A S S E S S M E N T   O F   L Y S I N E   D A M A G E

D U R I N G   F O O D   P R O C E S S I N G

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by

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- ABSTRACT -

The fluorodinitrobenzene (FDNB), succinic anhydride (SA), dansyl chloride (DAN), dye-binding lysine (DBL), total lysine (TL), ninhydrin (NIN) and Tetrahymena lysine (TET) methods were compared for their ability to assess available lysine in soyaprotein heated in the absence or presence of glucose, lactose or xylose and in formaldehyde-treated lactalbumin.

The reactive lysine methods showed comparable sensitivity to lysine damage in soyaprotein heated in the absence of sugar, the results indicating the presence of acid labile isopeptides and unidentified acid stable derivatives.

Results for soyaprotein heated with glucose, lactose or xylose showed that the type of sugar and the extent of heat treatment has a strong influence on the progress of the Maillard reaction. Furthermore since fructoselysine (F-L) and lactulosyl-lysine (L-L) are colourless up to 30% loss of available lysine can occur without any change in product colour. The FDNB method is the most sensitive for mildly damaged glucose-soya samples followed by DAN or DBL, SA and TL whereas for mildly damaged lactose-soya samples the order is DBL, FDNB, SA, TL and DAN. For severely damaged samples the DAN or SA methods were the most sensitive followed by DBL, FDNB and TL.

Formylation of lactalbumin occurred more readily at higher formaldehyde concentrations. Exposure time had less effect while pH (5 and 9) had no effect. Methylene derivatives reached maximum levels sooner than the methylol compounds. Lysine and tyrosine but not histidine formed methylene bridges while tyrosine was found to condense with free formaldehyde during acid hydrolysis raising questions as to the interpretation of similar studies reported in the literature. The FDNB, DBL and DAN methods were all very sensitive to this type of damage with the NIN and TL methods being less sensitive and the SA method being completely unsuitable.

The TET assay is unsuitable for 'early' Maillard damage since at low sample-N levels growth is stimulated by its ability to utilise unavailable F-L and L-L while at higher N-levels growth is inhibited.

No single method is most suitable for all types of damage. Furthermore, all except DAN and DBL are either too long, rather complicated, require expensive equipment or involve the use of dangerous chemicals. The DAN method appears promising but the problem of converting arbitrary fluorescence units to lysine values needs to be overcome. The DBL is recommended for routine analysis since it is simple, economical and highly sensitive to all lysine damage provided care is taken to optimise dye-binding for each type of material analysed.

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- PREFACE -

The experimental work described in this thesis was carried out in the
Institute of Physiology, Physiological Chemistry and Nutritional Physiology,
University of Munich, G.F.R., the Department of Applied Biology, University
of Cambridge, U.K., the Biochemistry Department, University of Zululand,
the National Food Research Institute, CSIR, Pretoria and the Biochemistry
Department, University of Natal, Pietermaritzburg.

These studies represent original work by the author and have not been
submitted in any form to another University. Where use was made of the
work of others it has been duly acknowledged in the text.

T. R. ANDERSON

- ACKNOWLEDGEMENTS -

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- ABBREVIATIONS -

AL   .....   available lysine

A - L   ....   ε-N-(β-L-aspartyl)-L-lysine

B   .....   browning (absorbance at 450 nm)

BPA   .....   bovine plasma albumin

BSA   .....   bovine serum albumin

BV   .....   biological value

CP   .....   'crude' protein (calculated as % nitrogen x 6,25)

DAN   .....   1-dimethylaminonaphthalene-5-sulphonylchloride or dansyl
                chloride

DBC   .....   dye-binding capacity

DBL   .....   dye-binding lysine

DL   .....   destroyed lysine

DNP   .....   dinitrophenyl

FDNB 1-fluoro-2,4-dinitrobenzene

F - L   ....   fructoselysine or ε-N-deoxy-fructosyl-L-lysine

FUR   .....   furosine or ε-N-(2-furosyl methyl)-L-lysine

G - L   ....   ε-N-(γ-L-glutamyl)-L-lysine

HAL   .....   τ-N-(2-amino-2-carboxyethyl)-histidine

HPLC   ....   high performance liquid chromatograph(y)

LAL   .....   ε-N-(2-amino-2-carboxyethyl)-lysine

LFL   .....   lysine as fructoselysine

L - L   ....   lactulosyl-lysine or ε-N-deoxy-lactulosyl-L-lysine

LLL   .....   lysine as lactulosyl-lysine

M - L   ....   ε-N-methyl-L-lysine

MSG   .....   monosodium glutamate

NIN   .....   ninhydrin

NPU   .....   net protein utilisation

PER   .....   protein efficiency ratio

ABBREVIATIONS (contd.)

RT	....	room temperature
SA	....	succinic anhydride
SCP	....	single cell protein
SDS	....	sodium dodecyl sulphate
TCA	....	trichloroacetic acid
TET	....	<u>Tetrahymena</u>-available lysine
TL	....	total lysine (determined by ion exchange chromatography)
TNBS	...	trinitrobenzene sulphonic acid
TNP	....	trinitrophenyl
<u>T</u>. <u>pyriformis</u>		<u>Tetrahymena</u> <u>pyriformis</u>
UHT	....	ultra-heat-treated (milk)

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CHAPTER 1

INTRODUCTION

The basic amino acid lysine was first isolated in 1889 and named in 1891 by E. Drechsel who demonstrated that it can exist both in free form and as a covalent-bound residue in proteins (Drechsel, 1889 & 1891). The synthesis of lysine by Fischer and Weigert (1902) gave the final proof of the chemical structure of the compound as α,ϵ-diamino-n-caproic acid. From the nutritional point of view a significant discovery was that of Osborne and Mendel (1914) who showed that lysine is an indispensable dietary amino acid required for normal growth and development. This amino acid is the most limiting essential amino acid in many plant and animal foods. Furthermore, the high nucleophilic nature of its epsilon amino (ϵ-NH_2) group renders it extremely susceptible to inter- and intra-molecular cross-linking in proteins as well as in reactions with carbohydrates, lipids, nucleic acids, polyphenols, vitamins or various food additives such as sulphites, formaldehyde and alkali (Anderson & Quicke, 1980 a; Hurrell, 1980). Since such adverse reactions can occur readily during both food storage and processing, the chemistry of lysine damage and its nutritional availability has been extensively studied over several decades.

Food processing which includes home-cooking has been an intricate part of food preparation since 3 000 BC when the Egyptians used the first ovens for the baking of bread. Processing is employed not only to preserve food while in storage, but to destroy pathogens, inhibitors and toxins, to inactivate enzymes, to augment digestibility particularly of plant proteins, to improve organoleptic properties and to produce more desirable physical properties and aesthetic characteristics. It is not surprising, therefore, that a variety of processes have been developed including heat sterilisation and pasteurisation, low-temperature chilling and freezing, dehydration, irradiation and numerous chemical treatments. For such processes to be of greatest benefit they should have a minimal effect on the nutritional quality of the product and it is therefore important to check for possible deleterious effects that might occur during processing, storage and transport of foodstuffs. Experience has shown that reduction in lysine availability is one of the most prevalent types of food damage and the monitoring of this type of damage is the focal point of the work reported in this thesis.

Since the ultimate availability of lysine and, for that matter, general protein quality in a foodstuff depends upon whether the protein is digested and the resulting amino acids absorbed and utilised in vivo, it would seem logical to use some selected bioassay for this purpose. Indeed three general categories of in vivo methods have been developed mainly using the rat, chick and mouse as experimental animals. These methods are based on:

(a) growth of animals (e.g. Calhoun et al., 1960; Mottu & Mauron, 1967; Carpenter & Opstvedt, 1976; Costa et al., 1977; Cave & Williams, 1980; Batterham et al., 1984) ;

(b) recovery of lysine in faeces (e.g. Guthneck et al., 1953; De Muelenaere & Feldman, 1960; De Muelenaere et al., 1967; Amato et al., 1975; Sibbald, 1980; Nitsan et al., 1981) ; and

(c) nitrogen balance (Linkswiler et al., 1958; Swartz et al., 1959).

Although these methods have been used with a fair degree of success, they are susceptible to the normally accepted variables associated with biological experiments. Factors such as variations in the physiological status of the animals, appetite and rate of passage of food through the gut, differences in mass, sex, age and species of animal within experimental groups, changes in environmental conditions, variations in quantities and ratios of dietary con-stituents, influence of intestinal microflora and a variety of other factors that are difficult to control or evaluate, render bioassays both unreliable and difficult to standardise. Faecal analysis which measures only the amount of unabsorbed lysine has the further disadvantage that assay procedures based on this approach make the questionable assumption that all the absorbed lysine will be utilised in body metabolism. Add to these difficulties the problem that lysine found to be nutritionally available to one species, is not necessarily available to a different species or to man (Bodwell, 1977) and it becomes clear that bioassays cannot be regarded as providing an abso-lute assessment of available lysine although the results are extremely useful indicators of lysine damage.

In view of the above problems and the time required for and cost effective-ness of in vivo methods, various in vitro methods were developed which, while not improving on the quantitative aspect of the assays certainly offered cheaper and more convenient ways of estimating relative lysine damage. Methods employing enzymic digestion (Mauron et al., 1955; Bujard et al., 1967; Rayner & Fox, 1978), plasma amino acid analysis (Wheeler & Morgan, 1958; Guggenheim et al., 1960; Rao & McLaughlan, 1967), microbiological assay

(Stott & Smith, 1960; Payne et al., 1977; El-Sherbiny et al., 1980) and chemical analysis (Anderson & Quicke, 1980 b; Mauron, 1980; Hurrell & Carpenter, 1981) have proved extremely useful despite numerous theoretical and practical problems.

Some companies have gone to the great expense of purchasing a conventional amino acid analyser for the nutritional evaluation of their products. This method cannot be relied on, however, for assessing the full extent of process-ing damage to food proteins. Processing may cause the formation of chemical linkages with amino acid side chains (Anderson & Quicke, 1980 a) that are resistant to mammalian digestive enzymes but labile to acid hydrolysis. The classic example is the transformation of lysine into fructoselysine, a compon-ent that is practically unavailable in vivo (Finot et al., 1977 b) but still liberates 50 % of its lysine content during conventional acid hydrolysis. Total amino acid analysis then is mainly useful for estimating amino acid destruction occurring during food processing and measures the maximum possible amount of amino acid that could be nutritionally available.

In recent years various chemical methods utilising ϵ-NH_2-reactive reagents have become the most popular since they are not only capable of giving a good estimate of relative lysine damage but are generally simple, rapid and economical enough for routine laboratory programmes. Chemical methods of this sort are of course based on the assumption that if ϵ-NH_2 groups in a protein are inaccessible to the electrophilic reagent, either due to steric or chemi-cal blocking, then they will not be reached or recognised by lysine-specific proteases, and therefore will not be digested and hence not absorbed or utili-sed in body metabolism. This may not always be the case since all the chemi-cal reagents will be sterically smaller than enzymes and in some cases strongly electrophilic reagents (e.g. trinitrobenzene sulphonic acid and 1-fluoro-2,4-dinitrobenzene) have been found to react with nutritionally un-available and chemically-blocked lysine e.g. fructoselysine. By contrast, in some cases (e.g. ϵ-N-(Y-glutamyl)-L-lysine) lysine is chemically blocked from reaction with the electrophilic reagent but is totally available as a source of dietary lysine (Mauron, 1970; Waibel & Carpenter, 1972). Furthermore, after advanced Maillard reactions or other severe heat treatments, lysine units with free reactive ϵ-NH_2 groups may be rendered unavailable as they pass unabsorbed into the faeces as components of indigestible peptides (Hurrell et al., 1976). In view of the above problems the term 'reactive' lysine rather than 'available' lysine is preferred when referring to chemical estimates of lysine and, the suitability of such reactive lysine procedures

would be expected to vary depending upon the type of damage involved. Indeed this is clearly demonstrated in some of the results reported in this thesis.

Apart from the reactive lysine methods, only microbiological assays, usually employing the protozoan Tetrahymena pyriformis W, are still used on a regular basis for the estimation of available lysine. This is mainly due to the work of Stott and Smith (1966) and Baker et al. (1978) who have streamlined the method so that it has become more suitable for routine analysis. However, as discussed in Chapter 2 and substantiated in the results reported in Chapter 8, numerous problems are associated with this method.

The main objectives of the present studies, which are a substantial extension of the author's MSc. investigations (Anderson, 1980), were three-fold, namely:

(1) to carry out a thorough study of selected reactive-lysine methods and render improvements where these were deemed necessary;

(2) to compare the original or improved methods for their ability to detect certain types of lysine damage; and

(3) to draw some conclusions as to the importance of monitoring available lysine in the food industry and the general usefulness of reactive lysine methods for such evaluations.

Three of the more prevalent types of lysine damage namely, heat damage occurring in the absence (mainly isopeptides) and presence (Maillard reactions) of reducing sugars and that resulting from formaldehyde treatment were selected for these studies. In view of the increasing importance of soyaprotein isolates as protein foods rich in essential amino acids, especially lysine, and the exposure of this protein to heat damage during processing, extracted soyaprotein was used to prepare model samples representing two types of lysine damage. Likewise extensive use of formaldehyde in food processing (see § 2.1.3) stimulated investigation of the suitability of the selected analytical procedures as a means of detecting this type of damage. Lactalbumin was selected as the model for these studies as it contains relatively high levels of lysine and tyrosine residues both of importance in formaldehyde-type cross-linking reactions. Some samples were prepared under relatively mild conditions in order to simulate typical commercial processing procedures, while other samples were more severely treated in order to enhance the extent of lysine damage so that the various methods could be tested over a wide range of processing conditions.

Five different reactive lysine methods as well as the microbiological method
of Stott and Smith (1966) were selected for study. The well-established
fluorodinitrobenzene (FDNB) (Carpenter, 1960) and dye-binding lysine (DBL)
(Hurrell & Carpenter, 1976 a) methods were included for their importance as
reference procedures while the less-known succinic anhydride (SA) (Anderson
& Quicke, 1984), dansyl chloride (DAN) (Christoffers, 1976) and ninhydrin
(NIN) (Friedman & Broderick, 1977) methods were included in order to test
their relative performance with the selected types of lysine damage. The
FDNB method of Carpenter (1960) is considered of vital importance as a refer-
ence procedure in this thesis since it has often been shown to give results
that correspond closely with those from both biological evaluation and
in vitro enzymic digestion (Mauron, 1977 & 1980) and was awarded no less than
a 'B' rating by Hurrell and Carpenter (1974) for a variety of types of lysine
damage. It is important to stress, however, that the FDNB method like the
other reactive lysine methods is not necessarily ideal for all situations.
Originally the intention was to analyse all three types of lysine-damaged
material by each of the reactive lysine methods, but this became less import-
ant than solving the numerous problems encountered with some of the method-
ology and the interpretation of certain results. This was more especially
true for the Tetrahymena assay which yielded unexpected results for the
Maillard-damaged soyaprotein and was ultimately applied only to this type of
material.

Apart from the introduction (Ch. 1), literature review (Ch. 2) and materials
and methods (Ch. 3), this thesis constitutes six major chapters in which the
experimental results are presented and evaluated. Chapter 4 includes the
results of various investigations into ways of improving the DBL and DAN pro-
cedures while in Chapters 5 and 6 respectively, the various reactive lysine
methods are examined for their ability to assess lysine damage in soyaprotein
isolates heated in the absence or presence of various reducing sugars.
Chapter 7 examines the application of the reactive lysine methods to formal-
dehyde-damaged lactalbumin, while Chapter 8 deals with the Tetrahymena assay
for available lysine employing Maillard-damaged soyaprotein as test samples.
An attempt is made in Chapter 9 to integrate these several findings and draw
some general conclusions regarding the relative usefulness of the procedures
under investigation.

CHAPTER 2

LITERATURE REVIEW

Since both the types of lysine damage as well as methods for its analysis
have been extensively discussed elsewhere (Anderson, 1980; Anderson & Quicke,
1980 a & b; Hurrell, 1980), only those aspects relevant to this thesis are
reviewed in this chapter.

2.1 TYPES OF LYSINE DAMAGE CAUSED BY PROTEIN PROCESSING

2.1.1 Lysine damage in the absence of reducing sugars

In 1934 Astbury and Woods postulated the formation of ω-ε bonds (Fig. 1 a)
between the side chains of lysine and glutamic or aspartic acid residues in
wool keratin, but it was not until the work of Mecham and Olcott (1947) that
any experimental evidence was offered for the formation of such isopeptides
in heat-treated proteins. A number of workers (Lorand et al., 1968;
Matačić & Loewy, 1968; Pisano et al., 1968) subsequently established the
existence of ε-N-(γ-L-glutamyl)-L-lysine (G-L) in polymerised fibrin, while
Asquith et al. (1970) isolated both G-L and ε-N-(β-L-aspartyl)-L-lysine (A-L)
from keratin and established that both these crosslinks are formed during the
heating of proteins (Asquith & Otterburn, 1971; Asquith et al., 1971).

Studying the mechanism of isopeptide formation, Bjarnason and Carpenter (1970)
reported that ammonia was liberated from bovine plasma albumin heated at
115°C for 27 h. Furthermore, correlation between lysine binding in different
proteins, and ammonia liberation and amide changes led them to suggest that
the main reaction of epsilon amino (ε-NH$_2$) groups is with the amide groups of
asparagine and glutamine (Fig. 1 a) rather than with ω-carboxyl groups. Their
conclusions are supported by the work of Erbersdobler et al. (1969) who, in
studying the entry of amino acids from heated casein into portal blood of
growing rats, found that besides lysine, glutamine and asparagine showed the
lowest availability. In addition Otterburn et al. (1977) showed that the
formation of isopeptides was primarily dependent on the proximity of aspara-
gine or glutamine residues to ε-NH$_2$ groups rather than the quantity of these
residues in the whole protein. Egg-white lysozyme, with lysine residues
located almost directly opposite asparagine residues in the bioactive protein
molecule (Phillips, 1967) preferentially formed A-L on heating.

From the nutritional point of view, the availability of these isopeptides and the effect of the inter- and intra-chain crosslinks on general protein and amino acid digestibility and quality is of prime importance. Bjarnason and Carpenter (1970) found that the feeding of rats with heat-treated bovine plasma albumin in which intra-molecular isopeptide linkages occurred, resulted in increased faecal lysine but little urinary lysine suggesting both poor protein digestibility and also malabsorption. Hurrell et al. (1976), likewise found that the digestibility in rats of heated chicken muscle was greatly reduced and that this was associated with the presence of isopeptides, the amount present increasing with increasing severity of the heat treatment. Ford (1965) showed that the lower protein digestibility associated with iso-peptide formation resulted in a general decrease in the availability of all amino acids. The $\epsilon-\omega$ linkage (Fig. 1a), itself is also resistant to gut proteases (Kornguth et al., 1963; Finot et al., 1978b) and hence if any iso-peptides are released from the protein they are absorbed unmodified directly into the blood plasma (Waibel & Carpenter, 1972; Finot et al., 1978b). Although free A-L is absorbed it is not metabolised by rats and remains totally unavailable whereas surprisingly, its homologue-free G-L is hydrolysed in the kidney (Finot et al., 1978b) by an ϵ-lysine acylase (Leclerc & Benoiton, 1968) and can be utilised as a source of lysine (Mauron, 1970). Free G-L apparently becomes available to rats approximately 2 h later than free lysine due to the absorption of the isopeptide in the more distal part of the small intestine and its later hydrolysis in the kidneys (Finot et al., 1978b). Thus current knowledge indicates that protein-bound A-L is totally unavailable whereas protein-bound G-L is either unavailable or at best only partially available depending on the ability of gut enzymes to penetrate the highly crosslinked material and release the G-L, and on its subsequent absorption and hydrolysis in the tissues.

Other crosslinks may also occur when proteins are heated. Heat can cause fission of the disulphide bond of cystine, yielding dehydroalanine which may condense with the $\epsilon-NH_2$ of lysine to form lysinoalanine (LAL) (Fig. 1b) (Sternberg et al., 1975; Raunio et al., 1978). Although this reaction is more prominent in akaline heat-treated protein (Ch. 1), Bjarnason and Carpenter (1970) also suggested that a peptide bond could be ruptured at dehydroalanine to form an amide group and a pyruvyl derivative (Fig. 1c). The amide could then react with lysine with the liberation of ammonia (Fig. 1a). Heat could also cause the deamination of $\epsilon-NH_2$ groups which are lost as ammonia (Fig. 1d) (Bjarnason & Carpenter, 1970). The aminoadipic semialdehyde residues thus formed may either crosslink with themselves by aldol condensation
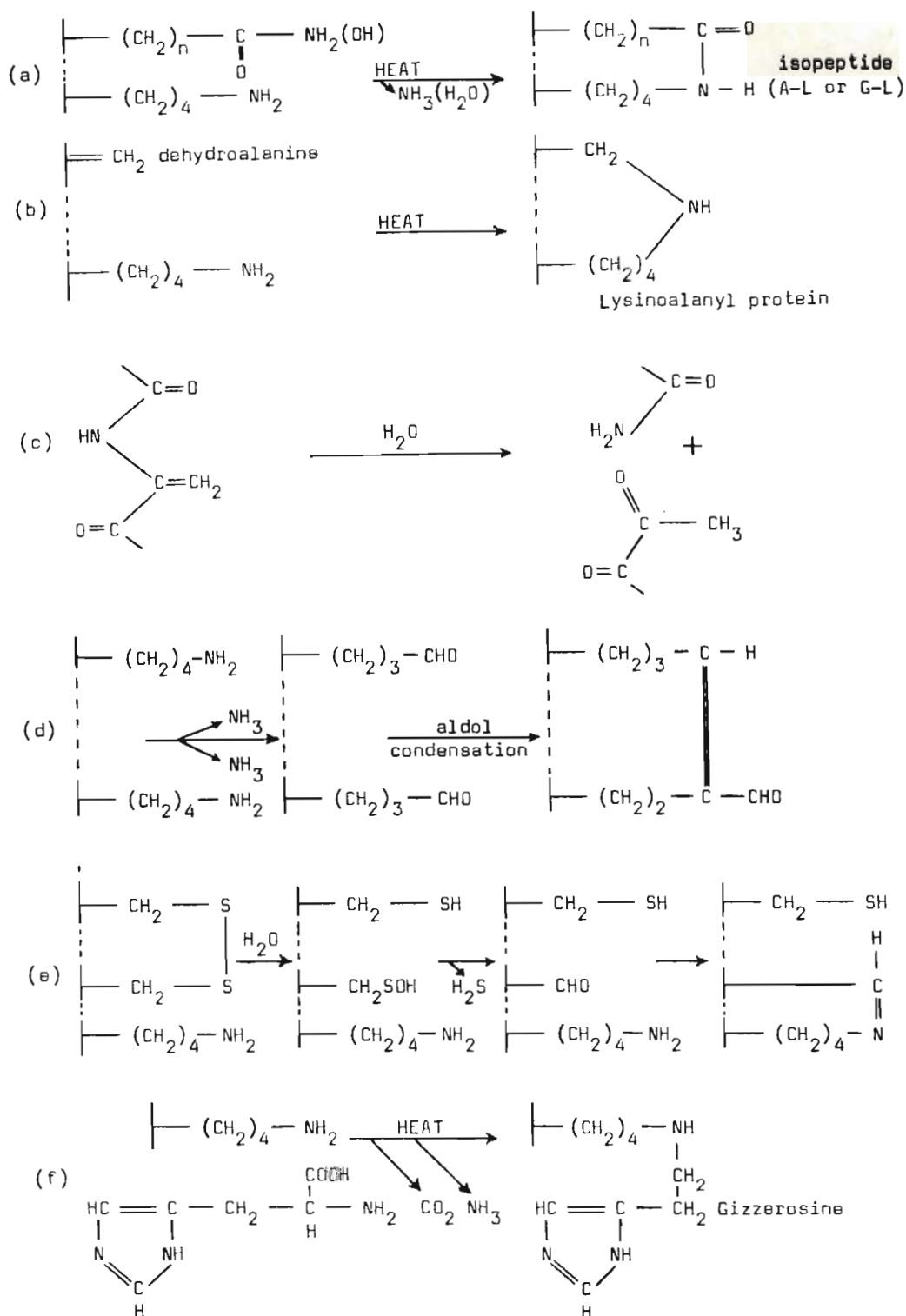
FIGURE 1 Isopeptide and other possible lysine interactions occurring during heat processing - proposed by: (a) Astbury & Woods (1934); Bjarnason & Carpenter (1970); (b) Sternberg et al. (1975); (c) Bjarnason & Carpenter (1970); (d) Bailey et al. (1970); Bjarnason & Carpenter (1970); (e) Philips (1936); (f) Okazaki et al. (1983). In (a): n = 1 (aspartate/asparagine); n = 2 (glutamate/glutamine)

(Fig. 1d) (Bailey et al., 1970) or with other lysine or hydroxylysine residues to form products such as dehydrohydroxylysine-norleucine, hydroxylysino-5-keto-norleucine and others (Bailey et al., 1974; Davis et al., 1975). Although these latter crosslinks have only been reported for collagen, it is by no means certain that they do not occur in food proteins. Philips (1936) proposed a crosslinkage between lysine and the aldehyde formed via cysteinsulphenic acid by the degradation of disulphide bonds (Fig. 1e).

Heat treatment of proteins may also cause racemisation of certain amino acids (Liardon & Hurrell, 1983) as well as peptide bonds (Hayashi & Kameda, 1980) which would resist attack by proteolytic enzymes (Friedman et al., 1981) and result in a general reduction in protein digestibility and quality (Hayase et al., 1975). The absorption of D-amino acids is also much slower than that of the corresponding L-form (Gibson & Wiseman, 1951) and even if digested and absorbed, many D-isomers including D-lysine are not utilised by man (Berg, 1959) or rats (Ohara et al., 1980).

Finally an important recent finding is that free histidine can crosslink with the $\epsilon-NH_2$ group of lysine residues, particularly in heated (130°C, 4 h) fish-meal to form 2-amino-9-(4-imidazolyl)-7-azononanoic acid or gizzerosine (Fig. 1f), a toxin shown to cause gizzard erosion in poultry (Okazaki et al., 1983). Little is known about this compound which could turn out to be one of a family of toxic products resulting from the heat treatment of protein in the absence of reducing sugars.

To summarise, heat treatment of proteins in the absence of reducing sugars can result in a variety of crosslinking reactions with $\epsilon-NH_2$ groups of lysine, the most prominent of which is probably with glutamine and asparagine sidechains to form the isopeptide residues G-L and A-L respectively. Whereas protein-bound A-L is totally unavailable, protein-bound G-L is either unavailable or at best partially available depending on whether any free G-L is released by gut enzymes from the highly crosslinked and therefore poorly digestible material. Numerous other lysine products may result from such processing but their nutritional and toxicological consequences remain to be established. Once established it will be important to monitor and control the problem products.

2.1.2   Lysine damage in the presence of reducing sugars

Lysine damage due to an amino-carbonyl reaction with reducing sugars was first observed by Louis-Camille Maillard, a French chemist who described the formation of brown pigments or melanoidins on heated glucose-lysine mixtures (Maillard, 1912). Although he did not study the nutritional effects of these reactions,

he urged that their biological implications should be examined (Maillard, 1916). These classical studies initiated an entire field of research on the chemistry, biochemistry and nutritional and toxicological consequences of the so-called Maillard reactions that has now spanned more than seven decades.

Early workers such as Block et al. (1946) found that after baking, the protein efficiency ratio (PER) of an ovalbumin-lactalbumin-sucrose cake-mix, was significantly decreased while Evans and Butts (1948) observed two types of lysine inactivation in autoclaved (4 h) soyabean oil cake meal. About 40% of lysine was destroyed while a further 20% was converted to a form from which active lysine was freed by acid but not in vitro enzymic hydrolysis. Sucrose was suggested to be involved in these reactions and it was subsequently found (Henry & Kon, 1950) that lysine was the only amino acid to manifest a significant reduction in availability after mild heating of casein with glucose.

For the non-reducing sugar sucrose, it became generally accepted that inversion is the rate-limiting step in the reaction responsible for heat damage, since the reducing monosaccharides glucose and fructose must first be released before any amino modification can take place (Karel & Labuza, 1968; Anantharman & Carpenter, 1971; Flink, 1983). Lysine was found to be stable for long periods in protein-sucrose mixtures held at not more than 37° C, but at higher temperatures (> 55° C), a slightly acidic pH (e.g. pH 5) and 1- 20% moisture content, more lysine was destroyed by sucrose than glucose on a mole to mole basis (Hurrell & Carpenter, 1977 a; Smith and Friedman, 1984). Noguchi et al. (1982) recorded up to 40% loss in fluorodinitrobenzene (FDNB)-reactive lysine due to sucrose-lysine interactions during extrusion-cooking of protein-enriched biscuits. Smith and Friedman (1984) found that starch also reacts with lysine but as expected, to a reduced extent, since starch possesses limited reducing ends and is more stable to hydrolysis than sucrose.

Most work, however, has focused on the effect of reducing sugars, milk and its products having received particular attention due to their high lactose content. Gupta et al. (1958) showed that lysine in roller-dried non-fat milk powder was much less available (68%) than that in spray-dried milk powder (92%) while Mottu and Mauron (1967) demonstrated that lysine in spray-dried milk was unavailable for digestion by enzymes in vitro. More recently Erbersdobler (1983 a) found that the FDNB-reactive lysine content of roller-dried skim milk stored at RT decreased by 6,7% per year over 9 years while spray-dried samples lost only 2,5% per year. This difference was explained by the higher initial water content in the roller-dried samples (4- 6%) as compared

to the spray-dried samples (< 4%). Roller-drying is apparently no longer used commercially to produce milk powders (Hurrell, 1980). Erbersdobler (1983 a) also found significantly higher lysine damage in milk subjected to pressure cooking (118° C) as against normal boiling (100° C), as well as smaller but definite losses of lysine in Ultra Heat Treated milk with increasing time and temperature of heating. He stressed the importance of keeping heat treatment to a minimum.

Glucose and other reducing monosaccharides may also drastically reduce available and total lysine during storage and heat processing. Considerable reduction (25 - 30 %) in lysine availability occurred even under mild conditions (37° C , 30 d) of storage of an ovalbumin-lactalbumin-glucose mix (Hurrell & Carpenter, 1974). By contrast tests with wheat flour showed added lysine to be stable during heating at 100 ° C for 2 h, but when glucose (10 %) was present only 19,1 % of the added lysine was recovered by amino acid analysis (Warthesen & Kramer, 1978). More recently Smith and Friedman (1984) recorded decreases in reactive lysine (FDNB and dye-binding lysine methods) of 35 % and 54 % respectively for casein heated with glucose at 37° C for 10 d and at 121° C for 1 h.

The basic chemical mechanism for the Maillard reaction was first described by Hodge (1953). Numerous subsequent studies have not significantly changed his basic scheme although many more intermediates have been isolated and identified (Olsson et al., 1981; Baltes, 1982; Namiki & Hayashi, 1983; Nursten & O'Reilly, 1983). Maillard reactions are clearly highly complex but were conveniently divided by Hodge (1953) into 'early' and 'late' (or advanced) reaction stages. The 'early' reaction (Fig. 2) which occurs during storage or mild heat treatment (< 60° C), involves the condensation between the carbonyl group of the reducing sugar and the amino group of free or protein-bound amino acids. The condensation product is rapidly converted via a Schiff's base and an aldosylamine intermediate to a colourless deoxyketosyl derivative which is the major Maillard product in processed foodstuffs. In view of the reversibility of the initial reactions (Fig. 2) the Schiff's base and aldosylamine derivatives are nutritionally available (Finot et al., 1977 b; Finot & Magnenat, 1981) whereas the deoxyketosyl compounds are more stable and unavailable to the rat as sources of lysine (Hurrell & Carpenter, 1981).

The deoxyketosyl compound fructoselysine (F-L), formed from the reaction between ϵ-NH_2 groups and either glucose or fructose, is totally unavailable to rats and humans (Finot et al., 1978 a; Mori et al., 1980). It seems that due to hindered protease action only 30 - 40 % of protein-bound F-L is released
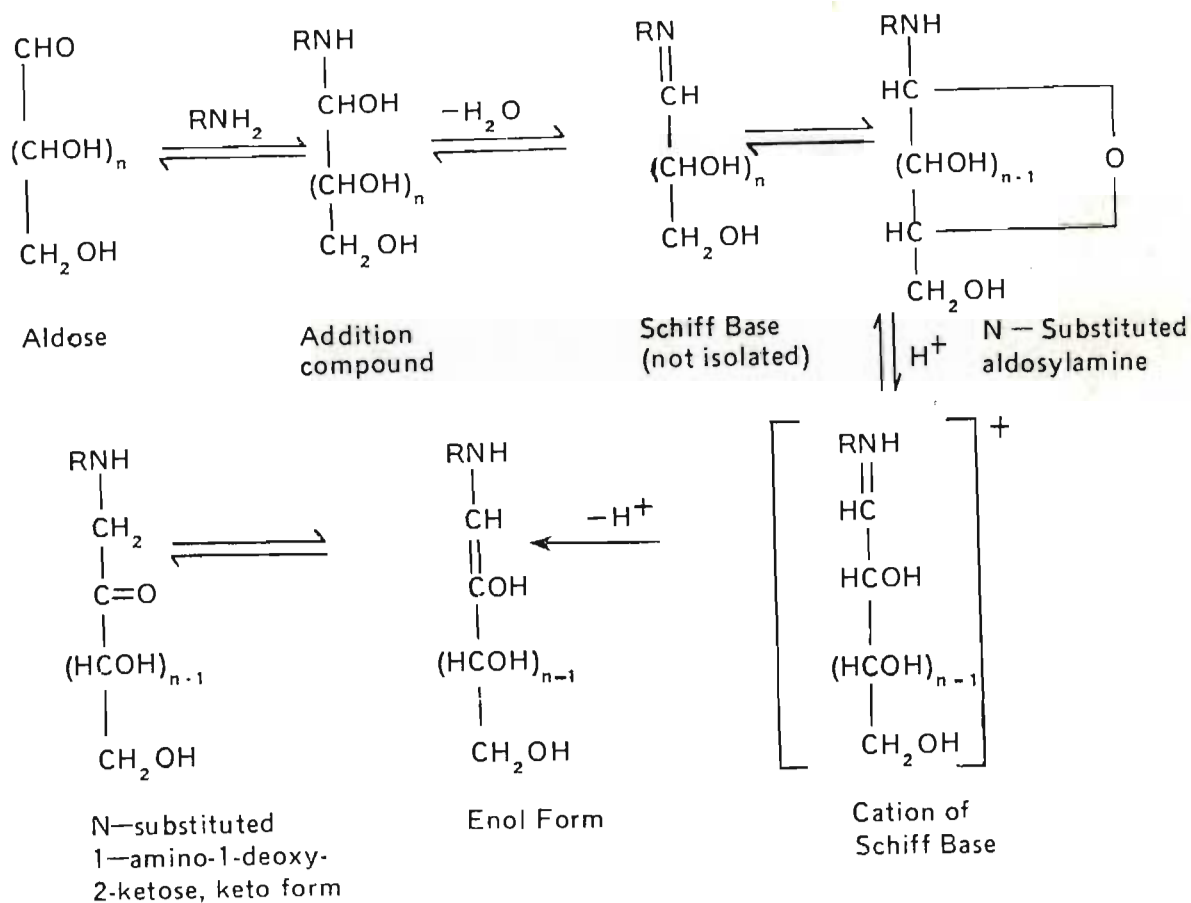
FIGURE 2 Detail of 'early' Maillard reactions (Hodge, 1953); R = Protein

(1) acid, (2) basic and (3) high temperature conditions
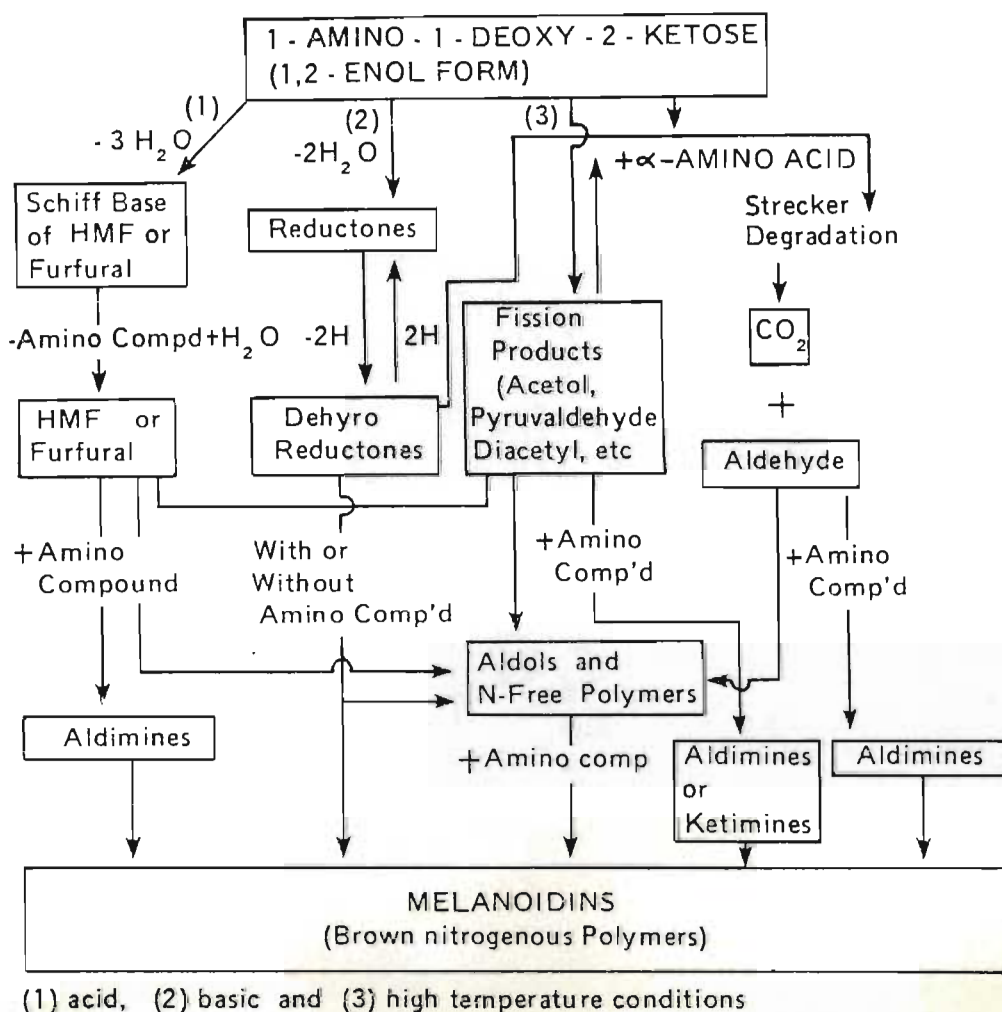
FIGURE 3 Summary of Hotel Maillard

during digestion in rats. Although most of the released F-L is absorbed by passive diffusion it is excreted unchanged in the urine (Erbersdobler, 1977; Mori et al., 1980). The undigested and unabsorbed F-L is either degraded by caecum microflora or excreted in the faeces (Erbersdobler et al., 1970; Finot et al., 1977b). Finot and Magnenat (1981) found similar results except that only 10 - 15% protein-bound F-L was digested and excreted. These results correspond well with those of Niederwieser et al. (1975) who found that 16% of protein-bound F-L given to babies in an infant formula was excreted in the urine. Recently Erbersdobler et al. (1984b) found that the majority of protein-bound F-L in grass cobs fed to dairy cows remained undigested or was destroyed by gut microflora with less than 10% being detected in the milk. Free F-L is more readily absorbed by rats, 60 - 70% being excreted in the urine (Finot & Magnenat, 1981). F-L may also inhibit disaccharidase activity in rats (Lee et al., 1977) and can accumulate in the kidneys (Finot & Magnenat, 1981) where it may be responsible for karyomegaly in the tubular epithelial cells of the pars recta in the outer stripe of the outer renal medulla (Erbersdobler et al., 1981; von Wagenheim et al., 1984) similar to that caused by LAL (Karayiannis et al., 1980), D-serine (Ganote et al., 1974) and D,L-2,3-diamino-propionic acid (Kaltenbach et al., 1979). Since Hayase et al. (1979) and Zumberge (1979) found that carbohydrates heated in the presence of proteins might promote amino acid racemisation, Friedman et al. (1981) suggested that the cytotoxicity observed by Erbersdobler et al. (1981) may be partly due to the presence of D-amino acids. Liardon and Hurrell (1983) however, found no racemisation at all in spray-dried milk that had undergone 'early' Maillard reactions suggesting that F-L is the main causative agent for the observed nephrocytomegalia.

The deoxyketosyl compound, lactulosyl-lysine (L-L), which is formed primarily in milk products by reaction between ϵ-NH$_2$ groups and lactose, is also unavailable to rats (Finot et al., 1977b), poorly digested (10 - 15%) out of proteins and excreted in the urine unchanged (Ford & Shorrock, 1971; Finot & Magnenat, 1981). The unabsorbed L-L is also mainly degraded by caecal microflora (Finot et al., 1977b). There have been no reports of L-L accumulation in organs or any form of toxicity.

With increasing severity of heat treatment (> 70° C) in the presence of carbohydrates, Maillard reactions go beyond the deoxyketosyl stage to the formation of numerous products including brown polymers or melanoidins (Fig. 3) (Labuza et al., 1977). Such reactions not only destroy lysine and other amino acids rendering them irrecoverable by either acid or enzymic hydrolysis

but also markedly reduce the digestibility of the protein as a whole, probably due to crosslinks formed between the protein chains and 'late' Maillard break- down products (Valle-Riestra & Barnes, 1970; Hurrell & Carpenter, 1977 b). Various authors (Sgarbieri et al., 1973; Lee et al., 1981) have, however, demonstrated that the poor nutritional quality of browned protein is not only due to losses in amino acids and decreased protein digestibility, since supple- mentation of the diet to make up for these losses does not restore the original biological value (BV). This suggests that certain 'late' Maillard products may also have anti-nutritional or even toxic effects. Indeed Lee et al., (1977) found that certain water-soluble brown products could inhibit disaccharidase activity in the gut of rats while the ingestion of other browned products have been reported to cause diarrhoea, caecum enlargement and a decreased rate of stomach emptying (Lee et al., 1981). These authors also observed other long- term (up to 12 months) adverse physiological effects but subsequent studies (Pintauro et al., 1983), suggested that Maillard-browned proteins as such are not toxic to rats, and that all previously observed changes in physiological and biochemical parameters were due to other nutritional and/or dietary factors. Nevertheless certain 'late' Maillard products have been found to possess bactericidal properties (Maeshige & Nakagawa, 1974), inhibit various gut enzymes (Oste et al., 1983), destroy vitamins in milk powder (Ford et al., 1983) and exhibit mutagenic effects (Omura et al., 1983).

