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THE EFFECT OF HEAT TREATMENT ON LYSINE AVAILABILITY AND DYE-BINDING CAPACITY OF SKIM MILK

A thesis presented in partial fulfilment
of the requirements for the degree
of Master of Technology in
Food Technology at
Massey University

Malcolm John Reeves
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ABSTRACT

The reported work on changes in lysine content in milk and dried milk is examined. The cause of these losses, the Maillard reaction, and the methods of lysine determination are discussed. All methods have recognised faults. Little information is available to the food processor regarding the kinetics of these losses, and the methods of their determination are not simple enough for routine quality control application.

Although the lysine content of milk products determined after acid hydrolysis is known to be higher than nutritional studies indicate the causes of this are being established. Therefore acid hydrolysis in conjunction with a GLC method of amino acid analysis was adopted after some modification. (It was found that dialysis of the milk prior to hydrolysis resulted in cleaner chromatograms and that as the recovery of several amino acids, such a proline, leucine, and isoleucine, was not affected by heat treatment then these were used as internal 'internal standards'.)

No simple rate expression could be found to fit the kinetics of the loss of acid released lysine. A first order model requiring the losses to be increased by a factor of 3.43 was devised and this could be used to satisfactorily predict values for acid available lysine in the heat treated milk. The possibility of the 3.43 factor being due to the regeneration of lysine by acid from Maillard intermediates, although requiring assumptions, was found to be not unreasonable.

The energy of activation of the reaction leading to a loss in acid released lysine at 31.5 Kcal/mole is similar to literature values while the model value of 37.2 Kcal/mole is rather higher.

The literature findings of little or no loss of lysine during pasteruization, evaporation, and sterilization of milk are supported.

The technique of protein determination by dye binding was examined and applied to following changes in lysine in heat treated milk. The inconsistencies in reported work on dye binding is of little consequence as relative changes only are required.

Changes in dye binding using amido black did not follow simple order kinetics, even when allowance was made for the constant binding by arginine and histidine. A first order model requiring the changes to be increased by a factor of 3.68 was developed. About 46% of this factor can be explained by assuming constant binding by arginine and histidine, the remainder of the factor possibly being due to Maillard intermediates binding dye, and/or a change in binding stoichiometry occurring. From the model it is possible to predict the observed changes in dye binding. Literature findings were supported.

The energy of activation for the dye binding changes is 28.6 Kcal/mole, and for the model, 30.8 Kcal/mole.

Ancillary investigations showed that the concurrent colour changes due to heat treatment have an energy of activation of about 30 Kcal/mole, and that there is a relationship between colour and dye binding capacity in heat treated milk.

The relationship between the Pro-Milk and a typical absorbance spectrophotometer was determined, and an expression found which would enable a spectrophotometer to be used for protein determination.

"I do not think that with products manufactured by modern advanced techniques the losses in protein value are of much importance. It is more important to avoid waste and prevent spoilage."

Dr.S.K. Kon

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December, 1976

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CHAPTER I

1.1 INTRODUCTION

Lysine deficiency is a common and serious problem, particularly in countries where the diet is based on cereals and cereal products. (Yannai and Zimmerman, 1970). The nutritionally available lysine content of foods can be severely reduced by improper processing, and poor storage conditions. This loss of nutritionally available lysine can result in some foods becoming nutritionally deficient in lysine, i.e. lysine can become the limiting amino acid.

It was observed that the biological value of the protein of dried skim milk stored at room temperature, but under conditions which excluded pickup of atmospheric moisture, decreased with the length of storage (Henry and Kon, 1945). After 18 months the biological value had fallen from 88.5 to 71.1. This observation resulted in further investigations into the cause of this loss. The subsequent research showed that the change in biological value was a result of the Maillard or non-enzymic browning (NEB) reaction between the aldehyde group of a reducing sugar (lactose in the case of milk powder), and the free amino groups of the protein which are largely the ϵ-amino groups of lysine, and the α-amino groups at the end of the protein peptide chains. (Henry, et al, 1946). The effect of the terminal α-amino will be small as there are so few (2%) in comparison to the number of the ϵ-amino groups of lysine.

This discovery of the involvement of the Maillard reaction in the nutritive deterioration of milk powder stimulated research into the Maillard reaction and changes in proteins in the presence of sugars, particularly reducing sugars. Amongst this early work was that of Henry et al, 1948; Lea and Hannan, 1949; 1950a, 1950b, 1950c, 1950d; Lea 1950; Hannan and Lea, 1951; Lea et al 1951 and Lea and Rhodes, 1952. This work, mostly with milk powder and casein-glucose models, showed thateven in relatively dry materials ($<$ 10% H_2O) the carbonyl groups of reducing sugars could react with the free ϵ-amino groups to form compounds

that had no nutritional value but from which free lysine could be released when hydrolysed by the strong acids, typically used in the hydrolysis of proteins prior to amino acid analysis. This explained, at least partly, why some materials had lower nutritive values than would have been predicted from the amino acid analysis of acid hydrolysates of the material. This discrepancy was noticed with materials, particularly milk products, that had been processed or stored under adverse conditions. (Block and Mitchell, 1946-47). More information about the role of the Maillard reaction in these nutritive changes and also a method of analysis that would indicate the amount of nutritionally available lysine was needed.

