamine) for hydrolysis experiment, run 12

Label	Peak Height (mm) a	b	Mean	Concentration L-glu (mM)
0	1	-	1	
10	29	28	29	0.125
20	48	53	51	0.25
30	74	77	76	0.375
40	97	94	96	0.50
50	115	115	115	0.625
60	123	132	128	0.75

Appendix 4.2: N-terminal amino groups determination standards (fluorescamine) for hydrolysis experiment, run 13

Label	Peak Height (mm) a	b	Mean	Concentration L-glu (mM)
0	2	2	2	-
10	30	32	31	0.125
20	57	57	57	0.25
30	83	79	81	0.375
40	98	103	101	0.50
50	130	128	129	0.625
60	149	138	144	0.75

Appendix 4.2: N-terminal amino groups determination standards (fluorescamine) for hydrolysis experiment, run 14

Label	Peak Height (mm) a	b	Concentration L-glu (mM)
0	1	0.5	-
10	29	22	0.125
20	42	46	0.250
30	61	62	0.375
40	86	82	0.50
50	95	100	0.625
60	118	119	0.75

Appendix 4.2: N-terminal amino groups determination standards (fluorescamine) for hydrolysis experiment, run 15

Label	Peak Height (mm) a	b	Mean	Concentration L-glu (mM)
0	6	4	5	-
10	34	34	34	0.125
20	64	64	64	0.25
30	91	91	91	0.375
40	116	119	118	0.50
50	139	150		0.62
60	123	166		0.75

Appendix 4.2: N-terminal amino groups determination (fluorescamine) for hydrolysis experiment, run 1

Sample	Peak Height (mm) Aa	Ab	Ba	Bb	Mean (σ_{n-1})	Concentration* L-glu in reactor (mM)
B1	41	51	47	54	49	13.0
	43	53	48	54	(5)	
1	46	45	42	40	44	11.7
	47	46	43	42	(2)	
2	44	41	45	40	43	11.4
	44	43	46	39	(3)	
3	43	49	46	45	46	12.2
	46	49	45	44	(2)	
4	53	60	50	50	54	14.4
	58	60	48	51	(5)	
5	70	71	61	59	66	17.6
	71	71	63	61	(5)	

* Calculation includes correction for sample dilution.

Appendix 4.2: N-terminal amino groups determination (fluorescamine) for hydrolysis experiment, run 2

Sample	Peak Heights (mm) Aa	Ab	Ba	Bb	Mean (σ_{n-1})	Concentration* L-glu in reactor (mM)
1	22	22	14	19	19.5	7
	22	23	17	17	(3.25)	
2	113	112	103	101	106	49.5
	112	111	101	94	(7.1)	
3	119	80	92	98	97	45
	120	80	90	93	(15.5)	
4	60	73	67	71	68	30.5
	62	72	66	67	(4.7)	

* Calculation includes correction for sample dilution.

Appendix 4.2: N-terminal amino groups determination (fluorescamine for hydrolysis experiment, run 3

Sample	Peak Height (mm) Aa	Ab	Ba	Bb	Mean (σ_{n-1})	Concentration L-glu in reactor (mM)
B1	37	55	39	38	43	27
	37	59	41	40	(9)	
1	55	57	48	71	58	32
	55	58	48	73	(9)	
2	62	66	63	65	64	35
	63	65	63	67	(2)	
3	115	46	52	55	67	37
	116	45	52	55	(30)	
4	79	54	64	73	68	38
	79	56	65	73	(10)	
5	-	-	77	72	76	43
			81	72	(4)	

* Calculation includes correction for sample dilution.

Appendix 4.2: N-terminal amino groups determination (fluorescamine) for hydrolysis experiment, run 4

Sample	Peak Height (mm) Aa	Ab	Ba	Bb	Mean	Concentration*
1	5	9	12	6	8	36
	6	9	11	6		
2	16	11	15	14	14	78
	16	12	14	13		
3	24	21	22	31	24	147
	24	20	23	29		
4	43	39	43	50	44	289
	45	40	42	48		
5	67	76	57	43	61	406
	67	79	56	44		

* Calculation includes correction for sample dilution.