Notwithstanding potential problems with Maillard damage many 'late' Maillard products contribute significantly to the desirable flavours and odours of food such as freshly-baked bread, coffee, cocoa, beer and cooked meats and vegetables (Fors, 1983) and are valuable as lipid antioxidants (Eriksson, 1982; Beckel & Waller, 1983; Lingnert & Waller, 1983). Consequently Maillard products will always be a part of daily diets but this does not negate the importance of selective control of the Maillard reaction and of the routine monitoring of its extent and rate.

Maillard reactions may be minimised by low-temperature processing and storage, and by lowering the pH of the solution and the concentration (increasing the water activity) of the final product (Feeney & Whitaker, 1982). Furthermore, substances that promote Maillard reactions such as the metal cations Cu^{2+}, Fe^{3+} and Fe^{2+} (Kato et al., 1981) as well as phosphates, carboxylic acids and their salts can be removed by various complexing and precipitating agents. Other approaches involve the removal of reducing sugar when it constitutes only a negligible part of the product, as in eggs and meat, by fermentation or enzym- atically with glucose oxidase. Treatment with sulphur dioxide which covalently

blocks the carbonyl group of the reducing sugar has also been used (McWeeny, 1981). However, if only the non-covalent salt, lysine sulphite (lysine : sulphurous acid, 2 : 1) is formed, the lysine is just as susceptible as free lysine to Maillard destruction although it is resistant to browning (Robbins & Baker, 1980). Lysine may also be protected from Maillard reactions by covalent attachment to either proteins (Li-Chan & Nakai, 1981) or other amino acids such as glutamic acid to form G-L (Finot et al., 1978 b) while at the same time maintaining the nutritional value of the lysine (Stegink et al., 1981).

To summarise, during 'early' Maillard reactions reducing sugars combine with $\epsilon-NH_2$ groups to form a colourless deoxyketosyl derivative via a Schiff's base and an aldosylamine derivative. Whereas the Schiff's base and aldosylamine intermediates are nutritionally available as sources of lysine, the deoxyketosyl compound is totally unavailable, can inhibit disaccharidase activity and in the case of F-L may be responsible for nephrocytomegalia in rats. With increased severity of heat treatment brown-coloured 'late' Maillard products are formed resulting in lysine destruction and reduced protein digestibility. Certain 'late' Maillard products also destroy vitamins in milk powder, affect gut enzymes and microflora, cause adverse physiological changes in rats and may even be mutagenic. It is acknowledged, however, that Maillard products are immensely important for the sensory quality of processed foods but this does not negate the importance of selective control of Maillard reactions and the routine monitoring of its extent and rate.

2.1.3   Lysine damage in the presence of formaldehyde

Over the years formaldehyde has been extensively used for a variety of purposes including the manufacture of disinfectants, urea-formaldehyde insulating foams, crease-resistant clothes, motor tyres, industrial filters, orthopaedic casts, photographic paper, glue, plywood, newsprint and cosmetics, the tanning of leather as well as in the pharmaceutical industry for the preparation of microbial toxoids (Feeney et al., 1975). In the food industry formaldehyde is used as a preservative and firming agent for wet proteinaceous materials such as fish before they are processed into concentrates, for the inactivation of tannins during the extraction of protein from sorghum (Daiber & Taylor, 1982) and for the protection of dietary protein against microbial degradation in ruminants (Broderick, 1975 & 1977; McAllan et al., 1982; Ashes et al., 1984). However, although formaldehyde is a natural product of choline catabolism, there has been growing concern as to its safety both in respect of human and

animal consumption (Hurrell & Carpenter, 1978) and general exposure to the volatile chemical (Pearce, 1984).

In 1965 Dvořak and Vognarová found that the smoking of meat proteins at 20° C resulted in a decrease in lysine availability proportional to the duration of exposure and to the concentration of formaldehyde, a constituent of wood smoke. Exposure to formaldehyde was also reported to reduce the FDNB-reactive lysine in minced fish samples (Wessels & Marshall, 1975; Carpenter & Opstvedt, 1976) while a 15% loss of total lysine (TL) and a 25-35% decrease in FDNB-reactive lysine occurred when a blend of lactalbumin and ovalbumin was heated for 2 h at 80° C with 1% formaldehyde (Hurrell & Carpenter, 1978). The same protein mix showed a 25% decline in digestibility and a 50-60% decrease in available lysine values for rats and chicks. Hove and Lohrey (1976) found that formy-lated-casein diets had drastic effects on both the growth and protein digest-ibility of rats as well as on the in vitro digestion by trypsin. More recently Ashes et al. (1984) observed that formaldehyde and glutaraldehyde treatment of casein significantly reduced its absorption from the small intestine in sheep. Although there is no evidence for any toxicity of products produced by formal-dehyde-treatment of food proteins, inhalation of the free compound has indeed been shown to cause nasal cancer in rats and has been suggested to be respons-ible for the high incidence of brain cancer found among anatomists, pathologists, embalmers and morticians (Pearce, 1984).

There is a large amount of literature on the reaction of formaldehyde with amino acids and proteins (French & Edsall, 1945; Kelly et al., 1977) as well as its general chemistry (Walker, 1964). Most reviewers have emphasised that a variety of reactions may occur between formaldehyde and proteins, their relative importance varying depending upon the conditions used. For example, formaldehyde is capable of crosslinking reactions with the formation of methylene bridges between amino acid units, a property not shown by other monoaldehydes (Galembeck et al., 1977). It would appear that although it can also react with sulfhydryl and other groups, the first major reaction of formaldehyde with proteins under cold aqueous conditions close to neutral pH, is with the ε-NH$_2$ group of lysine to form a methylol derivative (Fig. 4). As in the case of F-L (see § 2.1.2), the ε-amino methylol derivative decomposes during acid hydrolysis in 6M HCl at 110° C (Bizzini & Raynaud, 1974) to release free lysine and thus, the TL method is not sensitive to this type of damage. Although it is not known whether animals or humans can utilise the methylol derivative as a source of lysine, it is unlikely that the relatively mild temperature and acidic con-ditions of the stomach together with the short time food spends in the stomach, would result in any significant decomposition of the derivative which is
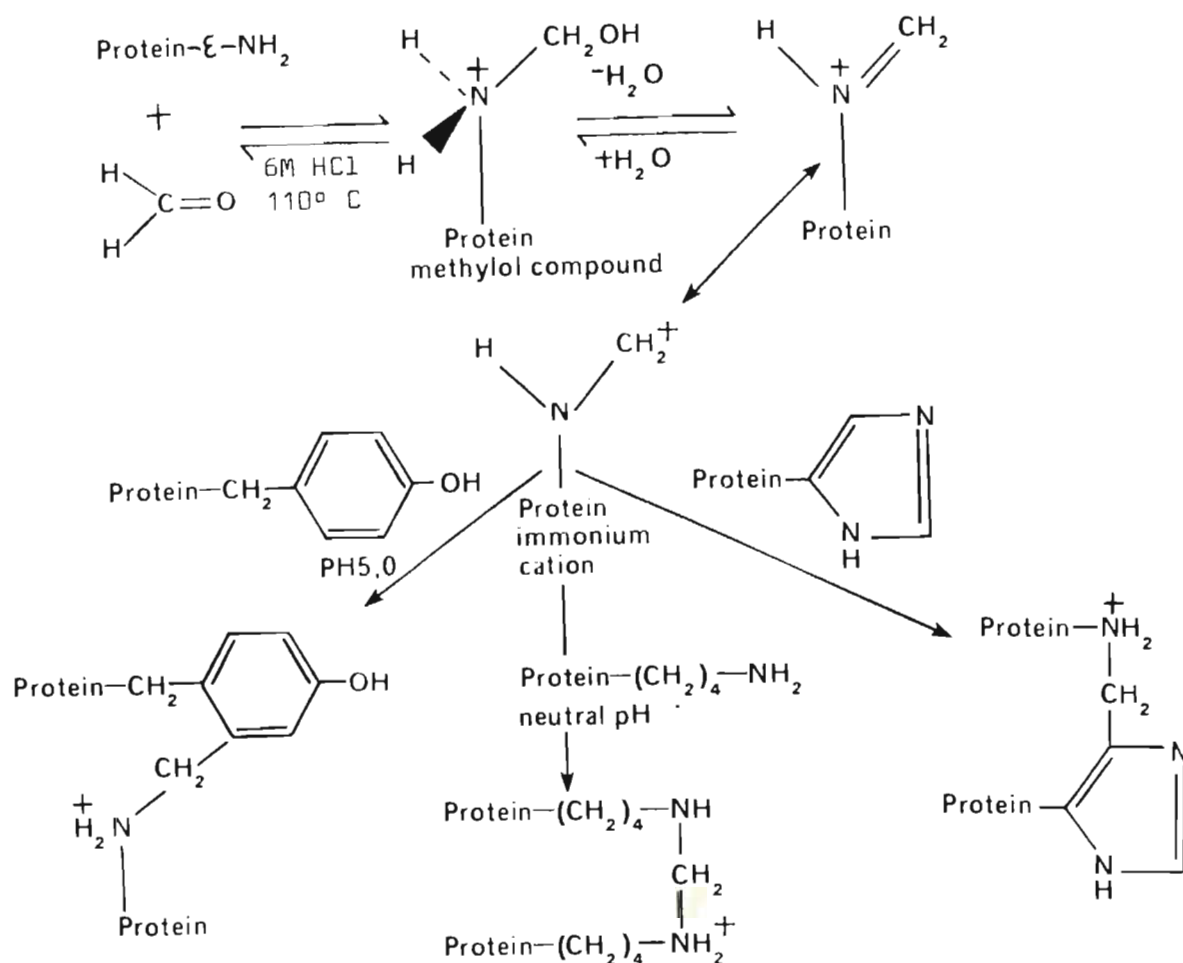
FIGURE 4   Protein-formaldehyde interactions

therefore probably nutritionally unavailable. Following methylol formation,
the derivative could then either become reduced to methyllysine (M-L)
(Reis & Tunks, 1973) which is almost totally unavailable to mice (Friedman &
Gumbmann, 1979), or more likely react further via the Mannich reaction (Fig. 4)
to form methylene bridges possibly with the ring carbons of tyrosine and
histidine, other ϵ-NH$_2$ groups (Fraenkel-Conrat & Olcott, 1948; Means & Feeney,
1971; Bizzini & Raynaud, 1974) or even with DNA (Siomin et al., 1973). To
date only the Lys-CH$_2$-Lys and Lys-CH$_2$-Tyr derivatives have been isolated, the
latter from hydrolysates of formaldehyde-treated tetanus and diphtheria toxins
(Means & Feeney, 1971). Crosslinking between lysine residues has been reported
to occur more readily at neutral pH, whereas that between lysine and tyrosine
occurs primarily at pH 5,0 (Warren et al., 1974).

Hurrell and Carpenter (1978) using borohydride to reduce the methylol com-
pounds to acid-stable M-L (Means & Feeney, 1968) found a relatively small
decrease in the lysine recoverable by acid hydrolysis compared to that detect-
ed by the direct FDNB method. This suggested that most of the methylol com-
pounds formed had proceeded to the crosslinkage stage in keeping with the
severely reduced digestibility of formaldehyde-treated protein samples. As
expected lysine in the acid-stable methylene-bridge form was also found to
be totally unavailable to ruminants (Ashes et al., 1984), chicks and rats
(Hurrell & Carpenter, 1978). Should the existence of the $Lys-CH_2-His$ deriva-
tive be confirmed, it may prove to be toxic since it is structurally similar
to both gizzerosine (Fig. 1 f) which causes gizzard erosion in chicks
(Okazaki et al., 1983) and histidinoalanine (Fujimoto et al., 1982) which
might have similar pathological effects as lysinoalanine (LAL).

To summarise, formaldehyde preferentially reacts with lysine residues in a
protein to form a methylol derivative which is unstable in 6M HCl (110° C)
but probably nutritionally unavailable as a source of lysine. Most of these
derivatives are either reduced to unavailable M-L or form methylene bridges
possibly with tyrosine, histidine or other lysines which lower both protein
digestibility and lysine availability. There is no evidence for toxicity
of these products but their structural similarity to other toxic compounds
as well as the publisised carcinogenic effects of free formaldehyde, strengthen
the importance of further investigation and if necessary control and monitor-
ing of the situation.

2.2   METHODS FOR THE DETECTION OF AVAILABLE LYSINE

Only the historical development and basic principles of the various methods
applied in this study are reviewed here. Available evidence regarding the
relative performance of the different methods for the different types of
lysine damage are discussed in later chapters where comparisons are made
with the results obtained in this thesis.

2.2.1   Fluorodinitrobenzene-reactive lysine

1-Fluoro-2,4-dinitrobenzene (FDNB) was the first reagent used for measuring
reactive lysine in foodstuffs (Carpenter & Ellinger, 1955; Carpenter, 1960)
(Fig. 5). It reacts with the free $\epsilon-NH_2$ groups of lysine in the protein mole-
cule and after acid hydrolysis, the yellow ϵ-dinitro-phenyllysine
(ϵ-DNP-lysine) produced is measured colorimetrically at 435 nm.

protein-bound—ε—dinitro-
phenyllysine
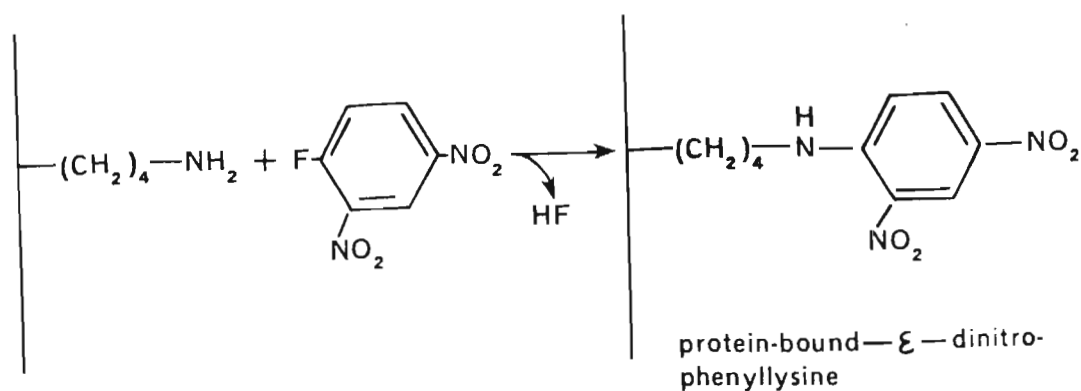
FIGURE 5   Reaction of fluorodinitrobenzene with lysine (Sanger, 1945;
            Carpenter & Ellinger, 1955)

Although this procedure has proved useful for the analysis of most animal
products (Carpenter et al., 1957; Carpenter, 1960), a number of modifi-
cations have been necessary, particularly in the case of plant materials and
milk products containing significant quantities of carbohydrates (Bodwell,
1976; Holsinger & Posati, 1975). Carbohydrates interfere in at least two
ways. They can reduce nitro groups in the chromophore during acid hydrolysis,
producing derivatives with altered spectroscopic properties (Friedman, 1982).
Secondly, they react with tryptophan during hydrolysis to form a brown preci-
pitate called humin which is reported to adsorb DNP-lysine thereby signifi-
cantly lowering its recovery (Matheson, 1968).

Nonspecificity of the dinitrophenylation reaction presents a further compli-
cation as coloured derivatives of other amino acid side chains may be formed.
Significant interference from DNP-arginine (Conkerton & Frampton, 1959) was
eliminated by using methoxycarbonyl chloride (Bruno & Carpenter, 1957), but
this led to interference from a coloured histidine derivative (Carpenter et al.,
1959) which was corrected for by extraction of the lysine-methoxycarbonyl
reaction product with diethyl ether and use of the resultant blank value to
correct for this interference (Carpenter, 1960).

DNP-lysine is also partially unstable in strong acid. Carpenter and co-workers
(Carpenter & March, 1961; Carpenter et al., 1963) applied a factor (1,09) to
correct for losses of approximately 8% of the DNP-lysine during acid hydro-
lysis. Booth (1971) improved the DNP-lysine yield by using a longer and more
complete DNP-protein hydrolysis and more thorough washing of the hydrolysates.
Different correction factors were suggested, namely 1,05 for carbohydrate-
free material, 1,09 for very soluble albumins, 1,20 for wheat and other cereals
and 1,14 for beans, ground nuts and maize. However, as pointed out by
Bodwell (1976) use of such correction factors may not always be satisfactory.

In attempts to overcome the effects of interfering compounds, many workers
resorted to separation of the DNP-lysine by means of paper (Baliga et al.,
1959), ion exchange (Hurrell & Carpenter, 1974), thin layer (Datta & Datta,
1977), or molecular exclusion (Datta, 1976) chromatography. Blom et al. (1967)
used chromatographic separation on polyamide powder and Amberlite CG-120
followed by polarographic detection of the eluted DNP-lysine. However, since
all these methods are based on the recovery and determination of DNP-lysine,
they are still subject to DNP-lysine losses mentioned above.

Despite the above difficulties the FDNB-reactive lysine method remains the
most widely used of all procedures for estimating available lysine and provided
suitable precautions are taken to minimise undersirable reactions, results can
correspond closely with those from both biological evaluation and in vitro
enzymic digestion (Mauron, 1977 & 1980; Hurrell et al., 1983). For this
reason the FDNB method was adopted in the present study as the standard pro-
cedure for comparative purposes.

2.2.2   Dye-binding lysine

Dye-binding procedures are simple, rapid and economical and can be successfully
semi-automated. They could therefore find wider application in the food indus-
try for the monitoring of the extent of heat damage to proteins in processed
foods. The acid azo dyes such as acid orange 12, orange G, remazol blue and
cresol red have proved most popular as they have the capacity to strongly bind
lysine, histidine and arginine residues of proteins in acid medium (Fraenkel-
Conrat & Cooper, 1944). This property termed dye-binding capacity (DBC = dye
bound by His + Arg + Lys) was used to provide indirect estimates of the total
protein content of food samples such as milk which generally have constant
proportions of the individual amino acids (Udy, 1971). Subsequently Bhatty and
Wu (1975) discovered that a high ratio of DBC to Kjeldahl 'crude' protein in
a sample could be used to select high-lysine cereal strains and this same ratio

was also found to be a sensitive indicator of protein quality in fish meal (Moran et al., 1963) and rapeseed meal (Goh et al., 1979).

Various acid azo dyes have also been used to estimate reactive lysine (Frölich, 1954; Pruss & Ney, 1972; Möller, 1973; Khan, 1978) however, results are seriously affected by the different proportions of basic amino acids in the different proteins, necessitating the correction of values for arginine and histidine binding. In an attempt to solve these problems, Hurrell and Carpenter (1976a) introduced their rapid dye-binding lysine (DBL) procedure. In this technique the dye (orange 12) binding capacity of a sample is measured before and after propionylation of a protein sample (Fig. 6), a step which masks the $\epsilon-NH_2$ groups of lysine and prevents them from reacting with the dye. The DBL is calculated by subtracting the DBC of the propiony-lated material (measuring histidine + arginine) from the DBC of the untreated material (measuring reactive lysine + arginine + histidine). This method was subsequently improved by Hurrell et al. (1979) and also semi-automated (Pro-meter MK II N. Foss Electric 69, Slangerupgade, DK-3400, Hillerod). The manual procedure has, however, proved to be more popular since the Pro-meter system is both expensive and less precise (Hurrell, 1980, pers. commun.).

Other procedures based on similar principles, but blocking the lysine groups with ethyl chloroformate (Sandler & Warren, 1974), trinitrobenzene sulphonic acid (Jones & Lakin, 1976), or fluorodinitro benzene (Hurrell & Carpenter, 1981) have also been developed but do not seem as practicable for routine analysis since the methods are generally much longer and interference from coloured compounds is greater than in the case of the Hurrell and Carpenter (1976) procedure. More recently Hurrell (1980, pers. commun.) has replaced propionic anhydride by acetic anhydride as acylating agent and it is this modi-fied procedure that is evaluated in this thesis.

2.2.3 Succinic anhydride-reactive lysine

Succinic anhydride is a non-volatile, reasonably stable solid which has been widely used for the chemical modification of purified proteins for the purposes of studying a variety of properties and structural aspects (e.g. Klapper & Klotz, 1972; Boosman & Chilson, 1976; Fraser et al., 1976; Kidwai et al., 1976; Strong & Keana, 1976). In food chemistry, succinylation has also proved useful for the improvement of the functional properties of various isolates. Succinylated isolates are more water-soluble, less heat-coagulable in water, lighter in colour and show higher oil absorption, emulsion capacity, gel strength, water hydration, water retention and viscosity, all of vital
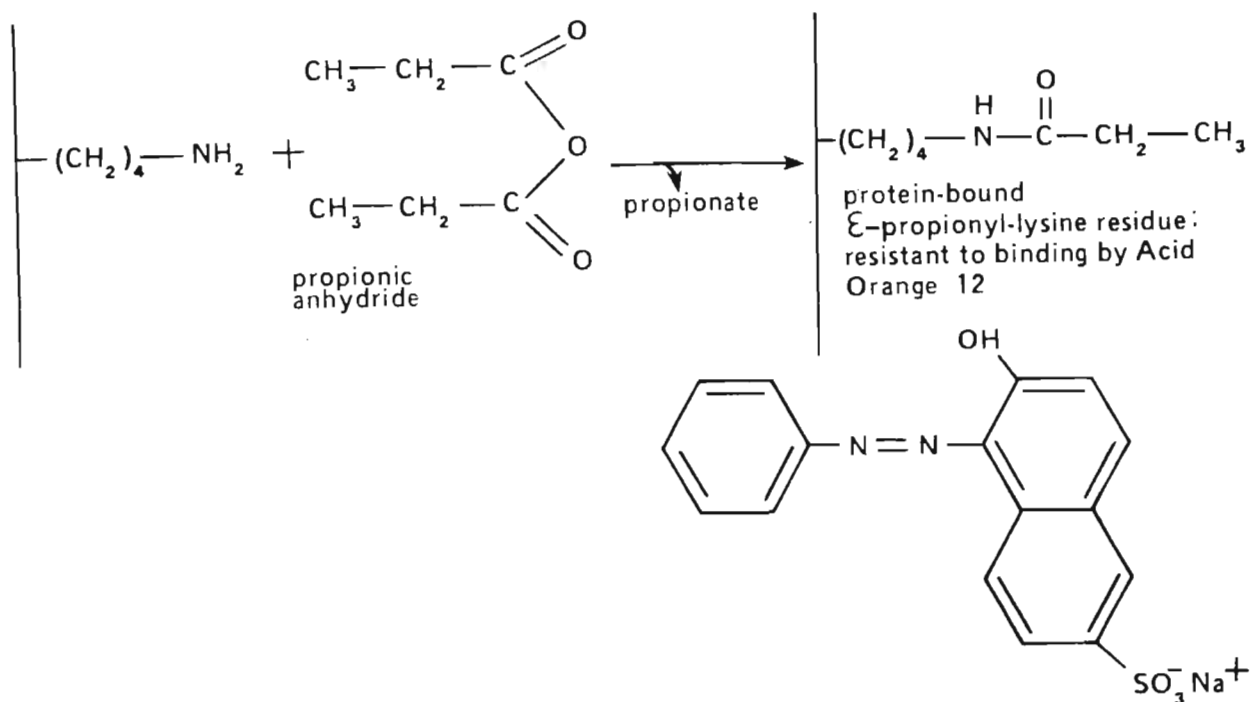
FIGURE 6   Propionylation of lysine residues as part of the DBL method
            (Hurrell & Carpenter, 1976 a)

importance in the food industry (McElwain et al., 1975;   Childs & Park, 1976;
Miller & Groninger, 1976;   Choi et al., 1981 & 1982;   Shukla, 1982).
Succinylation could also find future application as a means of protecting
lysine from processing damage and degradation by ruminant microflora
(Friedman & Broderick, 1977).

Succinic anhydride is known to preferentially react with alpha amino ($\alpha-NH_2$)
and $\epsilon-NH_2$ groups (Habeeb et al., 1958) (Fig. 7 a) but also simultaneously forms
O-succinyl esters of serine, threonine and tyrosine (Gounaris & Perlmann,
1967) (Fig. 7 b - d), S-succinyl esters of cysteine (Simon & Shemin, 1953)
(Fig. 7 e) and succinylimidazole derivatives of histidine (Stadtman, 1955)
(Fig. 7 f).   The fact that the tyrosyl and cysteinyl esters desuccinylate
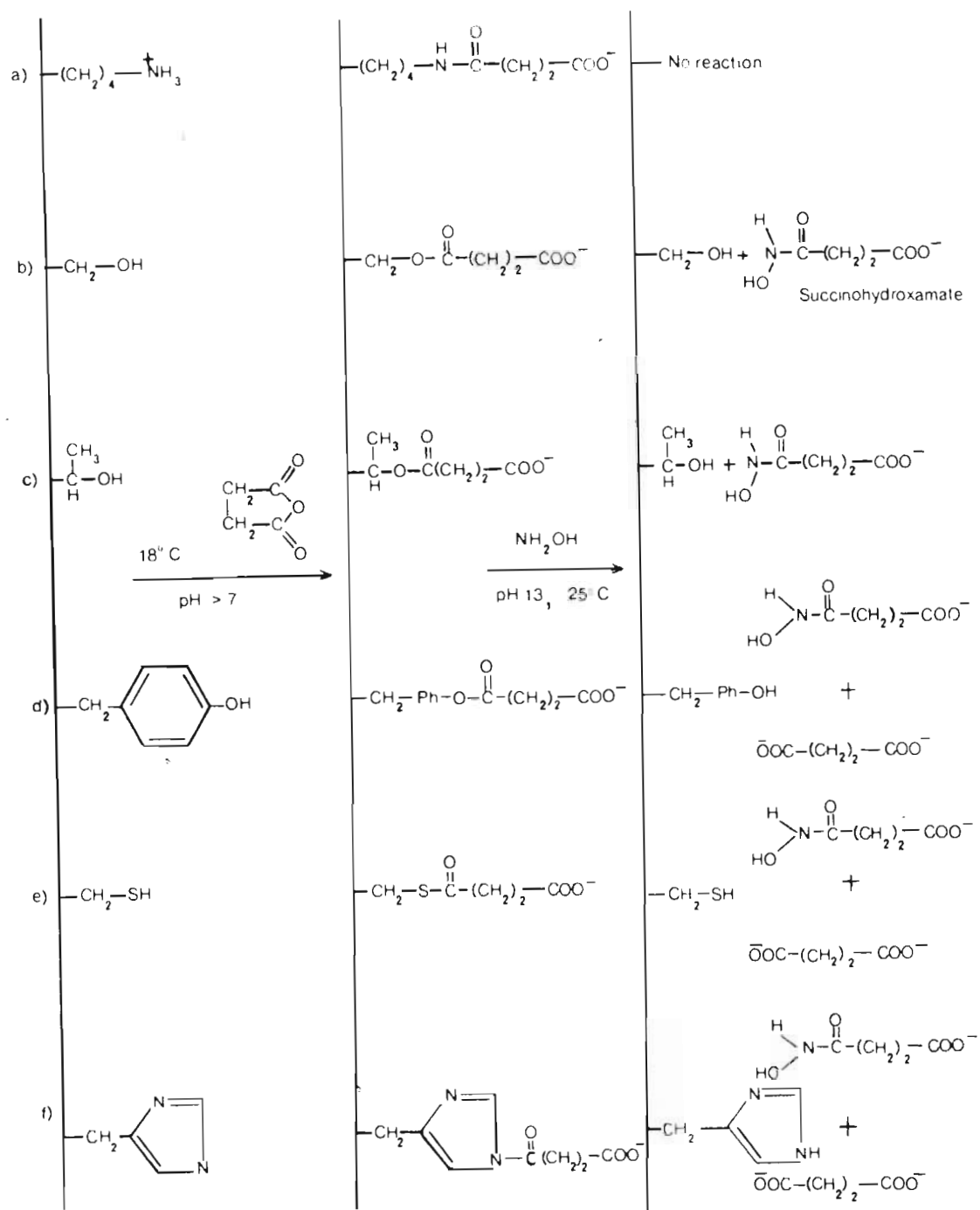
FIGURE 7   Succinylation followed by reaction with alkaline hydroxylamine of
(a) protein-bound lysine;   (b) serine;   (c) threonine;
(d) tyrosine;   (e) cysteine;   and (f) histidine.   O-succinyl tyro-
sine, S-succinyl cysteine and possibly succinylimidazole
derivatives also desuccinylate spontaneously within 3- 4 h to
give free succinic acid residues (Anderson & Quicke, 1984).
Ph = phenyl

spontaneously within 3 - 4 h (Simon & Shemin, 1953; Gounaris & Perlmann, 1967) and that alkaline hydroxylamine deacylates all the above esters (Fig. 7) whereas under these conditions the N-succinyl groups are stable (Gounaris & Perlmann, 1967), prompted the present author to develop a reactive-lysine method (Anderson, 1980; Anderson & Quicke, 1984) based on the specific modification of ϵ-NH$_2$ groups with succinic anhydride.

In the succinic anhydride (SA)-reactive lysine method (Anderson and Quicke, 1984) proteins are succinylated with an 80-fold molar excess of $[^{14}C]$-succinic anhydride relative to total lysine residues and then treated with hydroxylamine (pH 13, 25° C, 5 - 10 min) to remove unwanted O-succinyl esters. Precipitation of the N-succinylated protein with trichloroacetic acid is followed by thorough washing with absolute ethanol to remove any residual label. The extent of amino-labelling is then measured in a scintillation counter and the results expressed in terms of reactive-lysine per unit mass of protein material.

The method has given close to theoretical values for lysyl residues in a variety of pure model proteins (Anderson & Quicke, 1984). However, the method has not yet been tested on either plant or animal foodstuffs and more specifically the different types of lysine damage that can occur during food processing (Anderson & Quicke, 1980 a). This aspect is examined in some detail in the results of this thesis.

2.2.4   Dansyl chloride-reactive lysine

The use of extrinsic fluorescence probes has found wide and invaluable application in both the study of protein structure (Cantor & Timasheff, 1982) and in the cytochemical elucidation of the behaviour, interaction and spatial organisation of specific cellular components in living cells (Wang et al., (1982). The most useful extrinsic probes have absorption peaks at a wavelength longer than 300 nm since this eliminates interference due to the intrinsic fluorescence of the protein molecules.

Although derivatisation of amino acids with fluorescent reagents such as 1-dimethylaminonaphthalene-5-sulphonyl chloride (dansyl chloride or DAN) has been widely used for the identification and determination of free amino acids including lysine (Roth & Jeanneret, 1972; Smith & Best, 1981; Tapuhi et al., 1981), only two such methods have been published for the estimation of protein-bound reactive-lysine residues (Christoffers, 1976; Goodno et al., 1981). This is primarily due to the susceptibility of the procedures to a variety of complicating factors. Observed fluorescence is not only dependent on intensity of light source and absorbance of the material as in absorption spectroscopy,

but also on the quantum yield which is strongly affected by the presence of other fluorescent and non-flourescent substances. The quantum yield is dependent on the relative rates of the fluorescence process and of processes that can depopulate the excited singlet state nonradiatively such as internal conversion, phosphorescence and quenching due to collision with other molecules (Cantor & Timasheff, 1982). As pointed out in section 4.4, these factors create specific problems when analysing insoluble suspended protein material and when comparing results obtained for different materials containing different proportions of interfering substances.

Goodno and co-workers (1981) used ε-amino group modification with o-phthalaldehyde to produce a fluorescent adduct whose fluorescence intensity, corrected for N-terminal amino-group contributions, was a linear function of the lysine content of various proteins and peptides. Although the method performed well with 'pure' model proteins, its full usefulness will depend on its ability to determine the reactive lysine content of foodstuffs containing a variety of potentially interfering substances. Optical quenching of fluorescence by insoluble material could present a major problem, as sodium dodecyl sulphate (SDS) and mercaptoethanol employed in their method, are unlikely to solubilise all food proteins (see also § 4.4.1).

Christoffers (1976) used dansyl chloride modification of $\varepsilon-NH_2$ groups (Fig. 8) for the specific analysis of reactive lysine in both model proteins and various legumes and cereals. Consecutive treatments of sample with sodium bicarbonate solution and absolute ethanol are used to denature the protein thus rendering the $\varepsilon-NH_2$ groups more accessible to the dansyl reagent applied in the next step. The bicarbonate is also important for maintaining a basic medium during the dansylation step as well as in the cuvette since dansyl-protein does not fluoresce under acidic conditions. The fluorescence intensity of an aqueous suspension of the labelled material is read in a filter fluorometer specially designed by Christoffers (1976) to minimise optical quenching by insoluble suspended material. This method, which was briefly tested by Christoffers (1976) on various grains shows promise as a simple, rapid and inexpensive method for available lysine but first the problem of relating fluorescence intensity to reactive lysine values must be overcome. This thesis describes some attempts to solve this and other related problems and compares values obtained with this procedure for various types of lysine-damaged protein with those obtained by the more established methods.
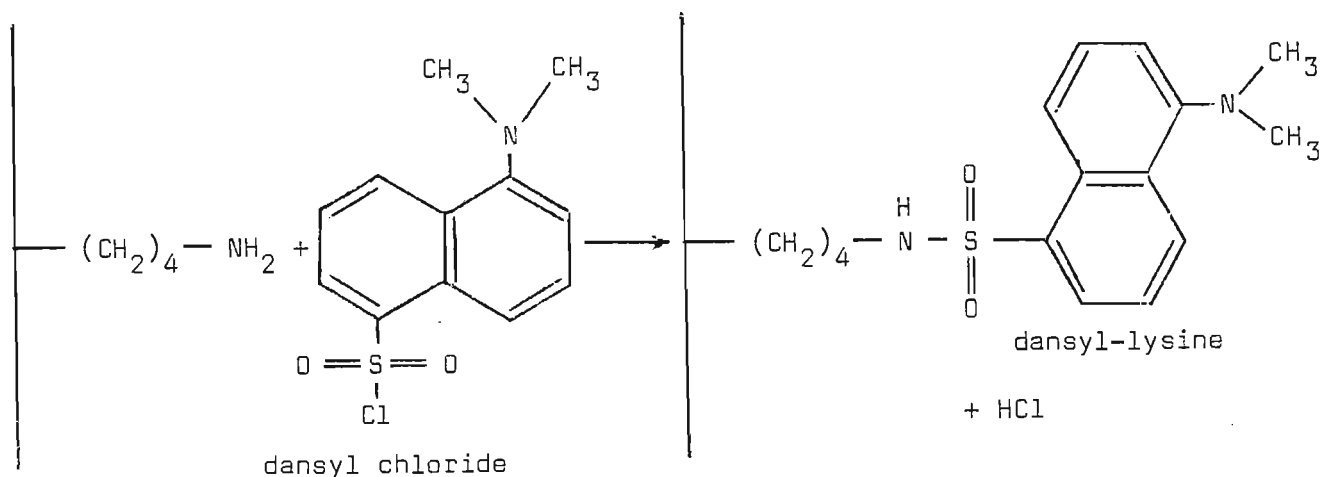
FIGURE 8 Reaction of 1-dimethylaminonaphthalene-5-sulphonyl chloride
with lysine

2.2.5 Ninhydrin-reactive lysine

In extensive studies on the mechanism, kinetics and stoichiometry of the nin-
hydrin reaction, Friedman and co-workers have shown that it is possible
to quantify lysine residues in a protein by reaction with ninhydrin and measure-
ment of the liberated coloured complex without the necessity of prior hydrolysis
and chromatographic analysis (Friedman & Sigel, 1966; Friedman & Williams,
1973 & 1974). However, the mechanism of this reaction in contrast to that
involving free amino acids, remains unelucidated and because protein amino
groups give a considerably lower ninhydrin colour than those of free amino acids
(Slobodian et al., 1962; Friedman & Williams, 1973; Anderson, 1979, unpublished
data), it is necessary to incorporate a correction factor for this into any
formula designed to express the results in terms of 'amino acid equivalents'.
Furthermore, a correction for $\alpha-NH_2$ group contributions should also be made
particularly if the results for different proteins are to be compared.

In the present study, the ninhydrin (NIN) method used by Friedman and Broderick
(1977) for assessing the extent of amino modification of casein was adapted
with minor modifications for the estimation of reactive lysine. As a preliminary
study this method was then tested for its ability to detect lysine damage in
formaldehyde-treated lactalbumin (Ch. 7). Since the above work was completed,
Friedman et al.(1984) published a new NIN-reactive lysine method with some
interesting similarities and differences to the presently reported procedure.

In the latter more extensive study Friedman et al. (1984) found that on a molar
basis the ninhydrin colour produced by a variety of model proteins varied from

63 to 109% of that of Ruhemann's purple derived from free leucine. This result indicates the importance of determining a new correction factor for each type of protein (see § 2.7.4). They suggested that in the case of mixed food proteins, the correction factor applicable to the most predominant protein could be applied. This may, however, introduce serious errors in some results since certain minor ninhydrin-positive substances could make major contributions to the overall ninhydrin colour of the sample. Interestingly the present author found a correction factor of 67,8% for lactalbumin which compares very favourably with 63,8% value obtained by Friedman et al. (1984) for the same protein. Those workers also corrected for a 9,17% α-NH$_2$ group colour contribution for lactalbumin which is similar to the 7,74% correction applied by the present author (see § 3.9.3). In the case of materials containing significant quantities of free amino acids and other ninhydrin-positive compounds, a further correction would of course be necessary, although none of the correction factors would be important if only the relative changes in reactive lysine of one specific material due to the effects of food processing were being examined.

To conclude, the Friedman et al. (1984) and modified Friedman and Broderick (1977) methods show promise as new reactive lysine methods but before their full usefulness can be gauged it will be necessary to apply them to all the different categories of lysine-damaged materials. As a beginning the present author has applied the modified Friedman and Broderick (1977) method to formaldehyde-damaged lactalbumin the results of which are presented and discussed in Chapter 7.

2.2.6   Microbiological assay of lysine availability with
         Tetrahymena pyriformis W

Almost 40 years ago Rockland and Dunn (1946) used the microbe Tetrahymena gellii to determine the tryptophan content of unhydrolysed casein. Subsequently other microbiological assays employing the organisms Clostridium perfringens (Boyd et al., 1948), Streptococcus faecalis (Halvay & Grossowicz, 1953), Streptococcus zymogens (Ford, 1962; Boyne et al., 1975) and Aspergillus flavus (Mohyuddin et al., 1978) were developed for amino acid and protein quality. Unfortunately all these organisms lack an absolute requirement for lysine. By contrast the ciliated protozoan Tetrahymena pyriformis W (T. pyriformis) which was first used by Pilcher and Williams (1954) for protein quality determinations, has an essential amino acid requirement similar to those of both the growing rat (Kidder & Dewey, 1961) and humans (Evans, 1978). This characteristic

encouraged the extensive use of this organism for the assessment of both pro-
tein quality (e.g. Evans et al., 1979 a and b; Wang et al., 1980; Bookwalter &
Kwolek, 1981; Janitz & Grodzka-Zapytowska, 1982) and the bioavailability of
various amino acids especially lysine (e.g. Stott & Smith, 1966; Boyne et al.,
1967; Shorrock, 1976; Shepherd et al., 1977; El-Sherbiny et al., 1980;
Hurrell et al., 1983). Of great advantage is that T. pyriformis is strongly
proteolytic (Dickie & Liener, 1962; Boyne et al., 1967) and can utilise intact
protein as sole source of amino acids (Rockland & Dunn, 1946). Despite this
however, various workers have found that enzymic pre-digestion of test samples
is necessary to obtain similar results to those of rat and chick assays
(Shorrock, 1976; Shepherd et al., 1977; Baker et al., 1978).

Over the years there have been significant improvements in the Tetrahymena (TET)
assay particularly the way in which cell viability is measured in the presence
of interfering food particles. Earlier workers such as Fernell and Rosen (1956)
used differential centrifugation in sucrose solutions and electromigration tech-
niques to separate food particles from the protozoa whose N-content was related
to the number of organisms. Since this technique showed limited success many
workers preferred to measure growth in their test cultures by bacterial counting
in a haemocytometer. However, in view of the tediousness of this approach
Wang et al.(1979) recommended one of the following chemical techniques for esti-
mating T. pyriformis growth: reduction of colourless 2,3,5-triphenyl tetra-
zolium chloride to red triphenyl-formazan by endogenous dehydrogenase enzymes
(Kaestner et al., 1976), O_2 uptake by cell cultures (Hill, 1972) or assessment
of endogenous ATP with firefly luciferase (Forsberg & Lam, 1977). Although the
use of these criteria need further investigation, Wang et al. (1979) have shown
that values obtained with all three techniques were highly correlated with
those obtained by a rat PER test. In 1977 Shepherd et al.showed that there is
a linear relationship between the number of T. pyriformis cells and their endo-
genous concentration of tetrahymanol, a pentacyclic terpene that can be extract-
ed and determined by gas chromatography. Unfortunately the technique is rather
laborious and therefore not suitable for routine analysis. Other workers in
using the relationship between cell count and endogenous concentrations of
2-aminoethyl-phosphonic acid (Kandatsu & Horiguchi, 1962) which can be assayed
with ninhydrin (De Koning, 1966), found that this technique gave poor corre-
lations with rat net protein utilisation (NPU) values (Maciejewicz-Ryś &
Antoniewicz, 1978).

All the above techniques with the exception of the haemocytometer method, may
however, be considered as indirect methods of assaying culture growth and

therefore will be more prone to error than any direct counting technique.
Currently therefore electronic counting with a Coulter counter is most favoured
and has been used with great success by Evancho et al. (1977) who also used a
channeliser attachment to correct for errors due to differences in cell size.
However, the Coulter counter does not discriminate between cells and undigested
food particles while difficulties may be encountered through blocking of the
aperture with debris (Ford, 1981). To remove this interfering material,
Teunisson (1971) advocated an elutriation procedure to separate cells from debris
prior to counting, whereas Baker et al. (1978) successfully used coarse filtra-
tion of their bromelain digestate prior to inoculation. The latter method of
course makes the assumption that the particulate material that remained un-
digested would also not be utilised by T. pyriformis.

Results have been reported from time-to-time revealing the unsuitability of
T. pyriformis for use as a test organism in routine bioassays (e.g. Bergner
et al., 1968; Evans et al., 1977; Lee et al., 1978) and its inconsistency
between laboratories (Bookwalter & Kwolek, 1981). This may be due to the dose-
dependent inhibition of Tetrahymena cultures by other food components such as
fat and various additives including spices, propionates, benzoates, sorbates
and the meat-curing adjuncts nitrate, erythorbate and ascorbate (Janitz &
Grodzka-Zapytowska, 1982). On the other hand, LAL which is totally unavailable
to rats (Erbersdobler, 1980), can be partially utilised by Tetrahymena as a
source of lysine (Sternberg & Kim, 1979). Since the 'early' Maillard product
F-L which is totally unavailable to the rat and human (Finot et al., 1978 a;
Mori et al., 1980), has been found to inhibit disaccharidase activity (Lee et al.,
1977) and cause nephrocytomegalia in rats (Erbersdobler et al., 1981), while
'late' Maillard products appear to be mutagenic (Omura et al., 1983), possess
bactericidal properties (Maeshige & Nakagawa, 1974) and cause adverse physiolo-
gical changes in rats (Tanaka et al., 1977), it was important to test the effect
of such material on Tetrahymena growth and thus the usefulness of this organism
for the assessment of available lysine in Maillard-damaged material. This
specific problem was tackled in this thesis, the results of which are presented
and discussed in Chapter 8.