1.2 THE MAILLARD REACTION

After the initial work of Lea, Hannan and co-workers of the late 1940's and early 1950's there have been continuing investigations into the browning reaction, particularly in the 'dry' state using model protein-sugar systems (such as casein and lactose). Included in these studies is the work of Hodge, 1953, (Chemistry of browning reactions in model systems), Richards, 1963, 1965 (Degradation of lactose by interaction with casein), Cole, 1967 (The Maillard reaction in food products; carbon dioxide production. This was in a liquid model system), Spark, 1969 (Role of amino acids in nonenzymic browning), and Ferretti and Flanagan 1970 and 1971 (Nonenzymatic Browning in a lactose-casein model system). There have also been a number of specific investigations into restricted areas such as the formation of specific compounds (e.g. 2,3 - Dihydro -3,5 - dihydroxy-6-methyl-4H-pyran-4-one) (Mills et al, 1970) and into the kinetics of the reaction (Song et al, 1966).

There have been a number of comprehensive reviews covering the Maillard reaction in detail (Hodge, 1967, Hodge et al, 1972; Reynolds 1963, 1965, 1969, 1970; Greenshields and Macgillivray, 1972), some with particular reference to foods and food flavours.

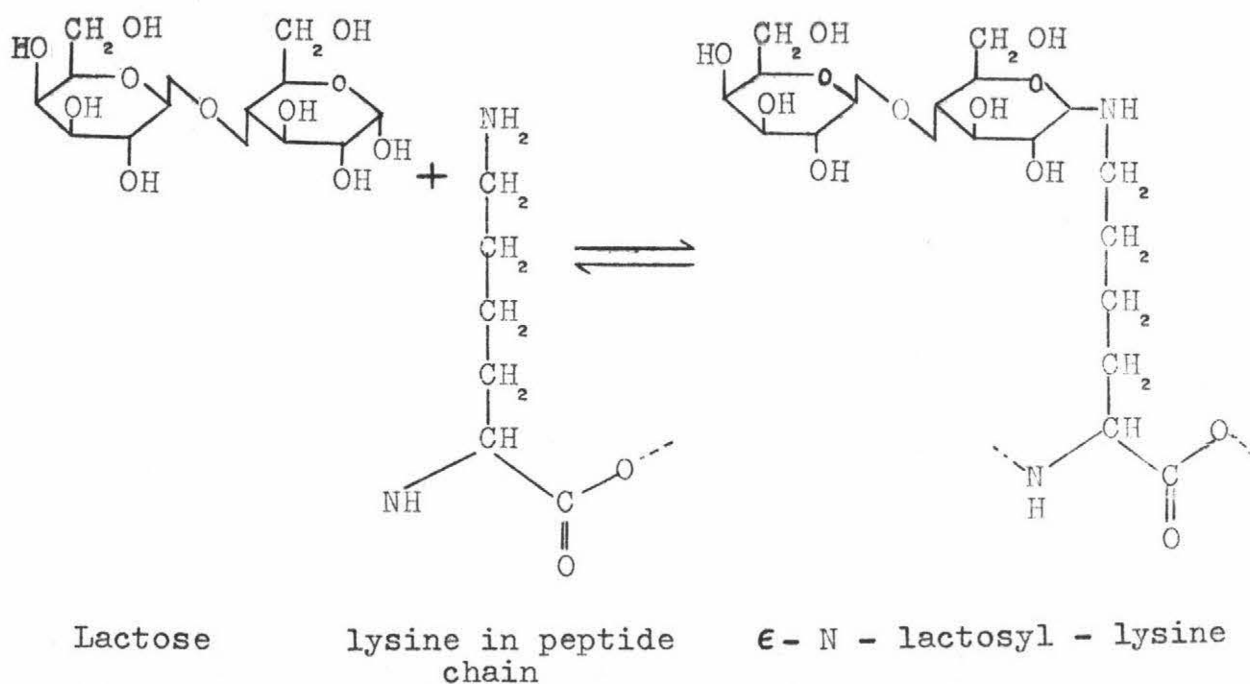
While free amino acids can become involved with reducing sugars in the Maillard reaction, in foods the free amino groups are largely the ϵ-amino groups of lysine, as

apart from the few terminal α-amino groups, the amino groups of the other amino acids are incorporated in the peptide chain. It is not clear what ultimately happens to the amino groups involved in the Maillard reaction but nitrogen seems to be incorporated in some of the pigments and melanoidins formed. In the initial stages the amino acid acts as a catalyst but the reactions they undergo after release is not clear.