Appendix 4.2: N-terminal amino groups determination (fluorescamine) for hydrolysis experiment, run 5

Sample	Peak Height (mm) Aa	Ab	Ba	Bb	Mean (σ_{n-1})	Concentration* L-glu in reactor (mM)
B1	47	47		50	49	
	50	48		52	(20)	3.2
1	48	51	53	50	51	
	51	51	52	49	(1.6)	3.3
2	53	51	5[illegible]	55	55	
	56	57	5[illegible]	55	(1.8)	3.6
3	53	55	55	57	55	
	54	56	54	56	(1.3)	3.6
4	59	60	57	59	59	
	62	60	59	59	(1.4)	3.9
5	73	63	66	59	65	4.4
	64	70	62	63	(4.5)	

* Calculation includes correction for sample dilution.

Appendix 4.2: N-terminal amino groups determination (fluorescamine) for hydrolysis experiment, run 6

Sample	Peak Height (mm) Aa	Ab	Ba	Bb	Mean	Concentration* L-glu in reactor (mM)
1	5	8	7	7	7	3.4
	8	7	7	7		
2	8	9	8	8	8.5	4.5
	9	9	9	8		
3	13	15	16	15	15	9.8
	14	15	16	15		
4	24	23	25	24	24.5	17.5
	26	24	25	25		
5			44	44	44	33.4
			43	44		
6	69	69	67	67	68	53.5
	69	69	66	68		
7	89	87	88	87	88	69.2
	89	87	86	91		
8	98	94	95	99	96	75.8
	98	94	95	97		

* Calculation includes correction for sample dilution

Appendix 4.2: N-terminal amino groups determination (fluorescamine) for hydrolysis experiment, run 7

Sample	Peak Height (mm) Ba	Bb	Aa	Ab	Mean (σ_{n-1})	Concentration* in reactor L-glu (mM)
B1	64	63	63	56	61	48
	64	62	65	54	(4)	
1	40	41	56	54	48	37
	40	42	57	54	(8)	
2	51	50	48	51	50	39
	52	50	48	52	(2)	
3	53	49	53	51	51	40
	46	49	54	51	(3)	
4	55	59	54	55	56	44
	55	59	54	55	(2)	
5	53	53	60	49	53	41
	52	53	54	49	(3)	

* Calculation includes correction for sample dilution.

Appendix 4,2: N-terminal amino groups determination (fluorescamine) for hydrolysis experiment, run 8

Sample	Peak Height (mm) Aa	Ab	Ba	Bb	Mean (σ_{n-1})	Concentration in reactor L-glu (mM)
B1	13		5	4	6.7	20
	11		4	3	(4.2)	
1	8	10	5	5	7	24
	8	10	5	5	(2.3)	
2	9	15	8	15	11.6	60
	8	15	8	15	(3.6)	
3	15	18	16	20	17	104
	15	17	6	19	(1.9)	
4	32	29	36	46	36	260
	32	30	37	45	(6.5)	
5	63	64	71	62	65	496
	64	65	69	62	(3.3)	

* Calculation includes correction for sample dilution.

Appendix 4.2: N-terminal amino groups determination (fluorescamine for hydrolysis experiment, run 9

Sample	Peak Height (mm) Aa	Ab	Ba	Bb	Mean (σ_{n-1})	Concentration* in reactor L-glu (mM)
B1	6	7	6	7	6.75	0.7
	7	7	7	7	(0.46)	
1	11	12	11	6	10.13	1.08
	11	11	12	7	(2.30)	
2	12	15	13	11	12.63	1.36
	12	1.	13	11	(1.41)	
3	18	15	15	16	16.13	1.78
	18	15	15	17	(1.36)	
4	17	18	13	13	15.25	1.68
	17	18	13	13	(2.43)	
5	17	16	15	20	16.88	1.88
	16	16	15	20	(2.03)	

* Calculation includes correction for sample dilution.