CHAPTER 3

MATERIALS AND METHODS

3.1 PREPARATION OF MODEL PROTEIN SAMPLES

3.1.1   Soyaprotein heated in the absence of reducing sugars

Isolated soyaprotein (Brand Purina Assay Protein RP100) was employed in these studies and found to contain on a dry matter basis 96% 'crude' protein (CP), 1,4% ash, 0,3% fat, 0,2% 'crude' fibre and 2% N-free extracts. This material was used to prepare 12 samples in which 90 parts by mass soyaprotein was heated with 10 parts by mass water in sealed metal containers (250 ml) at temperatures of 90 , 110 or 130° C for either 0,5 , 1 , 2 or 4 hours. A further 3 samples were prepared by heating equal masses of isolated soyaprotein and water in large open vessels for 24 h at either 95 , 138 or 160° C. The latter 3 samples were prepared in bulk for the animal experiments carried out by Erbersdobler (see Table 5 and Erbersdobler & Anderson, 1983). Hence the large open vessels. All 15 samples were milled (Retsch Ultracentrifugal Mill ZM1) to pass an 80 μm sieve before being analysed for TL and by the FDNB, DBL, SA and DAN procedures.

3.1.2   Maillard-damaged soyaprotein

A second series of 36 samples was prepared by heating 80 parts by mass of that soyaprotein isolate (see § 3.1.1) in sealed metal containers at different temperatures (90 , 110 , 130° C) for different periods of time (0,5 , 1 , 2 , 4 h) in the presence of 10 parts by mass of glucose or molecular equivalents of lactose or xylose and 10 parts by mass of water. All samples were milled (Retsch Ultracentrifugal Mill ZM1) to pass an 80 μm sieve and then analysed for TL as well as by the FDNB, DBL, SA and DAN procedures. Thirty-two of these samples (i.e. all except those heated at 130° C in the presence of xylose) were also used in the Tetrahymena assays reported in Chapter 8.

3.1.3   Formaldehyde-damaged lactalbumin

All chemicals were of analytical reagent grade.

0,2M Sodium acetate buffer (pH 5,0).   Sodium acetate-3 hydrate (8,8452 g) was dissolved in 250 ml of distilled water, 2 ml of glacial acetic acid added and the whole made up to 500 ml with distilled water. The pH was adjusted as required with either 40% (w/v) NaOH or concentrated HCl.

0,2M Sodium borate buffer (pH 9,0).   Boric acid (6,183 g) was dissolved in 200 mℓ of distilled water, 1,48 g sodium hydroxide pellets added, allowed to dissolve, cooled and then made up to 500 mℓ with distilled water.   The pH was adjusted as required with 40 % NaOH or concentrated HCl.

Formaldehyde solutions.   Solutions containing 0,5 , 1,5 or 8,0 g of formaldehyde per 100 mℓ of buffer were prepared by mixing 2 , 6 or 32 mℓ of 37 % (w/v) formaldehyde with 150 mℓ of either 0,2M sodium acetate buffer (pH 5) or 0,2M sodium borate buffer (pH 9,0).

Treatment of lactalbumin with formaldehyde.   Eighteen formaldehyde-damaged lactalbumin samples were prepared by mixing (magnetic stirrer) 20 g of lactalbumin (Sigma, L 7252), 81,88 % CP at 24° C with 50 mℓ of formaldehyde solution (0,5 , 1,5 or 8,0 % w/v) for 1 h, 12 h or 5 days at a pH of either 5 or 9.   In terms of 'crude' protein the lactalbumin samples had actually been treated with 1,5 , 4,5 or 24,1 g of formaldehyde per 100 g of CP.   Two control samples were prepared by mixing 20 g of lactalbumin with 50 mℓ of either pH 5 or pH 9 buffer for 1 h at 24° C.

After mixing for the specified time all reaction mixtures were treated with 200 mℓ of absolute ethanol to give a final concentration of 80 % ethanol and thoroughly mixed.   The resulting mixture was filtered through a Whatman no. 44 (ashless) filter, washed with two 20 mℓ volumes of absolute ethanol (to remove any residual electrostatically-bound formaldehyde), lyophilised, milled with a Retsch Ultracentrifugal Mill (ZM 1) to pass an 80 μm sieve and then stored at −20° C under nitrogen in McCartney bottles.   After equilibration with the atmosphere these samples contained on average 7,5 % moisture.   All samples were analysed for total amino acids as well as by the FDNB, DBL, DAN and NIN procedures.

3.1.4   Commercial soyabean samples

Twenty-seven defatted commercial soyabean samples containing between 35,5 and 50,5 % CP and 4,79 and 6,98 g lysine per 16 g N were milled with a Retsch Ultracentrifugal Mill (ZM 1) to pass an 80 μm sieve and shaken on a Fritsch Analysette sieve shaker fitted with a 75 μm brass sieve (internal diameter 20 cm).   The material that remained behind on the sieve was deemed to have a mean particle size of $\frac{80 + 75}{2} = 77,5$ μm .   This material was used in the analyses described in section 4.4.3 (see Table 3).

3.1.5   Soyabean samples of different particle size

Three of the 27 commercial soyabean samples, described in section 3.1.4 above, containing 5,79 , 6,34 and 6,85 g lysine per 16 g N and 46,1 , 48,6 and 46,6 % CP respectively, were each used to prepare 11 sub-samples ranging in mean particle size from 45 to 275 µm .   As illustrated in Figure 9 a Retsch Ultra-centrifugal Mill ZM1 was used to prepare 4 lots of material of particle size less than 80 , 120 , 200 and 500 µm respectively.   This material was then placed

Coarse Soyabean Meal

Retsch Ultracentrifugal Mill

milled to

80 µm   120 µm   500 µm   200 µm

300 µm
250 µm
150 µm
125 µm
106 µm
90 µm
75 µm
63 µm
53 µm
45 µm
pan
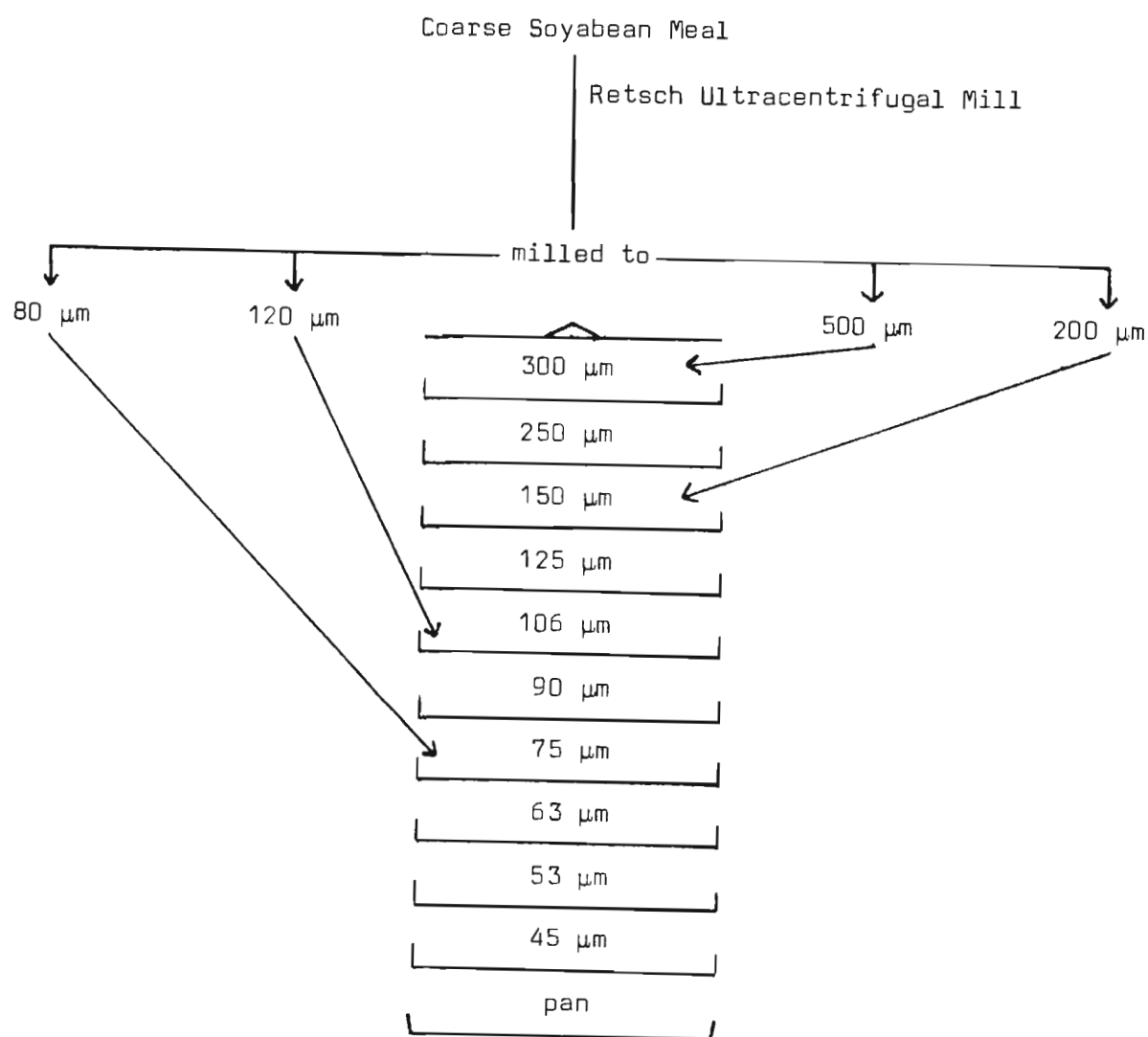
FIGURE   9   Preparation of soyabean meals of varying particle size in the range of 49 to 400 µm . Arrows indicate the point of introduction of the four milled fractions into the sieve stack. For details, see text

at appropriate levels in a stack of 10 brass sieves (internal diameter 20 cm)
(Fig. 9) and shaken for 60 min with a 2 s pause every 30 s on a Fritsch Analy-
sette sieve shaker. Probably due to the electrostatic problems associated
with the dry sieving of charged protein material, a negligible amount of
material passed through the 45 µm sieve into the pan and therefore this sample
was eliminated from the experiments described in section 4.4.3. The mean
particle size of the material in each sieve compartment was calculated by
averaging the sieve sizes immediately above and below each compartment, e.g.
the mean particle size between sizes 300 and 250 µm was taken as
$\frac{300 + 250}{2} = 275$ µm . The prepared samples were used exclusively for the study
of the effect of particle size on the extent of fluorescence quenching in the
DAN procedure (§ 4.4.3).

3.1.6   Model proteins

Eleven 'pure' proteins were used to obtain the standard curve presented in
Figure 15 (§ 4.4.3). The proteins studied are listed in Table 1 together with
all relevant details. The total lysine values were obtained as described in
section 3.3.

TABLE 1   Details of various model proteins discussed in section 3.1.6

Protein	Source	Molecular Mass	Total Lysine µmoles per g
Bovine insulin	Sigma No. I-5500	5 733	152,83
Ribonuclease A	Sigma No. R-5500	13 690	588,10
Egg white lysozyme	Sigma No. L-6876	14 400	383,92
Papain	BDH No. 39030	20 700	287,54
Casein	BDH No. 44020	23 600	483,09
α-Chymotrypsin	Miles-Serevac	25 637	489,99
Pepsin	Koch-Light Laboratories	34 600	24,91
Bovine β-lactoglobulin	Sigma No. L-0130	37 700	725,58
Ovalbumin	Sigma No. A-5503	44 000	396,62
Bovine serum albumin	Sigma No. A-7638	66 500	795,45
Yeast hexokinase	Sigma No. H-5000	96 600	558,84

3.2 ROUTINE ANALYSES

3.2.1   Moisture

An empty porcelain crucible (5 mℓ) complete with lid was heated in a hot-air
oven at 105° C for approximately 8,5 h (i.e. to constant mass), then immediately
placed in a vacuum dessicator, allowed to cool for exactly 1 h and its mass
determined. Approximately 100 mg of the protein sample was then added, its
mass accurately determined and then heated, cooled and weighed as described
above. The moisture content was taken to be the mass loss of the sample after
the heat treatment. All samples were analysed in duplicate.

3.2.2   'Crude' protein

The 'crude' protein (CP) content of all samples was estimated as N x 6,25 .
N-content was determined in duplicate using a macro-Kjeldahl digestion
(potassium sulphate-mercuric oxide catalyst). After treatment with sodium
thiosulphate to neutralise the effect of mercuric ions, digests were either
treated with NaOH and the released ammonia distilled into saturated boric acid
solution containing a mixed indicator and titrated with 0,1M-HCl, or in the
case of lactalbumin digests, ammonia was determined with an Auto Analyser
(Technicon Instruments Co. Ltd., Hamilton Close, Basingstoke, Hampshire, U.K.)
using the nitroprusside-phenate-hypochlorite reagent system (Fleck & Munro,
1965).

3.3 TOTAL AMINO ACIDS

All soya preparations were analysed for total lysine (TL) by conventional
amino acid analysis (Biotronic LC 6000 or Kontron Liquimat III) following
digestion of 500 mg samples under nitrogen with 800 mℓ of 6M HCl (110° C, 24 h)
using norleucine as internal standard.

Formaldehyde-damaged lactalbumin samples and the model proteins (Table 1) were
analysed for total amino acids on a Beckman 119 C amino acid analyser following
hydrolysis in vacuo of 25 mg of material with 3 mℓ of 6M HCl and using
norleucine as internal standard. All results were expressed as g amino acid
per 16 g N.

3.4 FRUCTOSELYSINE AND LACTULOSYL-LYSINE FROM FUROSINE

Furosine (FUR) concentration was determined in acid (300 mℓ 7,8-M HCl) hydrolysates of the Maillard-damaged soya samples (500 mg) according to the procedure of Brüggemann and Erbersdobler (1968a) with a Biotronik LC 6000 or Kontron Liquimat III amino acid analyser (Erbersdobler et al., 1979). FUR can be simultaneously determined with lysine, lysinoalanine and pyridosine on a long column (Erbersdobler et al., 1979). Since hydrolysis of fructoselysine (F-L) or lactulosyl-lysine (L-L) with 7,8-M HCl consistently releases 50% of their lysyl residues as lysine, 40% as FUR and 10% as pyridosine (Erbersdobler, 1970) (Fig. 10), this relationship was used to calculate the concentrations of F-L (Fig. 29), L-L (Fig. 31), lysine present as fructose-lysine (LFL; Fig. 18) or lysine present as lactulosyl-lysine (LLL; Fig. 19). The following relationships were used:

$$\text{moles (FUR)} \times \frac{100}{40} = \text{moles (F-L) or moles (L-L)}$$

$$\text{i.e. moles (FUR)} \times 2,5 = \text{moles (F-L) or moles (L-L)} .$$

By correcting for differences in residue molecular mass one gets:

$$\frac{\text{mass (F-L)}}{308} = \frac{\text{mass (FUR)}}{254} \times 2,5 \quad \text{i.e.} \quad \text{mass (F-L)} = 3,03 \times \text{mass (FUR)}$$

$$\text{or} \quad \frac{\text{mass (L-L)}}{470} = \frac{\text{mass (FUR)}}{254} \times 2,5 \quad \text{i.e.} \quad \text{mass (L-L)} = 4,63 \times \text{mass (FUR)} .$$

Thus mass (LFL) or (LLL) $= \dfrac{\text{mass (FUR)}}{254} \times 2,5 \times 146,2$

$$= \text{mass (FUR)} \times 1,4 .$$

The LFL or LLL values can be used to calculate the 'available lysine' (AL) presented in Figures 18 and 19:

$$\text{Mass AL} = \text{TL}_{\text{heated sample}} - \frac{\text{mass (LFL) or (LLL)}}{2} .$$

Destroyed lysine (DL)-values (Figs 18 & 19) were calculated as follows:

$$\text{DL} = \text{TL}_{\text{unheated sample}} - \text{TL}_{\text{heated sample}} .$$
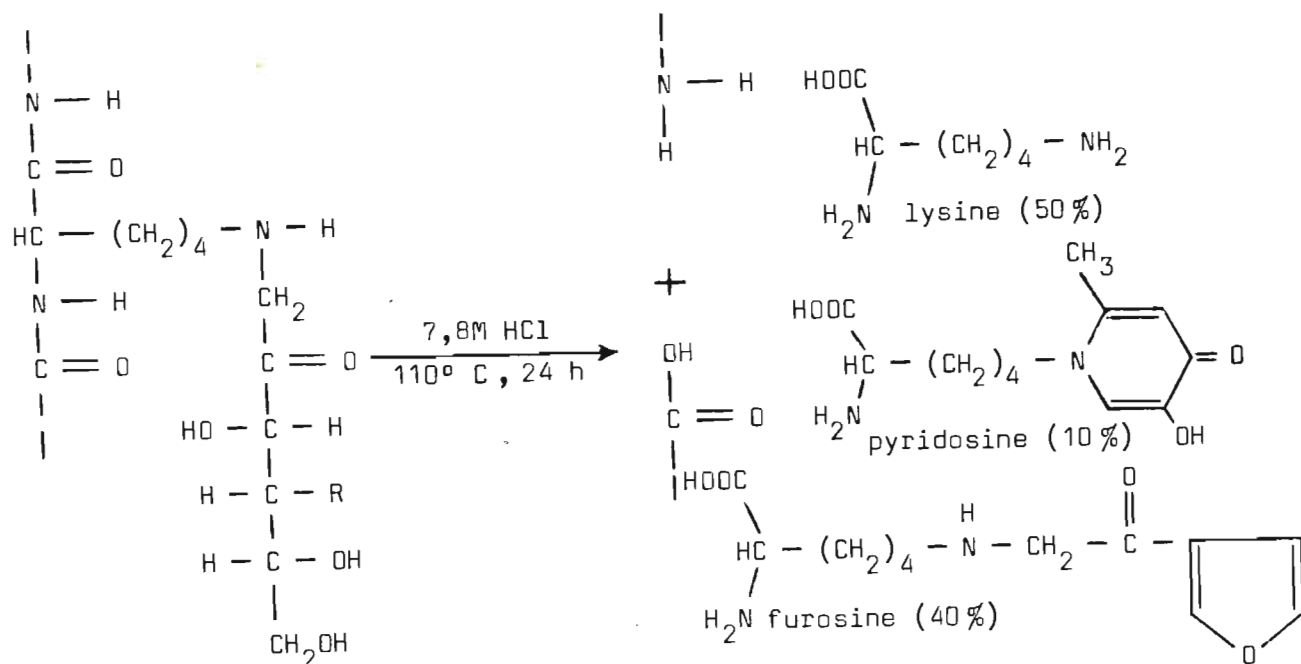
FIGURE 10   Formation of furosine from the acid hydrolysis of protein-
bound ε-N-deoxy-fructosyl-L-lysine (R = OH) or
ε-N-deoxy-lactulosyl-L-lysine (R = O-galactosyl) residues
(Erbersdobler, 1970)

3.5 FDNB-REACTIVE LYSINE (Booth, 1971)

3.5.1   Reagents

All chemicals were of analytical reagent grade.

Standard solution.   Mono ε-N-dinitrophenol-lysine hydrochloride monohydrate
(mol mass 366,77 ; Sigma Chemical Company) (340 mg) was dissolved in 250 mℓ
of 8,1 M-HCl by magnetic stirring for 6 h . To prepare the working standard
10 mℓ of this solution was diluted to 100 mℓ with distilled water. A 2-mℓ
aliquot was calculated to contain the equivalent of 0,1 mg of lysine.

FDNB reagent.   Approximately 0,4 mℓ of 1-fluoro-2,4-dinitrobenzene (Merck)
in 15 mℓ of absolute ethanol was used for each sample analysed. Fresh solu-
tion was prepared for each day's determinations. In view of the potential
vesicant effects of FDNB, polythene gloves were worn when using this reagent.

1M Sodium bicarbonate solution.   Sodium bicarbonate (84 g) was dissolved in
1 litre of distilled water.

Phenolphthalein solution.   Phenolphthalein (40 mg) was dissolved in 100 mℓ
of 60 % ethanol.

3M Sodium hydroxide solution. Sodium hydroxide pellets (120 g) were dissolv-
ed in 1 litre of distilled water.

Carbonate buffer (pH 8,5). Sodium bicarbonate (19,5 g) and sodium carbonate
(1 g) were dissolved in 250 ml of distilled water and the pH adjusted to 8,5
by the addition of small amounts of either 4M-NaOH or concentrated HCl.

Diethyl ether. It was important to use peroxide-free material. This was
prepared by thoroughly shaking 100 g ferrous sulphate (Merck) with 1 litre
of diethyl ether (GR grade; 0,0001% peroxide), followed by purification of
the ether by distillation.

Methyl chloroformate (Merck). This reagent was used undiluted but stored
in the refrigerator to delay decomposition.

3.5.2   Procedure

It was convenient to carry 3 samples in duplicate simultaneously through the
following procedure. A sample containing about 12 mg of reactive lysine was
gently shaken by hand with 10 ml of sodium bicarbonate solution and 4 anti-
bump glass balls in a 100 ml round-bottomed flask (B29 socket and 8 cm long
neck) until the material appeared fully wet. FDNB reagent (15 ml) was added,
the flask stoppered and shaken for 2 h on a mechanical shaker with a gentle
horizontal swirling motion. The ethanol (not the water) was then removed by
evaporation under reduced pressure (water venturi pump) in a Buchi rotavapor-
ator (waterbath, 40° C; condenser with circulating tap water). As a check
that no water was lost, the flask should not have lost more than 12,5 g in
mass.

When the mixture had cooled, 30 ml of 8,1 M-HCl was added to neutralise the
bicarbonate and give a final acid concentration of 6M. The mixture was
gently refluxed (30 cm Liebig condenser) for 16 h on a heating mantle where-
after the condenser was rinsed down with distilled water and the hot contents
of the flask filtered through Whatman no. 541 paper into a 250 ml volumetric
flask. When the filtrate had cooled, it was made up to volume with distilled
water and mixed. Sometimes a precipitate of dinitrophenol formed in which
case time was allowed for it to sediment before proceeding further. Any
unsedimented dinitrophenol that was transferred to the next stage was removed
in the ether extraction.

Aliquots (2 ml) of clear filtrate were pipetted into each of two 10 ml volu-
metric flasks, A and B (blank). The contents of flask B were extracted with

about 5 mℓ of peroxide-free diethyl ether and as much as possible of the
ether layer was removed with the aid of a pasteur pipette connected to a
water pump. The flask was placed in hot water (80° C) until effervescence
due to residual ether had ceased, and then cooled. A drop of phenolphtha-
lein solution was added followed by sufficient NaOH solution to just turn
the solution a pink colour. Carbonate buffer (2 mℓ) and 50 µℓ of methyl
chloroformate were added, the flask stoppered and vigorously shaken. After
about 8 min, 750 µℓ of 8,7M HCl was added slowly with agitation to prevent
frothing and the remaining gas removed by gentle shaking. The solution was
extracted three times with diethyl ether as described above, the flask
cooled and made up to volume with distilled water.

During the pauses between the manipulation of flask B, the contents of
flask A were extracted three times with diethyl ether, the residual ether
removed, the flask cooled and made up to volume with 1M HCl. The absorbances
of both solutions A and B were read in a Hitachi 220 Spectrophotometer
(1 cm cuvette) at 435 nm against distilled water. The difference in absor-
bance (A − B) is the net absorbance attributable to DNP-lysine.

Aliquots (2 mℓ) of DNP-lysine working standard were pipetted into each of
2 flasks C and D and carried through the procedure as described above for
flasks A and B respectively. This was done routinely along with each set of
analyses until confidence was gained in the reproducibility of the results.
The working standard gave a net absorbance of about 0,4 and a blank value of
0,01 at 435 nm in a 1 cm path length cuvette.

3.5.3   Calculation of results

The results were calculated as described by Booth (1971):

$$\text{FDNB-reactive lysine} = \frac{Ms \times At \times v \times 100 \times 100 \times Cf}{Mt \times As \times a \times CP}$$
$$(g \text{ per } 16 \text{ g N})$$

where Ms = mass of lysine equivalent to the DNP-lysine in 2 mℓ of
                 standard solution i.e. 0,1 mg lysine per 2 mℓ standard
                 solution (§ 3.5.1);

          Mt = mass of test material in mg ;

          As = net absorbance of standard;

          At = net absorbance of test sample;

           v = total volume of filtered hydrolysate i.e. 250 mℓ ;

           a = aliquot of filtrate taken for analysis i.e. 2 mℓ ;

          CP = percentage 'crude' protein (%N x 6,25) in test sample; and

          Cf = correction factor for hydrolytic losses (see below).

As recommended by Booth (1971) a correction factor of 1,05 was used for soya heated in the absence of reducing sugars and 1,2 for the carbohydrate-rich Maillard-damaged soya samples. A factor of 1,09 was used for the formy-lated lactalbumin (Hurrell & Carpenter, 1978).

3.6 DYE-BINDING LYSINE (Hurrell et al., 1979)

3.6.1 Purification of acid orange 12

Acid orange 12 (C.I. 15970, Sigma Chemical Company) (100 g) was dissolved in 500 mℓ of boiling distilled water, 500 mℓ of absolute ethanol added, the solution brought to the boil and then left to cool for 15 h at 5° C (refriger-ator). The crystallised mixture was filtered under vacuum on a Buchner funnel, washed with absolute ethanol and dried in an oven at 100° C. The recovery at this stage was about 82,5%. The above purification procedure was repeated twice more with recoveries of 70,0% and finally 62,0%. The dye was stored in a vacuum dessicator and kept in the dark at RT. Hurrell (pers. commun.) regarded the dye as 'pure' when a 0,04 mM-solution gave an absorbance at 475 nm of 0,800 in a 1 cm cuvette. The present author, however, found a lower absorbance of 0,75 at 475 nm but recorded a higher reading of 0,83 at 483 nm (see Fig. 11). None of these values changed with repeated recrystallisations.

3.6.2 Dye reagent

Buffer solution (pH 1,3). Distilled water (1 litre), 300 mℓ of glacial acetic acid, 8,5 mℓ of 85% o-phosphoric acid and 5 mℓ of propionic acid were mixed in a 5-litre volumetric flask. Potassium dihydrogen phosphate (17 g) and 14 g of oxalic acid dihydrate were separately dissolved in 1 litre of distilled water at 60° C and then mixed with the contents of the 5-litre volumetric flask. When cool, the whole was made up to volume with distilled water. If necessary the pH was adjusted to 1,3 by the addition of glacial acetic acid or potassium hydroxide.

Dye solution. To prepare 5 litres of dye solution (4 mM), 7,0060 g of acid orange 12 (molecular mass 350,3) was dissolved in 1 litre buffer solu-tion at 60° C and when cool made up to volume with the buffer solution. Sufficient dye solution was prepared to permit the use of a single solution for each batch of analyses. The dye solution was stored at 4° C in a 10-litre plastic aspirator protected from the light by a black refuse bag.

3.6.3   Other reagents

Sodium acetate solution (5 g/100 mℓ).   82,9453 g of sodium acetate tri-
hydrate (molecular mass 136,08) was dissolved in 1 litre of distilled water.
Glacial acetic acid and acetic anhydride were obtained from Merck and used
without dilution.   All other reagents were of analytical reagent grade.

3.6.4   Absorbance spectrum and calibration of dye

An absorbance spectrum was run on a 40 µmole ℓ^{-1} solution of acid orange 12
dye which had been prepared by accurately pipetting 0,5 mℓ of 4 mM-dye stock
solution (§ 3.6.2) into a 50-mℓ volumetric flask and making up to volume
with pH 1,3 buffer solution.   The spectrum was run over the wavelength 400
to 550 nm in a Beckman DU-7 Double Beam Recording Spectrophotometer using a
1 cm cuvette and pH 1,3 buffer solution as blank.

Hurrell et al. (1979) suggested that dye absorbance be read at 475 nm whereas
Walker (1979) used a wavelength of 482 nm.   In the present studies, acid
orange 12 (40 µmole ℓ^{-1}) gave an absorbance peak at 483,35 nm (Fig. 11) while
475 nm corresponded to the steep rising edge of the peak.   All absorbance
readings were therefore taken at a wavelength of 483 nm.

To calibrate the dye reagent, 10 , 20 , 30 and 40 mℓ aliquots of 4 mM dye
solution were pipetted into separate 50 mℓ volumetric flasks and made up to
volume with buffer solution.   Triplicate 0,5 mℓ aliquots of these solutions
and of the 4 mM-dye stock solution were diluted with buffer solution and the
absorbances read at 483 nm.   The calibration equation was calculated by
simple linear regression analysis from the absorbance readings and the corres-
ponding concentrations of dye solution prior to the final dilution step
(i.e. 0,8 , 1,6 , 2,4 , 3,2 and 4,0 mmole ℓ^{-1}).   A new calibration equation
was calculated for each fresh batch of dye reagent.   All equations reflected
excellent linearity over the working concentration range with high correlat-
ions between co-ordinates.

The following is a typical linear regression line:

$$Y_{\text{residual dye}\atop\text{concentration}} = 5,088\ X_{\text{absorbance}\atop\text{483 nm}} - 0,144\ (r = 0,99981;\ n = 15)\ .$$

3.6.5   Procedure

The dye-binding capacity (DBC) values of both acetylated (DBC-B) and untreat-
ed samples (DBC-A) were assayed simultaneously and in duplicate.
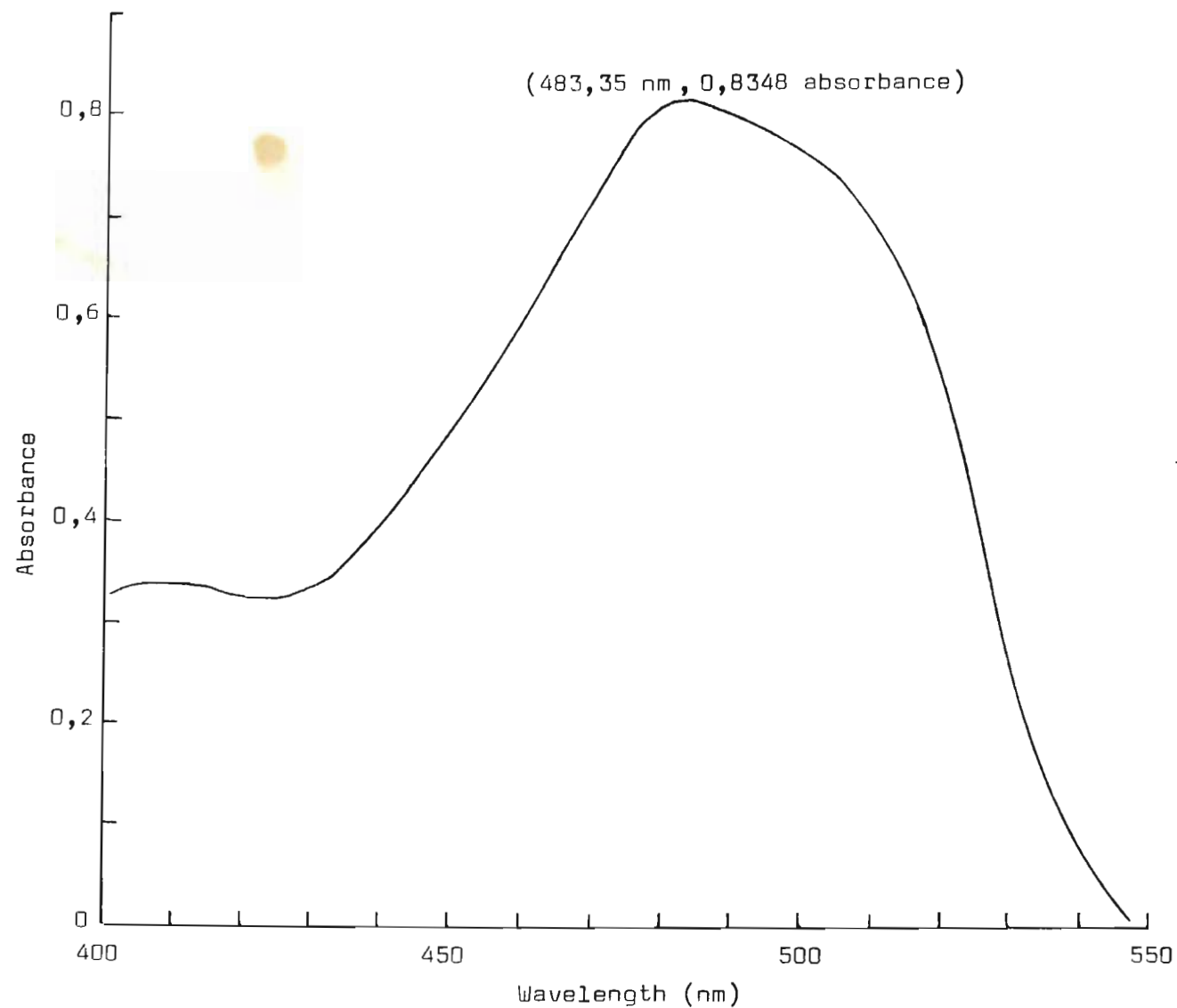
FIGURE 11   The absorbance spectrum of acid orange 12 (40 µmole ℓ^{-1}) run at a scan
speed of 100 nm/min, chart speed 10 cm/min with slit width of 0,2 in a
Beckman DU-7 Double Beam Recording Spectrophotometer

DBC-A (without pretreatment). Approximately 70 mg (equivalent to approximately 7,5 mg of 'lysine + arginine + histidine') of test material was accurately weighed directly into a 50 mℓ glass centrifuge tube and 2,0 mℓ of 5% sodium acetate solution, 0,4 mℓ of glacial acetic acid and 20,0 mℓ of 4 mM-dye solution added. The tube was sealed with a rubber stopper and the contents mixed by end-over-end rotation in a Heidolph Reax 2 axial Mixer for a period of time sufficient to attain maximum dye binding of the sample. As demonstrated in section 4.2, this time period varied considerably for different types of food materials. The reaction with maize was complete after 1 h whereas fishmeal needed 3,5 h , soya 5 h and lactalbumin 7 h. When the reaction was complete, the solids were centrifuged down at 5000 g for 10 min. Aliquots (0,5 mℓ) of the supernatant were diluted to exactly 50 mℓ with buffer solution and the absorbance measured at 483 nm in a 1 cm path length plastic disposable cuvette.

DBC-B (after acetylation). Approximately 140 mg (equivalent to approximately 7,5 mg of 'arginine + histidine') of each test material was accurately weighed directly into specially designed acetylation vessels. As shown in Figure 12 these were made from 20 mℓ polypropylene syringes (lubricated with silicon grease) by cutting off the delivery tip and cone (Fig. 12 (a)) and then inserting a cutdown piston (5 cm long, cut to a point to fit the cup of a Heidolph Whirly Mixer) completely into the base so that the vessel (Fig. 12 (b)) could stand on its base during sample weighings and solution deliveries. Sodium acetate solution (2 mℓ) followed by 0,4 mℓ of acetic anhydride was then added to the sample in the acetylation vessel and a second (uncut) piston inserted into the open end of the vessel which caused the shorter piston to move outwards slightly leaving a total volume between pistons of approximately 10 mℓ (Fig. 12 (c)). The reaction vessels were then vortexed (Heidolph Whirly Mixer) at 2,5 min intervals for 15 min (see § 4.2). Afterwards the shorter piston was removed and the acetylated sample transferred quantitatively to a 50 mℓ glass centrifuge tube by pushing down the longer piston. Dye solution (20 mℓ) was added and, as described for the DBC-A determination above, the tube was stoppered, subjected to axial mixing, centrifuged and the absorbance of a 100-fold dilution of the supernatant measured at 483 nm.

cut

cut

(a)

(b)

(c)
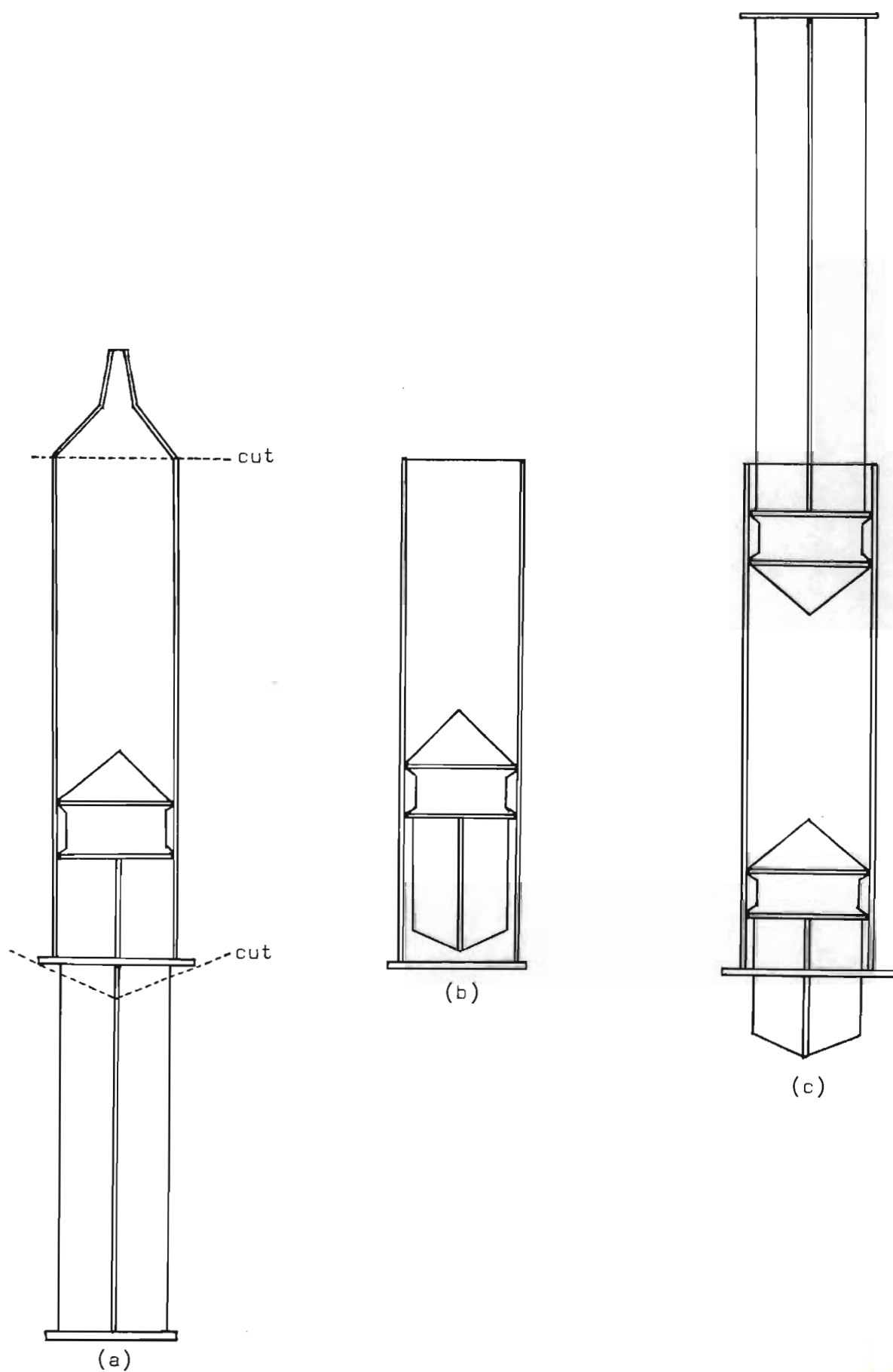
FIGURE 12 Design of the acetylation vessel used in the DBL method. For
detailed explanation, see text section 3.6.5

3.6.6 Calculation of results

The regression line of the calibration curve (see § 3.6.4) was used to convert DBC-A and DBC-B absorbance readings to their corresponding values for the concentration of residual (unreacted) dye. As the dye concentration at equilibrium influences the amount of dye bound by the protein (Hurrell et al., 1979), readings were only accepted when the residual dye concentration was in the range 1,1 - 1,6 mmole ℓ^{-1} and where the A and B readings did not differ by more than 0,3 mmole ℓ^{-1}. If this was not the case the analysis of either A or B was repeated with appropriate adjustment of sample mass.

DBC (mmoles per 16 g N) was calculated as follows:

$$\left[\left(4 \times \frac{20}{22,4} \right) - \frac{\text{residual dye}}{\text{concentration}} \right] \times \frac{22,4}{1000} \times \frac{100}{\text{sample mass (g)}} \times \frac{100}{\% \text{ CP}}$$

or $\left[80,00 - 22,4 \text{ (residual dye concentration)} \right] \times \dfrac{10}{\text{sample mass (g)} \times \% \text{ CP}}$.

Dye-binding lysine (DBL) (mmoles per 16 g N) was calculated as

mean DBC-A value — mean DBC-B value.

3.7 SUCCINIC ANHYDRIDE-REACTIVE LYSINE (Anderson & Quicke, 1984)

3.7.1 Dilution of $\left[1,4 - {}^{14}C \right]$-succinic anhydride

$\left[1,4 - {}^{14}C \right]$-succinic anhydride (specific activity 105 μCi μmole^{-1} ; radiochemical purity 97,5%) was obtained from the Radio-chemical Centre, Amersham, England. This material (250 μCi) was diluted with unlabelled succinic anhydride (Merck, GR grade), to give a specific activity of approximately $1,7 \times 10^{-3}$ μCi μmole^{-1} according to the following formula:

$$W = Ma \left(1/A' - 1/A \right)$$

where
W = mass (mg) of 'carrier' compound to be added;

M = molecular mass of labelled compound;

a = total activity in sample in mCi ;

A = molar specific activity of labelled compound supplied in mCi mmole^{-1} ; and

A' = required molar specific activity of diluted compound in mCi mmole^{-1}.

In the present case: $W = 103 \times 0,25 \left(1/1,70 \times 10^{-3} - 1/105 \right)$
$= 15,1468$ g of 'carrier' succinic anhydride.

Labelled and 'carrier' succinic anhydride was dissolved in a minimum volume
of hot redistilled acetic anhydride (b.p. 139 - 140° C), crystallised by
cooling in ice, filtered, washed with sodium-dried diethyl ether and rapidly
dried and stored in a vacuum desiccator. Earlier work by the author
(Anderson, 1980; Anderson & Quicke, 1984), employing a melting point deter-
mination and IR spectroscopy, showed that the above dilution procedure gave
consistently 'pure' preparations of succinic anhydride.

The specific activity of the preparation was assayed by dissolving suitable
quantities of the compound in 250 mℓ of distilled water. Four, 1 mℓ aliquots
were each mixed with 10 mℓ of Insta-Gel scintillator (Packard Instrument
Company, Inc., Johannesburg) in 20 mℓ glass vials and counted either in a
Packard Tri-carb or Beckmann Model LS 3801 Liquid Scintillation Spectrometer.
Both instruments use the 'H-number' method of automatic quench correction.
In the present study, 2 batches of labelled succinic anhydride were prepared.
Batch 1 had a specific activity of $2,60 \times 10^{-3} \mu Ci \ \mu mole^{-1}$ and was used to
analyse the soya isolate heated in the absence of sugar (Ch. 4) and the
Maillard-damaged soya protein samples (Ch. 6). Batch 2 had a specific acti-
vity of $1,63 \times 10^{-3} \ \mu Ci \ \mu mole^{-1}$ and was used for the analysis of the commer-
cial soyabean meals in Chapter 4.