It is generally accepted that the Maillard reaction follows the following reaction steps (considering aldose sugars only).

a) Aldose + amino acid $\rightleftharpoons$ Aldosylamine

For lysine, in a peptide chain, reacting with lactose

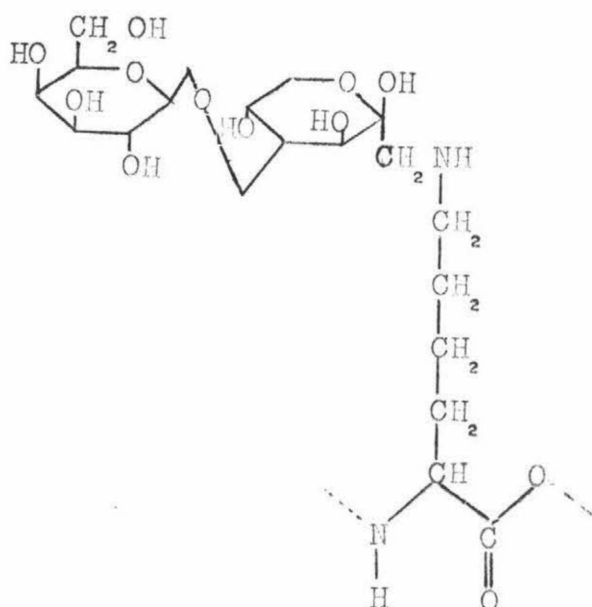


Lactose | lysine in peptide chain | ε - N - lactosyl - lysine

(The α-amino group is in the peptide chain and is not involved in the reaction).

b) Aldosylamine $\xrightarrow[\text{(rapid)}]{\text{Acid catalyst}}$ Ketoseamine

This is known as the Amadori Rearrangement. The aldosylamines are unstable and rapidly undergo this rearrangement to form ketoseamines (also termed deoxyketoseamines). In the reaction between lysine and lactose in food the first stable product formed is ε - N - (1-deoxy-D-Lactulosyl) - L - lysine (also called lactulose lysine) where

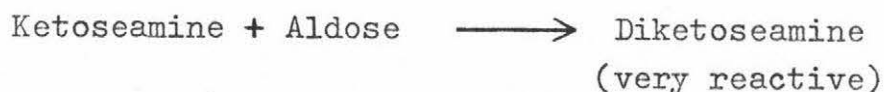


ϵ - N - (1 - deoxy - D -lactulosyl) - L - lysine
(Lactulose lysine)

the glucose moiety of the lactose molecule has rearranged to give a fructose moiety associated with the lysine.

Ketoseamines as a class are stable colourless solids but in solution they are reactive, rapidly forming a variety of products.

c) The ketoseamines can undergo a variety of reactions giving a wide range of products.



Ketoseamine + Aldose ⟶ Diketoseamine
(very reactive)

d) The various aminosugar moieties (ketoseamine, aldoseamine, and diketoseamines) formed in the first three steps can individually undergo a series of degradation reactions to both amino and non-amino compounds. These may have distinctive flavours or react further to give flavours or brown colours. It is this series of reactions and the wide range of compounds formed that has attracted the attention of many carbohydrate and food flavour chemists.

The degradation proceeds by two distinct pathways, viz. 1,2 enolization and 2,3 enolization.

(i) 1,2 enolization of a ketoseamine gives the corresponding 1,2 enol with the amino group in the C1 position. The 1,2 enol can then undergo β elimination of the C3 hydroxyl group to give the 2,3 enol, which being a Schiffs base is readily hydrolyzed to the enolic form of a 3-deoxyosulose, or else by β elimination of the C4 hydroxyl group gives the Schiffs base form of an unsaturated osulose. These products can then react further to give a variety of furfural and pyrrole type compounds.

The 3-deoxyosulose forms have been found in foods and are stable. The unsaturated osuloses though are much less stable.

The degradation via 1,2 enolization appears to be the main pathway to brown colours in foodstuffs while the osuloses subsequently formed can also give flavours via the Strecker degradation involving α-amino acids. (Flavours produced directly as a result of 1,2 enolization of ketoseamines are very limited.)

(ii) 2,3 enolization of ketoseamines is not very significant in food in terms of yield, but is important in terms of flavour production. (Reynolds, 1970). It is therefore unlikely to be of importance in terms of changes in lysine status in the protein.

e) Condensation reactions occur in which many of the compounds formed in step d) are involved. These may also involve additional amino compounds which can become incorporated into the brown pigments produced. Further details of these colour and flavour producing reactions are covered in the reviews listed earlier. (See also section 6.1)

The chemistry of the Maillard reaction is not yet completely understood (Finot, 1970) but for lysine in milk proteins the major steps appear to be (a) and (b), involving the formation of

(i) aldosylamines and the Schiffs base forms

(ii) deoxyketoseamines

In heated milk, and milk powders both of these states

exist (Finot, 1970) which contradicts Reynolds (1970) who states that the aldosylamines are unstable and immediately form ketoseamines.

1.3 METHODS FOR DETERMINATION OF LYSINE

With the realisation that conventional acid hydrolysis of some protein resulted in high estimates of nutritionally available lysine attention was given to finding a method for measuring only the nutritionally available lysine by chemical means. (It was possible to make an assay by microbiological means, and the reference method was by growth studies.) Although there are various biological methods each having faults and limitations (FAO, 1969, Reeves, 1973) the ultimate evaluation must involve an "in vivo" test in some way. However, if a chemical test could be developed which correlated highly with a biological test then it may be possible to routinely use the chemical method.