Appendix 4.2: N-terminal amino groups determination (fluorescamine) for hydrolysis experiment, run 10

Sample	Peak Height (mm) Aa	Ab	Ba	Bb	Mean (σ_{n-1})	Concentration* L-glu in reactor (mM)
B1	6	4	4	3	3.9	0.7
	4	4	3	3	(1.0)	(low sample dough ball)
1	9	6	6	6	6.5	2.6
	7	6	6	6	(1.1)	
2	13	9		9	9.7	5.0
	7	10		9	(1.5)	
3	18	17		18	17.6	11.0
	18	17		18	(0.5)	
4	28	29		29	28.5	19.3
	28	29		30	(0.9)	
5	48	52		51	51.6	37.0
	50	53		54	(1.9)	

* Calculation includes correction for sample dilution.

Appendix 4.2: N-terminal amino groups determination (fluorescamine) for hydrolysis experiment, run 11

Sample	Peak Height (mm) Aa	Ab	Ba	Bb	Mean (σ_{n-1})	Concentration L-glu in reactor (mM)
B1	17	13	19	11	14.6	57
	15	13	17	12	(2.8)	
1	12	10	16	14	13.0	47
	12	10	16	13	(2.4)	
2	9	10	10	13	10.3	30
	9	10	9	12	(1.5)	
3	9	10	10	8	9.1	24
	9	9	10	8	(0.8)	
4	9	9	9	8	8.4	20
	9	8	8	7	(0.7)	
5	9	8	7	8	7.9	16
	9	8	7	7	(0.8)	

* Calculation includes correction for sample dilution.

Appendix 4.2: N-terminal amino groups determination (fluorescamine) for hydrolysis experiment, run 12

Sample	Peak Height (mm) Aa	Ab	Ba	Bb	Mean (σ_{n-1})	Concentration* L-glu in reactor (mM)
B1	52	58	42	47	51	23.5
	56	60	42	49	(7)	
1	56	62	93	120	60	28.3
	57	65	91	125	(4)	
			2 x flmn suspected			
2	82	84	88	104	90	46
	86	86	87	106	(9)	
3+	130	134	129	119	128	75
	134	134	125	120	(6)	
3+	41	37	35	31	36	64
	38	39	35	34	(3)	
4	40	42	44	46	44	80
	42	40	46	48	(3)	
5	46	52	48	47	49	90
	47	54	48	48	(3)	

+ Determination at different dilution.

+ Calculation includes correction for sample dilution.

Appendix 4.2: N-terminal amino groups determination (fluorescamine) for hydrolysis experiment, run 13

Label	Peak Height (mm) Aa	Ab	Ba	Bb	Mean (σ_{n-1})	Concentration* L-glu in reactor (mM)
B1	36	36	38	34	36	16.5
	37	35	37	34	(1.5)	
1	33	35	65	56	47	21.0
	32	35	66	57	(15)	
2	54	45	48	55	45	20.3
	56	43	50	56	(16)	
3	85	110	89	60	86	40.4
	83	110	90	61	(19)	
4	65	72	77	73	72	33.5
	68	72	75	73	(3.8)	
5	97	95	87	88	92	43.3
	101	93	86	90	(5.3)	

* Calculation includes correction for sample dilution.

Appendix 4.2: N-terminal amino groups determination (fluorescamine) for hydrolysis experiment, run 14

Sample	Peak Heights (mm) Aa	Ab	Ba	Bb	Mean (σ_{n-1})	Concentration* L-gin in reaction (mm)
B1	23	29	19	23	24	14.3
	23	30	18	23	(4)	
1	24	25	27	24	26	15.5
	23	27	30	26	(2)	
2	35	29	34	29	32	19.3
	31	30	34	30	(2)	
3	32	32	40	28	34	20.5
	34	33	43	28	(5)	
4	45	43	33	35	39	23.7
	45	43	33	32	(6)	
5	55	57	66	64	62	38.2
	58	61	70	66	(5)	

* Calculation includes correction for sample dilution.