3.7.2   Reagents

All reagents were of analytical reagent grade.

Hydroxylamine reagent.   83,39 g of hydroxylamine hydrochloride (molecular
mass 69,49 ; Sigma Chemical Company) was dissolved in 600 mℓ of distilled
water and titrated with 3,5 M NaOH to a pH of 13.

6M guanidine hydrochloride.   143,25 g of guanidine hydrochloride (molecular
mass 95,5 ; Sigma Chemical Company) was dissolved in 250 mℓ of distilled
water.

10 % (w/v) trichloroacetic acid.   50 g of anhydrous crystals were dissolved
in 500 mℓ of distilled water.

0,2 M sodium hydroxide solution.   8 g of sodium hydroxide (Merck) was dis-
solved in 1 litre of distilled water.

5M sodium hydroxide solution.   10 g of sodium hydroxide (Merck) was dissolv-
ed in 50 mℓ of distilled water.

Absolute ethanol and Insta-Gel scintillator were obtained from Merck and Packard Instrument Company, respectively.

3.7.3   Procedure

Duplicate portions (approximately 15 mg) of each sample were accurately weighed into 20 mℓ glass scintillation vials, a 10 mm magnetic stirring bar added, followed by 3 mℓ of 6M guanidine hydrochloride and the mixture stirred at 40° C for 15 min. After cooling to room temperature (ca. 25° C), 0,1 mℓ of 5M NaOH was added followed by solid $\left[1,4 - {}^{14}C\right]$-succinic anhydride in ca. 7 mg portions over a period of 30 – 45 min to give an 80-fold molar excess over total lysine content. Vigorous stirring was maintained through-out the process and a further two aliquots (0,1 and 0,05 mℓ) of 5M NaOH were added after 15 and 30 min respectively. Ten minutes after the last portion of succinic anhydride had dissolved, the temperature was raised to 30° C and 5 mℓ of hydroxylamine reagent added. The mixture was stirred for 5 min (10 min for the soyabean samples, Ch. 4), the stirring bar removed and the protein precipitated at 5% (w/v) trichloroacetic acid. The vials were capped, lowered into Schott-Mainz 50-mℓ centrifuge tubes, suitably cushioned with plastic polytops and centrifuged (5000 g) for 5 min. The supernatant was carefully decanted and the precipitate washed twice with 10-mℓ aliquots of absolute ethanol. The washed precipitate was then treated with 1 mℓ of 0,2 M NaOH and gently warmed if necessary to achieve a fine, fairly homo-geneous suspension of the partially soluble material. Insta-Gel (10 mℓ) was then added to each vial, the mixture vortexed and immediately counted either in a Packard Tri-Carb or Beckmann Model LS 3801 Liquid Scintillation Spec-trometer.

It was not necessary to use a solubiliser with the samples analysed as long as the vials were counted before the insoluble material had sedimented to the bottom of the vial. This technique gave reproducible estimates of count-ing efficiency for each type of material and satisfactory replicates within each sample. The relatively high (90%) counting efficiency obtained for all samples analysed, showed that it was not necessary to make use of bleach-ing agents (Neame, 1977) to reduce colour quenching as done previously when haemoglobin served as test protein (Anderson, 1980; Anderson & Quicke, (1984).

By stirring up to 8 vials simultaneously on one magnetic stirrer (vials spaced evenly around the perimeter of the stirrer plate), it was possible to analyse 16 samples in duplicate per day.

3.7.4   Calculation of results

Succinic anhydride (SA)-reactive lysine (g per 16 g N) was calculated as follows:

$$\text{cpm} \times \frac{100}{E} \times \frac{1}{\text{sp. act.} \times 2{,}22 \times 10^6} \times \frac{10^2}{m} \times \frac{10^3}{1} \times \frac{10^2}{\% \text{ CP}} \times \frac{1}{10^6} \times \frac{M_{Lys}}{1}$$

$$\text{or} \quad \frac{\text{dpm} \times 6{,}5855 \times 10^{-4}}{\text{sp. act.} \times m \times \% \text{ CP}} \quad ,$$

where   cpm = counts per minute recorded for the sample;

E = the counting efficiency which in the present study was automatically calculated using the 'H-number' method;

dpm = disintegrations per minute for the sample, a value that was conveniently calculated by the spectrometers using the respective cpm and E values;

sp. act. = the specific activity of the $\left[1,4-^{14}C\right]$-succinic anhydride expressed in μCi μmole^{-1};

m = the sample mass expressed in milligrams;

% CP = grammes of 'crude' protein per 100 g of sample; and

M_{Lys} = the molecular mass of lysine.

3.8   DANSYL CHLORIDE-REACTIVE LYSINE (Christoffers, 1976)

3.8.1   Reagents

All reagents were of analytical reagent grade.

0,5 M sodium bicarbonate solution.   42 g of sodium bicarbonate (Merck) was dissolved in 1 litre of distilled water. It was important that this reagent be at least 70 h old in order to allow the solution to attain the following equilibrium and consequently a constant pH value:

$$2 \text{ NaHCO}_3 \rightleftharpoons \text{Na}_2CO_3 + H_2CO_3 \quad .$$
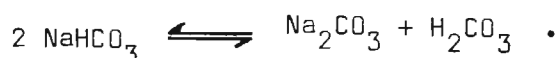
Christoffers (1976) found poor reproducibility of results if insufficient time is allowed for equilibration probably due to variations in reaction pH during dansylation.

<u>Dansyl chloride reagent.</u>   A 1 mg per mℓ solution of dansyl chloride (Sigma) in 95% (v/v) ethanol was freshly prepared before each batch of analyses. (Note: dansyl chloride decomposes in liquid media within 48 h.)

3.8.2   Procedure

Duplicate portions (approximately 20 mg) of each sample were accurately weighed into 15-mℓ glass centrifuge tubes, the material suspended in 1 mℓ of 0,5M sodium bicarbonate and vortexed to ensure maximum 'wetting' of sample. Absolute ethanol (4 mℓ) was added, vortexed again and centrifuged (bench-top centrifuge) at 5000 g for 1 min. After decanting the supernatant, dansyl chloride was added to the pellet to give a 2-fold molar excess of dansyl chloride over the total lysine content of the sample (see § 4.3). The mixture was vigorously vortexed 3 times over a period of 20 min, centri-fuged (5000 g , 1 min) and the supernatant decanted. The residue was washed twice with 4 mℓ aliquots of 95% ethanol, each followed by centrifugation (5000 g , 1 min) and decantation of the supernatant. Inclusion of a third wash as used by Christoffers (1976) did not change the values obtained. Finally, the residue was suspended in 5 mℓ of distilled water, quantitatively transferred to a cylindrical-shaped glass cuvette (diameter 55 mm) and the relative fluorescence intensity (arbitrary units) measured using a Biotronik Model BT 1010 amino acid-protein-filter-fluorometer. Readings could be taken up to 2 h later since the dansylated protein is not sensitive to photodecom-position (Christoffers, 1976).

The instrument was pre-calibrated against a built-in Schott GG 17 uranium glass standard to read 100 arbitrary units. With this setting, the detector response for quinine sulphate concentrations in the range 0 - 1 mg per mℓ of 0,05M H_2SO_4 was found to be linear up to a value of approximately 400 arbit-rary units. Protein samples giving readings higher than 400 units were re-analysed using lower sample masses (e.g. the model proteins described in § 3.1.5). For comparative purposes most results were expressed either as relative fluorescence intensity (arbitrary units) per 1,6 mg N (or 10 mg CP) or as lysine equivalents of another reactive lysine method (for details see § 4.4.4).

3.9 NINHYDRIN-REACTIVE LYSINE (Friedman & Broderick, 1977)

3.9.1   Reagents

All chemicals were of analytical reagent grade.

4M Sodium acetate buffer (pH 5,5).   136 g of sodium acetate hydrate was
dissolved with heating in 100 mℓ of distilled water, allowed to cool to RT,
25 mℓ of glacial acetic acid added and made up to 250 mℓ with distilled
water. If necessary the pH was adjusted to 5,5 with concentrated HCl or
NaOH.

0,125 M Sodium metabisulphite.   2,3763 g of sodium metabisulphite (Merck)
was dissolved in 100 mℓ of distilled water.

Dimethyl sulphoxide reagent (4 : 1 v/v).   400 mℓ of dimethyl sulphoxide
(Merck) was mixed with 100 mℓ of distilled water.

Ninhydrin reagent (3 g/100 mℓ).   3 g of ninhydrin (Merck) was dissolved in
25 mℓ of 4M sodium acetate buffer and mixed with 50 mℓ of dimethyl sulphoxide
and 25 mℓ of distilled water. Just before use, 13 mg of hydrazine sulphate
was dissolved in 10 mℓ of distilled water and mixed with 50 mℓ of the 3%
ninhydrin solution. The solutions were prepared in an amber-coloured bottle
away from direct sunlight.

Leucine standards.   L-leucine (molecular mass 131,2 ; Sigma) was used to
prepare 1 to 8 mM standard solutions by serial dilution from an 8 mM
(0,10496 g per 100 mℓ distilled water) stock solution.

3.9.2   Procedure

Duplicate portions (approximately 3 mg) of test material were accurately
weighed into 10-mℓ glass centrifuge tubes and 0,2 mℓ of 0,125 M sodium meta-
bisulphite and 3,8 mℓ of dimethyl sulphoxide reagent added. The mixture
was vortexed for 30 s , 1,2 mℓ ninhydrin reagent added, the tube capped with
a marble and heated in a waterbath at 100° C for 15 min. In the author's
experience, a 30-min heating period as used by Friedman and Broderick (1977)
gave lower absorbancy readings. After cooling rapidly by partial immersion
in cold water, the tubes were centrifuged at 1000 g for 5 min to remove any
insoluble material. Aliquots (1 mℓ) of each solution were diluted with 5 mℓ
of dimethyl sulphoxide reagent and absorbance read at 580 nm in a Beckman
Model DU-7 spectrophotometer against a blank that initially contained 3,8 mℓ

dimethyl sulphoxide reagent, 0,2 mℓ of distilled water and 1,2 mℓ ninhydrin reagent but had also simultaneously been carried through the above reaction and dilution process. A calibration curve for leucine was constructed by the identical treatment of a reaction mixture containing 0,2 mℓ of each leucine standard (see § 3.4.1), 3,8 mℓ dimethyl sulphoxide reagent and 1,2 mℓ of ninhydrin reagent. The absorbance readings of unknown samples were converted to NIN-reactive lysine values according to the published procedure of Friedman et al. (1984) the details of which are given in section 3.9.3 below.

3.9.3   Calculation of results

The ninhydrin absorbance readings for the various leucine standards were used to calculate a mean molar absorptivity constant of 14 000 ℓmole^{-1} cm^{-1} by substitution into the following general formula:

$$\text{Molar absorbtivity} = \frac{A}{b \times c}$$

where   A = absorbance at 580 nm ;

b = path length of cuvette (1 cm); and

c = concentration of leucine standards in moles per litre of final solution.

The correction factor for α-NH$_2$ groups recommended by Friedman et al. (1984) was calculated as follows for lactalbumin (mol. mass 17 400 ; total lysine content 10,02 g per 16 g N) :

$$\% \ \alpha\text{-NH}_2 \text{ groups} = \frac{1 \text{ terminal } \alpha\text{-NH}_2 \text{ group per mole} \times 100}{\text{No. of } \epsilon\text{-NH}_2 \text{ groups} + 1 \ \alpha\text{-NH}_2 \text{ group per mole}}$$

$$= \frac{1 \times 100}{\left[\dfrac{\text{TL(g 16 g N}^{-1}) \times \text{molecular mass of protein}}{\text{molecular mass of lysine} \times 100} \right] + 1}$$

$$= \frac{1 \times 100}{\left[\dfrac{10,02 \times 17 \, 400}{146,2 \times 100} \right] + 1}$$

$$= \frac{100}{11,93 + 1}$$

$$= 7,74\% \ .$$

The correction factor (CF) recommended by Friedman et al. (1984) for the lower NIN-colour contribution from protein amino groups of lactalbumin as compared to those of free amino acids (in this case leucine) was calculated as follows:

$$CF = \frac{\text{observed absorbance/mg protein} - \text{absorbance/mg protein due to } \alpha\text{-NH}_2 \text{ groups}}{\text{calculated absorbance/mg protein due to Lys } \epsilon\text{-NH}_2 \text{ groups}}$$

$$= \frac{0{,}226 - \left[7{,}74/100 \times 0{,}226\right]}{\left[\dfrac{\text{molar absorptivity of Leu-NIN complex} \times \text{TL (moles 16 g N}^{-1})}{\text{Final volume of reaction mixture (m}\ell) \times 100}\right]}$$

$$= \frac{0{,}226 - 0{,}0175}{\left[\dfrac{14\ 000 \times 0{,}0685}{31{,}2 \times 100}\right]}$$

= 0,678 Leu equivalents per Lys residue.

The NIN-reactive lysine content of all formaldehyde-treated lactalbumin samples (Ch. 7) was calculated as recommended by Friedman et al (1984):

NIN-reactive lysine (g 16 g N^{-1})

$$= \frac{\text{Corrected absorbance/mg protein}}{\text{molar absorptivity of Leu}} \times \frac{146{,}2}{10^3} \times \frac{10^6}{1} \times \frac{\text{Final volume of reaction mixture (m}\ell)}{CF}$$

or $\left[\text{observed absorbance/mg protein} - 0{,}0774 \times \text{absorbance/mg}\right] \times 48{,}0556$.

3.10 MICROBIOLOGICAL ASSAY OF LYSINE AVAILABILITY WITH Tetrahymena pyriformis W (Stott & Smith, 1966)

3.10.1 Preparation of the nutrient media

The constituents of the assay medium were as described by Stott and Smith (1966) with minor modifications (Robbins, pers. commun.). All details are presented in Appendix 1.

3.10.2   Standard lysine solutions

<u>Lysine stock solution.</u>   0,41667 g of lysine hydrochloride (Merck) was dis-
solved in 100 mℓ of distilled water.

<u>Lysine standard.</u>   1,19 g of HEPES was dissolved in 80 mℓ of distilled water,
the pH adjusted to 7,1 , exactly 10 mℓ of lysine stock solution added and
the solution made up to 100 mℓ.   This gave a working lysine hydrochloride
standard of 41,667 mg/100 mℓ which is equivalent to 33,334 mg lysine/100 mℓ.

3.10.3   Enzymic digestion of test materials

All samples were weighed out to contain approximately 32 mg of FDNB-reactive
lysine (Erbersdobler & Anderson, 1983) and digested with pronase E
(<u>Streptomyces griseus,</u> Type XIV, Sigma Chemical Co., activity 5,8 units per
mg solid) at a concentration of 8 mg enzyme per 100 mg sample N in 24 mℓ of
0,05 M HEPES buffer (11,915 g/ℓ adjusted to pH 7,5 with 30% NaOH) in a
shaking waterbath at 40° C for 20 h.   To suppress microbial growth, a 1:1:2
(v/v/v) mixture of chlorobenzene, ethylene dichloride and 1-chlorobutane
(Merck Chemicals) was added at a concentration of 0,5 mℓ per 100 mℓ of
buffer (Baker <u>et al.</u>, 1978).   The resulting suspensions were centrifuged at
40 000 g for 20 min to remove any insoluble material (dark brown) and the
percentage of digested N determined by Kjeldahl analysis (§ 3.2.2).   The
clear light-brown supernatant fractions were analysed by microbiological
assay as described in section 3.10.5.   The intensity of browning of these
fractions was determined by measurement of the absorbance at 450 nm, the
results being expressed as A_{450} per mg N.

3.10.4   Inoculum

Before inoculation, <u>T. pyriformis</u> W. stock culture medium was washed free of
its proteose-peptone broth by suspending the cells in 0,067 M potassium
phosphate buffer (pH 7,2), centrifuging at 1500 g for 15 s and decanting the
supernatant.   This procedure was repeated once more and then the cells were
finally re-suspended in sterile 0,067 M potassium phosphate buffer ready for
inoculation of the assay medium.

3.10.5   Assay procedure

All media preparations and inoculations were performed aseptically in a
sterile room in subdued light.

The supernatant fractions (§ 3.10.3) were assayed in duplicate at 3 different N-levels (between 0 - 10 mg N per 10 mℓ assay medium) selected to give suitable growth responses on the linear range of the standard curve. Suitable volumes of the lysine standards selected to cover the range 0 - 1,0 mg lysine per 10 mℓ assay medium in 0,25 mg increments were also simultaneously assayed in duplicate with each batch of analyses.

The assay medium consisted of the selected volumes of feed digest or standard lysine solution, 4 mℓ of solution 9 , 3 mℓ of solution 19 (see Appendix 1) and sufficient 0,05 M HEPES buffer (pH 7,1) to give a final volume of 10 mℓ. To minimise Maillard reactions the components of solution 9 (i.e. solutions 6 , 7 and 8 — see Appendix 1) were separately sterilised by autoclaving at 15 lbs for 10 mins, mixed to give solution 9 which was then added aseptically to the remainder of the assay medium which had been pre-sterilised as a whole solution.

After inoculation of the assay medium with 0,2 mℓ of a 3-day old <u>T. pyriformis</u> W. culture, the cultures were incubated for 4 days at 25° C with periodic agitation. Suitable aliquots of the cultures were then diluted to 200 mℓ with 0,35 % NaCl (prefiltered with a 0,45 μm Millipore membrane) exactly 0,5 mℓ of this solution was counted using a Coulter counter (Industrial Model D, Coulter Electronics, Florida, U.S.A.), fitted with an orifice tube of aperture diameter 200 μm. Results were calculated as described in section 3.10.6 below.

3.10.6   Calculation of results

Each diluted culture (in 0,35 % NaCl) was counted 4 times and the mean count corrected for coincidence counting according to the following procedure (Coulter Electronics Manual):

$$\text{Corrected mean count} = \text{mean count} + \text{coincidence correction}$$

$$= \text{mean count} + p\left(\frac{\text{mean count}}{10^3}\right)^2$$

where p = 20 , the coincidence factor (given).

The coincidence correction was kept below 10 % of the uncorrected mean count which meant that the uncorrected mean count needed to be kept below 5000. This was done by suitable dilutions of the test culture with 0,35 % NaCl.

The corrected mean count was then converted to organism counts per 10 mℓ culture medium as follows:

Counts per 10 mℓ culture medium

$$= \begin{array}{c} \text{corrected} \\ \text{mean count} \end{array} \times \frac{\begin{array}{c}\text{vol. of saline diluted}\\\text{culture medium}\end{array}}{\begin{array}{c}\text{vol. of saline diluted}\\\text{culture medium counted}\end{array}} \times \frac{\begin{array}{c}\text{total vol. of}\\\text{culture medium}\end{array}}{\begin{array}{c}\text{vol. of culture medium}\\\text{diluted with saline}\end{array}}$$

$$= \text{corrected mean count} \times \frac{200}{0,5} \times \frac{10}{\begin{array}{c}\text{vol. of culture medium}\\\text{diluted with saline}\end{array}} \quad .$$

The counts were converted to mg of lysine by substitution in a linear regression equation obtained for simultaneously assayed lysine standards, e.g.:

$$Y_{\text{mg Lys}} = 1,17 \times 10^{-7} \, X_{\text{counts}} - 0,14 \qquad (r = 0,987 \; ; \; n = 12) \quad .$$

The final result (glysine per 16 g N) was calculated as follows:

Available lysine (g 16 g N^{-1})

$$= \begin{array}{c} \text{mg Lys} \\ \text{(from regression} \\ \text{equation)} \end{array} \times \frac{\begin{array}{c}\text{total vol. of}\\\text{pronase digestate}\end{array}}{\begin{array}{c}\text{aliquot of digestate}\\\text{analysed}\end{array}} \times \frac{10^2}{\text{Sample mass (g)}} \times \frac{10^2}{\text{\% CP}} \times \frac{1}{10^3}$$

$$= \frac{\text{mg Lys} \times 240}{\text{aliquot of digestate} \times \text{original sample mass (g)} \times \text{\% CP}} \quad .$$

CHAPTER 4

PRELIMINARY INVESTIGATIONS OF THE
DBL AND DAN PROCEDURES

4.1 INTRODUCTION

During the course of this study a number of problems were encountered with some of the published procedures employed. In some cases this necessitated a re-examination of the entire procedure and the modification of various important aspects. This chapter therefore includes the results of various investigations into ways of improving some of the methods.

4.2 OPTIMISATION OF THE ACYLATION AND DYE-BINDING REACTIONS FOR THE DBL METHOD (Hurrell et al., 1979)

A variety of possible problems with the DBL method need to be investigated before making a decision on its suitability as a reliable reactive-lysine method. These include:

1. the dye-protein ratio and reaction time necessary to attain maximal dye binding;

2. the completeness of the acylation reaction;

3. the effect of acylation (propionylation or acetylation) of other amino acid side chains on the overall dye-binding capacity, e.g. steric and electrostatic effects, or the possible reaction of anhydrides with histidine to form 'dye-repelling propionyl- or acetyl-imidazole moieties similar to those formed by succinic anhydride (Stadtman, 1955);

4. non-specific dye-binding at α-NH_2 groups or localised electron-dense areas in the protein chain which may also include blocked ϵ-N-propionyl-lysyl or ϵ-N-acetyl-lysyl residues since the blocked amino group may still be sufficiently basic to facilitate dye binding; and

5. interference from other soluble coloured compounds.

In the present studies particular attention was paid to aspects 1. and 2., the optimisation of reaction conditions with respect to dye-protein ratio, reaction time and completeness of acylation. Furthermore, since Hurrell (pers. commun.) recently advocated the replacement of propionic anhydride

by acetic anhydride as acylating agent, studies were rather concentrated on the acetylation process.

The present author found that an 80-fold molar excess of succinic anhydride over estimated lysine content, added as a series of small portions, was needed to achieve complete ϵ-N-succinylation of various denatured (6M-guanidine HCl) model proteins (Anderson & Quicke, 1984). Furthermore, Walker (1979) had found it necessary to use a 16 - 24 h propionylation period for their work. It was therefore considered important to test whether a 15-min reaction time with a 70-fold molar excess of acetic anhydride over lysine content as recommended by Hurrell (pers. commun.) is sufficient to fully modify all ϵ-NH$_2$ groups, since an incomplete reaction would raise the DBC-B values and thereby lower the DBL results (§ 3.6.6). Using the 'acetylation vessel-vortex' method (Fig. 12 and § 3.6.5) and a sample of maize meal as model compound, the acetylation reaction was found to be complete after only 5 min. The sample gave a highly reproducible DBC-B value of 56,27 moles 16 g N^{-1} which did not appreciably change during a further 25 min of incubation with acetic anhydride. Increasing the anhydride-lysine ratio from 70 to 90-fold molar excess also had no effect on the results. Similar results were also obtained using an ultrasonic bath instead of the whirly mixer (§ 3.6.5) to enhance the acetylation reaction. For convenience it was decided to use a 15-minute acetylation time for all future analyses. The suitability of this procedure for the propionylation reaction was not tested.

Hurrell et al. (1979) used a time of 10 min for the dye-protein reaction in the Foss 'Double Reactor' (N. Foss Electric, Denmark) and recommended (Hurrell; pers. commun.) that this time be extended to 1 h if a laboratory shaker is used instead of a Double Reactor. Walker (1979) showed that the rate of dye uptake by various leaf protein concentrates varied for different samples with the reaction taking 8 - 16 h to reach completion on a laboratory shaker. It was therefore of prime importance to determine the optimal reaction conditions applicable to the different types of food proteins investigated in this study.

As seen in Figure 13 the four protein sources tested show large differences in the rate of uptake of acid orange 12. The reaction with maize was complete after 1 h followed by fishmeal (3,5 h), soya (5 h) and lactalbumin (7 h). These observations constitute a significant finding which should markedly influence future applications of the DBL method and certainly throw doubt on the validity of some of the work already published on this method. For instance the present findings could account for part of the large estimate
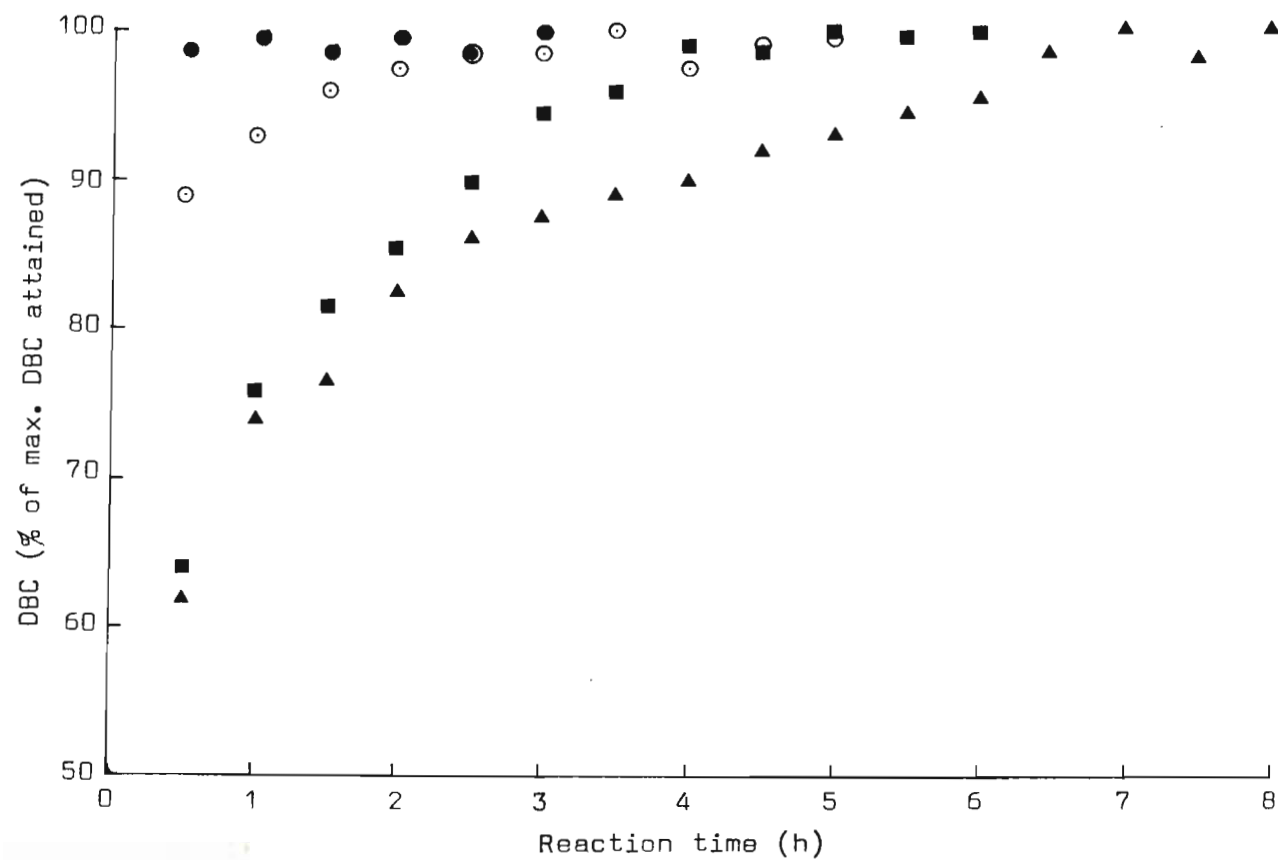
FIGURE 13 The progress of the dye-binding reaction between acid orange 12 and soya (■),
maize (●), fishmeal (○) and lactalbumin (▲). DBC values were determined as
described in text for DBC B analyses (§ 3.6.5)

of between laboratory variance and poor concordance of ranking of fishmeals
by different laboratories shown by the DBL method in a recent collaborative
trial (Barlow et al., 1984). The members of this collaborative trial were
instructed to employ a 1-h reaction time for their fishmeals which according
to Figure 13 would mean that the reaction was only 93% complete and the
rate of dye uptake was still fairly rapid.

To conclude, the results suggest that in order to ensure reproducible DBC
and DBL results rigid standardisation of the experimental procedure is
essential. In this regard, it is particularly important to check the time
required to attain maximum dye-binding for each type of material analysed,
bearing in mind the obvious effects of using different mixing devices.

4.3 EFFECT OF INCUBATION TIME, SHAKING PROCEDURE AND REAGENT CONCENTRATION ON THE DANSYLATION REACTION

In a time study Christoffers (1976) found that a 1-h incubation at 30° C with
a 3-fold molar excess of dansyl chloride over total lysine content of pro-
teins was sufficient to achieve maximum $\epsilon-NH_2$ group modification. In his
experiment reagent and precipitated protein and bicarbonate were thoroughly
mixed and then simply placed in a waterbath for the specified period without
any further mixing or agitation. It was considered surprising that the
reaction reached completion as the present author found that the precipitated
material rapidly (within a few minutes) formed a pellet at the bottom of the
tube, which presumably would be detrimental to rapid completion of the
reaction. It was therefore decided to investigate the conditions of the
reaction.

Experience with other protein modifying reagents (§ 4.2; Anderson & Quicke,
1984) prompted the author to explore the possibility of using 'end-over-end'
axial mixing (Heidolph Reax 2, see § 3.6.5) to enhance the rate and maybe the
extent of the dansylation reaction. Sub-samples of a soyabean preparation
(§ 3.1.4) were separately mixed with either a 2- or 3-fold excess of dansyl
chloride and shaken on a Heidolph Reax 2 Mixer at RT for 30 , 45 , 60 , 75,
90 , 105 and 120 min and then treated according to the standard procedure
(§ 3.8.2). The two '15-minute' samples were not mixed on the Reax Mixer but
were mixed initially and twice more on a whirly mixer during the 15 min
period. Two further samples were treated exactly as described by Christoffers
(1976), i.e. with a 3-fold excess of reagent and standing at 30° C for 1 h
without shaking.

A significant feature of the results (Table 2) is that the dansyl reaction reached completion after only 15 min or less with only brief intermittent vortex mixing. Longer incubation times with continuous shaking and higher reagent concentrations did not significantly increase the results. This was confirmed by an analysis of variance (Table 2), which demonstrated that there was no significant difference between values for different incubation times ($p > 0,05$) as well as the different reagent concentrations ($p > 0,01$). Furthermore, the interaction term (times x reagents) was not significant ($p > 0,05$) indicating that the 2 reagent concentrations gave similar values for every incubation time. The rapidity with which the reaction goes to completion probably explains why the mean value obtained with the original Christoffer's procedure (no mixing) was within the range of values obtained by the other procedures.

All subsequent dansylations were carried out at RT with a 2-fold molar excess of reagent over total lysine content and involved 3 vigorous vortexes of the reaction mixture over a period of 15 min. This meant a considerable reduction in the overall time taken for these analyses.

```
┌─────────────────────────┐
│        TABLE  2         │
│  (see following page)   │
└─────────────────────────┘
```

TABLE 2 Effect of incubation time and molar excess of
dansyl chloride over total lysine content on
the extent of dansylation of defatted soyabean
meal

Incubation time (min) [b]	RELATIVE FLUORESCENCE INTENSITY ARBITRARY UNITS PER 1,6 mg N [a]	
	Molar excess of Reagent	
	2	3
60 min, 30° C [c]	nd	158
15 [d]	161	154
30	158	152
45	159	159
60	161	157
75	156	156
90	159	155
105	153	155
120	158	150

a - means of duplicate determinations.

b - unless specified incubations were at RT (23° C)
employing a Heidolph Reax 2 mixer.

c - method of Christoffers (1976) employed.

d - sample vortexed 3 times over 15 min and not mixed
in Heidolph Reax 2 .

nd - not done.

ANALYSIS OF VARIANCE

Source of Variation	DF	F-test
Incubation times	7	ns ($p > 0,05$)
Reagent concentrations	1	ns ($p > 0,01$)
Times x Reagents	7	ns ($p > 0,05$)
Error	16	
Totals	31	

4.4 ATTEMPTED EXPRESSION OF FLUORESCENCE INTENSITIES (DAN VALUES) IN TERMS OF REACTIVE LYSINE

As pointed out in section 2.2.4, the observed fluorescence of a substance is strongly affected by its quantum yield which is in turn affected by quenching due to internal conversion, phosphorescence and collision with other fluorescent and non-fluorescent molecules (Cantor & Timasheff, 1982). These factors interfere with the fluorescence of N-dansylated proteins particularly when insoluble suspended protein material is analysed and when attempts are made to compare results obtained for different materials containing substantially different ratios of interfering substances (Christoffers, 1976). Values obtained are thus only arbitrary and cannot be expressed quantitatively. As it was hoped ultimately to be able to relate fluorescence intensity values to more meaningful units of reactive lysine it appeared desirable to minimise such interference. Several possible approaches were attempted as set out below.

4.4.1 Attempted solubilisation of dansylated feed proteins

Goodno et al. (1981) incorporated SDS (solubilising agent) and β-mercapto-ethanol (disulphide reducing agent) into their o-phthalaldehyde reagent in order to promote both sample solubilisation and amino group modification. For highly insoluble samples they advocated boiling in SDS and mercaptoethanol prior to the actual addition of the reagent but they only tested their procedure on relatively soluble, 'pure' model proteins. Solubilisation before dansylation is impractical since the procedure involves various precipitation procedures and dansylation itself is carried out in 95% ethanol which would probably re-precipitate the solubilised protein. Attempts to solubilise various feed samples after dansylation, by boiling with SDS/mercaptoethanol prior to measurement of fluorescence, were not successful, none of the test materials (i.e. soya, maize and fishmeal) being fully solubilised. Furthermore, the observed fluorescence obtained with treated proteins was almost the same as that obtained without treatment thus failing to solve the problem of fluorescence quenching.

4.4.2 Attempted use of an internal standard

The possibility of using an internal standard for the calculation of the efficiency of the fluorescence measurement for each sample, as done in liquid scintillation counting, was also investigated. For this purpose the highly fluorescent and UV-stable compound quinine sulphate was selected as internal

standard since it has frequently been used to calibrate fluorimeters (e.g. Goodno et al., 1981). Unfortunately nonsensical results were obtained. Further investigation revealed that dansylated protein only fluoresces in alkaline (bicarbonate) solution whereas quinine sulphate specifically requires an acid pH before it will fluoresce. No attempt was made to use alternative internal standards.

4.4.3   Estimation of reactive lysine from quench corrected fluorescence intensity (DAN) values and a regression equation obtained with 'pure' proteins

The aim of this approach was to fit a linear regression line to a plot of DAN fluorescence intensity versus sample particle size for each sample and extrapolate to zero particle size. The y-intercepts would yield fluorescence values independent of quenching due to insoluble suspended material. These values could then be converted to reactive lysine by substitution into another linear regression equation relating fluorescence intensity to total lysine content of a series of soluble 'pure' model proteins in which quenching would be presumably negligible.

As described in section 3.1.5, material from 3 different defatted soyabean samples were used to prepare 3 batches of samples with mean particle sizes ranging between 49 and 400 μm . All samples were then analysed in duplicate by the DAN method (§ 3.8) and the fluorescence results (arbitrary units per 10 mg CP) plotted against the corresponding mean particle size of each sample (Fig. 14). A computer programme ('GENSTAT') was used to calculate the 'best fit' curves and related statistical errors.

The results of the computer run revealed that quadratic functions (Fig. 14) rather than linear relationships fitted the experimental data the best with the equations for the 3 samples showing high correlation coefficients (r) of 0,97 , 0,93 and 0,96 respectively. The results clearly show the enormous effect of particle size on fluorescence with values for the same sample varying by up to 500 % over a 300 μm particle size range. This emphasises the importance of milling samples to similar particle sizes. Furthermore, contrary to the findings of Christoffers (1976), particles of less than 80 μm diameter still show significant quenching for which a correction would be important. Statistical analysis showed that there was no significant difference ($p > 0,05$; DF = 19) between the regression coefficients of the 3 quadratic equations indicating that the lines are more-or-less parallel (Fig. 14). From this one can make the assumption that all defatted soyabean meal at

The equations shown on the figure:

$$Y_A = 209,53 - 1,019x + 0,0015x^2 \quad (r = 0,96 \; ; \quad n = 20)$$

$$Y_B = 194,56 - 0,983x + 0,0015x^2 \quad (r = 0,93 \; ; \quad n = 20)$$

$$Y_C = 152,13 - 0,813x + 0,0013x^2 \quad (r = 0,97 \; ; \quad n = 20)$$
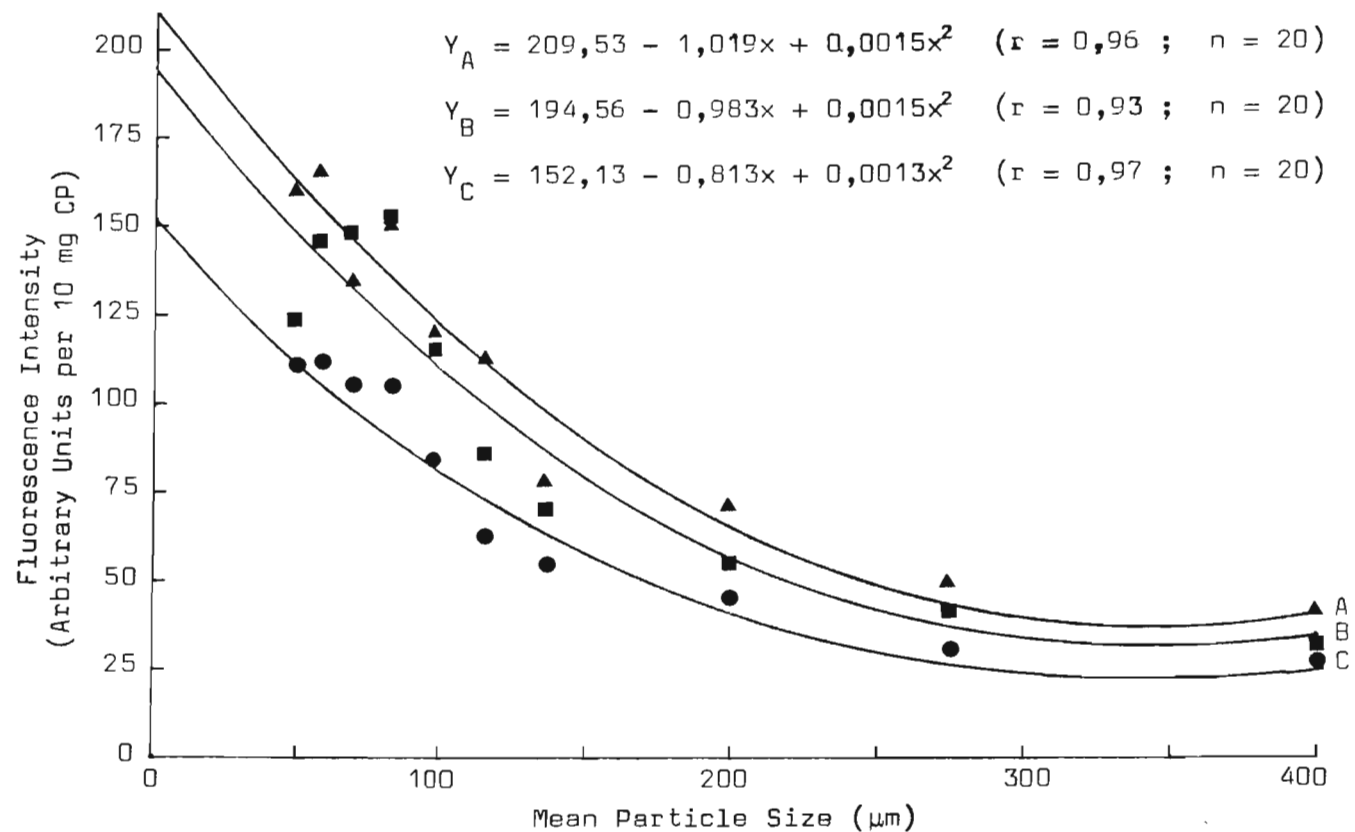
FIGURE   14   The effect of particle size on fluorescence intensity of 3 dansylated soyabean meal samples,   A (▲), B (■) and C (●).   Samples were prepared as described in section 3.1.4 and plotted co-ordinates are means of duplicate determinations

least of similar chemical composition should give a similar regression co-
efficient which would enable one to use one formula for such samples for the
conversion of observed fluorescence (FI_{OBS}) to fluorescence values indepen-
dent of quenching factors (termed true relative fluorescence intensity or
FI_{TRUE}).