One of the first modifications was to change the acid hydrolysis step to an "in vitro" enzymic hydrolysis method. Certain enzymic methods have been able to give results that are comparable with those given by growth tests. A review has been published recently covering "in vitro" methods. (Mauron, 1970) It is claimed that "the sophisticated "in vitro" tests have served to demonstrate the physiological basis of differences in protein quality but they do not provide an economical means of quality control in practice." (Carpenter, 1974)

Work was also directed towards finding a straight chemical method and from the increasing understanding of the Maillard reaction it was apparent that for lysine availability there was likely to be some relationship with the free ϵ-amino groups. Research concentrated on this aspect and various methods were examined.

a) Dye binding in which a coloured dye is reacted with the protein where the basic amino acids (histidine, arginine and free ϵ-amino lysine) bind the dye molecules. Typically acid orange 12, orange G, and amido black 10B have been used. Ney and Wirotama, 1970; Pruss and Ney, 1972 have examined

the use of Remazol Brilliant Blue but this method still needs refinement. (See Chapter 4 et seq.)

b) Modified Van Slyke procedure in which the free amino nitrogen content is determined. This has been used in casein/glucose studies (Lea, 1948, Lea and Hannan, 1950a, Richards, 1963) but requires special skill (Carpenter, 1974).

c) Trinitrobenzenesulphonic acid (TNBS) which reacts with the free ϵ-amino groups to give trinitrophenyl (TNP) lysine derivative (Kakade and Liener, 1969, Hurrell and Carpenter, 1974). Unfortunately the reaction is not specific and will give significant values for lysine moieties involved in the Maillard reaction and therefore requires modification or correction for satisfactory use with milk powders and milk.

d) Fluorodinitrobenzene (FDNB), which undergoes the Sanger reaction with free amino groups forming dinitrophenyl (DNP) derivatives. After acid hydrolysis and separation, or extraction the yellow DNP derivatives can be determined spectrophotometrically. Various modifications to the procedure have been devised to overcome problems encountered during hydrolysis (the presence of sugars during hydrolysis of the FDNB-protein can drastically reduce the recovery of DNP-lysine), and to overcome the problem of high apparent readings due to the presence of other DNP derivatives other than lysine. A full review of the FDNB method is presented by Carpenter, 1973 and further information by Finot and Mauron, 1972.

It has been used in modified forms extensively, and has been found to give, in some cases, a good correlation with "in vivo" and "in vitro" evaluations. (Bujard et al., 1967); Mottu and Mauron, 1967; Creamer et al., 1976 . Even so it has been reported that FDNB will give an indication of free ϵ -amino lysine for pure Maillard compounds such as α-formyl-ϵ-deoxyfructosyl lysine (Finot and Mauron, 1972; Hurrell and Carpenter, 1973, 1975). FDNB still seems to be the preferred reagent in many studies.

e) Methylisourea (MIU) has also been examined (Mauron and Bujard, 1964; Finot and Mauron, 1972; Hurrell and Carpenter, 1974; Creamer et al., 1976), and has been found to give similar results to those by the "FDNB-difference" method

(Roach et al., 1967). In the MIU reaction a guanidine derivative of lysine is formed which on subsequent hydrolysis yields homoarginine. No homoarginine is formed from lysine with the ε-amino groups bound to a sugar moiety and for this reason it is preferred by Finot and Mauron (1972). It was also recommended (Creamer et al., 1976) because only one amino acid analysis is necessary, as well as its freedom from side reactions.

f) Sodium borohydride has been found to give results comparable with the FDNB and TNBS methods (Couch and Thomas, 1976) in the testing of bovine serum albumin and various cottonseed meals. As these do not contain significant amounts of reducing sugars, and as milk products were not examined, its suitability for milk products is uncertain. Its principle is similar to that of the FDNB method.

1.4 ACID HYDROLYSIS OF HEATED MILK PRODUCTS

Although there are problems with the methods outlined above in predicting the nutritive values of protein containing foods, some have been found to indicate more precisely the nutritive value than does the conventional method of analysis after 6N HCl acid hydrolysis. It has been found that some Maillard reaction products (particularly lactulosyl lysine, and fructosyl lysine) which contain nutritionally unavailable lysine, released lysine on hydrolysis with 6N HCl. An examination of the acid hydrolysates of overheated milk powder revealed a basic amino acid not present in the original protein (Erbersdobler and Zucker, 1970). This compound was prepared, its chemical structure determined and given the name "Furosine" (Finot et al., 1971) while chemically the compound is ε- N -(2-furoyl-methyl) - L - lysine. During the preparation of furosine a second compound, termed "pyridosine" (ε -(3-hydroxy-4-oxo-6-methyl-1-pyridinyl)-L-norleucine) was also detected.

Furosine and pyridosine are isomers and are derived by the elimination of 3 molecules of water from ε-N-(1-deoxy-D-fructosyl)-L-lysine, also termed fructose lysine (a ketoseamine produced in step (b) of the Maillard reaction as outlined earlier).