Appendix 4.2: N-terminal amino groups determination (flourescamine) for hydrolysis experiment, run 15

Sample	Peak Height (mm) Aa	Ab	Ba	Bb	Mean (σ_{n-1})	Concentration* L-glu in reactor (mM)
B1	54	60	64	24	51	19.5
	55	61	64	23	(17)	
1	109	151	79	70	102	24.5
					(34)	
	109	150	78	70	74	
					(5)	
2	89	64	75	80	78	31.2
	87	66	78	83	(9)	
3	88	85	68	64	77	30.8
	89	87	66	65	(12)	
4	91	93	92	86	91	37.0
	91	92	92	88	(2)	
5	122	128	124	91	121	52.0
	121	129	127	123	(12)	

* Calculation includes correction for sample dilution.

Appendix 4.5: Analysis of standards for determination of N-terminal amino groups using fluorescamine for determination of total available peptide bonds

VOLUME CF 2.5 mM L-glu (µl)	PEAK HEIGHT (mm)		CONCENTRATION (mM)
	Duplicate a	Duplicate b	
0	1	1	0
5	15	15	0.0625
10	29	28	0.125
20	51	50	0.25
30	71	76	0.375
40	94	96	0.50
50	110	107	0.625
60	137	131	0.75

Appendix 4.6: Analysis of standards for determination of ammonia in determination of total available amide bonds

VOLUME OF 4.0 mM NH_4Cl (µl)	CONCN. IN SAMPLE (mM)	ABSORBANCE (A_{340})			
		First Determination		Second Determination	
		Duplicate a	Duplicate b	Duplicate a	Duplicate b
0	0	2.286	2.302	2.308	2.308
5	0.2	2.196	2.190	2.189	2.181
10	0.4	2.080	2.089	2.069	2.059
20	0.8	1.837	1.858	1.829	1.854
30	1.2	1.646	1.636	1.593	1.593
40	1.6	1.384	1.376	1.351	1.357
50	2.0	1.157	1.146	1.147	1.150
60	2.4	1.132	1.131	1.115	1.133

Appendix 5.1: Absorbance (A_{340}) for ammonia standards for total deamidation of spray dried product preparations

VOLUME OF STANDARD (4 mM NH_4Cl) (μl)	ABSORBANCE A_{340} (a)	(b)	CONCENTRATION OF AMMONIA IN SAMPLE (100 μl) (mM)
60	1.182	1.176	2.4
50	1.181	1.177	2.0
60	1.347	1.366	1.6
30	1.605	1.597	1.2
20	1.866	1.852	0.8
10	2.106	2.133	0.4
5	2.223	2.241	0.2
0	2.344	2.347	0

Appendix 5.2: Absorbance (A_{340}) for samples from total deamidation of spray dried preparations

REACTION TIME (hrs)	2		4		6			STANDARD	
SAMPLES	a	b	a	b	a	b	MEAN	DEVIATION	DILUTION
(11)	1.743	1.749	1.821	1.845	1.808	1.787	1.792	0.040	1/10
(10)	1.450	1.420	1.480	1.491	1.477	1.483	1.467	0.027	1/10
(9)	1.547	1.555	1.574	1.568	1.572	1.575	1.565	0.012	1/10
(8)	1.561	1.516	1.514	1.502	1.555	1.572	1.536	0.029	1/10
(7)	1.247	1.221	1.242	1.279	1.305	1.232	1.254	0.032	1/10
(6)	1.683	1.689	1.724	1.700	1.796	1.760	1.725	0.045	1/20
(5)	1.715	1.698	1.684	1.686	1.742	1.695	1.703	0.022	1/20
(4)	1.668	1.658	1.732	1.706	1.718	1.678	1.693	0.030	1/20
(3)	1.716	1.722	1.746	1.696	1.777	1.712	1.728	0.029	1/20
(2)	1.702	1.641	1.676	1.760	1.736	1.699	1.702	0.042	1/20
(1)	1.788	1.770	1.763	1.703	1.770	1.711	1.751	0.035	1/20
(7) (Repeat)	1.823	1.857	1.843	1.831	1.856	1.836	1.841	0.014	1/20