Since the 3 plots in Figure 14 showed similar regression coefficients and
all 27 samples had been milled to a mean particle size of 77,5 μm (§ 3.1.4)
the following formula for the conversion of FI_{OBS} to FI_{TRUE} values indepen-
dent of quenching due to insoluble material could be derived:

$$Y = c + bx + ax^2 \quad ,$$

or

$$FI_{OBS} = FI_{TRUE} + (\text{mean regression coefficient 'b'} \times \text{mean particle size}) +$$
$$(\text{mean regression coefficient 'a'}) \times (\text{mean particle size})^2 \quad ,$$

or

$$FI_{OBS} = FI_{TRUE} + \left(\frac{-0,983 - 0,813 - 1,019}{3}\right) 77,5 + \left(\frac{0,0015 + 0,0015 + 0,0013}{3}\right) (77,5)^2 \quad .$$

$$\therefore \quad FI_{TRUE} = FI_{OBS} + 64,11 \text{ arbitrary units per 10 mg CP} \quad .... \quad \underline{/1\underline{/}}$$

FI_{TRUE} values calculated with this formula are theoretically independent of
quenching due to insoluble material and thus should be comparable to FI
values obtained for soluble 'pure' model proteins of known lysine content
and 100 % reactivity. When FI values (arbitrary units per 10 mg CP) for
11 different model proteins (§ 3.1.6) were plotted against TL (μmoles lysine
per 10 mg CP) (Fig. 15) linear regression analysis unfortunately gave a poor
correlation coefficient, i.e.:

$$Y_{FI_{TRUE}} = 44,41 X_{TL} + 32,74 \quad (r = 0,754 ; \quad n = 11) \quad .... \quad \underline{/2\underline{/}}$$

However, closer examination of the co-ordinates in Figure 15 revealed that
the poor correlation was mainly due to the results for lysozyme,
α-chymotrypsin and ovalbumin. The remaining 8 model proteins gave an excel-
lent linear relationship, i.e.:

$$Y_{FI_{TRUE}} = 46,60 X_{TL} - 25,69 \quad (r = 0,97 ; \quad n = 8) \quad .... \quad \underline{/3\underline{/}}$$
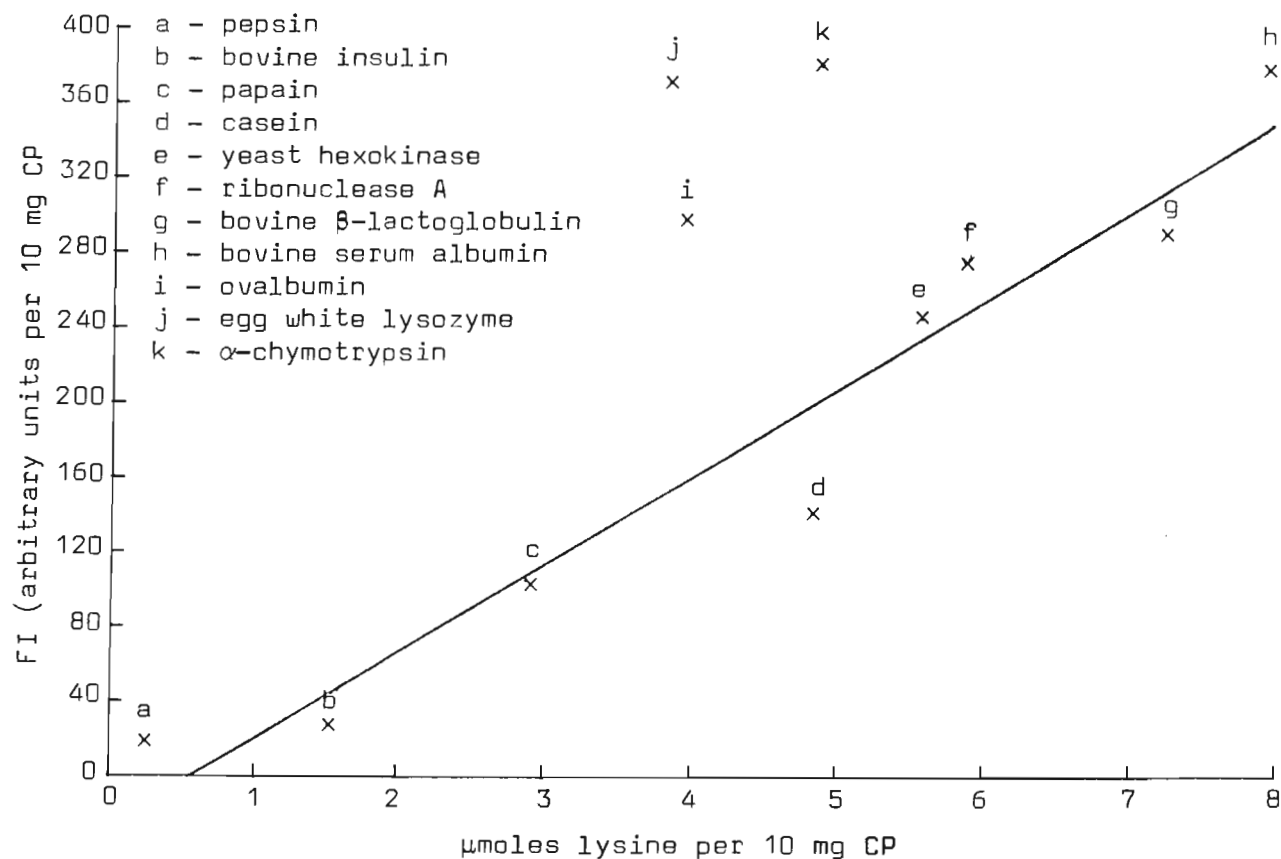
FIGURE 15 Relationship between fluorescence intensity of various dansylated model proteins and their total lysine content. FI was determined on 10 mg samples as described in section 3.8 while TL was analysed as detailed in section 3.3. The regression equation for all 11 proteins was: $Y_{FI_{true}} = 44,41x_{TL} + 32,74$ (r = 0,754)

while that for 8 proteins excluding co-ordinates i, j and k was: $Y_{FI} = 46,60x_{TL} - 25,69$ (r = 0,97) (see text)

Since no acceptable explanation could be found for the 3 exceptions it was decided to ignore these 3 co-ordinates and use equations $\underline{/1\_7}$ and $\underline{/3\_7}$ for the derivation of a formula for converting FI_{OBS} values (arbitrary units per 10 mg CP) to DAN-reactive lysine (g 16 g N^{-1}):

By substituting equation $\underline{/3\_7}$ into equation $\underline{/1\_7}$:

$$46,60 \ (TL) - 25,69 = FI_{OBS} + 64,11 \quad .$$

Rearranging:

$$TL \ (\ \mu moles \ per \ 10 \ mg \ CP) = 2,146 \times 10^{-2} \ (FI_{OBS}) + 1,927$$

$$\therefore \quad TL \ (\ g \ per \ 100 \ g \ CP) = (2,146 \times 10^{-2} (FI_{OBS}) + 1,927) \times \frac{146,2}{10^2}$$

$$= 3,137 \times 10^{-2} \ (FI_{OBS}) + 2,817 \quad .$$

This formula, however, only holds for 'pure' proteins of 100% reactive lysine content, (i.e. TL = DAN values). Therefore for other samples of lower lysine reactivity:

$$DAN\text{-reactive lysine (g 16 g } N^{-1}) = 3,137 \times 10^{-2} \ (FI_{OBS}) + 2,817 \quad \ldots \ldots \underline{/4\_7}$$

Equation $\underline{/4\_7}$ is of course only valid for defatted soyabean meal of similar chemical composition, milled to have a mean particle size of 77,5 μm and where FI_{OBS} is expressed in arbitrary units per 10 mg CP. Reproducibility of results would also depend on minimal instrument variation and most important the stability of the Schott GG 17 uranium glass standard used to calibrate the Biotronik BT 1010 (see 8 3.8.2).

In order to test the validity of this approach the above formula (equation $\underline{/4\_7}$) was applied to FI values obtained with 27 defatted commercial soyabean samples (8 3.1.4) which came from the same batch as and were similar in chemical composition to the 3 used in obtaining Figure 14 and hence equation $\underline{/4\_7}$. The results (Table 3) were disappointing in that all DAN values (indicated as DAN (1) in Table 3) correlated poorly with and consistently overestimated both TL (r = 0,63) and SA-reactive lysine (r = 0,44) results. Analysis of variance also showed that the values obtained by the SA and DAN (1) methods were significantly different (p < 0,001).

On re-examination of Figure 14 it was noted that there were large fluctuations in values below 69 μm particle size probably due to the poor ability of the micro-sieves to discriminate between small differences in particle

TABLE 3   DAN-reactive lysine values for 27 soyabean samples compared and
          correlated with those obtained by the TL and SA-reactive lysine
          procedures[a]

SAMPLE NO.	TL	SA	DAN (1)[b]	DAN (2)[c]
1	6,48	6,67	7,22	6,02
2	6,64	6,58	7,15	5,95
3	6,45	6,32	7,55	6,35
4	6,33	6,21	7,54	6,34
5	6,10	5,95	7,18	5,98
6	5,79	4,90	6,12	4,92
7	6,34	5,45	7,54	6,34
8	6,11	5,20	6,73	5,53
9	5,92	5,41	7,07	5,87
10	6,59	6,21	7,37	6,17
11	6,58	6,34	7,43	6,23
12	6,34	5,65	7,14	5,94
13	6,19	5,59	6,86	5,66
14	6,21	5,35	6,95	5,75
15	6,70	5,35	7,77	6,57
16	6,30	5,40	6,66	5,46
17	6,20	5,90	6,31	5,11
18	6,23	5,67	6,99	5,79
19	6,98	6,04	7,39	6,19
20	6,85	5,72	7,61	6,41
21	6,36	5,56	7,16	5,96
22	6,37	6,03	6,62	5,42
23	6,46	6,05	7,17	5,97
24	6,50	5,58	7,11	5,91
25	5,77	5,67	7,00	5,80
26	5,83	5,76	6,78	5,58
27	4,79	5,25	6,42	5,22
± S.E. (mean)	–[d]	0,15	0,10	0,10
Correlations (r) with:				
TL (n = 27)	–	–	0,63	0,63
SA (n = 27)	–	–	0,44	0,44

a – Results are expressed in g lysine per 16 g N and are means of duplicate
    determinations.

b – DAN (1) calculated from equation $\sqrt{4\_7}$ i.e.

$$\text{DAN-lysine} = 3,137 \times 10^{-2} \ (FI_{OBS}) + 2,817 \ .$$

c – DAN (2) calculated from equation $\sqrt{6\_7}$ i.e.

$$\text{DAN-lysine} = 3,137 \times 10^{-2} \ (FI_{OBS}) + 1,613 \ . \quad \text{(See text.)}$$

d – TL results were single determinations.

ANALYSIS OF VARIANCE

Source of Variation	DF	SA vs DAN (1)	SA vs DAN (2)
Methods	1	S ($p < 0,001$)	ns ($p > 0,001$)
Samples	26	S ($p < 0,001$)	S ($p < 0,001$)
Methods x Samples	26	S ($p < 0,001$)	S ($p < 0,001$)
Error	54		
Totals	107		

size over such narrow sieve size ranges. This problem would have been
enhanced by electrostatic interactions between the charged protein material
and the fine metal mesh of the sieves. On the other hand, the 400 μm
particle size material was observed to be very heterogeneous and could have
also yielded erroneous results. It was accordingly decided to investigate
the effects of eliminating these extremes. Attempts to find linear sections
between these two extremes gave the following 3 regression equations between
mean particle sizes of 98 – 275 μm in which there was no significant differ-
ence (p > 0,05) between their respective regression coefficients:

$$Y_A = -0,258x + 97,987 \quad (r = -0,92 ; \quad n = 10)$$

$$Y_B = -0,360x + 133,376 \quad (r = -0,90 ; \quad n = 10)$$

$$Y_C = -0,378x + 149,469 \quad (r = -0,92 ; \quad n = 10) \ .$$

As done previously for equation $\underline{/1\_7}$, the regression coefficients of the
above equations were used to derive the following formula:

$$FI_{TRUE} = FI_{OBS} + 25,73 \text{ arbitrary units per 10 mg CP} \quad \dots\dots \quad \underline{/5\_7}$$

Substitution of equation $\underline{/3\_7}$ into $\underline{/5\_7}$ gave the formula:

$$\text{DAN-reactive lysine (g 16 g N}^{-1}) = 3,137 \times 10^{-2}(FI_{OBS}) + 1,613 \quad \dots. \quad \underline{/6\_7}$$

When the values for DAN-reactive lysine were recalculated using equation $\underline{/6\_7}$
the results (see DAN (2) values, Table 3) were in the same range as both TL
and SA results. Furthermore, there was no significant difference
(p > 0,001) between the values obtained by the two methods although the
interaction term (methods x samples) indicated that the methods did not give
similar values for every sample (p < 0,001). Unfortunately the DAN (2)
results correlated poorly with both TL (0,63) and SA (0,44) but this was
probably due to the fact that the soyabean samples covered an extremely
narrow range of lysine values.

To summarise, these results have demonstrated that sample particle size has
a profound effect on fluorescence intensity readings for dansylated soya
protein and that quenching remains significant for particles below 80 μm in
diameter. For comparative purposes it is therefore of paramount importance
to mill samples to similar particle size. Attempts to correct results for
quenching and to convert values into units of reactive lysine were only

partly successful. Nevertheless it is considered that this has been a useful exercise which warrants further attention.

4.4.4   Expression of fluorescence intensity in terms of reactive lysine

Notwithstanding the above difficulties, in order to facilitate a comparison with other methods (see §§ 5.2 , 6.3 and 7.3) it was desirable to express fluorescence intensity values for the DAN method in terms of reactive lysine.

Christoffers (1976) suggested that one could select a high quality standard of 100% reactive lysine content and use the relationship between its DAN fluorescence value and its total lysine content to convert DAN fluorescence values of other samples to their corresponding relative lysine values. However, by using a single reference standard, this procedure assumes that a linear regression line relating DAN fluorescence to TL for a series of standards (all with 100% reactive lysine content) would pass through the origin. In fact such a regression equation may have any value for its regression coefficient (slope) implying that this procedure will be most accurate for DAN fluorescence values closest to that of the standard and becomes less reliable further away. Alternatively it is highly unlikely that a satisfactory range of, for example, soya samples could be found that had sufficiently different total lysine contents but where all the constituent lysine was reactive. This approach was therefore not attempted.

A more acceptable approach which suggested itself was the use of regression analysis as a means of expressing fluorescence values in terms of reactive lysine values obtained by selected reference procedures, e.g. 'FDNB-reactive lysine equivalents'. In such a case it would be important to ensure that the mean particle size of 'unknown samples' was the same as that of the standard samples used to derive the regression equation in order to minimise differences in the extent of fluorescence quenching. Furthermore, since quenching factors may vary for different materials, a different regression equation may be required for each type of protein feed. All converted DAN results would of course be affected by the limitations of the selected reference method and the extent of the correlation between DAN and reference procedures.

To test this approach the DAN results (arbitrary fluorescence units) obtained for 3 different types of lysine-damaged samples were separately correlated with the corresponding reactive lysine values obtained in each case by 4 different chemical methods. On the basis of the results in Table 4 it was decided to express all DAN results (see §§ 5.2 , 6.3 and 7.3) in terms of reactive lysine equivalents according to the method with which it correlated

TABLE 4 Simple linear regression results for various protein samples correlating dansyl-reactive lysine with lysine as determined by other methods [a]

Sample	Method x	A	B	r
Soyaprotein heated alone [b] (n = 15)	TL	+ 14,19	− 34,45	0,62
	FDNB	+ 10,66	− 5,50	0,86 [e]
	DBL	+ 7,77	+ 10,25	0,85 [e]
	SA	+ 6,78	+ 14,38	0,86 [e]
Maillard-damaged soyaprotein [c] (n = 36)	TL	+ 17,71	− 57,26	0,79
	FDNB	+ 15,40	− 32,27	0,87
	DBL	+ 14,08	− 21,12	0,93
	SA	+ 9,18	− 2,00	0,96
Formaldehyde-damaged lactalbumin [d] (n = 20)	TL	+ 31,76	− 214,17	0,81
	FDNB	+ 8,57	+ 22,55	0,95
	DBL	+ 7,11	+ 32,92	0,97
	NIN	+ 11,21	+ 13,97	0,93

a − Results in each row are related by the equation: $Y_{DAN} = Ax + B$ where DAN values are expressed as relative fluorescence intensity in arbitrary units per 1,6 mg N ;

A is the regression coefficient (slope);

B is the intercept on the y-axis;

r = correlation coefficient;

n = number of samples analysed in duplicate;

TL = total lysine;

NIN, FDNB, DBL and SA are the reactive lysine methods employing the reagents ninhydrin, fluorodinitrobenzene, acid orange 12 and succinic anhydride respectively.

b , c & d − Sample preparation described in sections 3.1.1 , 3.1.2 and 3.1.3 respectively.

e − no significant difference ($p > 0,05$).

the best. Since there was no significant difference (p > 0,05) between the
correlation coefficients for FDNB , DBL and SA results for soyaprotein
heated alone in this case the more established FDNB method was selected.
Thus DAN results for soyaprotein heated in the absence of sugar were express-
ed as 'FDNB reactive lysine equivalents' (see § 5.2), for Maillard-damaged
soyaprotein as 'SA reactive lysine equivalents' (§ 6.3) and for formaldehyde-
damaged lactalbumin as 'DBL equivalents' (§ 7.3).

Since the results in Table 4 have been extensively evaluated in later chapters,
no further discussion is necessary at this stage.

CHAPTER 5

EVALUATION OF SOYAPROTEIN ISOLATES SUBJECTED
TO LYSINE DAMAGE IN THE
ABSENCE OF REDUCING SUGARS

5.1 INTRODUCTION

As already discussed in detail in section 2.1.1, heat treatment of proteins in the absence of reducing sugars can result in a variety of cross-linking reactions with $\epsilon-NH_2$ groups of lysine (see Fig. 1), the most prominent of which is probably with glutamine and asparagine side chains to form the iso-peptides G-L and A-L respectively. Such crosslinks markedly lower protein digestibility (Hurrell et al., 1976) and general amino acid availability (Ford, 1965) and, since both G-L and A-L are poorly released by protein hydrolysis (Bjarnason & Carpenter, 1970) they are primarily unavailable in this form. Furthermore, the $\epsilon-\omega$ linkage (Fig. 1a) is resistant to gut enzymes (Finot et al., 1978b) and hence if any isopeptides are released from the protein they are absorbed unmodified into the blood where only free G-L is utilised as a source of lysine, free A-L remaining unavailable (Mauron, 1970). Thus current knowledge indicates that lysine present as protein-bound A-L is totally unavailable whereas that present as protein-bound G-L is either unavailable or at best only partially available, depending on the ability of gut enzymes to penetrate the highly crosslinked material and release the G-L, and on its subsequent absorption and hydrolysis in the tissues.

Since the formation of A-L and G-L readily occur in foodstuffs even at practical drying temperatures (> 100° C) (Bjarnason & Carpenter, 1970), it is obviously of paramount importance that the industry as well as nutrition researchers have at their disposal reliable and convenient methods sensitive to this type of damage. In view of the lability of isopeptides in 6M HCl (110° C) (Otterburn et al., 1977) quantitative determination of G-L and A-L is, however, difficult as it involves enzymic hydrolysis of the protein followed by long (7 or more) hours and laborious separations on specialised ion exchange systems (Otterburn et al., 1977; Schmitz et al., 1976). Although Weder and Scharf (1981) have recently published a shorter (4 h) modified procedure using a simpler conventional amino acid analyser system, quantitative problems remain since the effectiveness of enzymic hydrolysis depends both on enzyme specificity and the degree of steric hindrance, factors

which become important if the level of crosslinking is too high or if cross-linking is accompanied by racemisation (Milligan & Holt, 1977; Hayashi & Kameda, 1980).

The alternative to specific isopeptide analysis is assessment of ε-NH$_2$ lysine modification by reactive lysine analysis. This approach would have the added advantage of being sensitive to some of the other known (Fig. 1) or even unknown types of lysine damage. On the other hand, such methods would probably exclude that fraction of protein-bound G-L that is available. Thus the expected effect will be a slight underestimation of lysine availability although, this might be at least partially if not totally compensated for by the general drop in protein digestibility due to poor penetrability of the highly crosslinked material.

The aim of this aspect of the work then was to test the sensitivity of various reactive lysine methods to lysine damage occurring in protein in the absence of reducing sugar and to compare the results with those of the more establish-ed FDNB method (Carpenter, 1960) as reference method (§ 5.2). Three of the samples used in this study (see § 3.1.1) were also evaluated in trials carried out by Prof. H. Erbersdobler (Erbersdobler & Anderson, 1983) so that it has been possible to compare these results with the author's reactive lysine values. Changes in total and biological values for lysine, histidine, glutamic and aspartic acids in the above 3 samples are also used to speculate about the types of crosslinking that have taken place (§ 5.3).

5.2 SENSITIVITY OF VARIOUS REACTIVE LYSINE METHODS TO HEAT DAMAGE AND THEIR CORRELATION WITH FDNB AS REFERENCE PROCEDURE

For this aspect of the work 12 differentially heat-damaged soyaprotein iso-lates and an unheated control were prepared as described in section 3.1.1 and analysed in duplicate for TL, DBL and FDNB- , SA- and DAN-reactive lysine (expressed as FDNB equivalents - see § 4.4.4).

Statistical analysis of the results gave acceptable pooled standard errors of the means for the FDNB, DBL, SA and DAN values of 0,04 , 0,05 , 0,11 and 0,07 respectively. Mean reactive lysine values were plotted against the extent of heat treatment. From Figure 16 it is clear that all 5 procedures showed at least some measure of sensitivity to lysine damage and that gener-ally with increased severity of heat treatment a corresponding decrease in total and reactive lysine was detected. Generally the FDNB and especially the TL methods were, however, considerably less sensitive than the other
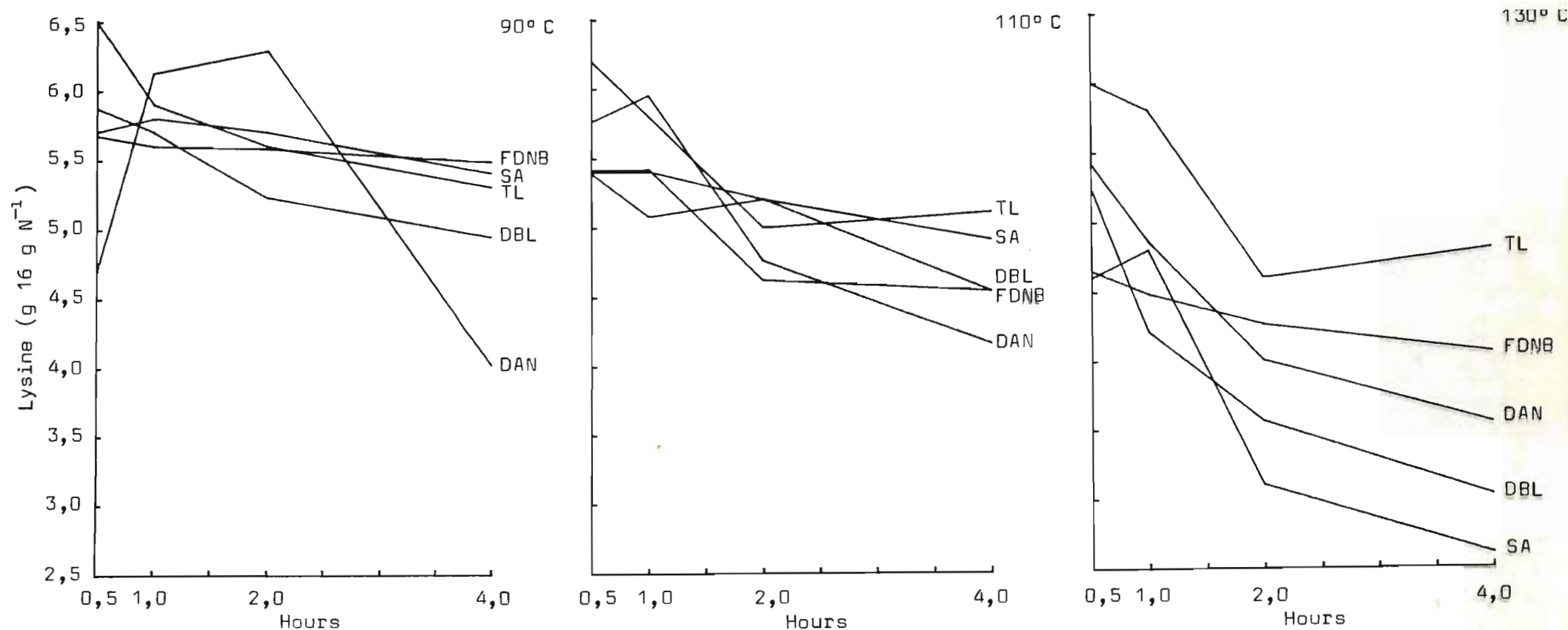
FIGURE 16 Comparison of the sensitivity of the FDNB, SA, TL, DBL and DAN methods to lysine damage in 12 differentially heat-treated soyaprotein samples (90 parts protein + 10 parts water). Samples were analysed as described in text and co-ordinates are means of duplicate determinations. DAN values are expressed in g 'FDNB lysine equivalents' per 16 g N. Unheated soyaprotein contained 6,0 g Lys per 16 g N

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methods, particularly for the most severely heated samples. By contrast, the DAN and DBL methods were consistently more sensitive over the entire range of heat treatments. Only the SA method did not show a consistent trend over the complete range of damage being insensitive to mild damage, but the most sensitive to severe lysine damage. The fact that DAN results expressed as 'FDNB equivalents' showed good sensitivity to heat damage with values within reasonable range of the other results, argues well for the acceptability of making such a conversion (see § 4.4.4).

Figure 17 shows a series of linear polynomials calculated from the data plotted in Figure 16. As could be expected, TL correlated very badly (r = 0,57) with FDNB lysine and gave a higher estimate of reactive lysine than FDNB at all levels of heat damage. One would, however, expect the TL values to have been higher since isopeptides are acid-labile and hence all lysine in this form is measured by total amino acid analysis (Otterburn et al., 1977). In fact if only isopeptide-type lysine damage was present in the samples studied, then there should have been no change at all in TL values over the entire range of samples. The results in Figures 16 and 17 therefore constitute strong evidence for the occurrence of other heat stimulated lysine modifications, the products of which must have been stable to the conditions of acid hydrolysis (§ 3.3). In this regard, any one or more of the reactions discussed in section 2.1.1 and detailed in Figure 1 could have taken place. Various other authors (e.g. Smith & Friedman, 1984; Rayner & Fox, 1978) have also detected a reduction in TL of protein heated in the absence of sugars, but none have attempted to identify the nature of any acid stable products that might be present. Of great significance though is the finding of Smith and Friedman (1984) that heating casein for 1 h at 121 , 200 and 300° C reduced total lysine by 4%, 19% and 30% respectively, these losses being accompanied by the appearance of 3 , 2 and 6 new ninhydrin-positive peaks on aminogrammes of the acid hydrolysed samples. The identity of these peaks, most of which eluted with the basic amino acids (as would be expected of many lysine derivatives including gizzerosine) should prove extremely interesting, although some could simply be thermal decomposition products since free lysine is pyrolysed if heated for 1 h at 250° C (Breitbart & Nawar, 1979).

In contrast to TL both the SA (r = 0,94) and DBL (r = 0,93) methods correlated well with the FDNB method (Fig. 17) but gave reactive lysine values (Fig. 16) that were in some cases very different from those of FDNB. Below a value of approximately 5,0 g per 16 g N , DBL and SA methods gave estimates of reactive lysine that were generally lower than that of FDNB
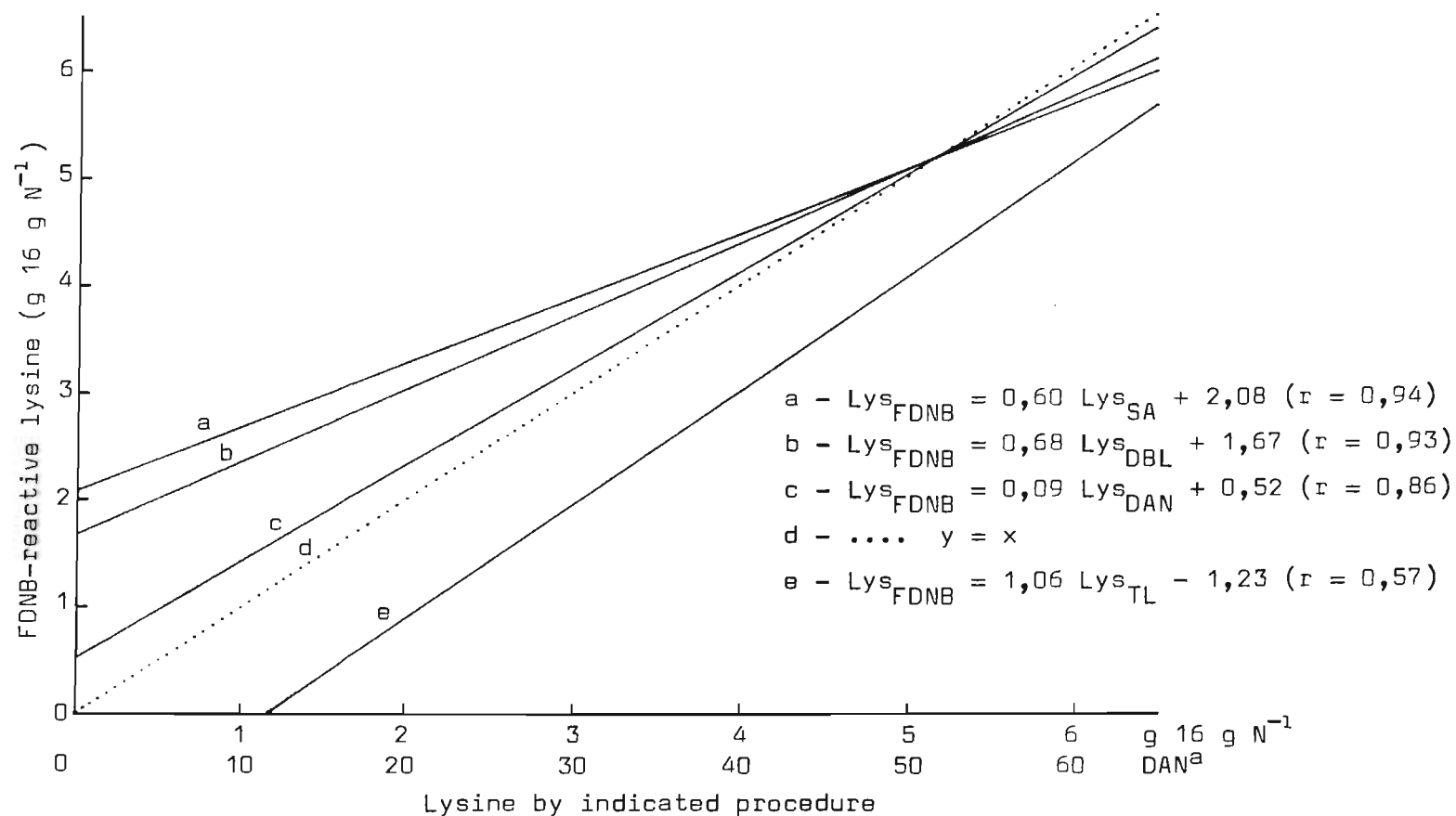
FIGURE 17 Correlation between FDNB-reactive lysine and TL, SA-, DBL- and DAN-reactive lysine values for 15 soyaprotein samples heated in the absence of reducing sugars (see Fig. 16). Samples were analysed as described in the text (Ch. 3).

[a]DAN values expressed as arbitrary fluorescence units per 1,6 mg N (see § 3.8.2)

whereas above 5,0 g per 16 g N their estimates were higher. The lower DBL and SA values (below 5,0 g per 16 g N) could be due to the failure of these reagents to fully penetrate the more damaged and hence highly crosslinked material. Succinylation and dye-binding are after all carried out under much milder conditions than is dinitrophenylation (see Ch. 3). Since gut enzymes also function under mild conditions, the SA and DBL methods may better reflect the influence of poor digestibility on lysine availability, although no allowance is made for the possible partial availability of lysine present as protein-bound G-L (Finot et al., 1978 b). The higher DBL and SA values (Fig. 17; above 5,0 g per 16 g N) for less damaged material could be due to more complete modification of free ϵ-NH$_2$ groups by the respective reagents reflecting the care the present author took to optimise both SA and DBL reactions. The SA method requires an 80-fold molar excess of succinic anhydride relative to TL residues in order to attain maximal ϵ-NH$_2$ modification (Anderson, 1980; Anderson & Quicke, 1984), and optimal dye-binding with soya is only achieved after 5 h with this type of material (see § 4.2 and Fig. 13). It was not established whether the FDNB method achieved complete dinitro- phenylation of ϵ-NH$_2$ groups but this was assumed in view of the decades of development of this procedure (see § 2.2.1). The study by Smith and Friedman (1984) on casein heated for 1 h at 37° C , 121° C , 200° C and 300° C gave almost identical results for FDNB and DBL methods but unfortunately it is not clear whether their 2 h dye-reaction time was long enough for optimal binding of the dye to the casein.

To summarise, all methods studied showed at least some measure of sensitivity to heat damage but there were large variations in the results obtained with different methods. In view of the acid lability of G-L and A-L, the TL method was the most insensitive to lysine damage. A small reduction in TL values suggested the presence of other types of crosslinks with lysine which are acid stable. SA (r = 0,94) and DBL (r = 0,93) methods correlated well with the FDNB method but were more sensitive than FDNB for the more severely heated protein (i.e. 130° C for 2 or 4 h). It is speculated that this may be due to failure of the milder succinic anhydride and acid orange 12 reagents to penetrate the highly crosslinked protein. SA and DBL values were higher than FDNB results for mildly damaged samples (e.g. 110° C , 2 h) possibly due to sub-optimal dinitrophenylation of ϵ-NH$_2$ groups. The DAN method showed trends and sensitivities to lysine damage similar to those obtained with SA and DBL methods. Expression of the DAN values as FDNB-reactive lysine equi- valents proved acceptable although a higher correlation (r = 0,86) between

fluorescence results and FDNB values would have been more desirable (see § 4.4.4). Finally, in view of the large variation in results between methods none of the procedures tested can be regarded as ideal for the assessment of available lysine but all methods except TL should prove useful as relative indicators of the extent of lysine damage in heat-damaged proteins.

5.3 COMPARISON BETWEEN CHEMICAL AND BIOLOGICAL METHODS

For this aspect of the work 3 differently heat-damaged soyaprotein samples were prepared in bulk as described in section 3.1.1 and analysed in dupli- cate for TL , DBL and FDNB- , SA- and DAN-reactive lysine contents. The same three samples were also analysed by Erbersdobler (see Grimm, 1973; Anderson & Erbersdobler, 1982; Erbersdobler & Anderson, 1983) for total histidine, aspartic and glutamic acids, plasma lysine and glutamic acid, as well as lysine- , histidine- , aspartic- and glutamic-acid digestibility. The aims of this study were firstly, to compare the various reactive lysine values with the corresponding biological values, and secondly, to obtain more information about the types of crosslinking that had taken place.

Despite the numerous problems associated with quantifying the results of animal experiments (see comments, Ch. 1), a comparison between reactive lysine and biological values is always useful and can at least give an idea of the generally expected trends of lysine damage. The results are summar- ised in Table 5 in which the values obtained by each procedure are expressed as a percentage of the corresponding value obtained with an unheated sample. As in the previous study (§ 5.2) all lysine methods reflected an increasing lysine loss with increased severity of heat treatment with the TL method being least sensitive and the SA method the most sensitive procedure. Plasma lysine and lysine digestibility also show the same trends although the results for the 138° C , 24 h sample are very different. The percentage decrease in plasma lysine for this sample is more comparable to that record- ed for the DAN and FDNB methods with DBL and SA results much lower and the corresponding TL results much higher. By contrast the percentage lysine digestibility for the 138° C, 24 h sample is much higher than all other results except that of TL. Although it is difficult to make any definite conclusion, the plasma lysine figure of 48% availability for this level of heat damage seems more realistic than that of lysine digestibility (80%) which makes the DAN and FDNB methods most suitable for assessing lysine damage at this level of heat treatment. By contrast, for the 160° C, 24 h

sample, the SA method gave results that were most comparable to both bio-
assays with the DBL, DAN and possibly the FDNB methods within the same range.
Only the DAN and FDNB methods therefore appear to be most similar to the bio-
assays over the entire range of heat treatments.

For the 95° C, 24 h sample, the reactive lysine results (Table 5) showed a
10 - 20 % reduction whereas the TL as well as both bioassays remained un-
changed relative to the unheated sample. These results indicate that in
this sample no acid-stable crosslinks have been formed but only small quan-
tities of isopeptides, the number of crosslinks being insufficient to signi-
ficantly affect either lysine digestibility or absorption into the plasma.
This is reflected in the small reduction in digestibility of aspartic and
glutamic acids (or their amides since glutamine and asparagine decompose to
their acids during acid hydrolysis). As expected total aspartic and glutamic
acid were unaffected by this or either of the more severe heat treatments,
since isopeptides are hydrolysed during conventional acid hydrolysis
(Otterburn et al., 1977). Total histidine for the 95° C, 24 h sample also
showed no change from the value for the unheated sample demonstrating its
lack of involvement in acid-stable crosslinks with lysine in this sample.

Table 5 also shows that with increased severity of heat treatment, first to
138° C, 24 h and then 160° C, 24 h, there was a significant reduction in all
lysine and histidine values as well as the bioassays for aspartic and glut-
amic acid. These results constitute strong evidence for the formation of
numerous isopeptides and acid-stable crosslinks that serve to significantly
lower the reactivity, digestibility and consequently the absorption and
utilisation of lysine in the soyaprotein. Furthermore, the large reduction
in all three histidine values, could be due to the formation of acid stable
histidine derivatives such as gizzerosine (Fig. 1) (Okazaki et al., 1983).
Smith and Friedman (1984) also found that losses in total histidine content
were associated with lysine losses for differentially heat-treated casein
while Rayner and Fox (1978) found similar trends for beef muscle heated at
121° C for 12 h. The latter authors recorded a 7% reduction in TL, a
15% reduction in FDNB lysine and in vitro pronase digestion losses of 31%,
21%, 58% and 36% for lysine, histidine, aspartic acid and glutamic acid
respectively. It was suggested that isopeptide interactions were the main
causes of reduction in pronase digestion, but the 7% loss of TL and the 21%
reduction in pronase digestion of histidine suggests that other acid-stable
lysine-histidine derivatives could have also been present. Finally,
Shorrock and Ford (1978) reported the inhibition of amino acid uptake in

TABLE 5 Chemical and biological evaluation of lysine, histidine, aspartic acid and glutamic acid in 3 differentially heat-damaged soyaprotein isolates[a]

ANALYSIS	S A M P L E		
	Soyaprotein 95° C, 24 h	Soyaprotein 138° C, 24 h	Soyaprotein 160° C, 24 h
TL	100	95	80
FDNB	85	56	29
SA	88	25	8
DBL	81	28	17
DAN	81	48	21
Plasma Lys[b]	113	48	0
Lys digestibility[b]	98	80	13
Total His[b]	100	85	77
Plasma His[b]	115	54	15
His digestibility[b]	94	88	23
Total Asp[b]	100	105	103
Asp digestibility[b]	94	89	21
Total Glu[b]	100	103	103
Plasma Glu[b]	100	23	6
Glu digestibility[b]	95	94	23

a - Results calculated as percent of corresponding values obtained for unheated sample except for digestibility values where due to presence of trypsin inhibitor, the unheated TL value (6,0 g 16 g N^{-1}) was taken as 100% .

b - Analyses done by Erbersdobler and partly published in Erbersdobler and Anderson (1983) and Anderson and Erbersdobler (1982); for plasma amino acid analysis, the concentration of free amino acids in portal plasma of adult rats was measured 45 , 60 , 75 , 90 , 120 and 180 min after intake of test protein and relative lysine availability calculated as described by Erbersdobler et al. (1968) and Grimm (1973); amino acid digestibility was determined with rats according to the method described in Brüggemann and Erbersdobler (1968 b) and Gunsser (1972).

everted sacs of rat small intestine by certain unavailable peptides isolated
from enzymic digests of heat-damaged (135° C, 20 h) cod fillet. Although
they did not identify any of the mentioned 'unavailable peptides' they
suggested that G-L may have been released by enzymic digestion and that this
may have interfered with the absorption of amino acids including lysine, by
blocking a mechanism involved in their transport across the mucosal barrier
(Buraczewski et al., 1967). Indeed it is highly likely that both G-L and
A-L were present in their 'unavailable peptide' fraction since both total
and available lysine as measured by microbiological assay were respectively
28% and 79% lower in the heated as compared to the unheated cod fillet.
Furthermore, typical to G-L, they reported that the 'unavailable peptides'
were only unavailable in the sense that they were relatively slow to digest.
Paralleling the above mentioned drop in lysine values there was also a 20%
and 56% loss of total and available histidine respectively. These results
also then suggest the presence of a lysine-histidine complex as part of the
'unavailable peptide mixture, this possibility being strengthened by the
fact that gizzerosine is readily formed in fish meal (Okazaki et al., 1983).

To summarise, the development of isopeptide-type lysine modifications in
heat-damaged soyaprotein is suggested by progressive and simultaneous reduc-
tions in reactive lysine, plasma lysine and plasma glutamate levels, as well
as indigestible lysine, aspartate and glutamate associated with increased
severity of heat treatment. The DAN and FDNB results gave the best general
agreement with the plasma lysine and lysine digestibility results although
the SA results compared the best in severely heated (160° C, 24 h) samples.
Most reactive lysine methods tended to indicate that the reduction in react-
ive lysine due to the unreactivity of the partially available isopeptide
G-L, is balanced out by the drop in digestibility due to those crosslinks
which affected the ability of the chemical reagents to fully penetrate the
material. The simultaneous reduction in values for chemical and bioassays
for lysine and histidine in the heat-treated soyaprotein, together with
results discussed in section 5.2 and the findings of other workers (see above)
constitute strong evidence for the existence of acid stable lysine-histidine
derivatives. Whether this includes gizzerosine will only be established
when at least a qualitative assay has been successfully developed.

CHAPTER 6

EVALUATION OF SOYAPROTEIN ISOLATES
SUBJECTED TO LYSINE DAMAGE IN
THE PRESENCE OF REDUCING SUGARS

6.1 INTRODUCTION

As already discussed in detail in section 2.1.2, heat treatment of proteins
in the presence of reducing sugars can involve lysine and other amino acids
in a variety of reactions which, for convenience, are divided up into 'early'-
and 'late'-type Maillard reactions. In 'early' Maillard reactions (Fig. 2)
reducing sugars combine with ϵ-NH_2 groups to form a colourless deoxyketosyl
derivative via a Schiff's base and an aldosylamine derivative. Whereas the
latter intermediates are nutritionally available as sources of lysine (Finot
& Magnenat, 1981), the deoxyketosyl compound is totally unavailable (Hurrell
& Carpenter, 1981), can inhibit disaccharidase activity (Lee et al., 1977)
and in the case of fructoselysine (F-L) may be responsible for nephrocyto-
megalia in rats (von Wangenheim et al., 1984). With increased severity of
heat treatment brown-coloured 'late' Maillard products (Fig. 3) are formed
resulting in lysine destruction and reduced protein digestibility (Hurrell
& Carpenter, 1977a). Certain 'late' Maillard products also destroy vitamins
in milk powder (Ford et al., 1983), affect gut enzymes and microflora (Oste
et al., 1983), cause adverse physiological changes in rats (Lee et al., 1981)
and may even be mutagenic (Omura et al., 1983).

Since Maillard reactions are particularly prevalent during heat treatment
and may even occur during storage, there is a need for reliable and conveni-
ent methods for the assessment of this type of lysine damage. While none of
the procedures currently available are ideally suited to the detection of
all phases of Maillard damage some are better than others for specific pur-
poses. Furosine (FUR) analysis is an excellent indicator of 'early'
Maillard damage (Erbersdobler, 1970), however its determination requires the
use of expensive equipment such as an amino acid analyser (Mauron, 1972;
Erbersdobler et al., 1979 & 1984a; Eichner & Ciner-Doruk, 1981; Eichner &
Wolf, 1983), a gas chromatograph (Büser & Erbersdobler, 1985) or a high per-
formance liquid chromatograph (HPLC) (Moll et al., 1982; Chiang, 1983).
Furthermore, release of FUR requires acid hydrolysis of the protein (§ 3.4)
and it is possible that further Maillard reactions may occur during this
process. The more economical gc method of Eichner and Wolf (1983) for

estimating volatile Strecker degradation products (Fig. 3) may prove more
useful since no acid hydrolysis is necessary and these products are good
'early' indicators of Maillard damage.

An alternative approach is to use chemical assays for reactive lysine, which
show good potential as relative indicators of both 'early' and 'late'
Maillard damage. However, in view of numerous procedural problems as well
as the observation (Hurrell & Carpenter, 1981) that some of the electro-
phillic reagents employed apparently react with the blocked unavailable
deoxyketosyl derivatives, further research is necessary to establish a
reliable and efficient method suitable for analysis.