In heated milk powders the major ketoseamine produced is ε-N-(1-deoxy-D-lactulosyl)-L-lysine, also termed lactulose lysine, which under acid hydrolysis can give the deoxyfructosyl lysine and galactose. The following is believed to be the reaction scheme:

a) Lactulose lysine $\xrightarrow[\text{hydrolysis}]{\text{Acid}}$ Fructose lysine + galactose

b) Fructose lysine $\xrightarrow[\text{conditions}]{\text{Acid}}$ Furosine + Pyridosine
$-3H_2O$ + Lysine

As stated earlier the ketoseamines are the first stable intermediate in the Maillard reaction and have been isolated from heated milk powders. Confirmation of lactulose lysine being one possible starting material for furosine and pyridosine was provided when pure lactulose lysine was prepared and then hydrolysed in 6N HCl. (Finot et al., 1971). The amount of lysine regenerated, furosine and pyridosine formed as a result of acid hydrolysis of lactulose lysine, was determined (Finot and Mauron, 1972). Their results expressed in terms of lysine recovered as a % of the original lysine in the lactulose lysine is given in Table 1.1.

TABLE 1.1 Lysine recovery from lactulose lysine by acid hydrolysis

Conditions of hydrolysis	% lysine regenerated	x % of lysine recovered as furosine	% of lysine recovered as pyridosine
6 N HCl	49.5	20.3	10.4
7.75 N HCl	43.8	29.3	11.4

(x These values are estimates based on chromatogram areas, and used lysine colorimetric factors, which may be not accurate for furosine and pyridosine.)

The proportions of the hydrolysis products were unaffected by the presence of reducing sugars during the hydrolysis, nor was there any statistically different recovery from fructose lysine and lactulose lysine. Only the strength of the hydrolysing acid was found to alter the proportions of the products. From this it was concluded

that approximately 50% of the lysine in the form of the Amadori derivatives will be recovered by the classical 6N HCl hydrolysis technique. This explains the higher than expected values for "total lysine" given for processed foods by this technique whereas in unprocessed foods, with no Maillard damage the "total lysine" is effectively the same as that given by nutritional studies. (As stated earlier lysine on the Maillard products lactulose lysine and fructose lysine is nutritionally unavailable (Finot and Mauron, 1972)).

1.5 THE RELATIONSHIP BETWEEN ACID DETERMINED AND NUTRITIONALLY DETERMINED LYSINE

The following discussion has been adapted mainly from the work of Bujard and coworkers which has been summarised by Finot (1973). From their work a number of relationships were derived regarding the availability of lysine, in heated milk products, as determined by acid hydrolysis, FDNB, "in vitro", and "in vivo" methods. The findings of their work can in part be summarised by figure 1.1.

It was found for heated milk products that

$$ALV = 2.70\ TLV - 167 \qquad (1.1)$$

$$(r = 0.99)$$

where ALV = enzymically available lysine, and
TLV = total lysine, by conventional acid hydrolysis

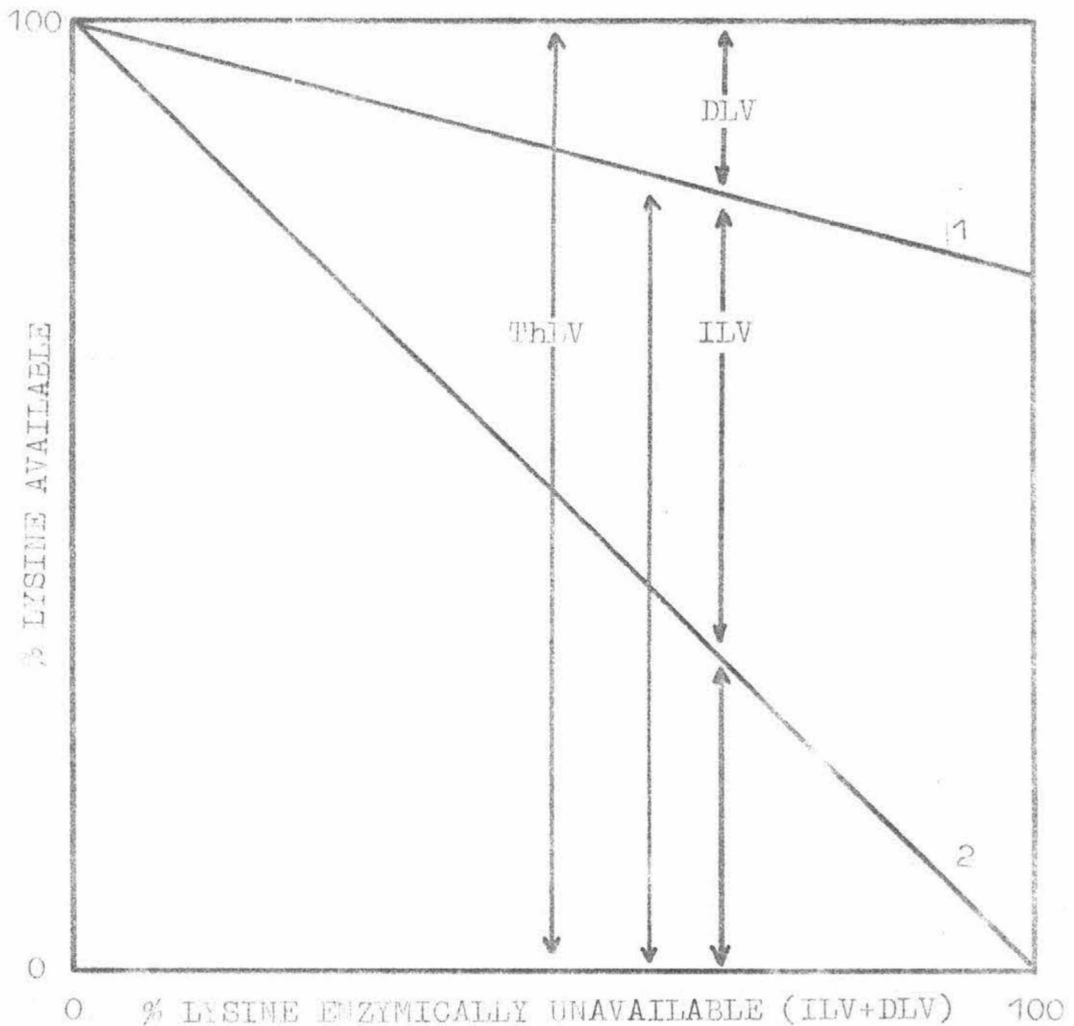
Unfortunately the equivalent equation for nutritionally determined ALV values is not given, and as their data is presented in summarised form only it is not possible to derive the relationship. It had also been found (Mottu and Mauron, 1967) that the "in vivo" ALV and enzymic ALV were related by