Appendix 5.3: Absorbance (A_{340}) for ammonia standards for stored liquid hydrolysates

VOLUME OF STANDARD (4 mM NH_4Cl) (µl)	ABSORBANCE (A_{340}) (a)	(b)	CONCENTRATION OF OF AMMONIA IN (100 µl) (mM)
60	1.158	1.155	2.4
50	1.166	1.164	2.0
40	1.374	1.374	1.6
30	1.626	1.627	1.2
20	1.837	1.857	0.8
10	2.074	2.082	0.4
5	2.195	2.176	0.2
0	2.290	2.271	zero

Appendix 5.4: Absorbance (A_{340}) for samples for stored liquid hydrolysates

SAMPLE	Aa	Ab	Ba	Bb	MEAN	STANDARD DEVIATION	DILUTION
(2)	-	1.855	1.767	1.914	1.913	0.057	1:40
(3)	-	1.921	1.899	1.936	1.919	0.019	1:40
(4)	-	1.903	1.989	1.980	1.957	0.047	1:40
(5)	-	1.908	1.892	1.892	1.897	0.009	1:40
(6)	-	2.034	2.011	2.012	2.019	0.013	1:40
(7)	-	1.764	1.696	1.714	1.725	0.035	1:40
(8)	1.363	1.261	1.206	1.333	1.291	0.071	1:40
(9)	1.424	1.388	1.334	1.309	1.364	0.052	1:40
(10)	1.368	1.494	1.375	1.553	1.448	0.091	1:66.7
(11)	1.897	1.453	1.535	1.468	1.588	0.209	1:100

Appendix 5.5: Absorbance (A_{340}) for ammonia standards for total deamidation of freeze dried product and hydrolysate of samples prepared without dialysis

VOLUME OF STANDARD (4 mM NH_4Cl) (µl)	(A_{340}) (a)	(b)	CONCENTRATION OF AMMONIA IN SAMPLE (100 µl) (mM)
60	1.107	1.111	2.4
50	1.178	1.131	2.0
40	1.462	1.351	1.6
30	1.658	1.576	1.2
20	1.910	1.817	0.8
10	2.290	2.139	0.4
5	2.295	2.309	0.2
0	2.510	2.498	0.0

Appendix 5.6: Absorbance (A_{340}) for samples from total deamidation of freeze dried product preparations

SAMPLE	Aaa	Aab	Aba	Abb	Baa	Bab	Bba	Bbb	MEAN	STANDARD DEVIATION	DILUTION
(2)	1.769	1.832	1.733	1.713	1.774	1.760	1.686	1.663	1.741	0.054	1:20
(4)	1.759	1.784	1.701	1.707	1.817	1.718	1.712	1.732	1.741	0.042	1:20
(5)	1.704	1.773	1.803	1.635	1.785	1.806	1.732	1.730	1.746	0.058	1:20
(3)	1.754	1.770	1.770	1.821	1.743	1.687	1.743	1.721	1.751	0.039	1:20
(6)	1.684	1.758	1.694	1.667	1.833	1.781	1.758	1.748	1.740	0.056	1:20
(7)	1.689	1.743	1.726	1.762	1.752	1.692	1.740	1.773	1.735	0.031	1:20
(8)	1.809	1.878	1.951	1.929	1.879	1.907	1.853	1.912	1.890	0.045	1:20
(10)	1.876	1.860	1.890	1.969	1.896	1.870	1.916	1.907	1.898	0.034	1:20
(9)	1.952	1.969	2.008	1.991	1.953	1.998	1.976	1.965	1.976	0.021	1:20
(11)	2.162	2.200	2.207	2.169	2.129	2.132	2.155	2.125	2.160	0.032	1:20
(12)	1.811	1.756	1.743	1.712	1.709	1.693	1.724	1.772	1.744	0.092	1:20
(13)	1.702	1.735	1.817	1.730	1.738	1.705	1.729	1.748	1.738	0.032	1:20