In order to test the effectiveness of the various reactive lysine methods
under examination in this thesis, it was necessary to prepare a suitable
series of Maillard-damaged samples. For this purpose 36 soyaprotein samples
were prepared by heating at either 90 , 110 or 130° C for 0,5 , 1 , 2 or 4 h
in the presence of glucose, lactose or xylose (see § 3.1.2 for details).
Attempts to elucidate the extent of lysine damage in each sample and to
identify some of the specific products involved are discussed in section 6.2.
These results are then used in section 6.3 to assist in the comparison of
the various reactive lysine methods.

6.2 ASSESSMENT OF EXTENT AND TYPE OF MAILLARD DAMAGE

In view of the variety of products that could exist in the Maillard-damaged
soyaprotein tested, it was important to first get an idea of the relative
proportions of 'early'- and 'late'-type Maillard products present in each
sample. For this purpose the approach used by Hurrell et al. (1983) for
examining the influence of heat on the progress of the Maillard reaction in
milk powders, was applied to the soyaprotein heated in the presence of glucose
and lactose (§ 3.1.2). As described in detail in section 3.4, 'early'
Maillard damage can be estimated by using the FUR content of samples to calcu-
late levels of the deoxyketosyl forms (F-L and L-L), the quantities of lysine
present in these two forms (i.e. LFL and LLL) as well as an estimate of avail-
able lysine (AL). Unfortunately this approach could not be used for xylose-
damaged soyaprotein since the existence of 'xylanosyl-lysine' has not been
confirmed and such samples do not release any FUR on acid hydrolysis.

An indication of the extent of 'late' Maillard reactions could be obtained
for all samples by estimating destroyed lysine (DL) and the degree of sample
browning. DL was calculated (§ 3.4) as the difference between TL values for

unheated and heated samples while the extent of sample browning (B) was esti-
mated by measuring the absorbance at 450 nm of the water-soluble fraction
(supernatant) of pronase-digested samples (see § 3.10.3), the brown colour
emanating mainly from pre-melanoidin pigments (§ 2.1.2).

The values obtained for the glucose- and lactose-damaged samples respectively
are presented graphically in Figures 18 and 19. Clearly with increased
severity of heat treatment there is an initial steep rise in the level of
lysine present as the deoxyketosyl form (LFL or LLL), followed by a subsequent
decrease back to zero as F-L and L-L are converted further to 'late'
Maillard products. Comparison of Figures 18 and 19 shows that F-L is produced
at a more rapid rate and peaks sooner than L-L, probably due to the greater
reactivity of glucose than lactose, particularly under milder heating con-
ditions where no hydrolysis of lactose to glucose and galactose has commenced.
It is also evident that DL remained fairly low notwithstanding increasing
LLL and FFL values but rose rapidly as soon as the deoxyketosyl levels start-
ed dropping. This is because acid hydrolysis will release lysine from the
L-L and F-L derivatives but not from 'late' Maillard products in which the
amino acid is often hardly recognisable. In most cases all the original
lysine units (6,0 g 16 g N^{-1}) were accounted for either as blocked lysine
(LLL or LFL), AL or DL (i.e. TL unheated sample = AL + DL + LLL or LFL in the
heated sample), which serves to confirm the validity of employing the
Hurrell et al. (1983) approach.

As expected, sample browning (B) remained low during the 'early' Maillard
phase but increased sharply as the colourless deoxyketosyl products (F-L and
L-L) were converted to brown-coloured 'late' Maillard intermediates. An
important observation is that up to 30% of lysine residues could be bound
in the unavailable L-L or F-L forms without any observable change in the
natural colour of the soya. Hurrell and Carpenter (1981) also found that
milk powder heated at 60° C showed no colour change after 9 weeks despite a
40% conversion of lysine residues to the bound L-L form. These findings are
of significance to the food industry which in the past has tended to judge
the nutritive value of a product solely on the basis of its physical appear-
ance.

Also as anticipated, AL values decreased continuously (Figs 18 & 19) with
increased severity of heat damage since both 'early' and 'late' Maillard
products are unavailable sources of lysine. The only exceptions were the 4 h,
130° C soya-glucose and the 4 h, 90° C soya-lactose samples whose AL and DL
values were respectively higher and lower than those of their corresponding
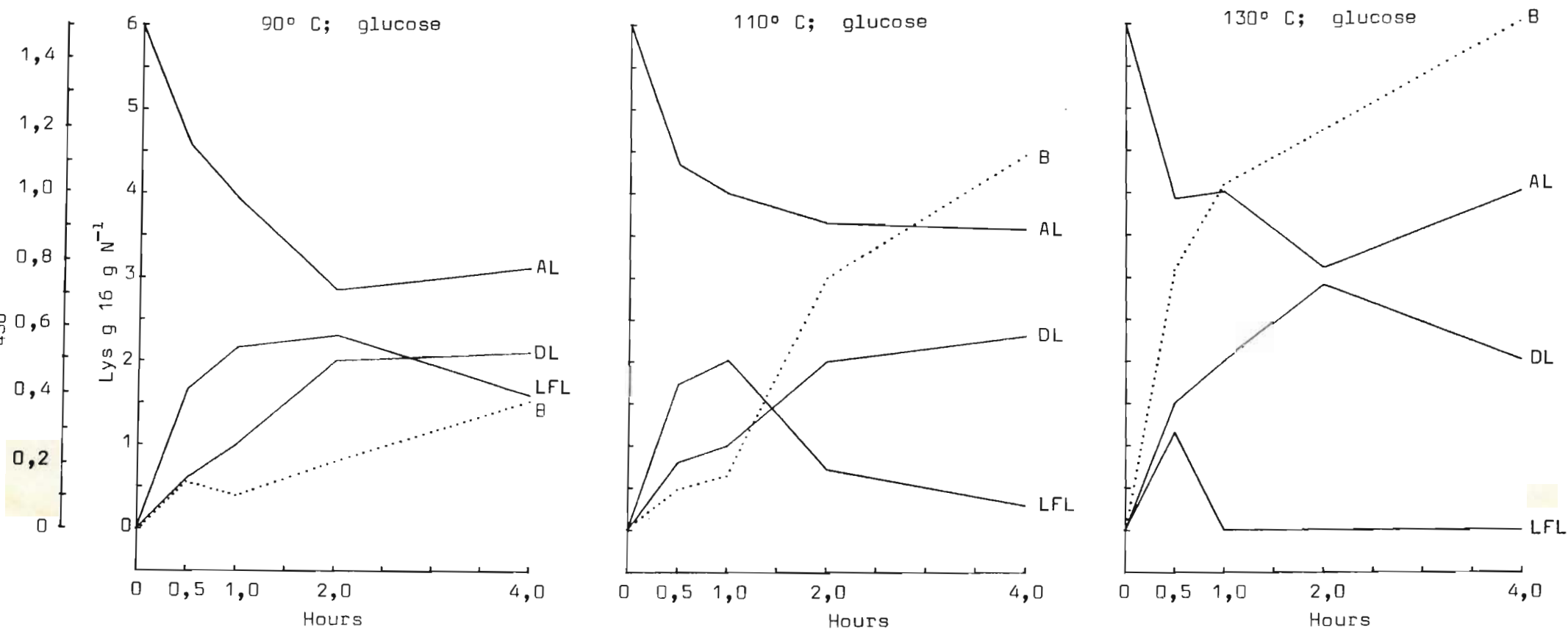
FIGURE 18 Levels of available lysine (AL), destroyed lysine (DL) and lysine present as fructoselysine (LFL) in relation to the extent of browning (B) of soyaprotein heated for 0,5 , 1 , 2 or 4 h at 90 , 110 or 130° C in the presence of glucose. The various parameters were calculated as follows:

$$DL = TL_{unheated\ sample} - TL_{heated\ sample}\ ;\quad LFL = FUR \times 1,4\ ;\quad AL = TL_{heated\ sample} - \frac{LFL}{2}\quad (see\ text).$$

Absorbance values are means of duplicates while other parameters were calculated from single TL and FUR values.
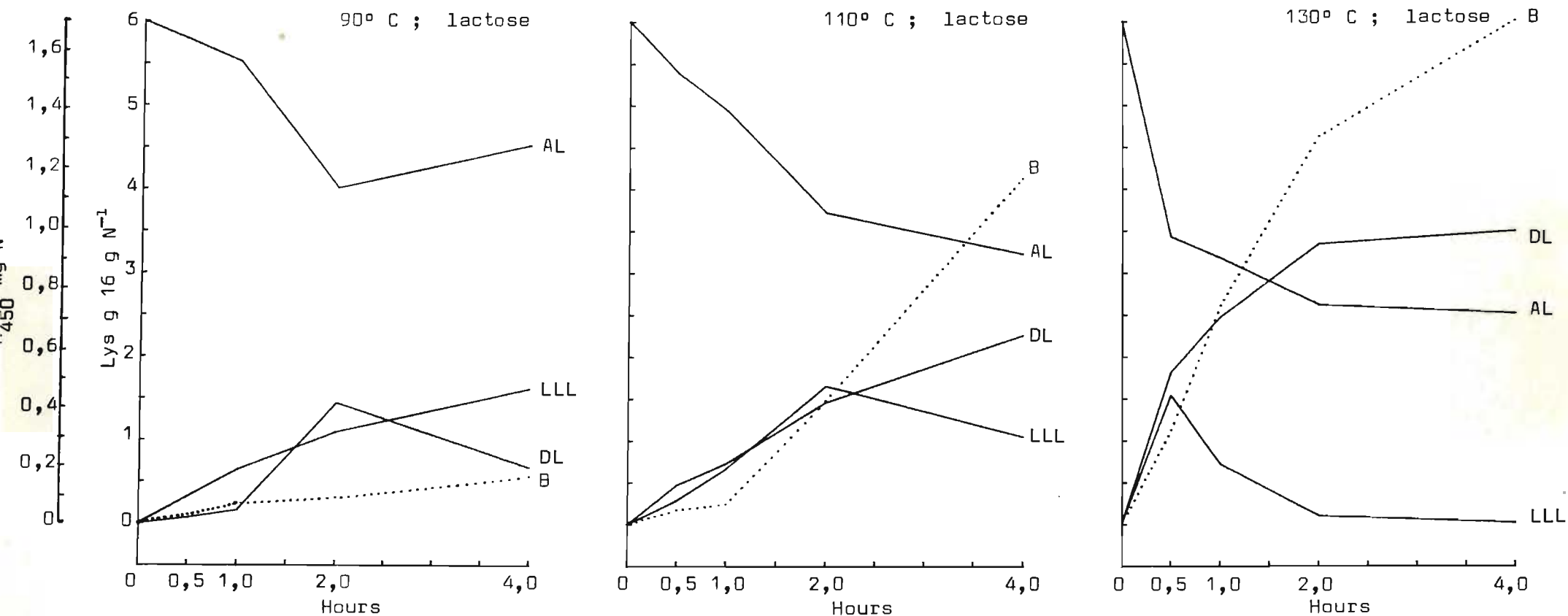
FIGURE 19 Levels of available lysine (AL), destroyed lysine (DL) and lysine present as lactulosyl-lysine (LLL) in relation to the extent of browning (B) of soyaprotein heated for 0,5 , 1 , 2 or 4 h at 90, 110 or 130° C in the presence of lactose. The various parameters were calculated as follows:

$$DL = TL_{unheated \atop sample} - TL_{heated \atop sample} \ ; \quad LLL = FUR \times 1,4 \ ; \quad AL = TL_{heated \atop sample} - \frac{LLL}{2} \ (see \ text).$$

Absorbance values are means of duplicates while other parameters were calculated from single TL and FUR values.

'2 h samples'. No satisfactory explanation could be found for these exceptions which were not manifestations of analytical error since the values were confirmed by repeat analyses. Perhaps significantly other workers (Hurrell et al., 1983; Smith & Friedman, 1984) have reported similar anomalous results but only for severely damaged materials. It was suggested that more severe heat treatment may be capable of converting certain previously acid-resistant Maillard products into intermediates that can release lysine for detection by amino acid analysis.

Figure 20 depicts the levels of DL in relation to the extent of browning in the xylose-damaged soyaprotein samples. Clearly the extent of lysine destruction and sample browning was much greater (note differences in A_{450} scales in Figs 18 - 20) for these samples than for the glucose or lactose-treated materials. This agrees with the findings of Lewis and Lea (1959) and Knipfel et al. (1983) who found that xylose reacts more readily than either glucose or lactose with ϵ-NH_2 groups probably because the proportion of the more highly reactive open chain form is greater in the case of pentoses than hexoses. Thus all xylose-damaged samples probably contained a greater proportion of 'late' Maillard products relative to 'early' products. Interestingly there was no marked increase in DL from 90° C to 110° C or 130° C indicating that the 'late' Maillard reactions proceeded very rapidly to completion even at 90° C. As in the case of glucose- and lactose-soya mixtures no satisfactory explanation could be found for the decrease in DL values in the 4 h compared with the 2 h sample (Fig. 20).

To summarise, it appears that glucose-soya and lactose-soya mixtures heated at 90° C up to 2 h or 110° C up to 1 h contain primarily 'early' Maillard products whereas more severe heat treatments tend to make 'late' Maillard products predominate. Since no xylanosyl-lysine as such has been identified, it was not possible to estimate the extent of 'early' xylose damage to soyaprotein, but certainly 'late' Maillard damage, as evidenced by browning, was more severe in all xylose-soya mixtures than in their glucose and lactose counterparts. No satisfactory explanation could be found for the lower DL values in the '4 h samples' relative to the '2 h samples' recorded for glucose- , lactose- and xylose-treated soyaprotein.

Scrutiny of LFL, LLL, DL and B values reveals, however, that it is not possible to classify samples into distinct groups having only 'early' or 'late' Maillard products since most contained both types of products with only the proportions varying.
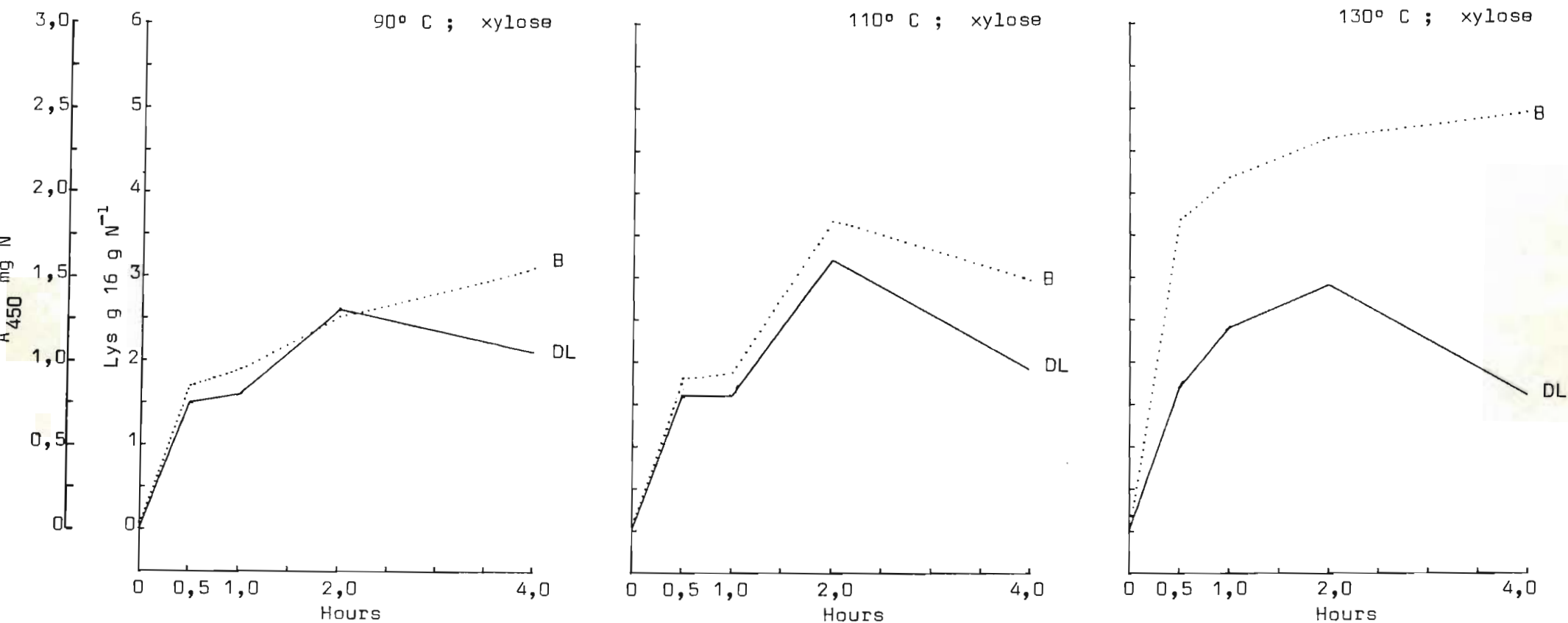
FIGURE 20   Levels of destroyed lysine (DL) in relation to the extent of browning (B) of soyaprotein heated for 0,5 , 1 , 2 or 4 h at 90 , 110 or 130° C in the presence of xylose.   DL = $TL_{unheated \atop sample} - TL_{heated \atop sample}$ (see text).

Absorbance values are means of duplicates while DL values were calculated from single TL determinations

6.3 SENSITIVITY OF REACTIVE LYSINE METHODS TO MAILLARD DAMAGE AND THEIR CORRELATION WITH FDNB AS REFERENCE PROCEDURE

The 36 Maillard-damaged soya samples described in section 6.2 were analysed in duplicate for DBL, FDNB- , SA- and DAN- (expressed as SA-lysine equivalents, see § 4.4.4) reactive lysine. Statistical analysis showed that the results for each method were highly reproducible with pooled standard errors of means of 0,06 , 0,05 , 0,11 and 0,05 respectively. To evaluate the sensitivity of each method to Maillard damage, the mean reactive lysine values were plotted against the extent of heat treatment (Figs 21 - 23). Correlations of the results obtained by the various methods with those obtained by the FDNB procedure are given in Figure 24.

As seen in Figures 21 - 23, the 5 procedures showed at least some measure of sensitivity to lysine damage in Maillard-type samples and in most cases increased severity of heat treatment was associated with a corresponding decrease in reactive lysine. The relative sensitivities of the methods varied with the degree and type of Maillard damage. For the mildly damaged glucose-soya samples (Fig. 21) with 'early' Maillard products predominating (e.g. samples heated at 90° C for 0,5 , 1 or 2 h and at 110° C for 0,5 and 1 h), FDNB was the most sensitive followed by DAN or DBL, SA and then TL methods. This order changed slightly for mildly damaged lactose-soya samples (Fig. 22) (e.g. samples heated at 90° C for 0,5 , 1 and 2 h and at 110° C for 0,5 and 1 h) in that DBL was more sensitive than FDNB and DAN was even less sensitive than the TL method. For the more severely damaged samples containing a predominance of 'late' Maillard products, the order of sensitivity was again different with DAN or SA showing greatest sensitivity followed by DBL, FDNB and TL. All severely damaged samples (i.e. glucose-soya and lactose-soya samples heated at 110° C for 2 and 4 h and at 130° C for 0,5 , 1 , 2 and 4 h) showed this order of sensitivity, the only exception being the lactose-soya sample that had been heated at 90° C for 4 h, whose sensitivity pattern matched that of the mildly damaged glucose-soya samples (see above).

Perusal of Figures 21 - 23 shows that TL was significantly reduced even after mild heat treatment. However, Figure 24 shows that TL values for the 36 Maillard-damaged samples correlated poorly (r = 0,77) with and gave a higher estimate of reactive lysine than FDNB at all levels of heat damage. The high TL values in the case of the less damaged samples could be due to their relatively high content of the 'early' Maillard products F-L or L-L which although poorly digested and totally unavailable as sources of lysine (Erbersdobler, 1980), release 40 - 50 % of their lysine content during acid hydrolysis (Finot & Mauron, 1972; Bujard & Finot, 1978). Since the existence of
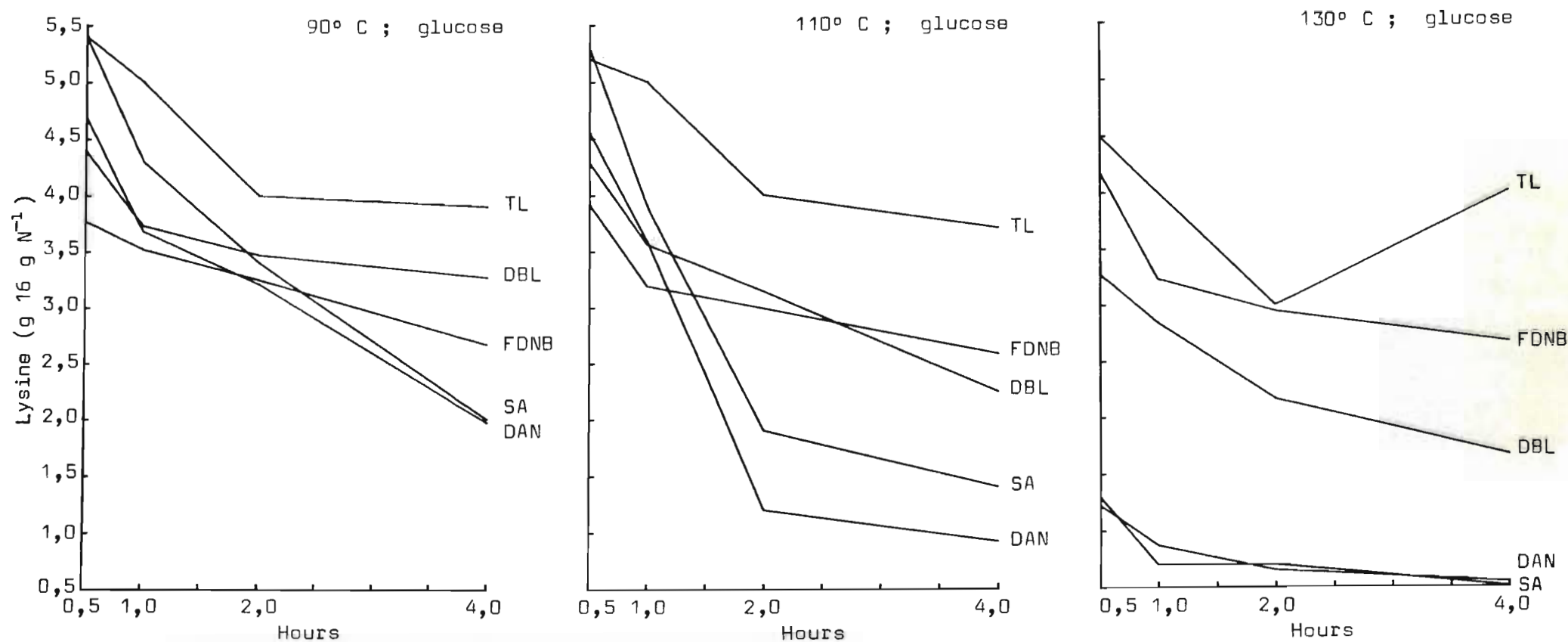
FIGURE 21   Comparison of the sensitivity of the FDNB, SA, TL, DBL and DAN methods to lysine damage in 12 model soya-
protein samples prepared by heating at 90 , 110 or 130° C for 0,5 , 1 , 2 or 4 h in the presence of
glucose.   DAN values are expressed in g 'SA-lysine equivalents' per 16 g N (see § 4.4.4).   Unheated soya-
protein contained 6,0 g Lys 16 g N^{-1}.   All results are means of duplicates except for TL which are
single determinations.
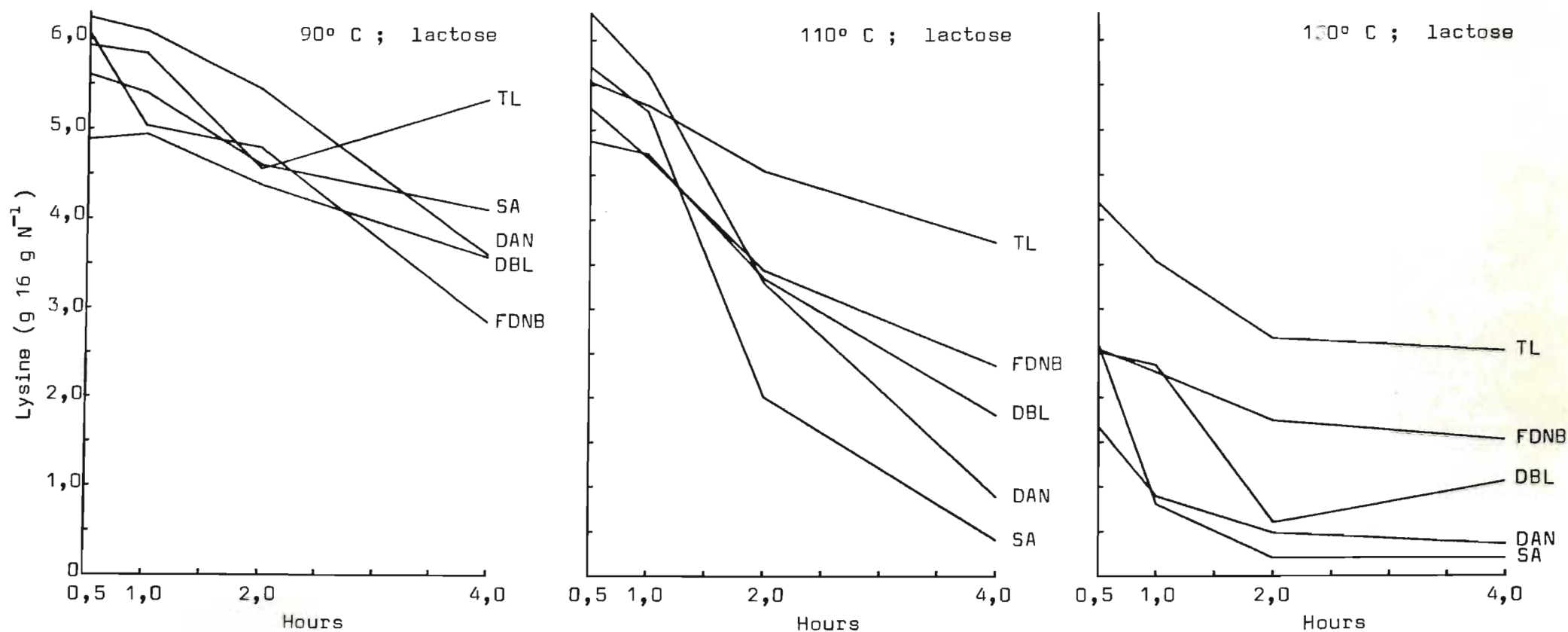
FIGURE 22   Comparison of the sensitivity of the FDNB, SA, TL, DBL and DAN methods to lysine damage in 12 model soya-
protein samples prepared by heating at 90 , 110 or 130° C for 0,5 , 1 , 2 or 4 h in the presence of
lactose. DAN values are expressed in g 'SA-lysine equivalents' per 16 g N (see § 4.4.4). Unheated soya-
protein contained 6,0 g Lys 16 g N^{-1}. All results are means of duplicates except for TL which are
single determinations.
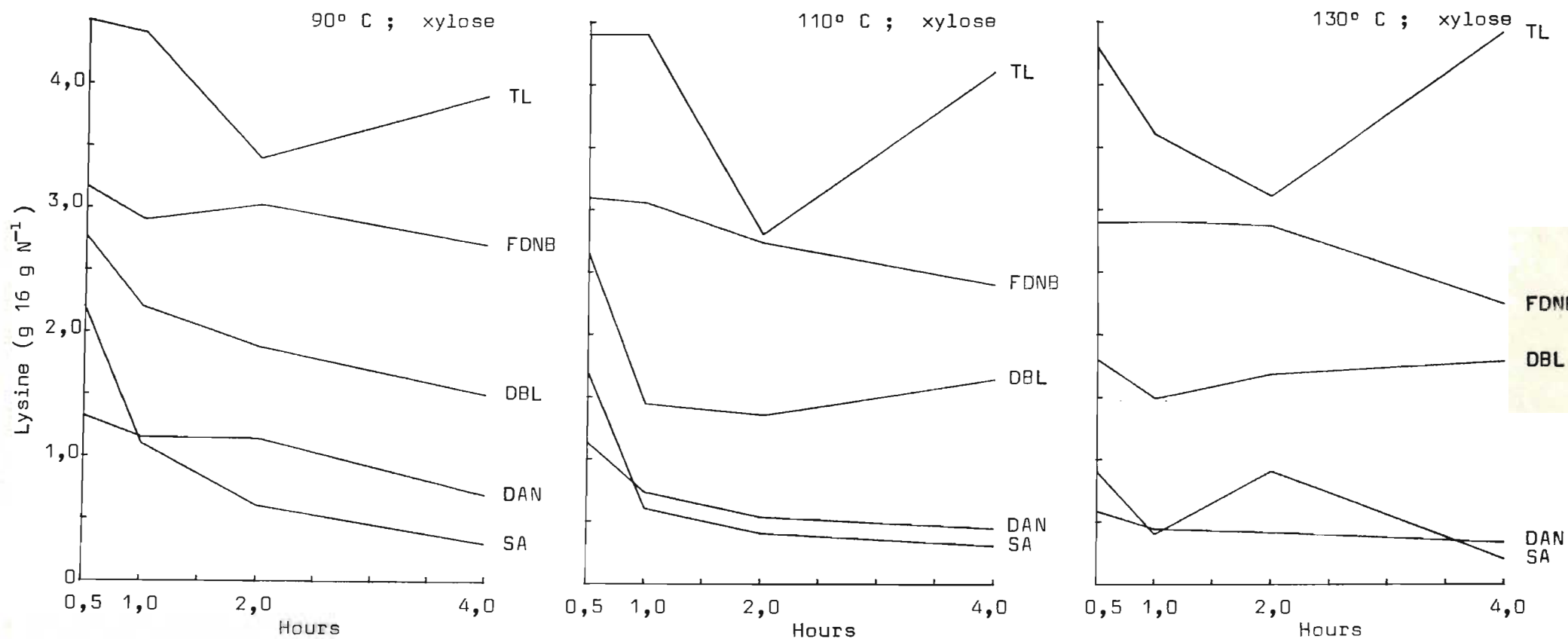
FIGURE 23 Comparison of the sensitivity of the FDNB, SA, TL, DBL and DAN methods to lysine damage in 12 model soya-protein samples prepared by heating at 90 , 110 or 130° C for 0,5 , 1 , 2 or 4 h in the presence of xylose. DAN values are expressed in g 'SA-lysine equivalents' per 16 g N (see § 4.4.4). Unheated soya contained 6,0 g Lys 16 g N^{-1} . All results are means of duplicates except for TL which are single determinations.
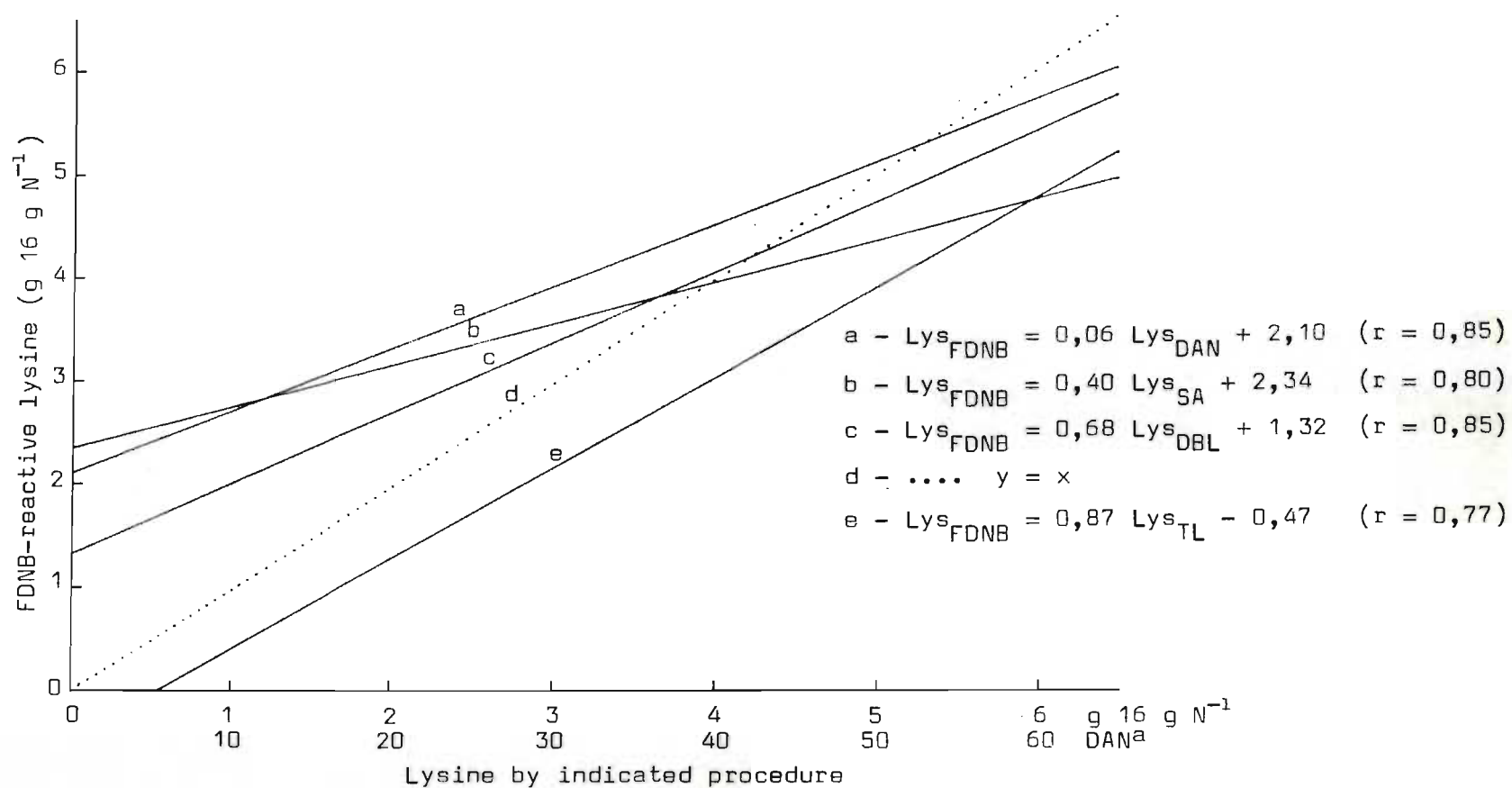
FIGURE 24   Correlation between FDNB-reactive lysine and TL, SA-, DBL- and DAN-reactive lysine values for 36 Maillard-damaged soyaprotein samples. Samples were analysed as described in text (Ch. 3). All lines were fitted by simple linear regression analysis employing a HP 33C calculator.

[a]DAN values expressed as arbitrary fluorescence units per 1,6 mg N (see § 3.8.2)

'xylanosyl-lysine' has as yet not been confirmed (see § 6.2), it is uncertain whether the same observation holds for xylose-soya samples although Figure 23 shows that TL values were higher than those obtained by other methods. Hurrell and Carpenter (1981) found that TL gave a good estimate of lysine availability in more severely damaged 'late' Maillard material since under these conditions most of the lysine is destroyed and the products do not release lysine on acid hydrolysis. In the present results, however, TL once again gave a higher estimate of reactive lysine than FDNB with TL for the glucose-130° C - 4 h, lactose-90° C - 4 h and all xylose-4 h samples actually increasing relative to their '2 h' counterparts. As already discussed in section 6.2 , this could be due to certain previously acid-resistant 'late' Maillard products being re-converted by the more severe heat treatment, into intermediates that can release lysine for detection by amino acid analysis. It is unlikely that this involved a reverse reaction yielding F-L or L-L (Fig. 3) since as shown in Figures 18 and 19 respectively, F-L was totally absent in the glucose-130° C - 4 h sample while the lactose-90° C - 4 h sample contained only low L-L levels.

Figure 24 also shows that DAN (expressed as arbitrary fluorescence units per 1,6 mg N) (r = 0,85), SA (r = 0,80) and DBL (r = 0,85) values all corre-lated reasonably well with FDNB although the actual values obtained with the SA and DBL methods were in some cases very different from those obtained with the FDNB method (see Figs 21 - 23). Below a value of approximately 4,0 g 16 g N^{-1} (primarily 'late' Maillard damage) both DBL and SA gave lower estimates of reactive lysine than FDNB while above this value (primarily 'early' Maillard damage) values were generally higher than FDNB. The higher SA and DBL values for 'early' Maillard damage could be explained by the suggestion of Finot and Mauron (1972) that certain NH_2-reactive reagents may react with the ε-NH linkage in F-L and L-L. The lower estimates for 'late' Maillard damage could once again (see § 5.2) be due to the failure of the two reagents to fully penetrate the highly crosslinked 'late' Maillard-damaged material. In this regard it should be noted that succinylation and dye-binding are carried out under much milder reaction conditions than is dinitro-phenylation (see Ch. 3). Since digestive enzymes also react under the mild conditions of the gut, the SA and DBL methods may better reflect the effect of poor digestibility on lysine availability than does the FDNB method. DBL values were, however, closer to FDNB values than SA values. Walker (1979) also found good agreement between FDNB and DBL values for Maillard-damaged materials. Hurrell et al. (1983) however, found that while the FDNB method was the most reliable for 'early' Maillard-damaged milk powders the DBL

procedure seriously underestimated lysine damage. The latter observation could be due to the short (1 h) reaction time used by Hurrell et al. (1983) which as explained in section 4.2, could result in unexpectedly high DBL values.

To summarise, the present results show large variations between methods for each sample, with the order of sensitivity depending on whether 'early' or 'late' Maillard products predominate in the sample. For mildly damaged glucose-soya samples FDNB was the most sensitive followed by DAN or DBL, SA and then TL whereas for mildly damaged lactose-soya samples, the order was DBL, FDNB, SA, TL and DAN. For the more severely damaged material virtually all samples showed the same trends with DAN or SA showing greatest sensiti-vity followed by DBL, FDNB and TL. The general insensitivity of TL and its poor correlation ($r = 0,77$) with FDNB was probably due to the acid lability of both 'early' Maillard products such as F-L, L-L and possibly 'xylanosyl-lysine' and certain 'late' Maillard lysine derivatives. DAN ($r = 0,85$), SA (0,80) and DBL (0,85) correlated reasonably well with FDNB but SA and DBL values were higher for mildly damaged material and lower than FDNB for severe damage. The higher values could be due to partial reaction of the reagents with the ε-NH linkage in F-L or L-L while the lower estimates may be a result of poorer penetration of the crosslinked material under the milder reaction condition for SA and DBL.

These results therefore clearly indicate that different methods are more suitable for different types of Maillard damage. However, considering all factors including method simplicity, speed and costs, the DBL method would be first choice since it is sensitive enough to give a satisfactory relative indication of all types of Maillard damage.

CHAPTER 7

EVALUATION OF LACTALBUMIN SUBJECTED
TO LYSINE DAMAGE IN THE
PRESENCE OF FORMALDEHYDE

7.1 INTRODUCTION

The detailed evidence presented in section 2.1.3 indicated that formaldehyde
appears to preferentially react with lysine residues in a protein to form an
ϵ-amino methylol derivative which although unstable in 6M HCl at 110° C
(Bizzini & Raynaud, 1974), is probably stable in the stomach and therefore
nutritionally unavailable as a lysine source. The methylol derivative can
then either become reduced to unavailable methyllysine (M-L) (Friedman &
Gumbmann, 1979) or forms methylene bridges possibly with tyrosine, histidine
or other lysines (Bizzini & Raynaud, 1974), which lower both lysine avail-
ability and general protein digestibility (Hurrell & Carpenter, 1978). These
known nutritional problems together with the possibility of toxic and carcino-
genic effects underline the need for a suitable method for monitoring such
damage. Unfortunately no method has been developed for the determination of
either methylol or methylene derivatives as such and, since the former is
acid labile, conventional amino acid analysis is not directly suitable.
However, reactive lysine methods once again show promise for allowing for
this type of lysine damage and were therefore evaluated on formylated lact-
albumin samples. The results of these studies are presented and discussed in
section 7.3. It will be noted that results for the SA method are absent
while the ninhydrin (NIN) method is evaluated here for the first time. This
is because the SA method gave highly erroneous results probably due to the
partial solubility of the succinyl-lactalbumin in the ethanol washing solvent
(see § 3.7.3 and Ch. 9) while the NIN values had been obtained during collabo-
rative research on the lactalbumin samples while the author was in Cambridge,
U.K. In order to fully test the various reactive lysine methods under study,
it was necessary to prepare a range of samples of varying extents of formalde-
hyde damage (§ 3.1.3). These samples are evaluated and discussed in section
7.2 where attempts are made to estimate the relative proportions of methylol
and methylene-type lysine derivatives.

7.2 ASSESSMENT OF THE EXTENT AND TYPE OF FORMALDEHYDE DAMAGE

As previously mentioned (§ 2.1.3), formylation may be responsible for the
formation of methylene bridges either between two lysines or between lysine
and tyrosine or histidine (Bizzini & Raynaud, 1974), although so far only the
Lys-CH_2-Lys and Lys-CH_2-Tyr derivatives have been isolated (Means & Feeney,
1971). Furthermore Warren et al. (1974) reported that the Lys-CH_2-Lys deriva-
tive occurs more frequently at neutral pH whereas Lys-CH_2-Tyr is formed
primarily at pH 5,0 . By contrast Galembeck et al. (1977) showed that methy-
lene bridge formation in both ribonuclease and lysozyme was negligible below
pH 7,0 but increased rapidly above this value. In the present research it
was therefore of interest to prepare a series of samples that would enable
study of the short and long term effects of exposing protein to different con-
centrations of formaldehyde under acidic and basic conditions. For this pur-
pose 18 samples were prepared by treating lactalbumin with 1,5 , 4,5 or
24,1 g formaldehyde per 16 g N at 24° C for 1 , 12 or 120 h at pH 5,0 or 9,0
(see § 3.1.3).

Based on the assumptions that the methylol and methylene derivatives are
respectively labile and stable in 6M HCl (110° C) and that the reactive lysine
methods are sensitive to both types of damage, the author derived the follow-
ing formulae for the estimation of the relative proportions of unmodified
lysine as well as methylol and methylene derivatives:

$$TL_u - TL_d = \text{methylene bridged lysine}$$

$$\text{and} \quad RL_u - RL_d = \text{methylol lysine + methylene-bridged lysine}$$

$$\therefore \quad \text{methylol lysine} = (RL_u - RL_d) - (TL_u - TL_d)$$

$$\text{and} \quad \text{unmodified lysine} = TL_u - (RL_u - RL_d)$$

where: TL_u and TL_d = total lysine content of undamaged and damaged
                        samples respectively;   and

   RL_u and RL_d = mean reactive lysine by DBL, FDNB and DAN methods
                        of undamaged and damaged samples respectively
                        (see Fig. 25).

Table 6 demonstrates that both formaldehyde concentration and duration of
exposure had marked effects on all the values (except histidine) with up to
about 70% of all ϵ-NH_2 groups being modified under the most severe conditions.