$$ALV_v = 1.02\ ALV_e - 0.23 \qquad (1.2)$$

$$(r = 0.98)$$

There is no statistical difference between the two ALV values, i.e. 1.02 is not significantly different from 1.00 and 0.23 is not statistically different from 0. The two

FIGURE 1.1 : THE RELATIONSHIP BETWEEN ACID AVAILABLE LYSINE AND NUTRITIONALLY AVAILABLE LYSINE (after Bujard et al., 1967)



1. Lysine determined after acid hydrolysis (TLV)

2. Lysine determined after enzymic hydrolysis (ALV)

ThLV Theoretical lysine Value

ILV Inactivate lysine value (available after acid hydrolysis, not available after enzymic hydrolysis.)

DLV Destroyed lysine value (not available after acid hydrolysis, or enzymic hydrolysis.)

methods are apparently measuring the same thing. Therefore equation 1.1 can be assumed to indicate the relationship between nutritionally available lysine and "total lysine".

These equations indicate that it should be possible to predict the nutritional availability of lysine in heated milk products (particularly skim milk powder) from the TLV of the milk product. Care may be needed in its widespread application as the data from which the relationship was derived included only one sample of Maillard damaged fluid milk, the remainder being powdered milk, although the range of ALV covered in the data was from 100% to 24%. It should also be noted that in equation 1.1 the values are expressed as percentages and it is therefore necessary to know the TLV of the non-damaged/unprocessed product before the percentage TLV in the damaged/processed product could be calculated. This will limit the usefulness of the relationship.

1.6 THE ROLE OF FUROSINE AND PYRIDOSINE

Equation 1.1 implies that the change in ALV is 2.7 times greater than the change in TLV,

i.e. $$\Delta \text{ALV} = 2.7\,\Delta \text{TLV} \qquad (1.3)$$

and in relation to figure 1.1,

$$\Delta \text{TLV} = \text{DLV}$$

and $$\Delta \text{ALV} = \text{DLV} \quad \text{ILV}$$

$$\therefore \quad \text{ILV} = 1.7\ \text{DLV} \qquad (1.4)$$

When the experimentally determined pairs of values for ILV, and DLV are correlated it was found (Finot, 1973) that

$$\text{ILV} = 1.93\ \text{DLV} - 6.2 \qquad (1.5)$$

(This includes the results of 2 other experiments as well as the results reported by Bujard et al., 1967, which explains the slight difference between 1.4 and 1.5.)

Now during the Maillard reaction in the initial stages the ϵ-amino groups of lysine are present in

(a) the free form,

(b) the aldosylamine (and its Schiffs base) form and,

(c) the deoxyketoseamine form.

On acid hydrolysis of Maillard damaged milk powder the lysine in the free amino form (the only nutritionally

available form of the three listed above), and in the aldosylamine (and its Schiffs base) form will be recovered 100%, but as shown in Table 1.1 only 49.5% of the deoxyketoseamine form (lactulose lysine) will be recovered.

On the basis of these recoveries, and equation 1.5, Finot concluded that in heated milk of the nutritionally unavailable lysine, 28.5% is present in the aldosylamine and Schiffs base forms, while 71.5% is present in the deoxyketoseamine form.

Finot and coworkers also used this information to derive the predictive relationship

$$ALV = 103 - 6.72\ FUR \qquad (1.6)$$

where FUR is the lysine recovered in the form of furosine, during hydrolysis of the milk with 6N HCl.