Appendix 5.7: Absorbance (A_{340}) for ammonia in hydrolysate of samples prepared without dialysis

SAMPLE	Aa	Ab	MEAN	Ba	Bb	MEAN
1	2.425	2.410	2.418	2.293	2.315	2.355
2	2.410	2.375	2.393	2.440	2.385	2.413
3a	2.145	1.966	2.056	2.310	2.345	2.328
3b	2.165	2.037	2.101	2.280	2.310	2.295

Dilution of A samples was 1:100

Dilution of B samples was 1:20

Appendix 5.8: Peak heights of L-glu standards for determination of N-terminal amino groups, using fluorescamine, of spray dried product preparations

VOLUME OF STANDARD (2.5 mM L-glu) (µl)	PEAK (mm)	HEIGHT (mm)	CONCENTRATION OF L-GLU IN SAMPLE (mM)
0	4	5	0
5	17	21	0.0625
10	39	40	0.125
20	58	54	0.25
30	79	80	0.375
40	102	101	0.50
50	120	121	0.625
60	143	138	0.75

Appendix 5.9: Peak heights for samples for determination of N-terminal amino groups, using fluorescamine, of spray dried product preparations

SAMPLE	PEAK HEIGHT (mm) Aa	Ab	Ba	Bb	MEAN	STANDARD DEVIATION	DILUTION
Gluten	8	8	38	40	23.5	17.9	1:40
(2)	19	20	18	19	19	0.8	1:40
(1)	17	17	17	17	17	0	1:40
(3)	13	12	19	19	15.8	3.8	1:40
(4)	15	18	17	17	16.8	1.3	1:40
(5)	19	19	174(1)	23	20.3	2.3	1:40
(6)	19	24	19	18	20	2.7	1:40
(7)	21	22	23	24	22.5	1.3	1:40
(8)	14	12	12	12	12.5	1.0	1:100
(9)	13	13	13	14	13.2	0.5	1:100
(10)	13	15	13	14	13.8	1.0	1:100
(11)	17	17	15	15	16	1.2	1:100

Note (1) Result excluded from calculations

Appendix 7.1: Absorbance, A_{340}, for ammonia standards for measurement of reaction with peptidoglutaminase enzymes

VOLUME OF STANDARD 4 mM NH_4Cl (µl)	ABSORBANCE A_{340} A_{340} (a)	(b)	CONCENTRATION OF AMMONIA IN SAMPLE (100 µl) (mM)
60	1.052	1.069	2.4
50	1.069	1.075	2.0
40	1.133	1.141	1.6
30	1.360	1.354	1.2
20	1.616	1.618	0.8
10	1.857	1.853	0.4
5	1.971	1.976	0.2
0	2.081	2.081	0

Appendix 7.2: Absorbance, A_{340}, for samples for measurement of reaction with peptidoglutaminase enzymes

REACTION TIME (MINUTES) / REACTION NUMBER	A_{340} 0	10	30	60
(1)	2.057	2.021	1.979	1.929
(2)	2.049	1.985	1.986	1.757
(3)	2.053	2.034	2.053	2.047
(4)	2.053	2.049	2.039	2.024
(5)	2.051	2.052	2.053	2.043
(6)	1.902	1.877	1.886	1.919
(7)	1.998	1.981	1.986	1.960
(8)	1.904	1.919	1.957	1.906
(9)	1.987	1.979	1.995	1.973
(10)	1.926	1.907	1.965	1.903
(11)	1.989	1.980	1.996	1.948
(12)	1.946	1.914	1.884	1.873
(13)	1.996	1.975	1.980	1.960
(14)	2.048	2.051	2.050	2.052