TABLE 6   Levels of unmodified lysine as well as ε-N-methylol and
           ε-N-methylene derivatives in relation to losses of tyrosine and
           histidine in 20 lactalbumin samples treated with 1,5 , 4,5 or
           24,1 g formaldehyde per 16 g N for 1 , 12 or 120 h at 24° C and
           a pH of 5,0 or 9,0

Formylation Conditions			mmoles per 16 g N				
				Lysine present as:			
Duration (h)	g HCHO per 16 g N	pH	Unmodified lysine [a]	Methylol derivative[a]	Methylene derivative[a]	Tyr losses [b]	His losses [b]
1	1,5	5	61,66	3,94	2,90	1,05	0
		9	61,39	3,25	3,86	1,60	0
	4,5	5	53,41	10,48	4,61	3,53	0,07
		9	53,79	9,62	5,09	3,86	0
	24,1	5	31,86	25,39	11,25	19,48	1,03
		9	33,02	25,26	10,22	20,25	0,71
12	1,5	5	55,19	6,92	6,39	3,97	0,45
		9	52,29	12,42	3,79	4,41	0
	4,5	5	39,77	22,13	6,60	15,95	0
		9	40,50	20,58	7,42	16,06	0,33
	24,1	5	23,20	38,84	6,46	20,75	0,39
		9	24,75	35,58	8,17	20,75	0,84
120	1,5	5	52,54	8,95	7,01	10,43	0,78
		9	50,26	10,55	7,69	10,49	1,23
	4,5	5	32,84	28,45	7,21	20,75	5,16
		9	33,05	25,29	10,16	20,75	1,16
	24,1	5	19,44	39,59	9,47	20,75	2,58
		9	20,76	38,95	8,79	20,75	5,03

a - Unmodified lysine $= TL_u - (RL_u - RL_d)$

   Methylol lysine $= (RL_u - RL_d) - (TL_u - TL_d)$

   Methylene bridged)
     lysine $\Big\} = TL_u - TL_d$

   where TL_u and TL_d = total lysine content of undamaged and damaged
                           samples respectively;

           RL_u and RL_d = mean reactive lysine by DBL, FDNB and DAN methods
                           of undamaged and damaged samples respectively
                           (see Fig. 25);   and

           TL_u and RL_u = 68,50 and 65,07 mmoles per 16 g N respectively.

b - Tyrosine and histidine values for undamaged lactalbumin were 20,75 and
    12,64 mmoles per 16 g N respectively.

Clearly though concentration rather than duration of exposure was responsible for the greatest lysine losses. For instance, treatment of lactalbumin with 1,5 , 4,5 or 24,1% formaldehyde for only 1 h lowered the unmodified lysine content by about 10 , 22 and 53% for 12 h by 22 , 41 and 65% and 120 h by 25 , 51 and 70%. Furthermore the greater part of the reaction occurred in the first 1 h and was virtually complete after 12 h with little or no change over the subsequent 108 h despite 30% of the ϵ-NH$_2$ groups remaining unmodified while there was still an excess of free formaldehyde (see § 3.1.3). Interestingly methylene bridge formation reached completion after only 1 h with 24,1% formaldehyde while the methylol derivative took 12 h with 24,1% formaldehyde to reach a peak. This is probably because only a limited number of methylene derivatives could be formed depending on the proximity of the relevant sidechains to each other.

Table 6 also shows that contrary to expectation, pH had no significant effect ($p > 0,05$) on any of the measured or calculated parameters. The rate and consequently the extent of methylol derivative formation (Fig. 4) was expected to be lower at pH 5 than at pH 9 since under acidic conditions the equilibrium between the $-NH_2$ and $-NH_3^+$ forms of the ϵ-amino group of lysine would favour the existence of the less reactive protonated species. It is of course possible that this effect is only detectable with shorter reaction times since the availability of unprotonated NH$_2$ groups not only depends on the pH but also the rate at which equilibrium is regained (i.e. Le Chateliers Principle). Indeed Galembeck et al. (1977) found that methylene bridge formation in lysozyme and ribonuclease was negligible below pH 7 probably due to the lower levels of methylol substrate formed during their shorter 30 min reaction period. Furthermore pH had no significant effect ($p > 0,05$) on losses of tyrosine and unmodified lysine which disagrees with Warren et al. (1974) who reported that the Lys-CH$_2$-Lys derivative forms more readily at pH 7 whereas Lys-CH$_2$-Tyr occurs primarily at pH 5 .

Also apparent from Table 6 is that whereas tyrosine and unmodified lysine levels decreased with increasing exposure time and formaldehyde concentration, there was little or no loss of histidine, except under the most severe treatments (e.g. 24,1% HCHO , 120 h, pH 9) and then only after all the tyrosine and significant quantities of lysine had been destroyed. Interestingly histidine levels were apparently not even lowered by condensation reactions with residual formaldehyde during acid hydrolysis (Gruber & Mellon, 1968). This possibly reflects the thorough removal of formaldehyde by washing with absolute ethanol after formylation. The only source of free formaldehyde

would therefore have been the relatively small amount of decomposing methylol derivatives. Since past authors have not removed residual formaldehyde before analysis it is possible that they have ascribed such losses to the involvement of histidine in Lys-CH$_2$-His crosslinks. Indeed this derivative has not been isolated and should it exist, the present results indicate that its formation is less favourable than that of the other derivatives.

In contrast to histidine, tyrosine appears to be involved in both methylene bridge formation and in condensation reactions with free formaldehyde released from methylol derivatives during acid hydrolysis. Evidence for the involvement in methylene bridges is found in Table 6 where tyrosine losses, which increased with increasing severity of formylation, corresponded well with both increases in methylene derivative levels and reductions in unmodified lysine. However, since on a mole-to-mole basis tyrosine losses in most cases far exceeded levels of the methylene derivative, the extra tyrosine was probably lost via condensation reactions. Further support for this is found in the observation that during the first 0,5 h of acid hydrolysis, a very intense but transitory pink colour was observed particularly for the most highly formylated samples (i.e. richer in acid labile methylol derivatives). Formaldehyde can form brightly-coloured polymers with aromatic or phenolic compounds. Free formaldehyde may also react with lysine during acid hydrolysis to form M-L (Reis & Tunks, 1973), but although total amino acid analysis was carried out no attempt was made to search for this product. No other amino acids showed any losses due to formaldehyde treatment.

To summarise, contrary to various reports, pH had no significant effect on either the ε-amino formylation reaction or the subsequent formation of methylene bridges with tyrosine or free ε-NH$_2$ groups. Furthermore histidine does not appear to participate in methylene bridge formation except perhaps after the bulk of tyrosine and lysine residues have reacted. Tyrosine appears to react readily and preferentially with methylol derivatives to form methylene bridges but also appears to be involved in condensation reactions with free formaldehyde released from methylol derivatives during acid hydrolysis. Since past authors have not removed residual formaldehyde before analysis it is possible that they have ascribed histidine losses due to condensation, with formaldehyde released by hydrolysis to the formation of Lys-CH$_2$-His. Finally, formaldehyde concentration rather than exposure time has the greatest enhancing effect on the formylation reaction with methylol derivatives reaching maximal levels after 12 h with 24,1% formaldehyde whereas methylene bridge formation is complete after only 1 h, probably because of the requirements of proximity of reactive sidechains.

Thus it is clear that none of the prepared samples contain exclusively either methylol- or methylene-type lysine, although their relative proportions do vary depending on the severity of sample treatment. Methylol derivatives, however, tend to predominate for all samples except the mildly-damaged lact-albumin (e.g. with 1,5% HCHO for 1 or 12 h) where levels of the two derivatives are comparable. It was therefore not possible to classify the samples into distinctly 'early' and 'late' formaldehyde-damaged groups so that the performance of the various reactive lysine methods could be separately assessed for each type of damage.

7.3   SENSITIVITY OF VARIOUS REACTIVE LYSINE METHODS TO FORMALDEHYDE DAMAGE AND THEIR CORRELATION WITH FDNB AS REFERENCE PROCEDURE

In section 7.2 attempts were made to describe the extent and type of damage present in the 18 formaldehyde-damaged samples. In this section the sensitivity of the various methods to formaldehyde damage is examined and results are correlated with those of FDNB as reference procedure. Since there was no significant difference ($p > 0,05$) between the samples treated at pH 5 or 9 , only the results for the 'pH 9 samples' are reflected in Figure 25.

The results presented in Figure 25 demonstrate that all reactive lysine methods as well as TL showed at least some measure of sensitivity to lysine damage and that all values decreased with increased severity of the formylation conditions. Results within each method were also highly reproducible with pooled standard errors of means for DBL, FDNB, DAN and NIN methods of 0,07 , 0,05 , 0,15 and 0,15 respectively. However, as already discussed in section 7.2 the TL method is only sensitive to changes in methylene derivative levels since the methylol derivative is decomposed during acid hydrolysis (Bizzini & Raynaud, 1974). Thus out of all methods TL is the least sensitive to general formaldehyde damage and consequently gave a higher estimate of reactive lysine than FDNB and showed a lower correlation ($r = 0,82$) with this method (see Fig. 26). These results agree with those of Hurrell and Carpenter (1978) who found only a 15% loss of TL in formylated ovalbumin as compared to a $50-60\%$ decrease in lysine availability to rats and chicks.

The NIN method was also less sensitive than the other reactive lysine procedures (Fig. 25) showing higher values at all levels of formaldehyde damage and a correlation coefficient of only 0,86 with FDNB values. This may be due to the partial instability of the methylol derivatives under the reasonably severe acidic conditions of the NIN assay (i.e. pH 5,5 , 100° C, 15 min).
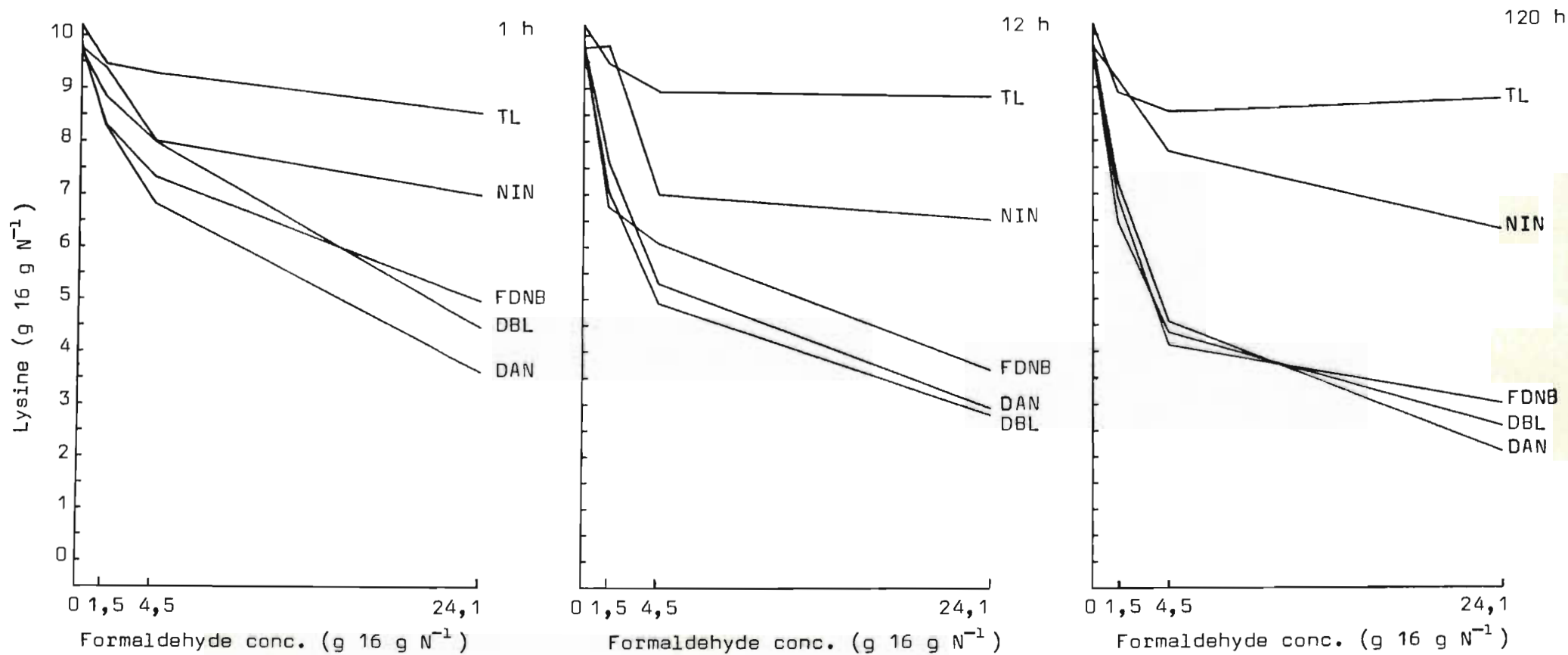
FIGURE 25   Comparison of the sensitivity of the TL, FDNB, DBL, NIN and DAN methods to lysine losses in
9 formaldehyde-damaged lactalbumin samples prepared by treating lactalbumin with 1,5 , 4,5 or
24,1 g formaldehyde per 16 g N for either 1 , 12 or 120 h at 24° C and a pH of 9 .
DAN values are expressed in g 'DBL equivalents' per 16 g N
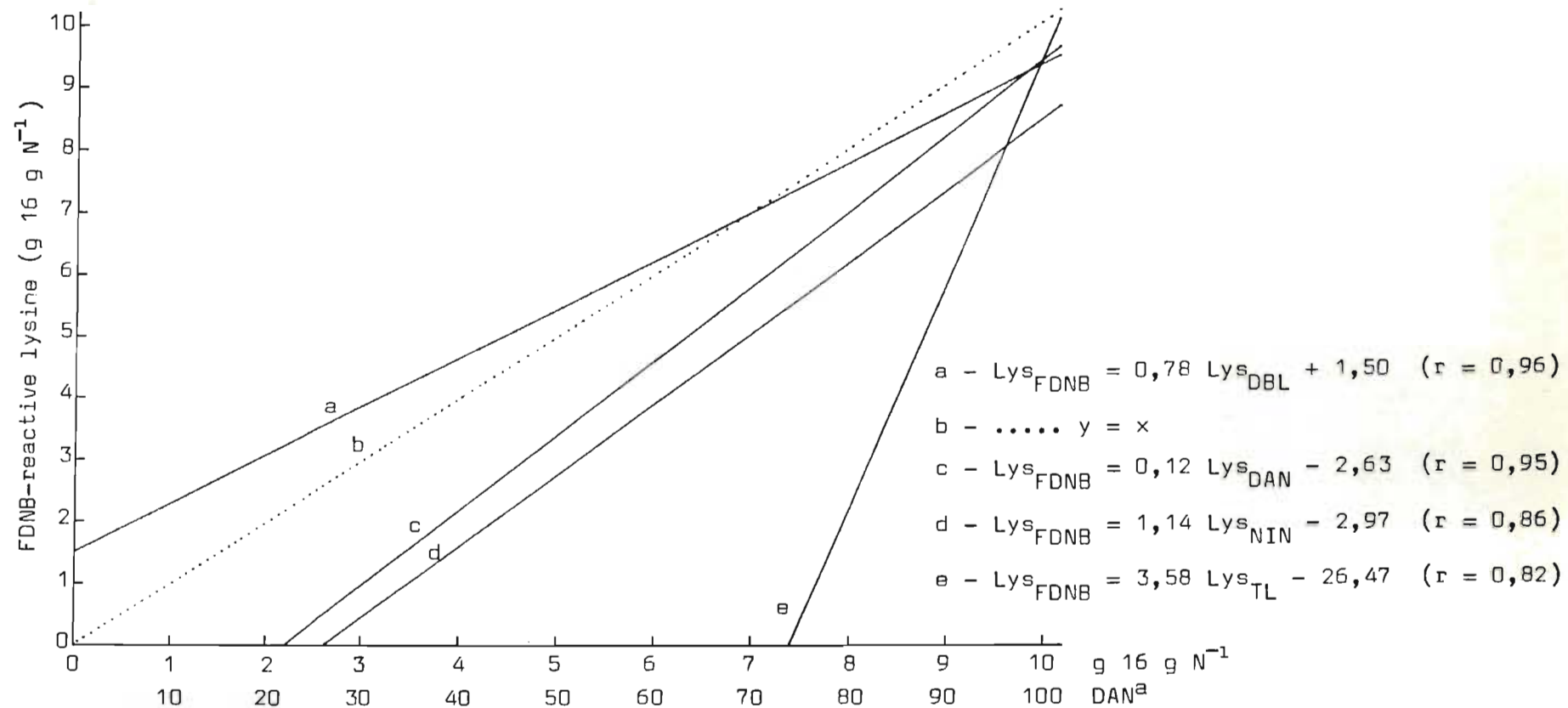
FIGURE 26   Correlation between FDNB-reactive lysine and TL, NIN-, DBL- and DAN-reactive lysine values
for 18 formaldehyde-damaged lactalbumin samples prepared by treating lactalbumin with
1,5 , 4,5 or 24,1 g formaldehyde per 16 g N for 1 , 12 or 120 h at pH 5 or 9 and 24° C.
[a]DAN values expressed as arbitrary fluorescence units per 1,6 mg N (see § 3.8.2)

The incubation time was unfortunately not extended to see if NIN values became more comparable to those obtained for the TL method.

All other reactive lysine methods (Fig. 25) were sensitive to both methylol- and methylene-type lysine damage with results for the FDNB, DBL and DAN (expressed as DBL equivalents) values being surprisingly similar, particularly for the 120 h samples containing maximal levels of both derivatives (Table 6). Consequently the DBL (r = 0,96) and DAN (expressed as arbitrary fluorescence units per 1,6 mg N) (r = 0,95) also correlated well with FDNB and gave similar regression coefficients (Fig. 26). However, since all 3 electrophilic reagents employed may partially react with the secondary amines of the methylol and methylene derivatives (Fig. 4), it is highly likely that even these methods do not reflect the full extent of lysine nutritional damage. Indeed, as already mentioned, Hurrell and Carpenter (1978) found that various reactive lysine methods were only sensitive to approximately half the 'true' reduction in bioavailability.

To summarise, all methods tested were at least partly sensitive to changes in formaldehyde damage of lactalbumin. However, the TL and to a lesser extent the NIN methods were less sensitive probably due to the acid lability of the methylol derivative. The FDNB, DBL and DAN methods were by contrast highly sensitive to damage, correlated well with each other and had similar absolute values. Whether or not these methods fully reflect losses in bioavailability however, remains to be confirmed with animal experiments which should also include feeding studies using synthetically-prepared methylol and methylene derivatives as sole dietary lysine sources.

CHAPTER 8

EVALUATION OF THE USEFULNESS OF
Tetrahymena pyriformis W FOR THE ASSESSMENT
OF MAILLARD - TYPE LYSINE DAMAGE

As already mentioned in the review in section 2.2.6, the essential amino
acid requirements of Tetrahymena pyriformis W are similar to those of both
the growing rat (Kidder & Dewey, 1961) and humans (Evans, 1978). This
characteristic has encouraged the use of this organism for the assessment
of overall protein quality of foods as well as the measurement of the bio-
availability of various amino acids, especially lysine (Stott & Smith,
1966; Shorrock, 1976; Shepherd et al., 1977). Although this organism can
utilise intact protein (Stott & Smith, 1966), enzymic predigestion of the
sample is necessary to obtain results similar to those of rat and chick
assays (Shorrock, 1976; Shepherd et al., 1977; Baker et al. 1978). In the
present study pronase was used for predigestion since evidence suggests that
its proteolytic abilities correlate well with in vivo digestion in man
(Hurrell & Carpenter, 1981).

The usefulness of this assay may be diminished due to the dose-dependent
inhibition of Tetrahymena cultures by other dietary components such as fat
(Evans et al., 1978) and various spices (Hsu et al., 1978). Food additives
such as propionates, benzoates, sorbates and the meat-curing adjuncts,
nitrate, erythorbate and ascorbate, also severely inhibit the growth of this
organism (Satterlee et al., 1979; Janitz & Grodzka-Zapytowska, 1982).
Further difficulties are that LAL which is totally unavailable to rats
(Erbersdobler, 1980), can be partially utilised by Tetrahymena as a source
of lysine (Sternberg & Kim, 1979) while the bioavailability and toxicity to
Tetrahymena of the many Maillard products resulting from food processing is
still very unclear.

The availability of suitable test materials prompted an extension of the
present study to include an evaluation of Tetrahymena for the assessment of
Maillard-type damage. Ideally one would wish to distinguish between the
effects on the organism of 'early' and 'late' Maillard products but as
already demonstrated (§ 6.2) the complexity of the Maillard reactions makes
it extremely difficult to prepare separate 'early' and 'late' Maillard
samples free from contamination by the other type of damage. For this reason
various authors (Adrian, 1974; Finot & Magnenat, 1981; Finot et al., 1978 a;

Lee et al., 1979) however, have worked with two fractions called
'melanoidins' and 'pre-melanoidins'. The pre-melanoidin fraction is water-
soluble and contains varying quantities of the deoxyketosyl derivative
(depending on extent of heat damage) as well as pale-brown low-molecular mass
products of the 'late' Maillard reaction. The melanoidin fraction on the
other hand, is insoluble in aqueous media and contains primarily dark-brown
high molecular mass polymers.

With the above considerations in mind, the pale-brown, water-soluble frac-
tions (supernatant) of pronase-digested glucose-, lactose- and xylose-damag-
ed soya samples (§§ 3.1.2 & 3.10.3) were selected for the assay of
Tetrahymena-available (TET) lysine. This fraction was expected to contain,
besides the usual nutrients, primarily the free and peptide-bound deoxyketo-
syl form in the case of the mildly damaged samples and less deoxyketosyl
intermediates but more isopeptides and pre-melanoidin products in the case
of the severely damaged samples. It was presumed the large polymers of
melanoidin pigments were removed in the dark-brown, insoluble fraction
(residue) (Lee et al., 1979). Furthermore, as already explained, pronase is
a powerful protease system and it was therefore unlikely that any of the
material removed in the insoluble fraction would have been utilised by the
organism.

Figure 27 shows that the pronase digestibility of 'crude' protein (see
§ 3.10.3) varied from 90% for the mildly damaged samples to approximately
20% for the severely damaged materials. The extent of reduction in digest-
ibility caused by heating with the different sugars also corresponded to
their respective reactivities as discussed in sections 6.2 and 6.3 (see also
Anderson & Erbersdobler, 1982). After mild heat (90° C) lactose-damaged
soya showed least reduction in digestibility, followed by glucose then
xylose, whereas under more severe conditions lactose showed either a compar-
able (at 110° C) or greater (at 130° C) reduction in digestibility than that
by xylose. This was ascribed to partial hydrolysis of the lactose which
considerably enhanced the reducing ability of those samples. As expected,
pronase-digestibility only dropped significantly when the level of F-L or
L-L started decreasing as these intermediates were converted to highly brown-
ed (see Fig. 27 as well as Figs 18 & 19) crosslinked and less soluble melan-
oidin products. It is of interest that Finot et al. (1978 a), separated a
strongly-coloured melanoidin fraction from a less-coloured lower molecular
mass pre-melanoidin fraction from a pronase digest of a heat-treated casein-
glucose mix. They found that the melanoidin fraction was almost completely

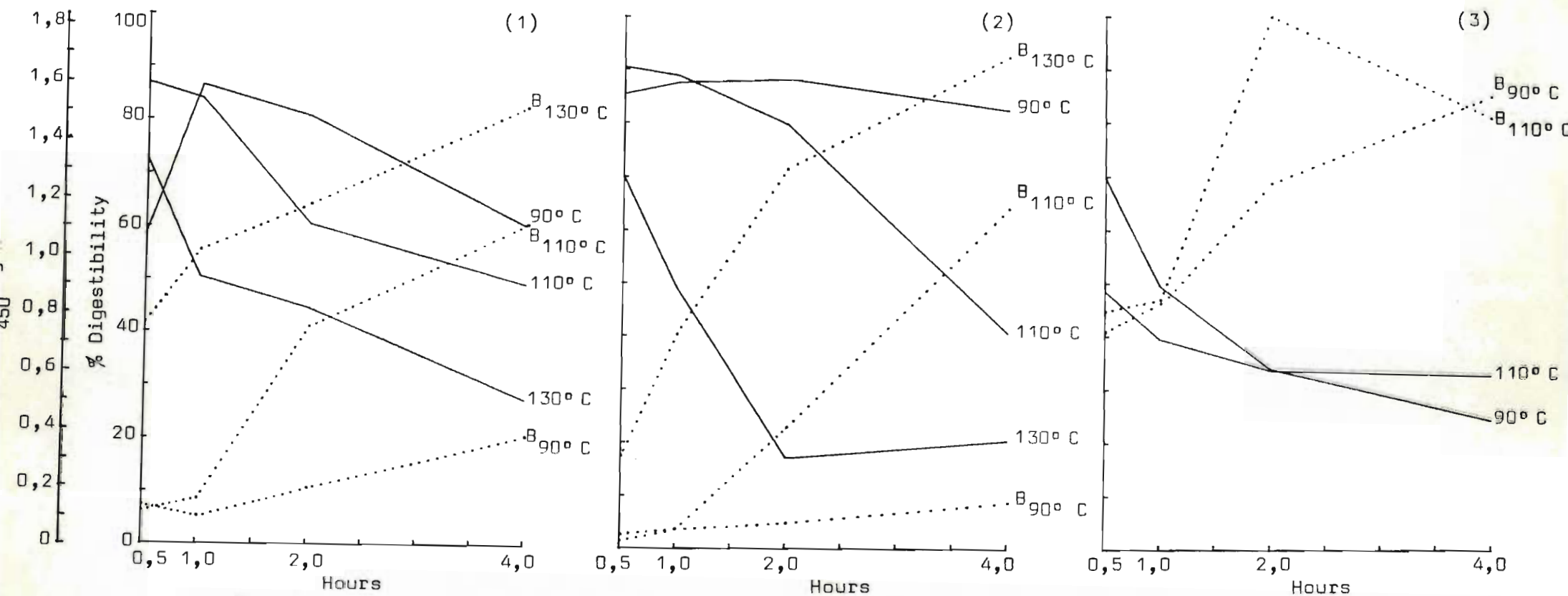FIGURE 27 Pronase digestibility (——) of various Maillard-damaged soyaprotein samples in relation to the extent of browning (B) expressed as absorbance at 450 nm per mg N (···) . Samples were heated at 90 , 110 or 130° C for 0,5 , 1 , 2 or 4 h in the presence of glucose (1), lactose (2) or xylose (3). Results are expressed in terms of pronase-digested N as a percentage of total sample N and are means of duplicate determinations

indigestible by rats, 90% of the material being recovered in the faeces, while the pre-melanoidin fraction was partially absorbed with 27% passing out in the urine unchanged.

The water-soluble fraction (supernatant) of each pronase-digested sample was then assayed for TET available lysine according to the standard procedure of Stott and Smith (§ 3.10). This included assaying each fraction in duplicate at 3 different sample-N levels (between 0 - 10 mg N per 10 ml assay medium - see § 3.10.5). The results for the soyaprotein samples heated in the presence of glucose are presented in Figure 28 where TET lysine values are plotted against the corresponding sample-N levels. In the absence of compli-cating factors it could be expected that the three values for a given sample when expressed per 16 g sample N, should have been similar with the means showing low standard errors. In other words, the data plotted in Figure 28 should have yielded more-or-less straight lines parallel to the x-axis. However, although the results for soyaprotein heated with glucose at 90, 110 or 130° C for 0,5 , 1 , 2 or 4 h, clearly show good sensitivity to in-creased severity of heat damage when the values are compared at any one sample N-level, the TET lysine values for each sample, particularly the mildly-damaged material, vary considerably at different N-levels making it impossible to assign any specific values. Clearly Tetrahymena growth is stimulated at low N-levels up to approximately 0,15 mg sample N per ml culture medium, but inhibited at higher N-levels (> approx. 0,2 mg per ml). In the case of the severely damaged samples (e.g. 110° C - 4 h and 130° C - 4 h) this trend is not so apparent as the curves are flatter thus allowing the results to be averag-ed as per normal procedure.

It is unlikely that glucose (or any other non-protein nutrient) could have stimulated the growth of Tetrahymena cultures since the level of glucose supplied via the enzyme digest was very low (approx. 4 mg) relative to that from the nutrient medium (approx. 150 mg), while the nutrient medium of Stott and Smith (1966) contained optimal levels of all the organism's require-ments except lysine. Indeed Evans (1978, 1979 a & b) showed that raising the levels of most nutrients above optimal had little or no effect on Tetrahymena growth while the high reproducibility of the standard curves obtained in the present studies strengthens the argument that the nutrient medium was optimal. The relevant regression equations were:

$$Y_{mg\ Lys} = 1,17 \times 10^{-7} \ X_{organism\ counts} - 0,14 \qquad r = 0,987 \qquad n = 12$$

$$Y_{mg\ Lys} = 1,15 \times 10^{-7} \ X_{organism\ counts} - 0,15 \qquad 0,977 \qquad 12$$

$$Y_{mg\ Lys} = 1,17 \times 10^{-7} \ X_{organism\ counts} - 0,14 \qquad 0,989 \qquad 12$$

$$Y_{mg\ Lys} = 1,33 \times 10^{-7} \ X_{organism\ counts} - 0,16 \qquad 0,993 \qquad 12$$
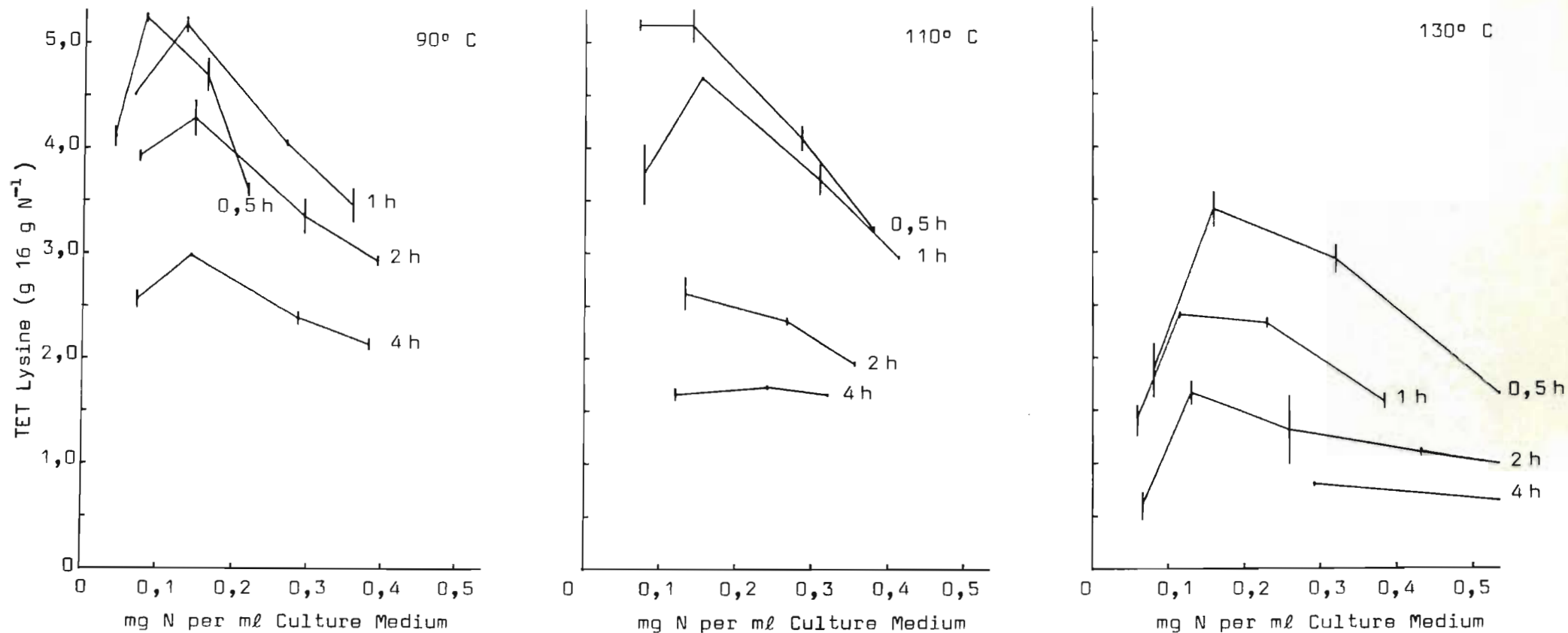
FIGURE 28 The effect of different sample-N levels on <u>Tetrahymena</u>-available lysine (TET) values for soyaprotein
samples heated at 90 , 110 or 130° C for 0,5 , 1 , 2 or 4 h in the presence of glucose. The error
bars span actual duplicate determinations with the line drawn to pass through the mean

The curves were linear up to the co-ordinates: 1 mg lysine, $9,9 \times 10^6$ cells per 10 mℓ culture medium.

Thus any stimulation or inhibition would probably be related to the lysine content of the sample. The observed stimulation of Tetrahymena cultures with increased sample N (and hence lysine levels), may have been due to the progressively increased ability of the organism to synthesise hydrolytic enzymes that will enable it to utilise the 'early' Maillard product F-L as a source of lysine. The enzyme concerned would probably be an export protein and therefore possibly not formed while lysine is limiting. Indeed it has been shown that Tetrahymena secretes various proteases for the utilisation of whole protein sources. Furthermore 'early' Maillard products have also been observed to stimulate the growth of both Lactobacillus (Rogers et al., 1953) and Aspergillus (Jemmali, 1965) cultures while, Griffiths and Pridham (1980) found that Escherichia coli (E. coli) had the ability to hydrolyse F-L by an extracellular, cell-bound 'F-L-ase' enzyme which appeared after an 8-hour lag period.

Support for the suggested stimulatory effects of F-L is presented in Figure 29 where the maximum TET values obtained for each sample are plotted on the same set of axes as the corresponding values obtained for the TL and DBL methods. Clearly the TET values for the mildly damaged samples (e.g. 90° C , 0,5 - 2 h; 110° C , 0,5 - 1 h) containing significant quantities of F-L and minimal browned 'late' Maillard products are close to the TL values implying utilisation of both unmodified and blocked (F-L) lysine residues (i.e. TL = free lysine + F-L). Furthermore the postulated influence of F-L on Tetrahymena growth is amply demonstrated by the observation (Fig. 29) that the F-L and TET curves are almost parallel over the ranges 90° C , 2 - 4 h and 110° C , 1 - 4 h. This ability of Tetrahymena to totally or partially utilise free or peptide-bound F-L, severely limits the potential usefulness of the TET assay for 'early' Maillard samples since F-L is totally unavailable to the rat and human (Finot et al., 1978 a; Mori et al., 1980).

The observed inhibitory effects on Tetrahymena cultures (Fig. 28) of mildly damaged samples above sample N levels of approximately 0,2 mg per mℓ, could be due to the inability of Tetrahymena to hydrolyse the higher F-L levels (Fig. 29) rapidly enough. Unhydrolysed F-L together with low levels of pre-melanoidin products (see level of browning, Fig. 29) may have had a significant toxic effect on the growth of the organism. It is also possible that higher levels of F-L and pre-melanoidins may have interfered with the absorption of other nutrients thereby indirectly inhibiting the growth of
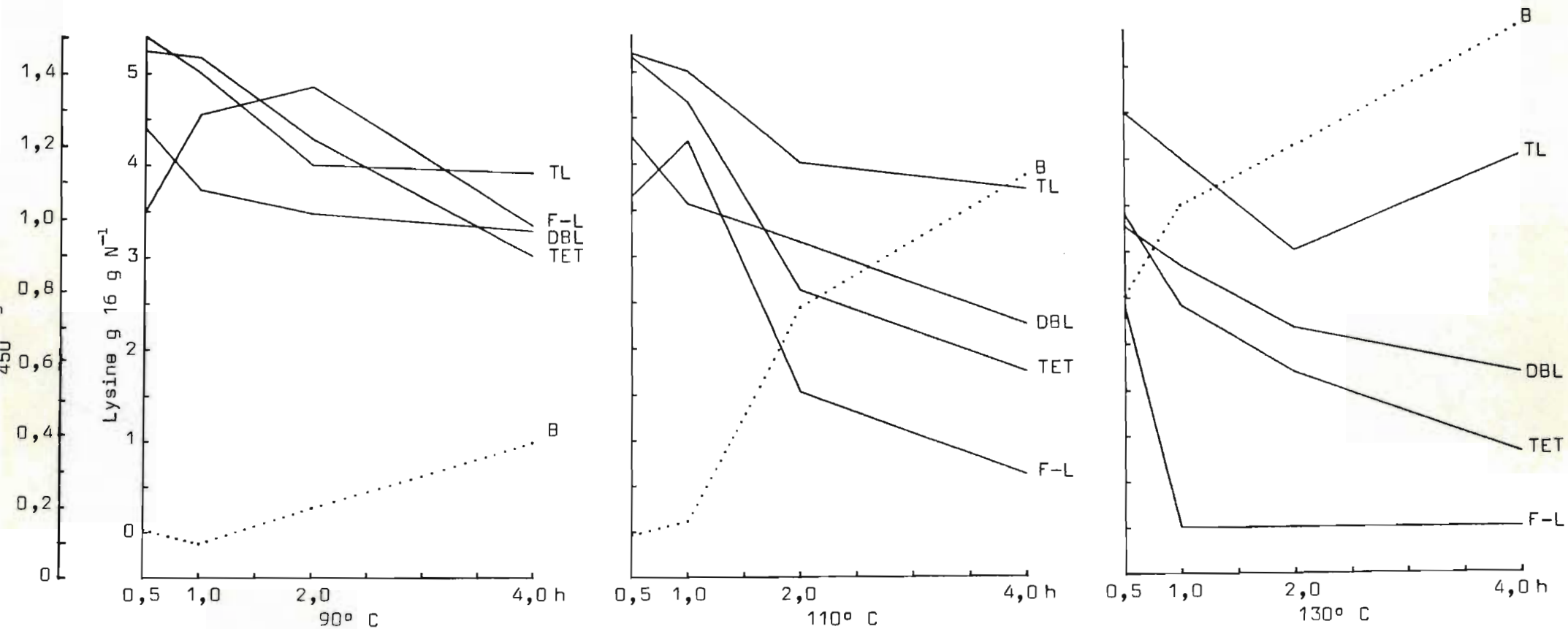
FIGURE 29 Total lysine (TL), dye-binding lysine (DBL), <u>Tetrahymena</u>-available lysine (TET), fructoselysine (F-L) and sample browning (B) values for soyaprotein samples heated at 90 , 110 or 130° C for 0,5 , 1 , 2 or 4 h in the presence of glucose. The mass of F-L was approximated by 3,03 × FUR. TL for unheated sample was 6,0 g 16 g N^{-1}

the organism. F-L has indeed been found to inhibit disaccharidase activity in rats (Lee et al., 1977). Furthermore the inhibitory effects may be partly due to the presence of poorly absorbed D-amino acids since, amino acid racemisation is apparently enhanced during Maillard reactions (Zumberge, 1979). Liardon and Hurrell (1983) however, found no racemisation at all in spray-dried milk that had undergone 'early' Maillard reactions.

Further studies were carried out to establish the effects of lactose- and xylose-damaged soya on Tetrahymena growth. Figures 30 and 32 clearly indicate similar stimulatory and inhibitory trends to those observed for glucose-damaged soya (Fig. 28) with the TET values also peaking at approximately 0,15 mg sample N per mℓ of culture medium. However, in the case of all the 'lactose-90° C samples' (Figs 30 & 31) the maximum TET values were in each case significantly greater than the theoretical maximum value of 6,0 g 16 g N^{-1} for undamaged soya, reaching as high as 9,0 g 16 g N^{-1} for the 90° C , 0,5 h sample. These results suggest that some other factor or sample constituent in addition to L-L was contributing significantly to the growth stimulatory effects. The identity of this other constituent was not established, but the stimulation was almost certainly not due to lactose as an energy source since as explained earlier, all growth media contained optimal levels of glucose (Stott & Smith, 1966). Despite these unexplained effects, evidence for the ability of Tetrahymena to utilise L-L as a source of lysine is presented in Figure 31 where the maximum TET values (Fig. 30) obtained for each sample are plotted on the same set of axes as the corresponding TL and DBL values. Since the TET values for the mildly damaged samples (e.g. 90° C , 0,5-4 h ; 110° C , 0,5-1 h ; 130° C , 0,5 h) containing significant quantities of L-L and minimal browned 'late' Maillard products (Fig. 31) are closer to the TL than the DBL values this implies that unmodified and blocked (L-L) lysine residues were utilised. Furthermore, as was the case for F-L in the glucose-soya samples, the strong influence of L-L on Tetrahymena growth is demonstrated by the observation that the L-L and TET curves are almost parallel over the ranges 110° C , 2-4 h and 130° C , 0,5-4 h. Recent studies by Hurrell et al. (1983) also suggest that both Tetrahymena and Pediococcus cerevisiae P60 can utilise L-L as a source of lysine. Interestingly some of their TET results for various milk powders rich in L-L were also higher than their corresponding TL values and the method in general grossly underestimated lysine damage. As was the case for the glucose-soya samples, the inhibition of Tetrahymena cultures (Fig. 30) by higher levels of the mildly damaged samples (e.g. 90° C , 0,5-4 h; 110° C , 0,5-2 h; 130° C, 0,5-1 h) could be due to the inability of the organism to

FIGURE 30   The effect of different sample N-levels on <u>Tetrahymena</u> (TET)-available lysine values for soyaprotein
samples heated at 90 , 110 or 130° C for 0,5 , 1 , 2 or 4 h in the presence of lactose.   The error
bars span actual duplicate determinations with the line drawn to pass through the mean

FIGURE 31   Total lysine (TL), dye-binding lysine (DBL), _Tetrahymena_-available lysine (TET), lactulosyl-lysine (L-L) and sample browning (B) values for soyaprotein samples heated at 90 , 110 or 130° C for 0,5 , 1 , 2 or 4 h in the presence of lactose.   The mass of L-L was approximated by 4,63 × FUR.   TL value for unheated sample was 6,0 g 16 g N^{-1}

cope with the corresponding increase in levels of both L-L and other toxic substances such as pre-melanoidins which then accumulate and reach toxic levels. Although L-L is unavailable (Finot et al., 1977 b) and poorly digested (10 - 15%) by rats (Finot & Magnenat, 1981) there have been no reports of L-L accumulation or toxicity in that animal and the present results are the first report of possible inhibitory effects by L-L on Tetrahymena.

The xylose-treated soya were generally the most severely damaged of all samples and over the narrower range of lysine values (i.e. 0 - 3 g 16 g N^{-1} for Fig. 32, as compared to 0 - 9,5 for Fig. 30 and 0 - 5,5 for Fig. 28) showed in some cases large variations between replicates for the TET assay. Despite this, stimulatory and inhibitory effects similar to those shown by the soya-glucose and soya-lactose mixtures were observed (Fig. 32). However, if the sensitivity of the TET-lysine scale in Figure 32 is compared with those in Figures 28 and 30, it is clear that the extent of stimulation and inhibition was less marked in the case of the xylose-damaged samples and that the values obtained with different size samples could be averaged. This could be due to the masking effects of the very high levels of browned products for all samples (see levels of DL and extent of browning - Fig. 20) which would of course be totally unavailable to Tetrahymena. As explained in section 6.2, 'xylanosyl-lysine' as such has not as yet been identified but should such an intermediate be present in these samples the minimal stimulatory and inhibitory effects on the cultures suggest that it was either in low concentration or unavailable as a source of lysine. Further evidence for the minimal utilisation of any 'early' Maillard products in the xylose-damaged material is shown in Figure 33 where the TET values are in every case closer to DBL than TL values.