This equation assumes that some lysine (28.5%) is present as the aldosylamine and Schiffs base forms. Reynolds (1970) states that these are unstable intermediates and readily form the deoxyketoseamine derivative. If there is little or no aldosylamine and its Schiffs base then equation 1.5 would become

$$ALV = 100 - 4.92\ FUR \qquad (1.7)$$

These relationships (1.6, 1.7) have the advantage over 1.1 in that the initial TLV is not required as the quantity of furosine is a direct indicator of the impairment of lysine availability. In the experimental work of this thesis the TLV of the unprocessed milk was determined and also as the characteristics of furosine in relation to gas chromatographic determination are unknown, the logical equation to use is 1.1.

All of these relationships apply only to heated milk products as they are based on the regeneration of lysine and the formation of furosine (together with pyridosine) from the deoxyketoseamine formed during the Maillard reaction. In pure proteins, or foods with a lesser content of reducing sugars the importance of the deoxyketoseamine as a form of nutritionally unavailable lysine is considerably reduced (Carpenter, 1973).

1.7 EXAMINATION OF LITERATURE DATA

Comparatively little work is available for examination in terms of fitting to the relationships 1.1 and 1.3 as few workers have determined both the ALV, and TLV for heated milk samples as percentages of the ALV and TLV of the unprocessed product.

Even less data is available than can be substituted into 1.7 as few workers apart from Finot and his coworkers have reported furosine values. Erbersdobbler (1970) reported furosine values but used 7.75 M HCl which as shown in Table 1.1, gives a higher yield of furosine, and therefore 1.7 cannot be applied. (It would be possible to derive a form of 1.7 applicable to the results of the hydrolysis using 7.75M HCl).

One set of values (De Vuyst et al., 1972) is in a suitable form, and for variously treated milk products (sterilised, pasteurised, sweetened condensed milks, and two powders) it is found that compared to the TLV value for fresh milk, on average for the products examined

$$\Delta \text{ALV} = 2.66\, \Delta \text{TLV}$$

The fit is not as good for another set of values, in the same paper, for a series of heated (at 100°) fluid milk samples for which

$$\Delta \text{ALV} = 1.98\, \Delta \text{TLV}$$

1.8 THE KINETICS OF THE MAILLARD REACTION

The difficulty in deciding what method of measurement, other than actual growth tests gives the best indication of nutritionally available lysine, has probably been the reason so little attention has been paid to the kinetics of the loss of availability. This applies not only to milk, but also to other food products. Further, many experiments have been conducted using a wide selection of treatment times and temperatures but without a consistent series being used. This makes the extraction of rate data very difficult. The prime reasons for this lack of attention are believed to be the complexity of the Maillard reaction, the fact that there are other mechanisms (especially in foods with little reducing sugar) that can cause lysine losses, and the difficulty of determining which measurement system should

be used. These problems have meant that attention has been focussed on understanding these other aspects first.

However, there has been some work which can be examined in terms of rate constants, even if it is not possible to determine the order of the reactions involved. A summary has been published (Carpenter and Booth, 1973) in which there is clear evidence that for 'dry state' (6% to 18% moisture) the rate of loss of lysine is highly dependent on the presence of reducing sugars. Generally the presence of a reducing sugar increases the rate of the reaction by a factor of about 10,000. Using the Arrhenius relationship

$$k = A e^{-\frac{E}{RT}} \qquad (1.8)$$

where
- k is the reaction rate constant
- A is a frequency factor, a constant for a given reaction.
- e is the exponential e
- R is the Universal Gas Constant
- T is the Absolute temperature
- E is the energy of activation

the following relationship can be derived.

$$E = \frac{R\,T_1\,T_2}{(T_2 - T_1)} \ln\left(\frac{k_2}{k_1}\right) \qquad (1.9)$$

and applying this to the graph published in the review (Carpenter and Booth, 1973) it is possible to calculate.that

(a) for the loss of reactive ϵ-amino groups in lysine in protein, in the presence of reducing sugars,

$$Ea = 27.6 \text{ Kcal.mole}^{-1}, \quad \text{and}$$

(b) for the same loss, but not in the presence of reducing sugar

$$Ea = 31.7 \text{ Kcal.mole}^{-1}$$

(As the plot of the logarithm of the rate constants against the reciprocal of the Absolute temperature (otherwise called an Arrhenius plot) was linear, for both systems,

over a range of about 100°, the Arrhenius relationship can be applied to the results.)

In calculating the rate constants from the published literature figures Carpenter and Booth assumed that first order kinetics were applicable. This is in effect the meaning of the statement "the rate of loss was assumed to be proportional to the concentration of reactive groups remaining "

$$\text{i.e.} \quad \frac{dc}{dt} = -k \ c \qquad (1.10)$$

which is the traditional rate form for a first order reaction. As the values calculated from this relationship resulted in a linear plot it seems that even if first order kinetics could not be applied exactly to the reaction system studied, any error introduced as a result is not significant in relation to the values found.