In the case of the more severely damaged samples (i.e. glucose - 110° C - 4 h, 130° C - 4 h; lactose - 110° C - 4 h, 130° C - 2 and 4 h; xylose - 90° C - 2 and 4 h, 110° C - 1, 2 and 4 h) containing little or no F-L or L-L but large quantities of browned 'late' Maillard products (Figs 29, 31 and 33) and therefore little recoverable lysine (except perhaps from G-L), the TET values are either similar or lower than the DBL values and their N-dosage curves (Figs 28, 30 and 32) are flatter and show no growth stimulation. Similar results were found by Warren and Labuza (1977) who compared TET values with those of the FDNB method. The reduction in digestibility by rats of highly damaged material due mainly to isopeptide crosslinks, does, however, not always account for the greater than expected decrease in nutritive value of the
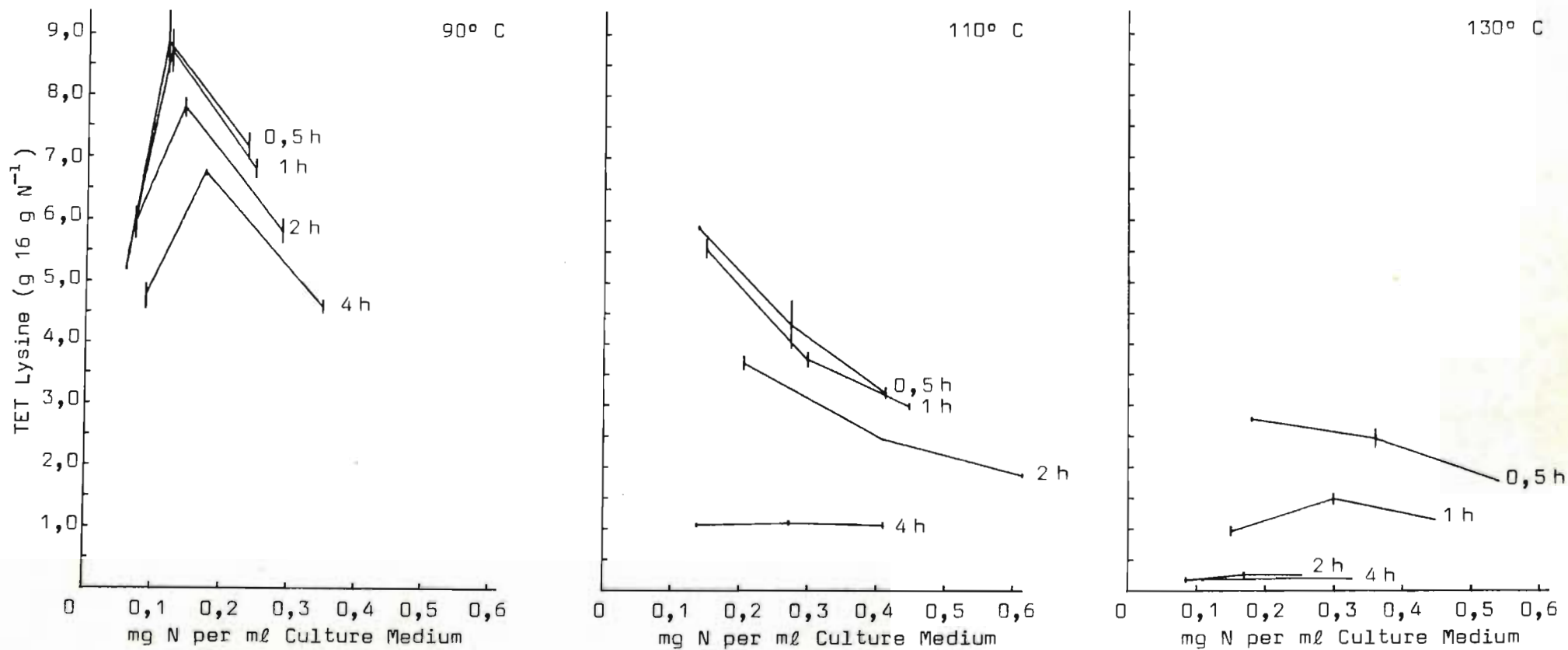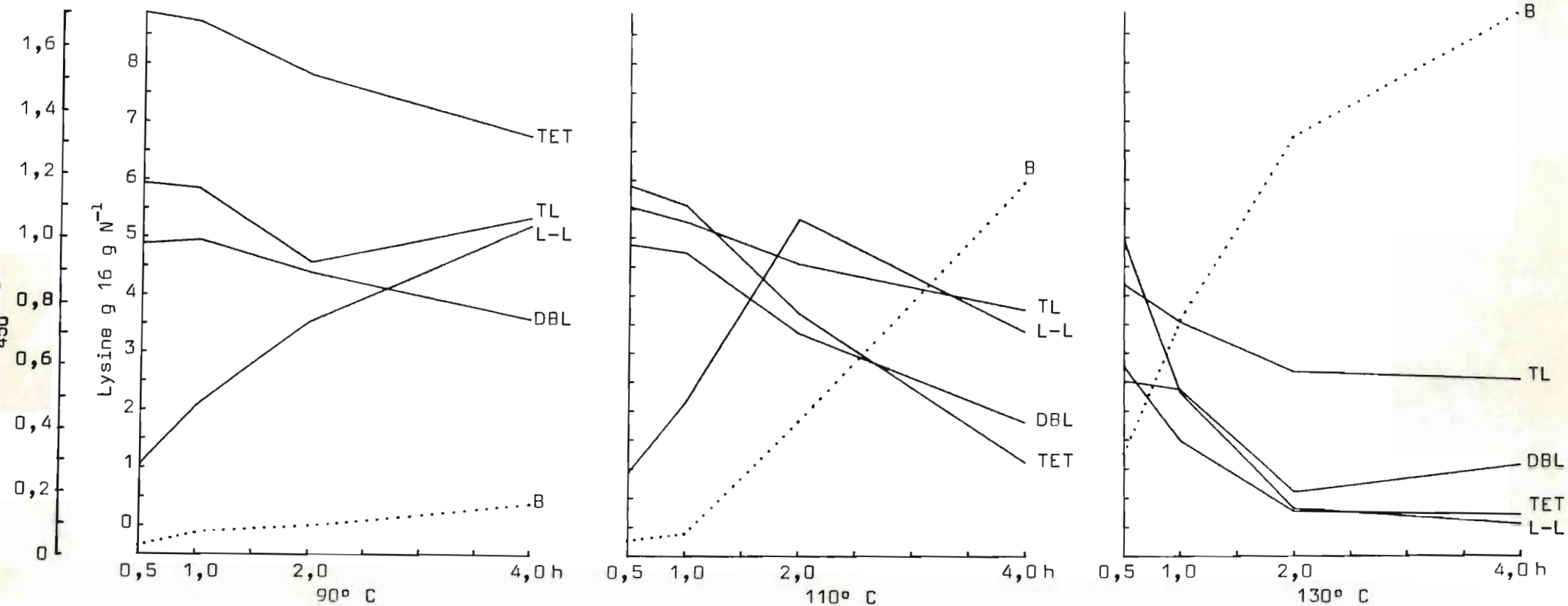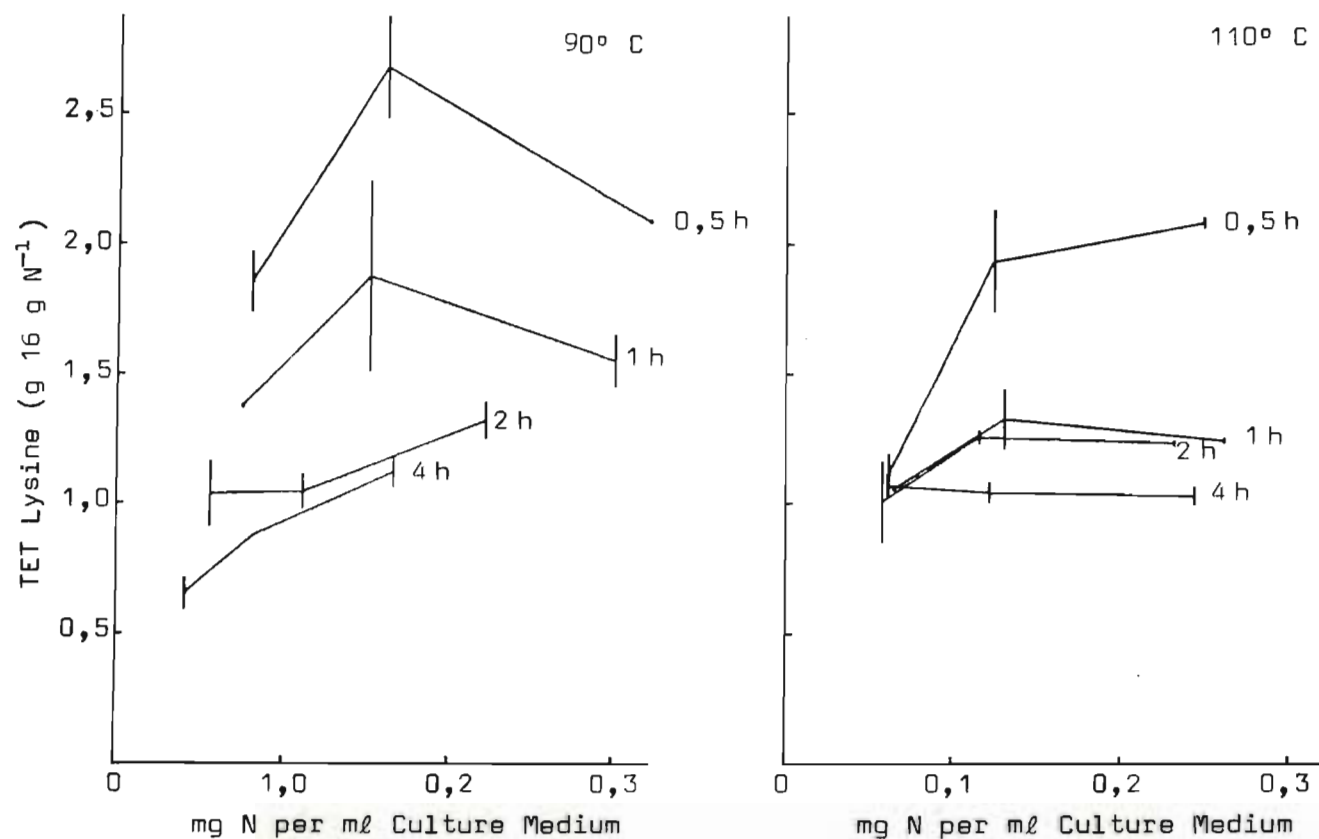
FIGURE 32 The effect of different sample N-levels on <u>Tetrahymena</u>-available lysine (TET) values for soyaprotein samples heated at 90 or 110° C for 0,5 , 1 , 2 or 4 h in the presence of xylose. The error bars span actual duplicate determinations with the line drawn to pass through the mean

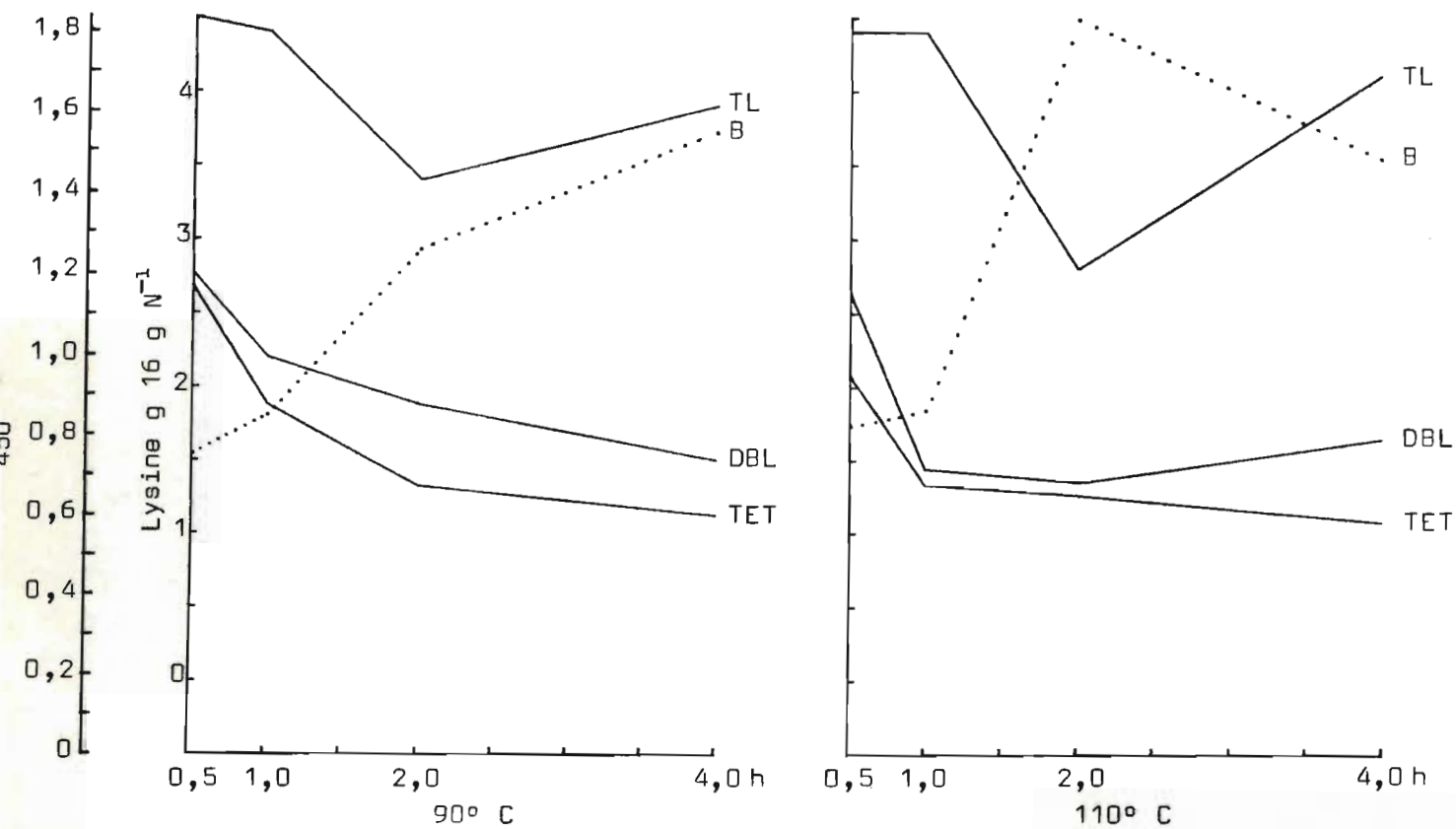FIGURE   33   Total lysine (TL), dye-binding lysine (DBL), Tetrahymena-available lysine
(TET) and sample browning (B) values for soyaprotein samples heated at
90 or 110° C for 0,5 , 1 , 2 or 4 h in the presence of xylose.   TL for
unheated sample was 6,0 g 16 g N^{-1} .   All values are means of duplicate
determinations

protein (Miller et al., 1965). It is possible that the amino acids that are absorbed by rats are excreted unmetabolised, but there is also evidence for the toxicity or growth-depressing effect of the pre-melanoidin products (Adrian, 1974). This would account for the observation (Figs 29, 31 and 33) that the TET values for the severely damaged samples are consistently below those of the DBL method.

To summarise, the pronase digestibility of 'crude' protein was found to vary from 90% for mildly damaged Maillard samples rich in deoxyketosyl deriva-tives to 20% for the severely browned samples low in deoxyketosyl deriva-tives but high in pre-melanoidins and possibly isopeptide linkages. Analysis of these samples for TET available lysine suggested that for 'early' Maillard material at culture-lysine levels sufficient for both growth as well as the synthesis of specific hydrolytic enzymes, Tetrahymena growth is probably stimulated by its ability to utilise F-L and L-L as a source of lysine. It is possible, however, that other factors in the samples may have also contributed to these effects since lactose-90° C samples in particular showed TET values much higher than the corresponding TL results. At higher sample N-levels, however, these stimulatory effects may be offset by inhibi-tion of Tetrahymena due to the toxic effects of higher levels of free or peptide-bound F-L, L-L or increasing levels of various pre-melanoidins and possibly other sample constituents. Xylose-damaged soya also showed similar but less marked stimulatory and inhibitory trends. The results do, however, suggest the existence of low levels of an 'early' Maillard product from which lysine can be recovered by Tetrahymena but, xylanosyl-lysine or some other such derivative remains to be identified. The TET assay is thus unsuit-able for the assessment of lysine availability in foods rich in 'early' Maillard products although results remain useful as relative indicators of processing damage. The method may, however, still be suitable for poorly digestible 'late' Maillard samples (low in deoxyketosyl derivatives) where it is more sensitive than the DBL method probably due to a simultaneous toxic effect by some of the pre-melanoidin products. Future studies in this area should involve more specific studies on the mechanism of absorption and metabolism in Tetrahymena of 'pure' peptide-bound and free deoxyketosyl preparations.

CHAPTER 9

GENERAL DISCUSSION

The prime aim of this thesis was to thoroughly study the total lysine, dye-
binding lysine, fluorodinitrobenzene, succinic anhydride, dansyl chloride,
ninhydrin and Tetrahymena methods, to render improvements where deemed
necessary and then to compare the perfected methods for their ability to
assess available lysine in various model compounds representing isopeptide,
Maillard and formaldehyde-type lysine damage. Since the main results of
this study have already been discussed in detail in the relevant chapters,
this general discussion will only review selected findings with emphasis
placed on the need for the surveillance of lysine damage in the food indus-
try and the suitability of the studied reactive lysine methods for routine
analysis in quality control laboratories.

It is clear from the extensive reviews in this thesis that numerous unde-
sirable reactions can occur during food processing which can have serious
nutritional and possibly toxicological consequences. Furthermore, many of
these reactions can occur without any noticeable changes in the physical
appearance and organoleptic properties of a product (§ 6.2; Hurrell, 1980;
Hurrell & Carpenter, 1981). Despite this, little has been done to devise
ways of inhibiting the reactions (Anderson & Quicke, 1985) while in general
the food industry has not included the monitoring of lysine availability in
its quality control programmes. More than this, protein quality is seldom
checked during the development of new products and processes.

Both livestock farmers and the consumer public can sometimes be grossly mis-
led by information such as total protein or lysine values obtained with
methods insensitive to certain types of nutritional damage. The argument
that there is no need to be concerned about product quality because most
people consume a more than adequate protein diet is not valid, and it is
important to ensure that claims made for the protein or amino acid contri-
bution of a product adequately reflect its nutritional availability. Such
information should be particularly important to animal feed manufacturers
since livestock farmers rely on precisely calculated diets designed for
optimal production at lowest cost.

Although there is no strong evidence that the products of protein damage
are a hazard to human health, it is obviously desirable to keep all potenti-
ally harmful substances to a minimum. This aspect has recently been

underlined in the animal feeds industry where the occurrence of the toxic product gizzerosine requires urgent attention. In the long term there is a need for the isolation and identification of all such potentially toxic products so that their true physiological and nutritional effects can be established. In the meantime, however, it is important to at least monitor any obviously undesirable changes occurring in protein during processing.

In seeking ways of monitoring protein damage, methods for the determination of available lysine are most appropriate as, a reduction in the availability of this amino acid is generally the first and most sensitive indicator of protein damage. Any such method would have to meet the needs of the industry by being simple, rapid and economical, and if possible suitable for 'on line' evaluations at critical points in a process. Since the ultimate test of whether a foodstuff is high in available lysine depends on whether it is digested, absorbed and utilised in vivo, it would seem logical to select one of the many bioassays for this purpose. Such assays are, however, subject to the normally accepted variables encountered with animal experiments and are too time-consuming and costly for routine use (see Ch. 1).

Some companies, in acknowledging the importance of monitoring amino acid levels in foods have invested in commercial amino acid analysers. However, various studies (e.g. Finot & Mauron, 1972) have revealed that acid hydrolysis releases lysine from some of its nutritionally unavailable bound forms so that such an analysis overestimates nutritionally available lysine. An alternative approach is the determination of chemically-reactive lysine. Although such methods cannot be expected to reflect the full extent of the nutritional availability of lysine, they do provide very useful indices of lysine availability which should satisfy the practical requirements of the livestock industry and other consumers.

The type of study undertaken for this thesis, although extremely useful in gauging the comparative capabilities of different procedures, does present difficulties when the most generally accepted reference method (in this case FDNB) is in itself not without problems. Attempts were made to use the method of Newell (1981) to assess the accuracy and precision of each method individually without the requirement of a standard reference procedure but unfortunately, the recommended computer programme was not compatible with available facilities. The possibility of using the Tetrahymena assay as standard reference procedure was also investigated but problems were encountered with the first batch of samples analysed, namely, the Maillard-damaged soyaprotein isolates (Ch. 8). Although the results for the various

Maillard-damaged soya samples showed good sensitivity to increased severity of heat damage when the values were compared at any one sample N-level, the TET lysine values for each sample, particularly the mildly-damaged material, varied considerably at different N-levels making it impossible to assign any specific values. TET growth was stimulated at low N-levels probably due to the ability of the organism to utilise 'early' Maillard products as sources of lysine while, at higher N-levels it was inhibited by toxic concentrations of the deoxyketosyl compounds and certain pre-melanoidin products.

In 1974 Hurrell and Carpenter classified selected reactive lysine methods with respect to their usefulness in assessing nutritionally-available lysine in different categories of processed proteins. Subsequently the present author (Anderson & Quicke, 1980 b) enlarged on this classification incorporating more methods and types of lysine damage. The comprehensive studies reported in this thesis now enable aspects of this classification to be reviewed. The new rating proposals are summarised in Table 7 where an indication is given of the duration of each chemical procedure for a single determination. Batch analyses obviously considerably speed up sample turnover.

For protein heated in the absence of reducing sugar (i.e. protein-protein interactions; Table 7) the TL method retains the D rating assigned by Hurrell and Carpenter (1974) since it is both the longest and least sensitive of all methods. This is mainly due to the 24 h acid hydrolysis step which releases lysine from some of its bound forms including unavailable A-L and partially available G-L (§ 5.2). The TL method may, however, qualify for a higher rating for more severely heated protein since it appears sensitive to certain unidentified acid-stable lysine products (§ 5.2). Various other authors (e.g. Rayner & Fox, 1978; Smith & Friedman, 1984) have also detected a reduction in TL of protein heated in the absence of sugars, but none have attempted to identify the nature of any acid stable products that might be present. Although the more rapid and economical NIN method has shown good sensitivity to various 'pure' and feed proteins (Friedman et al., 1984) it has not been tested on specifically heat-treated materials. However, in view of the proposed partial instability of the methylol derivative (formed as a result of formaldehyde treatment) during the ninhydrin reaction (100° C, pH 5,5 ; see § 7.3), it is possible that the acid labile (110° C, 6M HCl) isopeptides (Otterburn et al., 1977) may also be similarly unstable in which case only a C grading would be anticipated. Hurrell and Carpenter (1974) gave the FDNB method a B rating for protein

TABLE 7   A comparison of various chemical procedures with respect to
their usefulness in assessing available lysine in different
types of lysine-damaged proteins

Types of lysine damage	RATING OF PROCEDURE [a]						
	TL	FUR	NIN	FDNB	DBL	SA	DAN
Protein-protein	D	na	c	B	B	B	B
'Early' Maillard	D	B	c	B	B	B	B
'Late' Maillard	B	na	b	C	B/C	B	B
Formaldehyde	D	na	C	B	B	na	B
Estimated duration (h) [b]	30	30	1	20 – 22	2 – 8	2	1

a – Grading of usefulness for the 4 categories of damage indicated:

  A – a sensitive method believed to measure the full extent of
  changes in available lysine;

  B – a sensitive method but may not measure the full nutritional
  change;

  C – a less sensitive method but still capable of serving as an
  indicator of damage; and

  D – a method too insensitive for practical use.

  Symbols in lower case indicate anticipated grading for which experi-
  mental evidence is lacking.
  na = not applicable.

b – Time estimates are for single determinations and do not include time
  required for preparation of reagents, apparatus and the milling of
  samples. Sample turnover was more rapid for batch analyses.

heated in the absence of reducing sugars (i.e. protein-protein interactions; Table 7). In the present studies (§ 5.2) the FDNB method was found to be more sensitive than the other methods to mild heat damage but less sensitive to more severely heated protein. The other methods tested, namely, the DBL, SA and DAN procedures showed comparable sensitivities to this type of lysine damage. These methods including FDNB also agreed well with both plasma lysine and lysine digestibility (§ 5.3) suggesting that they could be sensitive to the full extent of lysine damage. It was therefore decided to assign B gradings to all four reactive lysine methods thus upholding the proposals of Hurrell and Carpenter (1974) for the FDNB method.

The unavailable 'early' Maillard products F-L and L-L are also partially acid-labile (6M HCl, 110° C) releasing on average approximately 50% of the bound lysine (Erbersdobler, 1970). Thus in view of the above discussion on the instability of methylol derivatives and isopeptides in acid media, it would be anticipated that the NIN method would once again be only partly sensitive to 'early' Maillard damage in which case only a C grading would be warranted. For a similar argument the TL method was dropped to a D rating (Table 7) as compared to the C rating assigned by Hurrell and Carpenter (1974) since as clearly demonstrated by the results in section 6.3, this method showed poor sensitivity to 'early' Maillard damage. Since F-L and L-L release constant proportions of FUR on acid hydrolysis (Erbersdobler, 1970) which can be detected on aminograms, analysis for FUR offers a useful alternative that is sufficiently sensitive (Figs 18 & 19) to warrant a B grading. Erbersdobler (1983 a) also found that FUR was a sensitive indicator of 'early' Maillard damage in various milk samples. The FUR method is obviously not applicable to other types of lysine damage since it depends on the presence of F-L or L-L. Based on the results for a mildly heated albumin-glucose mixture, Hurrell and Carpenter (1974) gave the FDNB method an A grading for 'early' Maillard damage. In the present studies (§ 6.3) the FDNB method was also the most sensitive to 'early' Maillard damage involving glucose followed by DAN or DBL and then SA methods. For mild damage caused by lactose, however, the order was somewhat different with DBL the most sensitive followed by FDNB, SA and then DAN. Interestingly, despite the latter high sensitivity of DBL, Hurrell et al. (1983) found that this method seriously underestimated 'early' Maillard damage in milk powders. The variations in the results between methods could be at least partly due to factors such as incomplete reaction of the specific electrophillic reagent with reactive amino groups or alternatively, due to the partial reaction of some of the reagents with the still basic ε-NH linkage in F-L and L-L (Finot & Mauron, 1972). In view of the above evidence and the more

detailed discussion in section 6.3, the four reactive lysine methods (Table 7) were assigned B ratings which in the case of FDNB represented a drop from an A grading as given by Hurrell and Carpenter (1974).

In most of the identified 'late' Maillard products lysine is no longer recognisable and presumably not recoverable even by acid hydrolysis. Under these circumstances one would expect TL to be highly sensitive to such damage and warrant an A grading. However, as shown in Figs 21 - 23 values can actually increase under more severe heat treatment probably due to certain previously acid resistant 'late' Maillard products being reconverted by the more severe heat into intermediates which can release lysine on acid hydrolysis. Thus despite the findings of Hurrell and Carpenter (1981) that TL gives a good estimate of 'late' Maillard material, the present results suggest that this method only warrants a B grading for this type of damage. The results in section 6.3 also show that for virtually all the 'late' Maillard-damaged samples the DAN and SA methods were the most sensitive followed by DBL and then FDNB. Under these circumstances SA and DAN were assigned B ratings while DBL was given a B/C and FDNB dropped to a C rating as compared to the B rating assigned by Hurrell and Carpenter (1974).

Although Hurrell and Carpenter (1978) found that both the FDNB and DBL methods were only sensitive to approximately half the 'true' reduction in bioavailability in a formaldehyde-damaged ovalbumin-lactalbumin blend, the results presented in section 7.3 show that in the present study these two methods as well as the DAN procedure were extremely and similarly sensitive to such damage. These methods are all therefore given B ratings (Table 7). By contrast the NIN and particularly the TL methods showed reduced sensitivity to formaldehyde damage and were therefore assigned ratings of C and D respectively. Both methods were probably sensitive to methylene type damage but as discussed in section 7.3, the methylol derivative is probably totally unstable during TL analysis and partially unstable during the ninhydrin reaction. The SA method unfortunately gave totally erroneous results probably due to the partial solubility of succinylated lactalbumin in the ethanol wash (see § 3.7.3 and Anderson & Quicke, 1984) and therefore is not recommended for this type of lysine damage.

Although the reactive lysine methods do not yield absolute values and therefore could not qualify for A gradings, Table 7 shows that all reactive lysine methods were reasonably sensitive to the various types of lysine damage, but no single method stood out as being most suitable for all types of lysine damage. While the SA method showed good ratings for the three most widely

encountered types of lysine damage, it cannot be used for any material solu-
bilised by succinylation (Anderson, 1980; Anderson & Quicke, 1984),
requires an expensive scintillation counter and in view of the precautions
that have to be taken with radioisotopic procedures, does not lend itself to
use in a routine quality control laboratory. The DAN method appears very
promising but cannot be recommended until the problems of converting arbit-
rary fluorescence units to lysine values (§ 4.4) can be overcome. The
renowned FDNB method is both long and laborious and special precautions have
to be taken when using the reagent due to its vesicant effects on the skin
while the NIN method due to the acid lability of various lysine derivatives,
appears unsuitable. On balance therefore, the DBL method, which qualifies
for a B rating for all types of lysine damage tested except 'late' Maillard
damage (B/C rating), appears to be the most useful procedure for routine
evaluation of available lysine. The procedure holds the further advantage
of being simple and very economical. It needs to be emphasised, however,
that the time required to achieve stable dye-binding is crucial and should
be established in each laboratory for each type of material being analysed
(see § 4.2) before the method is adopted as a routine analytical tool.

It is now 37 years since Kuiken and Lyman (1948) carried out the first avail-
able lysine determinations. During this period research involving studies
on the chemical, nutritional and toxicological consequences of protein damage
has developed to the extent that it has become virtually impossible to simul-
taneously study all aspects in any reasonable detail. Indeed even in select-
ing only 7 methods and 3 types of lysine damage, this thesis has generated
many unanswered questions and unsolved problems which it is hoped will stimu-
late further research in this field.

In the long term it will be mainly up to the chemists, biochemists and
nutritionists to investigate the more specialised problems of the chemistry
of lysine damage and the metabolic fate and toxicological consequences of
consuming such products - a very broad field indeed. Of more immediate
importance is the need for the food industry to recognise the deleterious
effect that food processing can have on protein quality and for the industry
to institute routine quality control programmes. While there is no single
ideal procedure for this purpose, some of the existing procedures do afford
reasonable assessments of available lysine which is the key factor in deter-
mining protein quality in many products. On the basis of the available
evidence the DBL procedure currently appears to be the simplest most widely
useful procedure for this purpose. The dansyl chloride procedure also shows

great promise provided a way can be found of expressing the results in terms of lysine and this aspect should therefore be the target of future research.

While this thesis has not produced final answers the results presented should provide some useful guidelines for future research in this field.

SUMMARY

The aim of this thesis was to investigate various reactive lysine methods,
render improvements where necessary and compare the perfected methods for
their ability to assess available lysine in certain specific types of
lysine damage.

The DBL method of Hurrell et al. (1979) was improved by measuring dye absor-
bance at 483 nm, acetylating for only 15 minutes and varying the duration of
the dye reaction to suit the protein being analysed. The DAN method of
Christoffers (1976) was shortened by dansylation at RT with a 2-fold excess
of reagent and 15 minutes of vigorous mixing. However, sample particle size
was shown to have a profound effect on fluorescence intensity readings for
dansylated soyaprotein with quenching remaining significant even for particles
below 80 μm in diameter. Attempts to correct results for quenching and to
convert values into units of reactive lysine were only partly successful.
The NIN assay of Friedman and Broderick (1977) was adapted for the analysis
of formaldehyde-damaged lactalbumin. Results were improved by using a
shorter (15 min) reaction time and correcting for a 9,17% α-NH$_2$ group and a
63,8% relative ϵ-NH$_2$ group colour contribution for lactalbumin.

The presence of the isopeptides aspartyl-lysine and glutamyl-lysine in heated
soyaprotein is suggested by the observed reduction in reactive lysine, plasma
lysine and glutamate, and lysine-, aspartate- and glutamate-digestibility.
Since isopeptides are acid labile, TL is the most insensitive of all methods
to this type of lysine damage. Results also suggest the presence of acid
stable derivatives which might include the poultry toxin gizzerosine. The
FDNB method was found to be more sensitive to mild heat damage but less
sensitive than the DBL, SA and DAN methods to severely damaged soya, the
latter three methods showing comparable sensitivities. All four methods
agreed well with both plasma lysine and lysine digestibility suggesting that
they could be sensitive to the full extent of lysine damage.

Results with soyaprotein heated with glucose, lactose or xylose show that
the type of sugar and the extent of heat treatment has a strong influence on
the progress of the Maillard reaction and this can be monitored by the analy-
sis of TL and FUR which can be used to estimate available and destroyed
lysine and fructose- or lactulosyl-lysine (F-L or L-L). Glucose-soya and
lactose-soya mixtures heated at 90° C up to 2 h or 110° C up to 1 h contain
primarily 'early' Maillard products whereas with more severe heat treatments

'late' Maillard products predominate. Since no 'xylanosyl-lysine' as such has been identified, it was not possible to estimate 'early' xylose damage, but 'late' Maillard damage was more severe in all xylose-soya mixtures than in their glucose- and lactose-soya counterparts. Since F-L and L-L are colourless, up to 30% loss of AL can occur without any noticeable change in product colour.

For mildly damaged glucose-soya samples FDNB was the most sensitive method followed by DAN or DBL, SA and then TL whereas for mildly damaged lactose-soya samples, the order was DBL, FDNB, SA, TL and DAN. For the more severely damaged material virtually all samples showed the same trends with DAN or SA showing greatest sensitivity to lysine damage followed by DBL, FDNB and TL. The general insensitivity of TL to Maillard damage was probably due to the acid lability of products such as F-L, L-L and certain unidentified 'late' Maillard lysine derivatives. It was suggested that variations in results between reactive lysine methods could be partly due to incomplete reaction of the specific electrophillic reagents with reactive amino groups, or alternatively due to partial reaction of some reagents with the basic ε-NH linkage in F-L and L-L.

Contrary to various reports, pH had no significant effect on either the ε-amino formylation reaction or the subsequent formation of methylene bridges with tyrosine or free ε-NH_2 groups in formaldehyde-treated lactalbumin. Furthermore formaldehyde concentration rather than exposure time had the greatest enhancing effect on the formylation reaction with methylol derivatives reaching maximal levels after 12 h with 24,1% formaldehyde and methylene bridge formation reaching completion after 1 h, probably because of the requirements of proximity of reactive side chains. Histidine does not appear to participate in methylene bridge formation, except perhaps after the bulk of tyrosine and lysine residues have reacted. Tyrosine by contrast readily forms methylene bridges but also appears to be involved in condensation reactions with free formaldehyde released from methylol derivatives during acid hydrolysis, raising questions as to the interpretation of similar studies reported in the literature. The NIN method and particularly the TL method showed reduced sensitivity to formaldehyde-damage probably because the unavailable methylol derivative is partially hydrolysed during the ninhydrin reaction and totally unstable during acid hydrolysis. The FDNB, DBL and DAN methods were all very sensitive to such damage while the SA method yielded erroneous results, probably due to the solubility of succinyl-lactalbumin in the ethanol wash.

The pronase digestibility of 'crude' protein was found to vary from 90% for mildly damaged Maillard samples rich in deoxyketosyl derivatives to 20% for the severely browned samples high in pre-melanoidins and possibly isopeptide linkages. These digests were also used to evaluate the usefulness of Tetrahymena pyriformis W for assessing Maillard-type damage. For 'early' Maillard material at low culture-lysine levels Tetrahymena growth was found to be stimulated by its ability to utilise F-L, L-L and possibly various unidentified 'early' xylose products as a source of lysine while at higher N-levels it is inhibited by toxic concentrations of the deoxyketosyl compounds and certain pre-melanoidin products. These trends were more profound for lactose-soya samples but considerably less marked in the case of the xylose-treated soya isolates. This assay is thus unsuitable for assessing lysine availability in 'early' Maillard material but remains suitable for 'late' Maillard damage.

The results reported in this thesis emphasise the importance of keeping all toxic products of protein processing to a minimum and incorporating reactive lysine analysis into protein quality control programmes in the protein industry. To meet the needs of the industry it is considered important to select a rapid, simple and economical method that can be performed on a routine basis and which yields sufficient information to satisfy the needs of farmers and other consumers. It is clear that different methods are the most suitable for different types of lysine damage, but the DBL method has the widest general applicability and most closely meets these requirements since the other reactive lysine methods are either too long, rather complicated, require expensive equipment or involve the use of dangerous chemicals.

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APPENDIX   1

PREPARATION   OF   THE   NUTRIENT   MEDIA   FOR
THE   <u>Tetrahymena pyriformis</u>   W   ASSAY
(SECTION   2.8.1)

VITAMINS

<u>Solution 1.</u>   Vitamin mix: Calcium pantothenate (12,5 mg), nicotinamide
(12,5 mg), pyridoxine hydrochloride (125 mg), pyridoxal hydrochloride
(12,5 mg), pyridoxamine hydrochloride (12,5 mg), riboflavin (12,5 mg),
thiamine hydrochloride (125 mg), inositol (12,5 mg) and p-aminobenzoic acid
(12,5 mg) (all obtained from Sigma) were accurately weighed out and dissolved
with brief boiling in 100 mℓ of distilled water.   The solution was divided
into five 20-mℓ amounts and stored in plastic containers in the deep freeze
until required.   All operations were carried out in subdued light.

<u>Solution 2.</u>   Choline chloride: 62,5 mg of material was dissolved in 10 mℓ
of distilled water.   The solution was freshly prepared before each batch of
analyses.

<u>Solution 3.</u>   Folic acid: 15,6 mg of material was dissolved in 7,5 mℓ of
0,1M NaOH and made up to 25 mℓ with distilled water.   The solution was
diluted 10 times before use.   If stored in the dark at 4° C the solution
remained stable for up to one month.

<u>Solution 4.</u>   Biotin: 15,6 mg of material was dissolved by boiling in
250 mℓ distilled water.   The solution expired after 3 months at 4° C.

<u>Solution 5.</u>   D,L-6,8-Thioctic acid: 10 mg material was dissolved in 50 mℓ
of distilled water. Small quantities of ethanol (96 %) were used if neces-
sary to assist in the dissolution. The solution was diluted 10 times before
use and could be kept for 3 months at 4° C.

<u>Solution 6.</u>   6,25 mℓ of Solution 1 and 1,25 mℓ aliquots of Solutions 2, 3,
4, 5 and distilled H$_2$O were mixed to give 12,5 mℓ of vitamin Solution 6
which was kept in the dark until used for the preparation of Solution 9.

<u>Solution 7.</u>   Amino acid mix: L-alanine (0,978 g), L-arginine hydrochloride
(0,930 g), L-aspartic acid (1,087 g), glycine (0,089 g), L-glutamic acid
(2,072 g), L-histidine hydrochloride (0,462 g), L-isoleucine (0,560 g),

APPENDIX 1 (contd.)

L-phenylalanine (0,890 g), L-methionine (0,303 g), L-proline (1,564 g), L-leucine (1,730 g), L-cysteine hydrochloride (0,293 g), L-serine (1,374 g), L-threonine (0,784 g), L-tryptophan (0,213 g) and L-valine (1,176 g) (all obtained from Sigma) were accurately weighed out and dissolved in 800 mℓ of distilled water to give Solution 7. The solution was adjusted to a pH of 7,1 with 30 % NaOH and divided up into four 200-mℓ amounts and frozen separately in plastic bottles until required.

<u>Solution 8</u>. Glucose: 37,5 g of glucose (Merck) was dissolved in 100 mℓ of distilled water.

<u>Solution 9</u>. Glucose-vitamin-amino acid mix: Solution 7 (200 mℓ) was mixed with 12,5 mℓ distilled water in a 500 mℓ conical flask, the container plugged and autoclaved at 15 lbs for 10 min. Solution 6 (12,5 mℓ) and Solution 8 (25 mℓ) were similarly autoclaved in separate glass test tubes. When cool, Solutions 6 and 8 were aseptically added to Solution 7 in the conical flask and mixed thoroughly.

MINERAL SALTS

<u>Solution 10</u>. 7 g of $MgSO_4 \cdot 7H_2O$ was dissolved in 50 mℓ of distilled water.

<u>Solution 11</u>. 3,125 g of $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ (Ferro-ammonium-sulphate) was dissolved in 50 mℓ distilled water and a drop of concentrated HCl added to prevent the formation of a Fe_3O_4 precipitate.

<u>Solution 12</u>: 62,5 mg of $MnCl_2 \cdot 4H_2O$ was dissolved in 50 mℓ of distilled water.

<u>Solution 13</u>: 12,5 mg of $ZnCl_2$ was dissolved in 100 mℓ of distilled water.

<u>Solution 14</u>: 600 mg of $CaCl_2 \cdot 2H_2O$, 60 mg of $CuCl_2 \cdot 2H_2O$ and 15 mg $FeCl_3 \cdot 6H_2O$ were dissolved in 200 mℓ of distilled water.

<u>Solution 15</u>: 1,75 g of KH_2PO_4 and 1,75 g of K_2HPO_4 were dissolved in 100 mℓ of distilled water.

<u>Solution 16</u>: 5 mℓ aliquots of Solutions 10, 11, 12 and 13 were mixed and made up to 50 mℓ with distilled water.

APPENDIX 1 (contd.)

Solution 17: 5 mℓ aliquots of Solutions 14 and 16 were mixed, diluted to approximately 40 mℓ with distilled water, 5 mℓ of Solution 15 added and made up to 50 mℓ with distilled water. The order of addition of these reagents was important for the minimisation of a precipitate caused by the addition of Solution 15.

Solution 18. Purines and pyrimidines: Uracil (25 mg), 50 mg of adenosine-2' (-3')mono-phosphoric acid, 75 mg of guanylic acid and 62,5 mg of cytidylic acid were dissolved in 40 mℓ of boiling distilled water, cooled and made up to 50 mℓ with distilled water.

Solution 19. Mineral salt-purine/pyrimidine mix: Equal quantities (50 mℓ) of Solutions 17 and 18 were mixed and the pH adjusted to 7,1 .

-oOo-