A further qualification is provided in the statement that "wherever possible, a period, (H, hours) in which there was a 15 to 35% loss was used for calculation" of the rate constant. The formula used was the integral form of equation 1.10, i.e.

$$k = \left(\frac{100}{H} \right) \ln \left(\frac{C}{C_o} \right) \qquad (1.11)$$

In the same review a summary is presented in graphical form for the loss of reactive ϵ-amino groups of lysine in foods. This shows that a close similarity exists between the loss rates in a food containing reducing sugars (milk powder) and the model systems containing reducing sugars (mostly casein/glucose or casein/lactose systems). Also there is close agreement between the rates for loss in protein foods which contain little or no reducing sugar (such as ground nut flour and dried fish), and the rates for loss in isolated pure proteins heated without reducing sugar. Therefore the model systems do reflect the changes that occur in foods.

The kinetics of the Maillard reaction are complicated and this is exemplified by the little published work on determining the order of the reaction. The kinetics of browning of glucose-glycine can be expressed as (Mollah, 1968)

$$Cp = k . C_{gly}^{2} . C_{glu} . t^{2} \qquad (1.12)$$

where Cp is concentration of pigment
k is the rate constant
C_{gly} is the concentration of glycine
C_{glu} is the concentration of glucose, and
t is time.

The form of this equation suggests that there is some rate limiting intermediate that involves at least glycine and possibly glucose, in which case the intermediate may be glucosylglycine of step (a) or fructoseglycine of step (b) in the Maillard reaction scheme outlined in Section 1.2. Both of these intermediates are required for the formation of colour.

In contrast for a fructose-glycine system the kinetics can be represented by

$$Cp = k.C_{gly} . C_{f} . t \qquad (1.13)$$

where C_f is the concentration of fructose.
Here the intermediate may be the only rate controlling moiety and the reaction is second order, with subsequent steps being zero order.

The complexity is further shown by the work of Richards, 1963, which showed that there was production of galactose, lactulose, tagatose, changes in reducing power, and the formation of deoxyketoseamines in the initial stages of the Maillard reaction occurring in both dried skim milk and a 'dry' lactose-casein mixture and after an initial increase there was a general decrease. This would imply that there are a number of sequential reactions occurring and that the system is not just a simple one step sugar/amino acid interaction. Carpenter and Booth circumvented this problem in their analysis of the literature data by considering only the initial stages of the reaction.

This and other data relating to browning has been summarised in the Table 1.2

TABLE 1.2 BROWNING REACTION KINETICS SUMMARY

Reaction System	Reaction Order	E (Kcal/ mole)	Reference
Loss of ϵ-NH_2 groups in casein/glucose, 0-90°, 70% ERH	Assumed initially to be zero order, but true order not determined	29	Lea and Hannan, 1949
Colour in a solution of bovine serum albumen and glucose	I.D.	30.3	Mohammad et al., 1949
Loss of ϵ-NH_2 groups in casein/glucose, (1:1.5 equiv.) at 70% ERH	Assumed second order; when glucose in excess (3:1) pseudo- first order	I.D.	Lea and Hannan, 1950
Formation of fructoseglycine during dehydration of potato, 13-17% H_2O	I.D.	26	Hendel et al., 1955
Colour in heated cows milk	zero	28.3	Burton, 1954
Colour in heated goats milk	zero	27.6	Burton, 1963
Fructose/glycine in a syrup containing 25% water	1st order initially	26	Reynolds 1963 and 1965
Destruction of lysine in heated soybean meal (100-127°)	I.D.	30	Taira et al., 1966
Colour in glucose/glycine 1M:0.25M) pH 5.5 - 5.6	Zero order for colour; pseudo first order for fructoseglycine; 1/2 order for colour with respect to glucose concentration	22.1	Song, et al., 1966

Table 1.2 (cont)

Reaction System	Reaction Order	E (Kcal/ mole)	Reference
Loss of free NH_2 in glycine/ reducing sugar solution (xylose; mannose; galactose; glucose)	Zero order when reactants present in excess	I.D..	Spark, 1969
Loss of reactive ϵ-NH_2 groups in casein/glucose (Literature data)	Assumed first order	27.6	Carpenter and Booth, 1973
Loss of reactive ϵ-NH_2 groups in heated casein (Literature data)	Assumed first	31.7	Carpenter and Booth, 1973
Colour formation in an intermediate moisture food containing mixed proteins and excess glucose	Zero order	I.D.	Schnickels et al., 1976

I.D. = Insufficient Data Available for calculation

From Table 1.2 for colour development, the reaction is of zero order, (although not indicated in Table 1.2 there is usually a lag phase first), while for the loss of free ϵ-amino groups a first order or pseudo-first order is commonly assumed, and sometimes reported. As shown by Song, et al., 1966 the reaction becomes very complex as it progresses and simple integral order kinetics is unlikely to apply to the reactions of the Maillard intermediates once formed. Further just as the conditions of the reaction (pH, temperature, type of sugar, protein, amino acid, water content, and ERH), all influence the rate of the reaction, the same factors could well affect the order and energy of

activation for the Maillard reaction.

It thus appears that the Maillard reaction is of a variable order, depending on what species are reacted, and the method used to follow the progress of the reaction.